A Study of Selected Newer Dual Action Angiotensin and Endothelin Receptor Blockers

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"A Study of Selected Newer Dual Action Angiotensin and Endothelin Receptor Blockers"** submitted by Mr. Ram Gupta, ID. No. 2003PHXF403 for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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Ram Gupta

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List of Abbreviations

Abbreviation	Description
ACE	Angiotensin converting enzyme
Ang	Angiotensin
ARB	Angiotensin receptor blocker
ARF	Acute renal failure
AT_1	Angiotensin type 1
AT_2	Angiotensin type 2
AUC	Area under curve
BK	Bradykinin
BP	Blood pressure
CHF	Congestive heart failure
C_{max}	Maximum plasma concentration
CO	Cardiac output
CRF	Chronic renal failure
CVD	Cardiovascular disease
DMSO	Dimethyl sulphoxide
DOCA	Deoxycorticosterone acetate
DRC	Dose response curve
ECE	Endothelin converting enzyme
ECG	Electrocardiogram
ECM	Extracelular matrix
ED_{50}	Effective dose 50 (dose capable of producing 50 percent of maximal response)
ED_{75}	Effective dose 75 (dose capable of producing 75 percent of maximal response)
ET	Endothelin
ET_A	Endothelin type A
ET_B	Endothelin type B
hERG	Human ether-a-go-go-related gene
HPLC	High performance liquid chromatography
i.v.	Intravenous
IC_{50}	Inhibitory concentration (dose capable of inhibiting 50 percent of maximal response)

ICH	International conference on harmonisation
JNC7	Seventh report of the joint national committee
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MAP	Mitogen activated protein
MBP	Mean blood pressure
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NCE	New chemical entitie
NEP	Neutral endopeptidase
NP	Natriuretic peptide
NO	Nitric oxide
PAMPA	Parallel artificial membrane permeation assay
PBS	Phosphate buffer saline
PK	Pharmacokinetic
p.o.	Per oral
PR	Peripheral resistance
PRA	Plasma renin activity
QTc	Corrected QT (correction in QT interval of ECG for variability in heart rate from one subject to other)
RAS	Renin-angiotensin system
RMANOVA	Repeated measures analysis of variance
RSNA	Renal sympathetic nerve activity
SHR	Spontaneously hypertensive rat
SHRsp	Spontaneously hypertensive rat stroke prone
SNS	Sympathetic nervous system
$t_{1/2}$	Half life
TGF- β	Transforming growth factor- β
T_{max}	Time to reach C_{max}

Abstract

There is evidence based on large scale clinical studies which clearly point to the fact that even a modest fall in blood pressure in hypertensive population can retard end-organ damage and improve the morbidity and mortality associated with the disease. Despite multiple therapies currently available, substantial pockets are still far from adequately controlled. African American population is known to be resistant to many of the existing lines of treatment for hypertension. Besides, co-morbidities like diabetes pose additional challenge in terms of achieving the target blood pressure. It is becoming increasingly recognized that multifactorial condition like hypertension needs to be addressed by targeting not one but multiple interacting pathways.

Angiotensin II and endothelin participate in a manner involving closely interwoven pathways in increasing blood pressure and inducing end-organ damage. We tested the hypothesis that blocking angiotensin type 1 (AT_1) and endothelin type A (ET_A) receptor simultaneously would have a potentiating effect in reducing high blood pressure when tested in a salt loaded spontaneously hypertensive rat stroke prone (SHRsp) model that has both the components of activated renin-angiotensin and endothelin systems.

The dose response of an AT_1 receptor blocker, losartan and ET_A receptor blocker, ZD1611 was obtained after single dose administration followed by measuring effect on mean blood pressure upon combining minimally effective antihypertensive dose of ZD1611 with the ED_{75} dose for losartan. Minimally effective antihypertensive dose of ZD1611 could significantly potentiate the antihypertensive activity of ED_{75} dose of losartan. In the same model, the dose response of another ET_A receptor blocker, ZD4054 was followed by combining its different doses with the reported maximally effective dose of another AT_1 receptor blocker, candesartan, administered repeatedly once daily for 8 days. ZD4054 at doses of 1 and 3 mg/kg administered once daily for three days was able to potentiate the effect of maximal antihypertensive dose of candesartan. The measured plasma concentration of ZD1611 and ZD4054 at the lowest potentiation doses was substantially below the recorded levels in toxicological studies with ZD1611 and ZD4054. Upon successfully proving the hypothesis, a series of fifty new chemical entities were designed and synthesized in Torrent Research Centre. These new chemical entities were designed to be selective dual AT_1 and ET_A receptor blockers. Selectivity of new chemical

entities toward AT_1 and ET_A receptor was established in an *in vitro* assay performed at AstraZeneca, Sweden. Seven NCEs were selected out of the series for further *in vivo* profiling on the basis of their IC_{50} values and ratios of ET_A to AT_1 receptor IC_{50} values. Selected new chemical entities were screened *in vitro* for their solubility, permeability, plasma protein binding and rat liver microsomes mediated metabolism followed by their *in vivo* AT_1 and ET_A receptor blocking activity in anesthetized rat model infused with angiotensin II, having ET_A receptors blocked or with big endothelin-I having AT_1 receptors blocked respectively. Three potential new chemical entities identified from activity screening were further screened for their *in vivo* antihypertensive efficacy in salt loaded SHRsp after establishing their oral bioavailability. In all the *in vitro* and *in vivo* assays and screening procedures, another dual action receptor blocker BMS346567 was evaluated as reference to our new chemical entities. TRC23029, the most efficacious antihypertensive molecule identified during the screening process was tested for its neuropharmacological, cardiac and respiratory safety in appropriate animal models.

We successfully demonstrated that targeting together the renin-angiotensin system through AT_1 receptor blockade and endothelin system via ET_A receptor blockade is more efficacious antihypertensive strategy than targeting renin-angiotensin system alone through AT_1 receptor blockade. We also showed that the potentiation in antihypertensive effect observed while targeting AT_1 and ET_A receptors together is achievable while maintaining plasma levels of ET_A receptor blockers well below their recorded toxic levels. As a part of new drug discovery program, chemical entities designed to be dual AT_1 and ET_A receptor blockers were successfully screened for activity and efficacy in appropriate animal models. The potential drug candidate, TRC23029 was identified and developed up to the stage of preliminary acute animal safety study ready for further detailed chronic preclinical safety and toxicity studies before proceeding for human clinical trials.

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Introduction

1. Introduction

Hypertension is defined as “a persistent systolic blood pressure (BP) of at least 140 mmHg and/or diastolic BP of at least 90 mmHg or BP that is controlled to guideline recommended levels using antihypertensive medication” (Fields, et al., 2004;Rosamond, et al., 2008).

Despite recent progress documented by awareness, detection, prevention and treatment of high BP; it continues to remain an important public health challenge (Chobanian, et al., 2003). The worldwide prevalence of hypertension had been estimated at approximately 1 billion individuals (Epstein, 2008;Kearney, et al., 2005;Perkovic, et al., 2007) and is expected to grow to ≈1.5 billion (≈30% of the global population) by year 2025 (Perkovic, et al., 2007).

The prevalence of hypertension rises with age and in developed and developing countries alike, up to 60-70% of hypertensives are those beyond the seventh decade of life (Staessen, et al., 2003). Its prevalence is increasing in both men and women across the age span, but more rapidly in women. Indeed, the age adjusted prevalence of hypertension is higher in women than in men and is also higher in black people than in members of other racial groups (Flack, 2007). The recent data for the United States estimated that the prevalence of hypertension is 41.8% in African American men compared with 30.6% in white men and 45.4% in African-American women compared with 31.0% in white women (Ferdinand and Saunders, 2006).

The overall burden of hypertension and hypertension related diseases had been reported to rise rapidly in Asian countries like India (Gupta, 2004) and China (Gu, et al., 2002). The prevalence of hypertension; systolic, diastolic and mean BP varies tremendously from one Asian country to another and from one community to another community in the same country depending upon the economic development and affluence. However, overall prevalence of hypertension in different countries of Asia is estimated to be 5-35% (Perkovic, et al., 2007).

Currently about 32% urban population in Indian (Gupta, 2004) and 27.2% Chinese population (Gu, et al., 2002) is hypertensive. These data indicate that the prevalence of hypertension has shown a trend toward increased by 18% and 42% in India and China respectively, during past decade. The prevalence of hypertension in India and China is estimated to exceed that of many developing countries and is similar to that in industrial countries. Chinese epidemiological data highlights a trend

of increase in prevalence of hypertension in Chinese of younger age group which is much greater in comparison with older age groups, with increase of 74% in men and 62% in women of age 35 to 44 years and 18% in men and 15% in women of age 65 to 74 years (Gu, et al., 2002). The scenario is further complicated by the fact that the percentage of awareness, treatment and control of hypertension is unacceptably low in the Indian (Gupta, 2004;Israili, et al., 2007) and Chinese (Gu, et al., 2002) adult population.

Economic development, changes in lifestyle and diet and an increase in life expectancy are the factors suggested to contribute to the rapid increase in the prevalence and absolute number of hypertensives (Gu, et al., 2002;Gupta, 2004), leading to a stage where without an effective intervention program, an epidemic of hypertension would be inevitable in the near future.

Hypertension is known to have impact beyond mere increased BP recording. Incrementally higher levels of BP, especially systolic BP is reported to cause or promote microvascular and macrovascular diseases (Flack, 2007) and is one of the most important modifiable risk factors for cardiovascular disease (CVD) as stroke, coronary heart disease, congestive heart failure (CHF) and renal disease in western and Asian populations (Epstein, 2008;Gu, et al., 2002;He and Whelton, 1999a;Rodgers, et al., 1998).

Data from a meta analysis of 61 prospective studies involving almost 1 million individuals suggest that even when BP is normal, the increase in risk of ischemic heart disease and stroke mortality is progressive and linear from systolic BP levels ≥115 mmHg and diastolic BP levels ≥75 mmHg (Ferdinand and Saunders, 2006). Accordingly, for individuals aged 40-70 years, each increment of 20 mmHg in systolic BP or 10 mmHg in diastolic BP is suggested to double the risk of CVD across the entire BP range from 115/75 mmHg to 185/115 mmHg (Epstein, 2008). In addition, longitudinal data obtained from the Framingham Heart Study have indicated that BP values in the 130 to 139/85 to 89 mmHg range are associated with a more than 2-fold increase in relative risk from CVD compared with those with BP levels below 120/80 mmHg. Report from The World Health Organization and other sources had indicated that poor BP control is largely responsible for about 62% of cerebrovascular disease and 49% of ischemic heart disease (Chobanian, et al., 2003) contributing approximately 7.1 million deaths (Israili, et al., 2007) and 64 million disability adjusted life years every year (Perkovic, et al., 2007). Race and ethnicity had also been factors affecting the outcome of hypertension, with the middle aged European and Americans suffering from coronary heart disease as the predominant hypertensive

complication whereas Asians and older age group hypertensives encounter stroke as most common complication (Staessen, et al., 2003).

The importance of maintaining BP within normal range is emphasized by studies which suggested that even a modest decrease in BP could have the potential to significantly reduce hypertension related morbidity and mortality. It has been estimated that lowering systolic BP by 5 mmHg across the population would reduce the incidence of fatal stroke by 14%, fatal coronary heart disease by 9% and all cause mortality by 7%. Indeed, it is suggested that if the population levels of systolic BP were lowered by 2 mmHg, this would translate into approximately 7% lower mortality from ischemic heart disease or other vascular causes and 10% lower risk of stroke mortality in middle age (Ferdinand and Saunders, 2006).

Hypertension is an outcome of a number of metabolically linked risk factors. A large number of pathophysiologic factors have been implicated in the genesis of primary hypertension which include increased sympathetic nervous system (SNS) activity, overproduction of sodium (Na^+) retaining hormones and vasoconstrictors such as endothelin (ET) and thromboxane, chronically elevated Na^+ intake, inadequate dietary intakes of potassium and calcium (Ca^{2+}), increased or inappropriate renin secretion, deficiencies of vasodilators such as prostaglandins and nitric oxide (NO), diabetes mellitus, insulin resistance, obesity, increased activity of vascular growth factors and altered cellular ion transport, to name the most studied pathways so far.

Although our understanding of the pathophysiology of elevated arterial pressure has increased, in 90 to 95% of cases the etiology (and thus potentially the means of prevention or cure) is still largely unknown (Fisher and Williams, 2005). According to the Seventh Report of the Joint National Committee (JNC7) on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, only about 34% of patients undergoing treatment for hypertension have their BP controlled to recommended limits (Chobanian, et al., 2003) and of uncontrolled hypertensives, a substantial portion is contributed by blacks (Bakris, et al., 2002;Douglas, et al., 2003).

Substantial amount of hypertensive population show altered renin-angiotensin system (RAS) activity, which has also been implicated in the development of various CVD. It is now clear that angiotensin (Ang) II, the effector molecule of RAS is involved in the development of cardiac hypertrophy and remodeling and in the pathophysiology of hypertension. Therefore, drugs that interfere with the RAS, such as angiotensin converting enzyme (ACE) inhibitors and angiotensin type 1 (AT_1)

receptor blockers have been shown to be of great therapeutic benefit in the treatment of such cardiovascular disorders (Kentsch and Otter, 1999).

ETs are family of most potent endogenous vasoconstrictor identified to date. ET-1 is the predominant isoform of the ET peptide family which exerts diverse biological effects through two distinct G-protein coupled ET type A (ET_A) and ET type B (ET_B) receptors. Stimulation of ET_A receptors results in vasoconstriction and cell proliferation, whereas ET_B receptors mediate clearance of ET-1, inhibition of endothelial cell apoptosis, release of NO and prostacyclin and inhibition of endothelin converting enzyme (ECE)-1 expression (Barton and Kiowski, 2001).

ET has been implicated in the pathogenesis of hypertension (Epstein, 2008) and increased ET-1 content in the vasculature of deoxycorticosterone acetate (DOCA)-salt hypertensive rats, Dhal salt sensitive rats and salt loaded spontaneously hypertensive rat stroke prone (SHRsp) has been identified (Pinto-Sietsma and Paul, 1998;Schiffrin, 2005). Studies have demonstrated that ET-1 and its receptors are also involved in the regulation of Na^+ and water reabsorption and excretion and therefore, suggested to contribute to the development and/or maintenance of hypertension (Grubbs and Ergul, 2001). In addition to acute vascular effects ET-1 has a direct mitogenic action on cardiovascular tissues, as well as co-mitogenic actions with a wide variety of growth factors and vasoactive substances (Chua, et al., 1992;Fujitani and Bertrand, 1997;Grainger, et al., 1994). ET receptor blocker also seems to have pressure independent properties that could either prevent or regress end-organ damage in the context of hypertension (Barton and Kiowski, 2001;Epstein, 2008).

That the ET system and RAS are upregulated in hypertensive patients have been clearly shown by elevated plasma levels of ET-1 and plasma renin activity (PRA) (Ergul, 2000;Ergul, et al., 1998;Ibrahim, 2006;Kim and Iwao, 2000;Pinto-Sietsma and Paul, 1998). Similarly, most of the obese and diabetic hypertensives have overactive plasma/tissue (adipose tissue & kidney respectively) RAS and ET system (Chan, et al., 2005;Engeli, et al., 2000;Engeli and Sharma, 2001;Goossens, et al., 2003;Lteif, et al., 2007;Mather, et al., 2002;Sarafidis and Bakris, 2007). All these evidences suggest that abnormality in the RAS and ET system plays a major role in the pathophysiology of resistant and difficult to treat hypertension (Epstein, 2008; Shreenivas and Oparil, 2007).

Angiotensin receptor blocker(s) (ARB)s are well established and safe drugs for antihypertensive treatments (Fisher and Williams, 2005). ET receptor blockers have been shown to have antihypertensive effects in hypertensive patients (Attina, et

al., 2005;Epstein, 2008) and to prevent end-organ damage in hypertensive rats (Kassab, et al., 1998). Emerging experimental evidence from various studies suggests that Ang II and ET-1 participate in a manner involving closely interwoven pathways in increasing BP and inducing end-organ damage (Boemke, et al., 2001;Gómez-Garre, et al., 1996;Herizi, et al., 1998;Montanari, et al., 2003;Yoshida, et al., 1992). The physiological benefit of Ang II and ET-1 blockade has been demonstrated in hypertensive transgenic rats that over express the human renin gene (Bohlender, et al., 2000). A combination of AT_1 receptor blocker and nonselective ET_A/ET_B receptor blockers reduced the mean blood pressure (MBP) more than monotherapy in spontaneously hypertensive rat (SHR), SHRsp and Dahl salt sensitive hypertensive rats (Ikeda, et al., 2000). Thus, concomitant blockade of both Ang and ET pathways is expected not only to enhanced BP reductions but also retard the end-organ damage directly and indirectly.

However, given the opposing actions of the ET_A and ET_B receptors, therapeutic opportunity and threats associated with selective blockade of ET_A receptor and nonselective blockade with nonselective ET_A/ET_B receptor blocker needs to be carefully assessed. ET_A receptors are present on smooth muscle cells and are responsible for mediating contraction in response to ET-1. ET_B receptors are present on the endothelium and upon stimulation induce release of NO and prostacyclin, which relax the underlying vascular smooth muscle. However, stimulation of ET_B receptors present on vascular smooth muscle cells of large vessels has been shown to mediate vasoconstriction in animals and humans (Schiffrin, 1995). In addition to its vasodilatory function, the ET_B receptor also acts as a clearance receptor for circulating ET-1 (Dhaun, et al., 2006). Potential role of ET_B receptors in physiological regulation of renal salt and water excretion has also been pointed out (Pollock, 2005). ET receptor blockers that can block either ET_A or ET_B receptors or both have been developed and the blockade of ET_B receptors have been shown to impair the pulmonary clearance of ET-1 and reduces NO-mediated vasodilatation (Luscher and Barton, 2000). In same line, systemic blockade of the ET_B receptor have been shown to increase peripheral vascular resistance in healthy men (Strachan, et al., 1999) and ET_B receptor deficiency to be associated with hypertension in mice (Luscher and Barton, 2000). However, in most experimental (Kaddoura, et al., 1996;Mulder, et al., 1997) and clinical studies (Kiowski, et al., 1995;Sutsch, et al., 1998;Wenzel, et al., 1998) nonselective ET_A/ET_B receptor blockers improved cardiovascular function and structure, suggesting that therapeutic effects can be expected provided that ET_A receptors are blocked, regardless of

concomitant ET_B receptor blockade. Thus, upon reviewing various evidences, selective blockade of ET_A receptor seems more advantageous than nonselective ET_A/ET_B receptor blockade. Same have also been proved in studies directly comparing benefits of ET_A versus nonselective ET_A/ET_B receptor blockers in conditions as chronic renal failure (CRF) and heart failure (Goddard, et al., 2004;Leslie, et al., 2005).

While combining pharmacologically effective doses of AT_1 receptor blocker with ET_A receptor blocker may provide substantial reduction in BP, ET receptor blocker at higher doses are reported to be teratogenic involving craniofacial, great vessel, heart and thyroid malformations (Battistini, et al., 2006;Treinen, et al., 1999). Given the known role for ETs in development and concordant malformations observed in rats and rabbits, teratogenicity is suggested to be a class effect of ET receptor blockers (Treinen, et al., 1999). However, use of teratogenic antihypertensives is not unprecedented. ACE inhibitors, ARBs and direct renin inhibitors are contraindicated in pregnancy (Epstein, 2008).

Thus these studies were undertaken to establish whether antihypertensive effect of selective AT_1 receptor blocker can be potentiated further by adding minimally effective antihypertensive dose of selective ET_A receptor blocker which is also safe with respect to teratogenic potential. Further, upon the confirmation of our hypothesis we aimed to identify and develop novel antihypertensive molecules, which, while acting as dual receptor blocker against AT_1 and ET_A receptors could be used to treat human hypertension.

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2. Review of Literature

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2.1. Etiology of hypertension

There is still much uncertainty about the etiology of hypertension and no single or specific cause is known in most cases of hypertension and the condition is referred to as primary hypertension (Beevers, et al., 2001). Few cases do exist wherein there is an underlying condition secondary to which hypertension develops. These are the cases categorized as secondary hypertensives and the condition is referred to as secondary hypertension.

2.1.1. Secondary hypertension

Only a small number of patients have an identifiable underlying cause for their raised BP, termed secondary hypertensives and the condition is referred to as secondary hypertension (Table1) (Beevers, et al., 2001;Mancia and Grassi, 2005). Secondary forms of hypertension are relatively uncommon and account for only 5 to 10% of all cases of hypertension. Findings as severe hypertension, sudden onset of hypertension and BP responding poorly to drug therapy are indicative of a secondary form of hypertension. Though secondary hypertension accounts for very small number of hypertension cases, it still must be investigated before initiation of antihypertensive therapy (Kjeldsen et al., 2006), as treating the underlying pathway then becomes essential.

Table 1: Classification of secondary hypertension according to its etiology.

Exogenous substances
Oral contraceptive pills
Glucocorticoides
Erythropoietin
Cyclosporine
Acute alcohol
Renal disease
Renovascular hypertension
Renal parenchimal hypertension
Endocrine disease
Corticoadrenal hypertension
Hyperthyroidism and hypothyroidism
Pheochromocytoma
Acromegaly
Pregnancy
Pregnancy induces hypertension
Neurological disease
Acute cerebrovascular ischemia
Sleep-apnea syndrome
Guillain-Barre syndrome
Quadriplegia

Adapted from: Mancia G and Grassi G (2005) Defination and classification of hypertension, in *Hypertension: Principles and practice* (Battegay EJ, Lip GYH; Bakris GL eds) pp 20, Trylor & Francis, Boca Raton.

2.1.2. Primary hypertension

Primary hypertension refers to a lasting increase in BP due to complex interaction between heterogeneous genetic and environmental factors accounting for approximately 90 to 95% of all hypertension cases (Calhoun et al., 2004). Systemic BP is the product of cardiac output (CO) and peripheral resistance (PR). Since persistent hypertension can develop in response to either an increase in CO or a rise in PR, defects may present in one or more of the multiple factors that affect these two contributors (Figure 1). The interplay of various derangements in factors affecting CO and PR may precipitate the disease and these abnormalities may differ in both type and degree in different patients (Kaplan, 2004). Genetic predisposition additionally is known to influence the incidence and severity of the hypertension.

2.1.2.1. Genetic predisposition

Genetic influences on primary hypertension have been well acknowledged and it has been suggested that genetic causes may initiate the cascade which could lead to permanent hypertension (Kaplan, 2004). Though, the efforts to identify common genes, having predominant influence on human hypertension have failed, results from various studies have suggested that several loci exist in general population, each contributing a little to overall BP and therefore primary hypertension has been considered to be polygenic in origin (Calhoun et al., 2004;Hsueh, et al., 2000;Hunt, et al., 2002;Kristjansson, et al., 2002;Levy, et al., 2000;Rice, et al., 2002).

Evidence for genetic influence on BP had been documented from variety of studies. Twin studies have documented greater concordance of BP in monozygotic than dizygotic twins (Feinleib, et al., 1977) and population studies have shown greater similarity in BP within than between families (Longini, Jr., et al., 1984).The latter observation was not attributable to a shared environment only, since adoption studies had demonstrated greater concordance of BP among biological siblings than adoptive siblings living in the same household (Biron, et al., 1976).

Monogenic forms of hypertension are rare form of human hypertension which display Mendelian mode of inheritance. They are caused by defects within a single gene sufficient to cause large change in BP (Hamet, et al., 2002). Mutations in ten genes that cause Mendelian form of human hypertension and nine genes that cause hypotension have been described (Lifton, et al., 2001;Luft, 2003;Wilson, et al., 2001). Monogenic forms of hypertension account for very small percentage of hypertensive cases and do not explain BP variability in the population at large (Staessen, et al., 2003). However, study of these rare disorders helps to elucidate pathophysiologic

mechanisms that predispose humans to more common forms of hypertension and suggest novel therapeutic approaches.

2.1.2.2. Pathophysiology of hypertension

Growing evidences suggest participation of structural and functional vasculature abnormalities, including endothelial dysfunction, increased oxidative stress, vascular remodeling and decreased compliance which may antedate hypertension and contribute to its pathogenesis. These factors contribute to altered cardiac, microvascular and macrovascular changes which culminate in the hypertensive state. A large number of pathophysiologic factors have been implicated in the genesis of primary hypertension, including perturbed SNS activity, overproduction of Na^+ retaining hormones and vasoconstrictors, deficiencies of vasodilators, increased activity of vascular growth factors and altered cellular ion transport (Calhoun et al., 2004).

The basic abnormality of primary hypertension originates from an imbalance between total PR and plasma volume (and thus CO). The former stems from abnormal regulation of contractility of vascular smooth muscle in resistance arteries and the latter from an abnormal regulation of extracellular fluid volume (Alderman, 2008;Semplicini and Monari 2002). Therefore, at given point of time the change in arterial blood volume per unit time represents the difference between rate at which blood is pumped into the arterial system by heart and the rate at which it leaves the arterial system through the resistance vessels. If arterial inflow exceeds outflow, arterial volume increases, the arterial walls are stretched more and arterial pressure rises (Berne and Levy 2002).

CO is influenced by the contractile properties of the heart, cardiac rate and rhythm, preload, autonomic nervous system activity and the functional integrity of the cardiac valves, where preload is determined by intravascular volume and venous tone and capacitance (Calhoun et al., 2004). The possible ways by which an increase in CO can contribute to hypertension could be either from an increase in body fluid volume or from an increase in contractility from neural stimulation of the heart (Vikrant and Tiwari, 2001). Vascular resistance, on the other hand is related to the viscosity of blood and the length of the arterial segment and inversely related to the fourth power of the luminal radius. The first two factors are relatively fixed, so in practical terms PR is almost entirely determined by the properties of the small distal resistance vessels, those with diameters of 500 μm or less. Smooth muscle in the walls of the resistance vessels is controlled by perivascular nerves and by

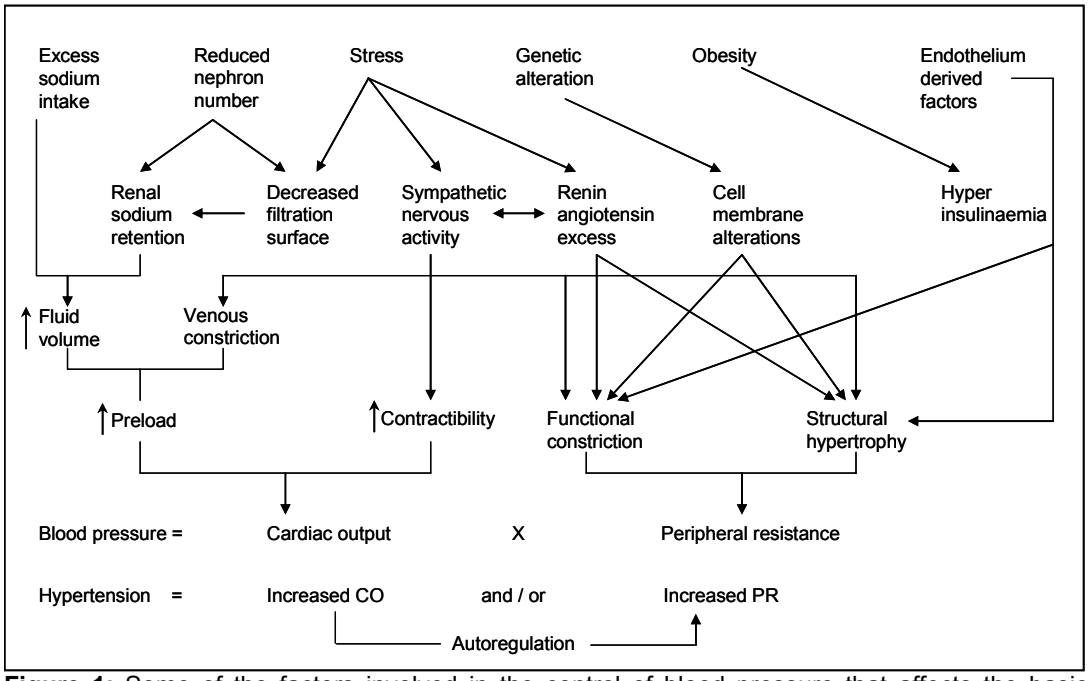


Figure 1: Some of the factors involved in the control of blood pressure that affects the basic equation: blood pressure = cardiac output x peripheral resistance. CO, cardiac output; PR, peripheral resistance.
Adapted from: Vikrant S and Tiwari SC (2001) Essential hypertension – Pathogenesis and pathophysiology. *Indian Academy of Clinical Medicine* **2**:140-161.

substances either in the circulation or produced by endothelial cells in their lining. A number other factors may also work on PR leading to functional contraction and structural remodeling and hypertrophy (Calhoun et al., 2004). Following discussion deals with factors which modulate one or more of the above mentioned components responsible for maintenance of BP.

2.1.2.2.1. Altered renal physiology and salt handling

In primary hypertension, physiologic and pathologic renal changes often precede changes identifiable in other organs, but whether they precede or follow the onset of the hypertension itself has not been clearly understood. Further, active role of excess Na^+ intake and its renal retention is also suggested in the genesis of hypertension (Kaplan, 2004; Vikrant and Tiwari, 2001).

Studies have estimated that approximately one half of all hypertensive patients have some degree of salt sensitivity, defined as a 10 mmHg drop in BP when consuming a low salt versus a high salt diet (Cushman, 2003). The mechanisms for salt sensitivity are not entirely clear, however, it has been suggested that salt sensitivity may occurs due to defects in cellular Na^+ transport, which is

variably contributed to abnormalities in the nephrons, RAS and SNS (Cushman, 2003;Weinberger, 1996).

It is suggested that Na^+ retention could arise due to decrease in filtration surface by a congenital or acquired deficiency in nephron number or function (Brenner and Anderson, 1992;Kaplan, 2004); resetting of pressure-natriuresis relationship wherein a rise in pressure invokes an immediate increase in renal Na^+ excretion, thereby shrinking fluid volume and returning the pressure to normal (Guyton, 1992) and nephrons heterogeneity, where due to subpopulation of nephrons that are ischemic, either from afferent arteriolar vasoconstriction or from an intrinsic narrowing of the lumen, show tonically elevated renin, which interferes with the compensatory capacity of intermingled normal nephrons to adaptively excrete Na^+ and consequently, perturbed overall BP homeostasis (Kaplan, 2004).

A decrease in the capacity of kidneys to excrete salt would cause salt and water retention, increased extracellular and plasma volume and increased BP. The ability of kidneys to excrete Na^+ declines gradually with age and even smaller increase in salt intake induce a rise in BP (Khalil, 2006). With increase in age beyond third decade of life, an accelerating fall in glomerular filtration rate (Meneton, et al., 2005) accompanied by a decline in functioning nephrons and progressive glomerulosclerosis has been documented. Thus, advancement into later decades of life warrants reduction in salt consumption, however, failure to do so results in elevation of BP to increase fractional Na^+ excretion and restore Na^+ balance (Khalil, 2006).

Results from studies conducted on hypertensive humans have demonstrated that acute changes in salt intake could change Na^+ levels not only in the plasma, but also in the cerebrospinal fluid (Huang, et al., 2004). Acute experimental increase in plasma or cerebrospinal fluid Na^+ >5 mM/kg raised BP independent of the extracellular fluid volume (Meneton, et al., 2005). Increased plasma Na^+ levels have been suggested to initiate Na^+-nucleic acid interaction in various cell types and increase the production of BP modulating hormones. In cultured vascular smooth muscle, an increase in Na^+ by 2-10 mM increases messenger ribonucleic acid (mRNA) expression of hypertrophy related factors and the number of AT_1 receptors (Gu, et al., 1998).

Renal NO contributes to the regulation of renal medullary flow and diuresis (Cowley, Jr., et al., 2003). It is suggested that dietary Na^+ may inhibit L-arginine transport in the renal medulla and thereby renal NO production and medullary flow (Zewde, et al., 2004). High-salt diet may also increase renal medullary osmolarity

and decrease NO synthase expression (Herrera and Garvin, 2005). Additionally, salt intake is suggested to be associated with increased oxidative stress and renal expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and decreased activity of superoxide dismutase (Kitiyakara, et al., 2003).

Consequently, individuals who are more Na^+ sensitive have been found to have more markers of endothelial damage, nondipping of nocturnal BP and increased mortality (Weinberger, et al., 2001) than do those who are less Na^+ sensitive and thus the relationship between dietary salt and hypertension has evolved from a possible association to the measurable relationship with other contributing factors.

Thus increased salt retention either due to compromised renal ability to get rid of excess salt or due to its increased intake, leads to a situation of increased body fluid volume contributing to increased CO as well as increased vascular resistance due to its involvement in expression of hypertrophy related factors directly and indirectly by triggering the expression of AT_1 receptors known to mediate vasoconstriction and hypertrophy upon stimulation with its ligand Ang II.

2.1.2.2.2. Altered vascular reactivity and remodeling

Peripheral vascular resistance is reported to be characteristically elevated in hypertension due to alterations in structure, mechanical properties and function of small arteries. Remodeling of these vessels contributes to the development of high BP and its associated target-organ damage (Oparil, et al., 2003). The elevated vascular resistance seen in hypertensive subjects is related to a combination of rarefaction (decrease in number of parallel-connected vessels) and narrowing of the lumen of resistance vessels. Examination of specimens obtained from gluteal skin biopsies of patients with untreated primary hypertension has uniformly revealed inward, eutropic remodeling (Calhoun et al., 2004).

Systolic BP and pulse pressure increase with advancing age mainly as a result of increased stiffness of the large conduit arteries. Arteriosclerosis in these arteries results from collagen deposition and smooth muscle cell hypertrophy, as well as thinning, fragmenting and fracture of elastin fibers in the media (Oparil, et al., 2003). Hypertension exacerbates these chances due to vascular remodeling thereby further affecting the performance of conduit arteries and initiating a vicious cycle of hypertension and arterial remodeling (Humar et al., 2005).

An important contribution comes from elevation of SNS activity which has been shown to raise BP in both normotensive and hypertensive subjects through

stimulation of the heart, kidney and peripheral vasculature, causing respectively, increase in CO, fluid retention and vascular resistance. Studies have demonstrated a positive correlation between heart rate and future development of hypertension. In 10 years long multicenter longitudinal Coronary Artery Risk Development in Young Adults (CARDIA) study (Kim, et al., 1999), heart rate was found to be an independent predictor of subsequent elevated diastolic BP. Since SNS predominantly controls heart rate, these results support the concept that chronic sympathetic over activity contributes to hypertension. Also, direct determination of peripheral or muscle sympathetic nerve activity using microneurography has shown that muscle sympathetic nerve activity is increased in hypertensive versus normotensive subjects (Anderson, et al., 1989;Yamada, et al., 1989). The baroreceptors when activated by a rise in BP or central venous pressure normally reduce heart rate and lower BP by vagal stimulation and sympathetic inhibition. When hypertension is sustained, these reflexes are shown to reset rapidly from both structural and functional changes so that given increase is BP evokes less decrease in heart rate (Chapleau, et al., 1995) and presumably associated with a reflection of increased CO (Kaplan, 2004). It is postulated that the decreased inhibition of the vasomotor center resulting from resetting of arterial baroreceptors may be responsible for increased sympathetic out flow and thereby in the perpetuation of hypertension (Vikrant and Tiwari, 2001).

Exposure to stress increases sympathetic outflow and it has been hypothesized that repeated stress-induced vasoconstriction results in vascular hypertrophy, leading to progressive increases in PR and BP. Laboratory stress testing suggests that persons with a family history of hypertension manifest augmented vasoconstrictive responses to laboratory stressors, such as cold pressor testing and mental stress, which may predispose them to the development of hypertension (Calhoun et al., 2004).

Sustained elevation of the sympathetic tone may lead to hypertension and diseases of the cardiovascular system (Borchard, 2001) by favoring cardiac and vascular hypertrophy (Julius, 2000). Sympathetic nerves increase the rate of synthesis of contractile protein in vascular muscle and are suggested to be the cause for greater resistance and enhanced vasoconstriction in hypertensive animals (Abboud, 1982). Same has been supported by studies where surgical removal of local sympathetic innervation prevented the pressure-related arterial hypertrophy in SHR. The vascular hypertrophy is important since the thick muscular (medial) layer diminishes the size of the lumen of the blood vessel. This is of minor hemodynamic significance when the vessels are dilated. However, during vasoconstriction, the wall

of such hypertrophic vessels abnormally and excessively encroaches upon the lumen and thereby elicits a steep increase in vascular resistance. This accelerates vasoconstrictive responses and becomes an important amplifier of hypertension (Julius, 2000).

The hypertrophy of coronary arterioles makes them more susceptible to spasms. Because of the thicker wall, hypertrophic coronaries are also less capable of vasodilation. The insufficient vasodilation is further aggravated by pressure-related endothelial dysfunction. These mechanisms lead to a substantial decrease in coronary reserve in patients with hypertension whose large coronary arteries are free of atherosclerotic disease (Julius, 2000).

Further, it has been shown that patients with relatively higher central sympathetic output are more likely to develop left ventricular hypertrophy than those with equivalent severity of hypertension having lower sympathetic output. In support of this, catecholamines have been shown experimentally to have trophic properties on cardiac myocytes and in the intact animal heart. Also, in human studies, indexes of left ventricular mass have tended to correlate with plasma norepinephrine values and cardiac noradrenaline spillover (Burns, et al., 2007;Greenwood, et al., 2001).

Though hypertrophy in the short term enhances the functional performance of cardiovascular organs, long-standing hypertrophy carries negative prognostic implications. A hypertrophic heart becomes stiffer, which impedes the diastolic filling and function. In due course, the hypertrophic myocardium outgrows its blood supply which eventually causes ischemic heart disease (Julius, 2000).

2.1.2.2.3. Cell membrane alterations

Evidence suggests that the cell membranes of hypertensives are altered in a primary manner, allowing abnormal movements of ions and thereby changing the intracellular environment to favour contraction and growth. Abnormalities of the physical properties of the membrane and of multiple transport systems have been implicated in the pathogenesis of hypertension (Russo, et al., 1997).

Intracellular Ca^{2+} is a major determinant of vascular smooth muscle cell contraction and a key element of cellular response to agonists, acting as a second messenger. In resting cells intracellular Ca^{2+} homeostasis is maintained by variety of Ca^{2+} mobilizing and Ca^{2+} homeostatic mechanisms like Ca^{2+} channels and Ca^{2+} pumps located in the plasma membrane and intracellular organelles. Results from circulating blood cells and vascular smooth muscle cells of hypertensive animal models and hypertensive humans suggest disruption in Ca^{2+} homeostasis, which

presents an elevated intracellular Ca^{2+} levels and/or larger Ca^{2+} transients to agonists. The abnormalities associated with primary hypertension include increased cell Ca^{2+}, increased Ca^{2+} uptake and decreased Ca^{2+} removal. The increased Ca^{2+} observed in hypertension is postulated to be result of either reduced binding of Ca^{2+} to the inner cell membranes or due to low activity of Ca^{2+} ATPase. Despite extensive investigations, a unifying explanation for abnormal Ca^{2+}metabolism in hypertension still does not exist (Semplicini and Monari 2002).

Similarly, cellular Na^+ levels are reported to be elevated in many cell types in primary hypertension, both in humans and animals. Evidences suggest that the Na^+ hydrogen (Na^+/H^+) exchanger is stimulated in hypertensive patients either by an increased cellular Ca^{2+} load or enhanced external Ca^{2+} entry. An increased Na^+/H^+ exchanger could play a significant role in the pathogenesis of hypertension, both by stimulating vascular tone and cell growth and possibly by increasing Na^+ reabsorption in renal proximal tubule cells (Semplicini and Monari 2002). Similar conclusions have been drawn from experiments studying apical membrane vesicles from the kidney of young prehypertensive Milan rats and SHR which show increased Na^+ uptake via Na^+/H^+ exchange. Also, transgenic mice overexpressing the Na^+/H^+ exchanger in renal tubules are found to develop hypertension during salt loading (Brooks, et al., 2001;Lorenz, et al., 1999).

Red blood cell membrane from hypertensives have shown increased cholesterol: phospholipid ratio in association with high Na^+-lithium transport (Freel and Connell, 2004) and increased ratios of fatty acid metabolites to precursors compared to those from age matched normotensives (Russo, et al., 1997). Such changes in lipids is suggested to produce a high membrane microviscosity and decrease in fluidity (Carr, et al., 1995), which may contribute to increased permeability to Na^+ and other alterations in Na^+ transport (Vikrant and Tiwari, 2001).

2.1.2.2.4. Endothelial dysfunction

The vascular endothelium synthesizes and releases a spectrum of vasoactive substances and therefore plays a crucial role in basal and dynamic regulation of the circulation and in the pathogenesis of hypertension.

NO released from endothelial cells in response to shear stress of blood flow and activation of variety of receptors, leads to relaxation of smooth muscle cells by activating guanylate cyclase, thereby increasing intracellular cyclic guanosine monophosphate and it also possess antithrombogenic, antiproliferative and leukocyte-adhesion inhibiting effects (Calhoun et al., 2004). Although, the role of NO

in the regulation of BP is uncertain, several studies have reported its influence on BP and renal haemodynamics (Cowley, Jr. and Roman, 1996). Hypertensive patients have been shown to have a reduced vasodilatory response to various stimuli of NO release that is independent of the origin of hypertension and degree of gross vascular structural alteration (Kaplan, 2004). In healthy human subjects, inhibition of NO syntheses by N monomethyl- L-arginine acutely increased BP, peripheral vascular resistance and fractional excretion of Na^+ (Vikrant and Tiwari, 2001). Impaired NO mediated vasodilatation is suggested to promote abnormal vascular remodeling and found to serve as a marker of future cardiovascular events (Kaplan, 2004).

Balance between various endothelial vasodilatory and vasoconstrictor has been often reported to be disturbed in hypertensives. The superoxide anions generated by Ang II activated NADPH oxidase or by cyclooxygenase can scavenge the vasodilatory NO to form the highly reactive peroxynitrite which can further damage cell membrane and oxidize lipids. In SHR, though NO synthase is upregulated, NO is inactivated by excess superoxide anions whereas, in salt-related hypertensive model, as DOCA salt hypertensive rats, NO production system is suppressed and the ET system is found to be upregulated (Spieker and Lüscher, 2005).

Insulin resistance is another factor which contributes to the endothelial and vascular dysfunction. Association between hypertension and hyperinsulinemia has been recognized, particularly with accompanying obesity but also in about 20% of non-obese hypertensive patients (Kaplan, 2004). Obese individuals have higher CO, stroke volume and central and total blood volume than non-obese individuals with similar BP. The increase in CO is proportional to the expansion of body mass and may be the primary reason for the rise in BP (Vikrant and Tiwari, 2001). Virtually all obese people are hyperinsulinemic secondary to insulin resistance and more so if the obesity is predominantly visceral, abdominal or upper body. The impairment in insulin action may in turn result from a defect in the usual vasodilatory effect of insulin mediated through increased synthesis of NO, which normally counters the multiple pressor effects of insulin. These pressor effects include activation of sympathetic activity, a trophic action on vascular hypertrophy and increased renal Na^+ reabsorption. The failure of vasodilation to antagonize the multiple pressor effects of insulin presumably eventuates in BP rise that may be either a primary cause of hypertension or, at least, a secondary potentiator (Kaplan, 2004).

Prominent role of RAS and the ET system in maintenance of BP by regulation of the fluid and electrolyte homeostasis, renal function, endothelial function, vascular reactivity and structural modulation on one hand and cardiac structure and function on the other is widely recognized. Contribution of the RAS and ET system to pathophysiology of hypertension is dealt with separately in the following sections in order to address in depth their functions individually and cross talk between the two.

2.2. The renin-angiotensin system

"Renin" was first described by Tigerstedt and Bergmann in year 1898. Since then, knowledge about renin and the RAS is continuously evolving (Mazzolai and Nussberger, 2005).

RAS is a bioenzymatic cascade that plays an integral role in cardiovascular homoeostasis by influencing vascular tone, fluid and electrolyte balance and the SNS (Dinh, et al., 2001). Traditionally, the RAS was viewed as a circulating endocrine system, where renin is expressed, stored and released in a regulated manner by the juxtaglomerular cells of the kidney (Mazzolai and Nussberger, 2005). Biosynthesis and release of renin, a step-limiting enzyme with a specific action on its substrate of cleavage, angiotensinogen (Volpe, et al., 2002), is released by juxtaglomerular cells of the kidney in response to renal hypoperfusion, decreased Na^+ delivery and sympathetic activation (Givertz, 2001). Renin secretion from juxtaglomerular cells is Ca^{2+} dependent. However, unlike other hormones, Ca^{2+} plays an inhibitory action on renin release (Della, et al., 1995). Renin cleaves the liver-derived macroglobulin precursor angiotensinogen, its only known substrate, to produce the inactive decapeptide Ang I, the only precursor of Ang peptides. Ang I is then cleaved by the endothelial cell associated or soluble dipeptidyl carboxypeptidase ACE to the biologically active octapeptide, Ang-(1-8)octapeptide or Ang II within the pulmonary circulation. Almost all the biological actions of RAS are mediated primarily by this highly active Ang II (Dinh, et al., 2001). A minor portion of Ang I is alternatively cleaved by tissue specific endopeptidases into the Ang-(1-7)heptapeptide, which is sequentially converted by ACE into Ang-(1-5)pentapeptide. Ang II is further cleaved by aminopeptidase A or carboxypeptidase P to form either Ang-(2-8)heptapeptide, that is Ang III or Ang-(1-7) heptapeptide, respectively; Ang III is further converted by aminopeptidase B into Ang-(3-8)hexapeptide, which is also called Ang IV (Mazzolai and Nussberger, 2005).

Several studies have shown that alternative pathways exist for production of Ang II. In addition to ACE, enzymes as tonin, trypsin, kallikerin, cathepsin G, chymotrypsin, chymase and tissue plasminogen activators (Araujo, et al., 2002;Hollenberg, et al., 1998;Ikeda, et al., 1988;Nishimura, et al., 1998) have been demonstrated to be able to transform Ang I into Ang II. However, the pathophysiological relevance of these alternative pathways still remains unclear.

In addition to the systemic (circulating) RAS, there are evidences to indicate that many tissues, including the vasculature, heart, kidney and brain, are capable of producing Ang II, which may thereby mediate autocrine, paracrine and intracrine

effects (Campbell, 1987;Johnston, 1992). Numerous studies have also shown that the requisite components of the RAS, such as angiotensinogen, renin and ACE are present in such tissues (Phillips, et al., 1993;Vinson, et al., 1995). Regardless of how it is formed, the effects of Ang II are mediated via its specific membrane-bound receptors.

2.2.1. Angiotensin receptors

The actions of Ang II are mediated by specific heterogeneous populations of Ang II receptors. Ang II is known to interact with at least two distinct Ang II receptor subtypes, designated as AT_1 receptor and angiotensin type 2 (AT_2) receptor (Dinh, et al., 2001).

2.2.1.1. AT_1 receptors

AT_1 receptors selectively bind biphenylimidazoles, including losartan, candesartan and irbesartan, with high affinity and are rather insensitive to tetrahydroimidazolpyridines, such as PD123319 and PD123177. The AT_1 receptor belongs to the class of G-protein coupled receptors with its gene localized on human chromosome 3. In contrast to human AT_1 receptor, two isoforms for the AT_1 receptor are described in rodents, termed AT_{1A} and AT_{1B}, which share 94% similarity. AT_1 receptors are primarily found in the brain, adrenals, heart, vasculature and kidney and are predominantly involved in the regulation of BP, fluid and electrolyte balance (Dinh, et al., 2001).

Activation of the AT_1 receptor by its ligand Ang II stimulates a variety of intracellular signal pathways, including those typically activated by G-protein coupled receptors, growth factor receptors and cytokines, as well as events leading to the regulation of receptor function, such as phosphorylation and internalization of the receptor (de Gasparo, et al., 2000;Hunyady, et al., 2000).

There are five suggested signal transduction mechanisms for the AT_1 receptor; activation of phospholipase A_2, phospholipase C, phospholipase D and L-type Ca^{2+} channels and inhibition of adenylate cyclase in different tissues. It has been reported that activation of the AT_1 receptor stimulates growth factor pathways, such as tyrosine phosphorylation and phospholipase C-β, leads to activation of downstream proteins. The growth-like effect is associated with increased tyrosine phosphorylation and activation of mitogen activated protein (MAP) kinase and related pathways, which results in increased expression of early response genes, such as c-*fos*, c-*jun* and c-*myc*, which control thymidine incorporation, cellular proliferation and

growth. Such actions have been linked to cardiovascular diseases, including hypertension, cardiac failure and atherosclerosis (Dinh, et al., 2001).

Ang II binding to AT_1 receptors also stimulates the internalization and processing of the ligand-receptor complex. Internalization of the AT_1 receptor occurs predominantly by endocytosis via clathrin-coated pits (Thomas, 1999). Internalization is important for controlling receptor function by regulating the number of available cell surface receptors and facilitating resensitization of membrane receptors that have been desensitized by GPCR kinase (GRK)-mediated phosphorylation (Ferguson, 2001). Dephosphorylation is suggested to occur within the endosomes after receptor endocytosis; subsequent recycling of the resensitized receptor to the cell surface maintains signal generation (Hunyady, et al., 2002).

2.2.1.2. AT_2 receptors

The AT_2 receptor is characterized by its high affinity for PD123319, PD123177 and CGP42112 and very low affinity for losartan and candesartan. Similar to AT_1 receptor, AT_2 receptor belongs to the family of G-protein coupled receptors, binds Ang II with similar affinity and shares 34% sequence identity with the AT_1 receptor. The AT_2 receptor gene has been mapped in humans to chromosome X, containing an intronless coding region. The AT_2 receptor is highly expressed during fetal development but its expression rapidly declines at birth. In the adult, AT_2 receptors are present in brain, heart, adrenal medulla, kidney and reproductive tissues (Dinh, et al., 2001).

Although the AT_2 receptor belongs to the 7-transmembrane family, it did not reveal any functional features commonly attributed to this class of receptors. AT_2 signaling does not modulate cytosolic Ca^{2+} and agonist binding does not induce receptor internalization (Mazzolai and Nussberger, 2005), instead, activation of AT_2 receptor signaling pathways induces activation of protein phosphatases and protein dephosphorylation, the NO-cGMP system and phospholipase A_2 (release of arachidonic acid). In particular, stimulation of AT_2 receptors leads to activation of various phosphatases resulting in the inactivation of extracellular signal regulated kinase (ERK), opening of potassium channels and inhibition of T-type Ca^{2+} channels. Importantly, MAP kinase plays a major role in cellular proliferation and the AT_2 receptor has been reported to block MAP kinase activation in rat neurons in culture by dephosphorylation of tyrosine phosphate by MAP kinase phosphatase-1 (MKP-1)

or serine/threonine phosphate by serine/threonine phosphatase-2A (Dinh, et al., 2001).

2.2.1.3. Atypical angiotensin receptors

Accumulating evidence indicates the existence of additional Ang receptors, which are pharmacologically distinct from AT_1 and AT_2 receptors. The recently designated AT_4 receptor is a novel binding site that displays high specificity and affinity for Ang IV but low affinity for Ang II (Wright and Harding, 1997). The binding of Ang IV to the AT_4 receptor is insensitive to both losartan and PD123319, but is selectively blocked by the peptide blocker divalinal-Ang IV (Krebs, et al., 1996). Three isoforms of the AT_4 receptor have been described, all of which are synthesized by a single gene on chromosome. AT_4 receptors are expressed primarily in the heart, placenta, kidney, small intestine and skeletal muscle. Certain level of AT_4 receptor is also found throughout the brain and is concentrated particularly in regions involved in cognition (Mazzolai and Nussberger, 2005). The functional role of the AT_4 receptor remains to be elucidated but studies suggest that it may play a role in mediating cerebral and renal blood flow, memory retention and neuronal development (Wright and Harding, 1997).

Studies have also demonstrated a unique binding site for the peptide fragment Ang-(1-7), which is also unaffected by losartan and PD123319 (Tallant, et al., 1997). Ang-(1-7) has been reported to mediate some physiological effects that are identical to those of Ang II, such as the stimulation of vasopressin and prostanoid release, via its own receptor and may also oppose the actions of Ang II by stimulating the release of vasodilator prostaglandins and NO (Chappell, et al., 1998;Ferrario, et al., 1997).

Yet another atypical Ang binding site, loosely termed the AT_3 receptor, has also been identified in cultured mouse neuroblastoma (Neuro-2A) cells, which binds Ang II with high affinity, but which has low affinity for Ang III and no affinity for losartan or PD123319 (Chaki and Inagami, 1992).

2.2.2. Role of renin-angiotensin system in hypertension and beyond

2.2.2.1. Role in hypertension

Angiotensinogen and Ang I are inactive peptides for which no function has yet been described. Angiotensins other than Ang II (Ang III, Ang IV, Ang-(1-7), Ang-(1-5)) are active peptides that are normally produced in small amounts, physiological

relevance for which is still not completely understood. Thus, the biological actions of the RAS are considered to be mediated primarily by the highly active peptide Ang II (Mazzolai and Nussberger, 2005).

The very fact that inhibition of RAS results in lowering of BP in hypertensives provide evidence for its involvement in the pathophysiology of hypertension (Ibrahim, 2006). Physiologically RAS is intimately involved in the maintenance of cardiovascular homeostasis. Ang II, the key hormone of the RAS, plays a pivotal role for both regulation of vessel width and Na^+/water reabsorption and it therefore importantly contributes to BP control (Mazzolai and Nussberger, 2005). Ang II is also demonstrated to constrict arterioles by direct stimulation of AT_1 receptors present on vascular smooth muscle cells (Dinh, et al., 2001).

AT_1 receptors in the heart are known to mediate the positive ionotropic and chronotropic effects of Ang II on cardiomyocytes (Moravec, et al., 1990). Ang II is also known to mediate cell growth and proliferation in cardiac myocytes and fibroblasts, as well as in vascular smooth muscle cells and can induce the expression and release of various endogenous growth factors, including fibroblast growth factor, transforming growth factor-β (TGF-β) 1 and platelet-derived growth factor (Huckle and Earp, 1994;Rosendorff, 1996). It is now well accepted that over activation of RAS leading to such long term trophic effects of Ang II lead to development of cardiac hypertrophy and remodeling seen in hypertension (Luft, 2001).

Results from various pharmacological investigations and clinical studies suggest that Ang II may play a central role not only in the etiology of hypertension but also in the pathophysiology of subsequent cardiac hypertrophy and remodeling, heart failure, glomerulosclerosis, vascular thickening and atherosclerosis in humans by virtue of activation of the tissue RAS (Kim and Iwao, 2000).

2.2.2.2. Role in cardiac pathology

Accumulating *in vitro* and *in vivo* evidence supports the concept that Ang II is involved in important processes resulting to pathological cardiac hypertrophy, including myocyte hypertrophy, myocyte gene reprogramming, fibroblast proliferation and extra-cellular matrix (ECM) protein accumulation. Although cardiac myocytes express both AT_1 and AT_2 receptors, almost all of the biological responses to Ang II reported so far are suggested to be mediated by AT_1 receptor (Kim and Iwao, 2000).

Ang II has been demonstrated to cause hypertrophy of cultured neonatal cardiac myocytes (Sadoshima and Izumo, 1993) and adult myocytes (Liu, et al., 1998). Although, all the links between Ang II and cardiac myocytes hypertrophy

remains yet to be established, it is thought to be channeled through Ang II mediated expressions of fetal phenotype of gene, such as those of β-MHC, skeletal α-actin, atrial natriuretic factor and expression of immediate-early genes (Sadoshima and Izumo, 1993).

In addition to cardiac myocytes, Ang II is also demonstrated to produce direct effect on cardiac fibroblasts, which, unlike cardiac myocytes retains ability to proliferate even in the adult heart and express only AT_1 receptors (Villarreal, et al., 1993). Ang II treatment stimulated the proliferation of rat cardiac fibroblasts, increased mRNA expression and protein secretion of collagen type I (Crabos, et al., 1994), TGF-β_1 and fibronectin (Iwami, et al., 1996).

In vivo infusion of Ang II in rats has been reported to induce cardiac hypertrophy which was completely inhibited upon treatment with AT_1 receptor inhibitor candesartan cilexetil but not by hydralazine. Suggesting that Ang II *in vivo*, via AT_1 receptor, directly induces cardiac myocyte hypertrophy-gene reprogramming and probably fibroblast proliferation and subsequent fibrosis as well, independent of the elevation of BP, indicating the key role of Ang II in the development of pathological cardiac hypertrophy (Kim and Iwao, 2000).

While working on TG1306 transgenic mice line, which express almost twice the normal concentration of Ang II in the heart, but with no significant increase in plasma Ang II, Mazzolai et al demonstrated that this modest increase in cardiac Ang II can led to significant right and left ventricular cardiac hypertrophy (Mazzolai, et al., 1998) that leads to an age-dependent decrease in cardiac function, development of dilated cardiomyopathy and increased mortality. These physiological changes were also found to be mirrored in cardiomyocytes isolated from TG1306 mice which were longer and wider than those from controls and exhibited a 30% to 40% decrease in contraction rate (Domenighetti, et al., 2005). Similarly, in mice with ≈2-fold increased cardiac AT_1 receptor demonstrated high in utero death associated with grossly enlarged atria and heart block (Hein, et al., 1997). This suggests that the AT_1 receptor could be the mediating factor in RAS mediated direct effects on the heart.

In a meta-analysis of 80 trials comparing the effects of antihypertensive drugs on left ventricular mass, ARBs were shown to induce the greatest reduction in left ventricular mass index. Comparing ARB (losartan) with β-adrenergic blocker (atenolol), in hypertensive patients with left ventricular hypertrophy, while achieving the same degree of BP lowering, ARB induced a greater decrease in electrocardiogram (ECG) voltage criteria of left ventricular hypertrophy (LIFE Study). In another study, ACE inhibitor (ramipril) produced a greater reduction in left

ventricular mass index assessed by echocardiography than dihydropyridine Ca^{2+} blocker (nifedipine) in spite of achieving same levels of BP (Ibrahim, 2006).

The beneficial effects of targeting RAS in inducing left ventricular hypertrophy regression may be due to blockade of the direct effects of this hormone unrelated to BP control or may be due in part to the influence of Ang II on cellular pathways and growth factors mediated through the AT_1 receptor (Carson, et al., 2001).

2.2.2.3. Role in renal pathology

Ang II concentrations within the kidney are reported to be ≈1000-fold higher than those in circulating blood (Ingelfinger and Dzau, 1991). Ang II in addition of its physiological role of glomerular filtration rate regulation is also shown to be involved in development of glomerulosclerosis by increasing glomerular capillary pressure caused by preferential constriction of efferent arterioles (Miller, et al., 1991). Besides its unique hemodynamic effects in the kidney, Ang II has been shown to have various important direct actions on mesangial cells, which exclusively express AT_1 receptor and play a crucial role in Ang II mediated glomerular injury (Miller, et al., 1991). In cultured murine mesangial cells, Ang II stimulated cellular hypertrophy, as indicated by increase in cell size, total protein content and synthesis (Anderson, et al., 1993) and mesangial cells proliferation (Gómez-Garre, et al., 1996). However, the molecular mechanism responsible for Ang II mediated mesangial cell growth is poorly understood. Further, parallel with mesangial cell hypertrophy and proliferation, Ang II is demonstrated to cause an increase in mesangial cell superoxide anion and TGF-β_1 production, which plays a key role in progression of glomerulosclerosis by directly enhancing mesangial cell hypertrophy and ECM production (Border and Noble, 1998;Jaimes, et al., 1998).

Continuous administration of Ang II in rats demonstrated an increase in glomerular mRNAs for TGF-β_1 and collagen type I (Kagami, et al., 1994) and dramatic upregulation of otherwise absent α-smooth muscle actin in glomerular mesangial cells and desmin in epithelial cells, indicating that Ang II caused the phenotypic changes in these glomerular cells (Johnson, et al., 1992).

Treatment of DOCA-salt hypertensive rats and SHRsp with ARB demonstrated the BP independent ability of these agents to significantly reduced urinary protein, albumin excretion and induced histological improvement in renal lesions, in association with decreases in renal cortical mRNA levels for TGF-β_1, fibronectin, laminin and collagen types I, III and IV. These findings provide evidence implicating involvement of Ang II, via AT_1 receptor, in renal injury in these models,

due to enhanced renal TGF-β_1 and ECM expressions. Similar improvement in renal function by targeting RAS is reported in glomerulosclerosis induced by hypertension (Kim, et al., 1994a), subtotal nephrectomy (Hamaguchi, et al., 1996;Junaid, et al., 1997), glomerulonephritis (Peters, et al., 1998) or cyclosporin A nephropathy (Shihab, et al., 1997).

At equal levels of BP lowering, RAS inhibitors have been proved to be more effective than other antihypertensive agents in retarding progression of renal damage in hypertensive patients with albuminuria (Ibrahim, 2006). Renoprotective actions that result from inhibition of the RAS include effects on endothelial cell function, glomerular hypertrophy, mesangial cell proliferation and matrix production (Carson, et al., 2001). Inhibition of the RAS is demonstrated to prevent or delay the development of microalbuminuria which is the main clinical expression in the short run of disease activity (Barnett, et al., 2004).

2.2.3. Effects associated with modulating renin-angiotensin system

Drugs targeting RAS at different levels have been developed and are used throughout the world. These drugs either act at the level of inhibiting converting enzyme which catalyses cleavage of Ang I to Ang II or by blocking AT_1 receptor through which Ang II mediates its effects. More recently “Aliskiren”, another member to the group of drugs targeting RAS has been introduced which act as a renin inhibitor by preventing interaction of renin with its substrate angiotensinogen (Fisher and Hollenberg, 2005;Muller and Luft, 2006). Although ACE inhibitors are able to suppress the RAS (Johnston, 2000), but this action is far from complete (Johnston, 2000;Martin, et al., 2002;Weber, 1997). Though, acute inhibition of ACE decreases Ang II levels but with chronic ACE inhibition, plasma Ang II returns to pretreatment concentrations, freely available to bind with its receptors. Studies in normal volunteers have shown that ACE inhibition is associated with an increase in the levels of both renin and Ang I, the latter is then partially converted to Ang II through both ACE and non-ACE pathways. Much of this ACE-independent conversion of Ang I to Ang II is suggested to result from the action of chymase, a chymotrypsin-type serine protease predominantly found in mast cells (Carson, et al., 2001). This gradual increase in Ang II levels while on ACE inhibitor therapy has been termed “ACE escape” (Hofman, et al., 2005).

Furthermore, ACE is not an enzyme with Ang I as its only substrate. The dual action of ACE inhibitors on the RAS and the kinin system, results in accumulation of bradykinin, which becomes important contributing factor to benefits, as vasodilation

(Huber, et al., 1997) and side effects, mainly chronic dry cough, observed with therapy of ACE inhibitors. Chronic dry cough, a class-specific side effects associated with use of ACE inhibitors is reported in a relatively high percentage of patients, leading to discontinuation of therapy in 8-14% of ACE inhibitor users (Davi, et al., 2000;Givertz, 2001;Huber, et al., 1997). ACE inhibitors are also reported to cause a reactive renin rise, which *per se* may represent a cardiovascular risk factor. The associations between renin levels and cardiovascular risk have been demonstrated in a study of 2902 hypertensive patients, where pretreatment plasma renin activity was found to be independently and directly associated with the risk for myocardial infarction (Fisher and Hollenberg, 2005;Muller and Luft, 2006).

AT_1 receptor blocker, on the other hand, binds to the AT_1 receptor, thereby providing more complete blockade of effects of Ang II than is possible with ACE inhibitors. The specificity of AT_1 receptor blockade also ensures that efficacy is achieved without inducing the side effect of cough that results from the nonspecific consequences of ACE inhibition. Preclinical and early clinical studies demonstrate that AT_1 receptor blockers produce at least the same degree of target organ protection as has been demonstrated for ACE inhibitors (Johnston, 2000).

Additional benefits of AT_1 receptor blockers are suggested to arise from their selectivity for AT_1 receptors thus leaving the AT_2 receptor unaffected available for occupancy. Though the importance of this selectivity has not yet been established but it has been postulated that the selective blockade by this class of drugs directly decrease the growth promoting actions of Ang II at the AT_1 receptor, while leaving the growth inhibitory effects of the AT_2 receptor unaffected (Weber, 1997). This state of affairs is viewed as salubrious by some but by no means all investigators. Thus, whereas some preclinical studies have shown that AT_2 receptor activation may be associated with beneficial effects other studies have suggested that AT_2 receptor mediated signaling pathways could lead to harmful effects such as vascular cell proliferation (Muller and Luft, 2006).

From a mechanistic perspective, therefore, AT_1 receptor blockers appear to have advantages over ACE inhibitors, in terms of a more complete blockade of Ang II effects, while also avoiding the specific side effects associated with ACE inhibition (Johnston, 2000). Theoretically, though, renin inhibition could render the RAS quiescent by suppressing all of the Ang I derived products, however, no preclinical or clinical data on these lines is yet available (Fisher and Hollenberg, 2005).

One can not look at the RAS system in isolation when considering a complex physiological phenomenon like BP regulation. Although, interventions targeted at

RAS controls BP reasonably and protects against a wide variety damage to end-organs, which alone does not undermine the role played by other factors in similar capacity either independent of RAS and/or through an interdependent synergism of actions. Of those factors which contribute to the regulation of BP and whose modulation may additionally influence the control of BP in hypertensive state, ET has been shown to initiate several of the pathway underlying the altered BP and organ damage.

2.3. The endothelin system

ET, one of the most potent vasoconstrictors yet known in biological systems, was identified in 1988 by Yanagisawa and colleagues (Yanagisawa, et al., 1988), a finding that was in harmony with previous research by Rubanyi and Vanhoutte (Rubanyi and Vanhoutte, 1985). ET-1 is the predominant isoform of the ET peptide family, which also includes ET-2, ET-3 and ET-4 (vasoactive intestinal constrictor), each one of 21 amino acids in length. However, few 31-residue ETs have also been described (Luscher and Barton, 2000;Remuzzi, et al., 2002).

The biological activity of ET-1 is regulated by a complex process of synthesis, release and breakdown. ET synthesis is regulated by physicochemical factors such as pulsatile stretch, shear stress, pH, exercise and hypoxia. ET-1 biosynthesis is stimulated by cardiovascular risk factors such as elevated levels of oxidized low-density lipoprotein, cholesterol and glucose, estrogen deficiency, obesity, cocaine use, aging and procoagulant mediators such as thrombin. Furthermore, vasoconstrictors as Ang II, growth factors, cytokines and adhesion molecules also stimulate ET-1 production. NO, prostacyclin, atrial natriuretic peptides and estrogens inhibit ET-1 synthesis (Luscher and Barton, 2000;Shreenivas and Oparil, 2007). ET-1 is synthesized predominantly, but not exclusively, in endothelial cells of blood vessels. Smooth muscle cells, cells in the kidney, heart, brain, lung, pancreas, spleen and parathyroid gland also synthesize ET-1 (Remuzzi, et al., 2002).

Three distinct ET related genes are located on different chromosomes in the human genome (chromosome 6p23–24 for ET-1, 1p34 for ET-2 and 20q13.2–13.3 for ET-3), each encoding a specific precursor of each mature isoform (Inoue, et al., 1989).

The translation of prepro-ET mRNA results in the formation of a 212-amino acid peptide, prepro-ET-1, which is cleaved by a furin convertase to the active 38-amino acid precursor peptide big ET-(1-38) or pro-ET-1 or big ET-1. Once formed, big ET-1 is processed to ET-(1-21) through cleavage by ECE -1 (Dhaun, et al., 2008), which exists in 4 isoforms (a, b, c and d) and by ECE-2a, ECE-2b, ECE-3 and chymase. In addition, chymase cleaves big ET-1 resulting in the formation of ET-(1-31) whereas; ECE-3 selectively converts big ET-1 into ET-3. ECE-1a, ECE-1c and ECE-1d are the isoforms localized at the cell surface, whereas ECE-1b resides intracellularly. The extracellular form of ECE-1 cleaves externally supplied big ET-1 from outside the cell, but endogenously produced big ET-1 is converted to active peptide by the intracellular enzyme. ECEs are localized in endothelial and smooth muscle cells, cardiomyocytes and macrophages. ECEs which belong to the

metalloprotease family, share functional and structural similarity with neutral endopeptidases (NEP) and do not have big ET-1 as their only substrate and are also known to hydrolyze peptides such as bradykinin, substance P and insulin (Luscher and Barton, 2000;Remuzzi, et al., 2002).

Two distinct secretary pathways for the transport and release of ET-1 have been proposed. First, ET-1 is continuously transported in and released from secretary vesicles by a constitutive pathway through a cyclic-adenosine monophosphate-independent mechanism; second, ET-1 is also stored in weibel–palade bodies (with other vasoactive compounds) and is released at the cell surface after appropriate stimuli, among which are fluid shear stress, TGF-β, interleukin-1 and Ang II (Remuzzi, et al., 2002).

Though ET is reported to remain bound to the ET_A receptor for up to two hours, resulting in its prolonged biological effects (Hoyer, et al., 1989), its plasma half life ($t_{1/2}$) is just 1-2 minutes (Gasic, et al., 1992) owing to its efficient extraction and clearance process particularly during its pulmonary circulation, although the splanchnic and renal circulation also contributes (Attina, et al., 2005;Dhaun, et al., 2008;Dupuis, et al., 1996b) via receptor and non-receptor mediated mechanisms. The extraction of ET is mediated by binding to cell surface clearance ET_B receptors, followed by internalization and degradation, probably within lysosomes. ETs are also degraded by NEP, which are mainly found in the brush border vesicles of the proximal tubules of the kidney. ET-1 is predominantly secreted abluminally and as such, exerts paracrine actions (Attina, et al., 2005;Benigni, 2000;Dhaun, et al., 2008). Therefore, the plasma concentration of ET-1 is very low. Indeed, effects on ET clearance rather than production are a major determinant of its plasma concentration.

2.3.1. Endothelin receptors

The effects of ET, which is too hydrophilic to cross the cell membrane, are mediated by binding to cell-surface receptors that belong to the family of G-protein coupled receptors. So far, two types of ET receptor have been identified in humans. The ET_A receptor, having 100 folds higher binding affinity for ET-1 and ET-2 than for ET-3 whereas the ET_B receptor binds the three isopeptides with comparable affinity. The ET_A and ET_B receptors are encoded by genes located on human chromosome 4q28 and 13q22, respectively. A putative ET receptor type C (ET_C) has been cloned, but has not been identified in the human genome (Remuzzi, et al., 2002). In humans, ET_A receptor mRNA is expressed primarily in vascular smooth muscle cells (particularly in aortic, cardiac, pulmonary and renal tissues) but not in endothelial

cells. Whereas, ET_B receptor mRNA is highly expressed in endothelial cells, but is also expressed in vascular smooth muscles obtained from human aorta, pulmonary artery and coronary artery (Cowburn and Cleland, 2001).

In addition to their vascular expression, considerable presence of ET receptors has been established in the heart, kidney and lungs (Remuzzi, et al., 2002). Binding of ET-1 to either ET_A or ET_B receptors produce different functions based on their location. ET-1 binding to these receptors results in activation of the phosphatidyl inositol phospholipase C pathway and initiates an array of intracellular events, with both short and long term effects, such as rapid increase in intracellular Ca^{2+} levels, activation of protein kinase C and nuclear signaling mechanisms (Attina, et al., 2005). The ET_A receptor expressed in the vascular smooth muscle upon interaction with ET-1 results in vasoconstriction, cell growth, adhesion, fibrosis and thrombosis (Rodriguez-Vita, et al., 2005). In contrast, the ET_B receptors, which are predominantly expressed on vascular endothelial cells, are linked to an inhibitory G-protein. Their activation results in NO induced vasodilatation and prostacyclin release, prevention of apoptosis and inhibits ECE-1 expression in endothelial cells. ET_B receptor is also present, at a much lower level, on the vascular smooth muscle cells, where its activation can contribute to vasoconstriction (Attina, et al., 2005;Burke, et al., 2000) and functions similar to ET_A receptor stimulation (Attina, et al., 2005;Haynes, et al., 1995).

Expression of all ET isopeptides and ET receptors has been demonstrated in the mammalian heart, particularly in cardiomyocytes, cardiac fibroblasts and endothelial cells of the coronary vessels. ET-1 remains the predominate cardiac isopeptide and of ET_A and ET_B receptors which are found on cardiomyocytes and fibroblasts, ET_A receptor represents ≈90% of all ET receptor present on cardiomyocytes (Kedzierski and Yanagisawa, 2001;Remuzzi, et al., 2002). ET-1 via acting through its receptors is responsible for maintaining basal vasomotor tone in coronary arteries (MacCarthy, et al., 2001) and is suggested to have positive inotropic and chronotropic effect (Goraca, 2002). Similarly, ET-1 remains the major renal ET isoform and in the human kidney, the only one that has been so far shown to be expressed at the protein level. Within the kidney, ET-1 is produced by glomerular epithelial and mesangial cells and renal tubular and medullary collecting duct cells (Dhaun, et al., 2006). ET receptors are widely distributed within the human kidney, with the ET_A subtype localized to vascular smooth muscle, notably in the glomeruli, vasa recta and arcuate arteries, whereas ET_B receptors are more numerous, almost double and more widespread, with a high concentration in the

collecting system (Wendel, et al., 2006). With respect to the renal system, ET-1 has a role in the paracrine/autocrine regulation of renal and intrarenal blood flow, glomerular hemodynamics, Na^+ and water homeostasis and acid-base balance. Indeed, the renal vasculature is more sensitive to the vasoconstricting effects of ET-1 than other vascular beds. Although exogenous ET-1 reduces total renal blood flow, a regional difference has been observed, with cortical vasoconstriction and NO-dependent medullary vasodilation. Exogenous ET-1 has also been shown to cause constriction of afferent and efferent arterioles, with a greater effect on the former and reduce filtration coefficient by mesangial cell contraction. In humans, a similar vasoconstrictor and pressor response has been demonstrated leading to a fall in total renal blood flow and a consequent reduction in glomerular filtration rate (Dhaun, et al., 2006;Rabelink, et al., 1996).

In the absence of disease, ET-1 actions result in a complex modulation of vasomotor tone, tissue differentiation and cell proliferation, driven by the interplay between effects on ET_A and ET_B receptors. However, experimental evidence suggests that, in pathological states, ET receptors are differently regulated, thus contributing to unbalanced effects tending towards vasoconstriction and cell proliferation (Attina, et al., 2005).

2.3.2. Role of endothelin system in hypertension and beyond

2.3.2.1. Role in hypertension

Initial evidence of a pressor action of ET-1 led to the speculation that it might be implicated in hypertension (Yanagisawa, et al., 1988). Production of vascular ET-1 is increased in some but not all the animal models of hypertension. Models where ET-1 production is augmented (mostly, but not exclusively, salt-dependent types), presents increased BP and vascular growth, which responds to both selective and nonselective ET receptor blockers (Schiffrin, 1999). Thus, ET in addition of being powerful vasoconstrictor also influence local vascular growth by mediating cellular hypertrophy (Chua, et al., 1992;Grainger, et al., 1994;Moreau and Schiffrin, 2003;Pinto-Sietsma and Paul, 1998;Schiffrin, 1995) and proliferation, most probably by playing role of a comitogen (Fujitani and Bertrand, 1997). Moreover, ET is suggested to possess ability to modify cell number by inhibiting apoptosis (Diep, et al., 2000), to modify the ECM by acting as a potent stimulus for collagen synthesis (Tharaux, et al., 1999) and thus, suggested to be involved in the process of fibrosis (Ammarguellat, et al., 2001). Therefore, these peptides are speculated to participate

through these two mechanisms in the pathogenesis of the elevation of BP and/or in the maintenance of hypertension in both experimental animal models and human primary hypertension (Schiffrin, 1995). To justify the involvement of ET in the pathophysiology of hypertension studies evaluating augmentation of the peptide levels, potentiation of its vasoconstrictor response and lowering of arterial BP in hypertensive diseases by reasonably selective ET blockers had been undertaken.

Administration of exogenous ET is demonstrated to elevate BP in various animals (Moreau and Schiffrin, 2003;Pinto-Sietsma and Paul, 1998) and healthy volunteers (Rabelink, et al., 1994). ET infusion transiently lowers BP, followed by a prolonged rise (Gratton, et al., 1995;Pinto-Sietsma and Paul, 1998). The initial BP lowering effect is suggested to be mediated by ET_B receptor activation followed by prolonged ET_A receptor mediated vasoconstriction that is sustained for up to two hours (Clarke, et al., 1989) because of slow dissociation of ET-1 from its receptors (Goraca, 2002).

In contrast to the expected elevated levels of ET in the vasculature or serum, most hypertensive animal models demonstrated either normal or only slightly increased plasma ET levels (Goto, 2001;Pinto-Sietsma and Paul, 1998). Certain animal models of experimental hypertension, in particular the salt sensitive and low renin forms and those having malignant hypertension, such as in the SHR treated with DOCA and salt or in the two kidney-one clip hypertensive rat treated with caffeine; significant elevations of plasma ET levels have been found (Pinto-Sietsma and Paul, 1998). Similarly, among animal models of hypertension, only the DOCA-salt rat, the DOCA-salt SHR and salt loaded SHRsp (Schiffrin, 2005) have increased ET-1 mRNA levels in the vessel wall and kidney, whereas, SHR have similar or lower ET-1 mRNA than normotensive controls (Schiffrin, et al., 1995). Estimation of vascular ET-1 levels is considered as important parameter because in hypertensive models, elevated vessel ET-mRNA may mediate important structural effects, such as vascular hypertrophy, due to its growth-promoting properties (Hirata, et al., 1989). Interestingly, DOCA-salt hypertensive rat arteries show severe vascular hypertrophy with prominent medial thickening and overexpression of the ET-1 gene. In contrast, little vascular hypertrophy and no ET-1 gene expression are seen in SHR (Pinto-Sietsma and Paul, 1998).

In the DOCA-salt hypertensive rat, ET receptor blockers lower BP and almost normalize vascular hypertrophy. ET receptor blockers are also reported to lower BP in the DOCA-salt treated SHR with malignant hypertension and in the SHRsp. In Dahl-salt hypertensive rats, ET blockade decreases but does not normalize BP. In

contrast, in the two kidney-one clip or one kidney-one clip hypertensive rat and in SHR, ET receptor blockers do not lower BP or affect vascular hypertrophy. This discrepancy is suggested to reflect strain/model specific mechanisms in the pathogenesis of hypertension and whether an ET receptor blocker influences BP possibly depends on the relative importance of the ET system in the specific hypertensive model (Pinto-Sietsma and Paul, 1998).

The first demonstration of a chronic antihypertensive action of an orally active ET receptor blocker was performed using the nonselective ET_A/ET_B receptor blocker bosentan and the treatment was found to be effective in salt-sensitive models, such as DOCA salt, aldosterone-salt, Dahl salt-sensitive and SHRsp rat models supplemented with salt. Although treatment lowered the BP in these models, their BP lowering efficacy was found to be moderate in magnitude. Thus it was suggested that in general, salt-sensitive and severe forms of experimental hypertension are at least partially responsive to ET receptor blockers in terms of BP reduction (Treinen, et al., 1999).

Direct evidence that ET-1 might cause hypertension in humans has been provided by a report describing two patients affected by malignant hemangioendothelioma and hypertension. Surgical excision of the tumours led to a reduction in plasma ET-1 levels and resolution of hypertension and in one patient recurrence of the tumour was associated with an increase of plasma ET-1 and BP levels (Letizia, et al., 1997). ET-1 gene expression has been detected in human pheochromocytoma and such patients were found to have significantly higher plasma ET-1 levels than observed in patients with primary hypertension. Another hypertensive disease reportedly accompanied by elevated ET-1 levels is preeclampsia or eclampsia with even higher levels in patients with severe pregnancy-induced hypertension. In contrast, plasma ET-1 and big ET-1 are not elevated in pregnant women with chronic hypertension. Patients receiving cyclosporine and erythropoietin also present increase ET levels in their plasma, suggesting contribution of ET in the etiology of such hypertension (Luscher and Barton, 2000).

However, similar to strain dependent heterogeneity observed in rats, data on human primary hypertensives have revealed both normal and elevated plasma ET-1 levels (Remuzzi, et al., 2002). In humans with mild to moderate hypertension, plasma ET-1 levels are normal or only slightly increased. Similarly, ET levels are higher in salt-sensitive primary hypertension and in obese hypertensives than in normotensive controls. Racial differences for ET levels in human hypertension have also been suggested. Thus, plasma immunoreactive ET-1 levels were found to be significantly

higher in black than in white hypertensives. In addition, Schiffrin et al have shown increased vascular ET-1 expression in patients with severe hypertension (Schiffrin, et al., 1997). Therefore, depending on the group of hypertensive patients studied, differences might or might not be found, as only a subset of the hypertensive population might have increased circulating levels of ET-1 as a contributing factor to their hypertension (Letizia, et al., 1997).

2.3.2.2. Role in cardiac pathology

In general, there are two broad lines of evidence that support a pathophysiologic role of activated ET in cardiovascular disease. The first line of evidence is derived from numerous studies that have demonstrated increased tissue expression and circulating levels of ET-1. The second line of evidence is derived from animal and clinical studies that have demonstrated beneficial effects of administration of ET receptor blockers.

Many studies employing various models of left ventricular dysfunction and CHF have documented, in conjunction with increased circulating ET-1 levels, increased tissue expression of ET in organ systems including the heart. Thus, in the hypertrophied rat heart induced by abdominal aortic banding (Kobayashi, et al., 1999;Sakai, et al., 1996b); in rats with CHF following myocardial infarction induced by coronary artery ligation (Sakai, et al., 1995) and in the canine model of pacing-induced CHF (Huntington, et al., 1998), cardiac ET-1 mRNA levels have been reported to be markedly increased. Though, increased prepro-ET-1 mRNA and ET peptide was observed in both the non-infarcted and infarcted area of the rat heart, but was found most abundant in the granulation tissue in the infarct region (Kobayashi, et al., 1999).

Beneficial effects of ET_A receptor inhibitor have been reported in rat model of chronic heart failure where treatment with BQ123 (selective ET_A receptor blocker) has decreased mortality and improved cardiac function. These benefits are accompanied by significant amelioration of left ventricular dysfunction and prevention of ventricular remodeling, in which there is usually an increase in the ventricular mass and ventricular cavity enlargement (Sakai, et al., 1996a). In rats with myocardial infarction, the surviving myocardium expressed greater amount of ET-1 as well as ET_A receptors (Kobayashi, et al., 1999), suggesting that endogenous ET-1 might help to support cardiac function through the up-regulation of the ET system (Sakai, et al., 1996b). Upregulation of the ET pathway may be beneficial in providing short term inotropic support for the failing myocardium in which β-adrenergic

responsiveness is frequently attenuated. However, prolonged stimulation by up-regulated ET pathways may have important maladaptive effects on myocardial structure and function, thereby leading to fatal events as CHF. Long term treatment with an ET_A receptor blocker, BQ123, by means of a subcutaneous osmotic mini pump not only greatly improved the survival rate of rats but also ameliorated left ventricular dysfunction and prevented harmful ventricular remodeling (Sakai, et al., 1996a). Likewise, the effectiveness of chronic treatment with orally active selective ET_A or nonselective ET_A/ET_B receptor blockers has been demonstrated in animal models of CHF due to myocardial infarction (rat) (Mulder, et al., 1997;Mulder, et al., 2000), cardiomyopathy (hamster) (Yamauchi-Kohno, et al., 1999), rapid ventricular pacing (dog) (Borgeson, et al., 1998;New, et al., 2000) and coronary microembolism (dogs) (Mishima, et al., 2000).

Myocardial infarction is documented to increases plasma ET not only in animals, but also in patients (Pinto-Sietsma and Paul, 1998). In patients with acute myocardial infarction, which often leads to CHF, the plasma level of ET-1 seems to be an indicator of long-term prognosis (Goto, 2001). The increase in myocardial ET_A receptor expression (Barton and Kiowski, 2001) and levels of circulating ET-1 have been correlated with the severity of symptoms (Rich and McLaughlin, 2003) and the long-term mortality in patients with CHF (Goto, 2001). This is further supported by observation that ET levels are lower in patients with early coronary artery reperfusion than in patients without early reperfusion (Pinto-Sietsma and Paul, 1998) and the levels of ET_A receptor regresses after improvement in left ventricular function with assist devices (Remuzzi, et al., 2002).

2.3.2.3. Role in renal pathology

The isoform ET-1 is also implicated in the pathology of both acute- and chronic- renal failure (Kedzierski and Yanagisawa, 2001;Orth, et al., 2001). Studies have suggested ET-1 as an important player in the pathogenesis of acute renal failure (ARF) due to its vasoconstrictive properties and the sustained regional vasoconstriction observed following renal ischaemia. The active involvement of ET-1 in ARF is supported by its increased renal release and concentrations observed in rat models of post-ischaemic ARF (Knoll, et al., 2000).

Both, antibody to ET-1 and non-peptide ET receptor blockers have been demonstrated to be effective in animal models of ARF (Knoll, et al., 2000). Administration of ET-1 antibody before renal artery clamping in rats prevented the increase in periglomerular arteriolar resistance, the reduction in single nephron

glomerular filtration rate and the histological changes that accompany proximal renal tubular necrosis. Also, orally active non-peptide ET receptor blockers, SB209670, LU135252, ABT627 were successfully shown to attenuate ischemia/reperfusion-induced renal dysfunction and histological damage in rats. Similarly, non-peptide ET blockers were demonstrated to be effective in dogs with ARF induced by bilateral renal artery occlusion and aortic cross-clamping induced ischemia and endotoxin. These results strongly suggest that overproduction of ET-1 plays an important pathophysiological role in the development of ARF of various origin (Goto, 2001).

It is suggested that renal actions of ET-1, if sustained chronically, could contribute to the development of progressive renal injury. Long-term effects of ET on the kidney include stimulation of messangial cell proliferation and ECM deposition as well as stimulation of vascular smooth muscle hypertrophy in renal resistance vessels (Kassab, et al., 1998). Similar results were obtained in mice overexpressing the human ET-1, despite normal BP, the transgenic mice demonstrated interstitial fibrosis, glomerulosclerosis and declining renal function (Kedzierski and Yanagisawa, 2001).

Beside vasoconstrictive effects, ET-1 via its non-haemodynamic effects is thought to play an important role in the progression of CRF. Glomerular sclerosis and interstitial fibrosis, prominent features in chronic renal injury, are suggested to be at least partly promoted by ET-1. There are reports demonstrating a pro-fibrotic and pro-proliferative effect of ET-1. Targets of the mitogenic properties of ET-1 are endothelial cells, fibroblasts, vascular smooth muscle cells and mesangial cells. ET-1 also enhances the accumulation of ECM proteins such as collagen IV or fibronectin and synergises or even potentiates the proliferative effect of certain cytokines and growth factors (Knoll, et al., 2000).

Renal ET-1 gene expression has been shown to increase in a parallel fashion with the progression of renal disease in different models of renal injury, including reduced renal mass, diabetic nephropathy, lupus nephritis and proliferative nephritis. Increased urinary excretion of ET-1 (Kassab, et al., 1998), cortical expression of prepro-ET-1 mRNA and ET_A receptor, but decreased ET_B receptor is reported in rats with reduced renal mass and was found to be accompanied by marked proteinuria, decreased creatinine clearance, high BP and decreased renal function. Administration of an ET_A receptor blocker, demonstrated significant improvement in all the symptoms (Goto, 2001). The Dhal salt rat, model that typically develops malignant hypertension and marked renal pathological changes in the form of glomerulosclerosis, renal tubule dilatation, vascular hypertrophy and renal

insufficiency in response to the high salt intake have been shown to have elevated renal ET-1 production which correlate with the extent of glomerulosclerosis observed in this model. Chronic blockade of ET_A receptor in this model, demonstrated protective effect against glomerular and tubular injury. Plasma and urinary levels of ET-1 have also been shown to be elevated in patients with CRF (Kassab, et al., 1998). Treatment of hypertensive subjects with CRF with selective ET_A receptor blocker BQ123 improved renal blood flow and reduced renal vascular resistance, in addition to decreasing systemic BP (Attina, et al., 2005).

Thus, abundant evidence exist which points to a pivotal role for ET-1 in the pathogenesis of cardiovascular and renal pathology. The peptide once produced can, in turn, act on different vascular beds to elicit vasoconstriction, proliferation, hypertrophy and/or ECM accumulation manifesting as various disorders of vasculature, heart and kidney.

2.3.3. Effects associated with modulating endothelin system

The last decade have seen the clinical development of a number of selective ET_A and nonselective ET_A/ET_B receptor blockers. The difference between selective and mixed blockers, however, is not pharmacologically well defined, making ET receptor blocker studies difficult to interpret. Thus, the degree of receptor selectivity achieved by any particular drug may depend on the dose used; with higher doses of modestly selective blockers, practically selective pharmacological blockade may no longer exist. Unfortunately, there have been no studies in humans to determine the functional selectivity of any of the so-called selective blockers (Dhaun, et al., 2008).

One of the first studies evaluating the effects of systemic ET receptor blocker on BP studied a cohort of 293 patients with primary hypertension for the effects of nonselective ET_A/ET_B receptor blocker bosentan and enalapril on BP. Bosentan significantly lowered diastolic BP and its effect was similar to enalapril. In addition there was no concomitant reflex activation of the RAS or the SNS (Attina, et al., 2005). In another study conducted in hypertensive patients and resistant hypertensive patients, darusentan, an oral selective ET_A receptor blocker, was efficacious in lowering both systolic and diastolic BP, compared to the placebo group (Attina, et al., 2005;Epstein, 2008).

Darusentan, a selective ET_A receptor blocker was evaluated in the HEAT-HTN (Hypertension Endothelin Antagonist Treatment) trial, a randomized, double-blind, placebo-controlled, parallel-group study in patients with Stage II hypertension. The placebo-subtracted reduction in BP was 11.3/8.3 mmHg at the 100 mg dose,

with a discernible dose response effect. Patients treated with darusentan were more likely to achieve their BP goal. Thus, the HEAT-HTN study confirms the feasibility of treating hypertension with a selective ET receptor blocker.

With currently available knowledge, a causal relationship between ET and hypertension is highly conceivable, hypertension being a heterogeneous disease entity, ET is clearly more relevant in some hypertensive disorders. Thus it is suggested that in salt-sensitive and severe forms of experimental hypertension and certain types of hypertension in humans, ET plays a more important role than in other. Its role in several cardio-renal pathologies suggests that similar pathways may also contribute to the pathophysiology of hypertensive disorder.

2.4. Crosstalk between pathways contributing to the pathophysiology of hypertension

Though there is little doubt that RAS and ET systems are important contributors to the pathogenesis and maintenance of hypertension in primary hypertensives, it is also clear now that these are, just two amongst many contributors. Indeed there exists a complex interplay between various contributory systems as RAS, ET, aldosteron, sympathetic system, salt status; which in addition to their independent action, work through an interdependent synergistic way to direct overall hypertensive status of individual. For example, the fact that BP falls in majority of primary hypertensive during treatment with ACE inhibitors or ARBs points to a contributory role of the RAS. Equally however, arterial pressure usually falls with extreme diet Na^+ restriction, administration of diuretic, β blocker, centrally acting α_2 agonist, Ca^{2+} channel blockers, ET receptor blockers etc. Hence, each factor though important by itself but one amongst several causes which should be considered together when studying this disease condition.

2.4.1. Crosstalk between renin-angiotensin and endothelin system

Ang II and ET-1 are powerful vasoconstrictors involved in the regulation of vascular tone and there are considerable evidences suggesting an interaction between the RAS and ET system. It has been hypothesised that Ang II, the active peptide of the RAS affects the synthesis of ET-1, which in turn, can influence the RAS (Dhaun, et al., 2006). Several lines of evidence support this hypothesis, where Ang II has been shown to increase synthesis and release of ET-1 both *in vitro* (Dohi, et al., 1992;Emori, et al., 1991;Imai, et al., 1992) and *in vivo* (Barton, et al., 1997;Ferri, et al., 1999;Moreau, et al., 1997).

Using northern blot analysis and *in situ* hybridization it has been demonstrated that Ang II treatment upregulates prepro-ET-1 mRNA level by threefold over control level as early as 30 min (Ferri, et al., 1999;Ito, et al., 1993;Paul, et al., 1995). Though several hours are required for synthesis of mature ET-1, release of ET-1 takes place within minutes from vessels *in vitro* or human vascular cultured cells exposed to Ang II, implying that preformed ET-1 is stored in cells and that its release may be Ang II dependent (Imai, et al., 1992;Montanari, et al., 2003).

Ang II has been shown to stimulate the expression and release of ET-1 in rat vascular smooth muscle cells and endothelial cells. Also, Ang II mediated release of ET-1 is shown in rat mesangial cells and cardiomyocytes (Sasser, et al., 2002). Similar findings have been reported with human cultured cells which have clearly

shown that Ang II promotes both synthesis and release of ET-1 by endothelial cells (Montanari, et al., 2003). The *in vitro* Ang II mediated release of ET-1 is shown to be abolished by candesartan, an inhibitor of the membrane-bound AT_1 receptor; by actinomycin D, an ribonucleic acid synthesis inhibitor and cycloheximide, a protein synthesis inhibitor; indicating that ET-1 release depend on AT_1 receptor subtype and *de novo* protein synthesis. This suggests that Ang II regulates ET-1 release by cultured endothelial cells through an AT_1 receptor-dependent pathway (Ferri, et al., 1999).

Evidence supporting *in vivo* stimulation of ET system by Ang II comes from studies involving chronic Ang II infusion in rats with and without ET_A receptor blocker pretreatment. Increase in arterial pressure in response to Ang II infusion in rats caused concomitant significant increase in expression of prepro-ET-1 mRNA, tissue ET-1 content and functional ECE activity (Alexander, et al., 2001;Barton, et al., 1997) in the systemic vasculature (Schiffrin, 2003) and kidney (Sasser, et al., 2002). Hypertension (Sasser, et al., 2002), decrease in glomerular filtration rate (Alexander, et al., 2001), increased tissue ET-1 content and functional ECE activity (Barton, et al., 1997) caused by this Ang II infusion was completely abolished in rats pretreated with the ET_A receptor blocker. Therefore, it has been suggested that in severe hypertension or when Ang II is infused exogenously at a high and steady rate, there is over expression of ET-1 by the systemic vasculature, renal vessels and glomeruli. However, when Ang II is generated endogenously and hypertension is not malignant, ET-1 is not stimulated due to the circadian variations that occur with endogenously generated Ang II. The exceptions are certain vascular beds, such as the coronaries, in which the endothelium may be particularly sensitive to stimulation by Ang II, resulting in enhanced expression of ET-1 in situations where endogenously generated Ang II is increased even in the absence of severe or malignant hypertension (Schiffrin, 2003).

Though, the mechanisms whereby ET-1 protein synthesis and release is increased in response to Ang II remain poorly defined, it has been postulated that this is due to Ang II receptor mediated mobilization of intracellular Ca^{2+} (Emori, et al., 1991), activation of protein kinase C (Emori, et al., 1991;Rajagopalan, et al., 1997) and consequent activation of c-*fos* and c-*jun* binding to activator protein-1 sites in the ET promoter (Imai, et al., 1992;Rajagopalan, et al., 1997). In fact, it has been shown that the 5′ gene-flanking region of the human ET-1 precursor contains consensus motifs for the binding sites of the *Fos-Jun* complex, activating protein 1 and nuclear

factor 1, the last two proteins being suggested to mediate the induction of ET-1 by Ang II (Remuzzi, et al., 2002).

Not only is Ang II reported to upregulate prepro-ET-1 mRNA, augment ET-1 production and ECE activity, but ET-1, in turn, has also been shown to stimulate both vascular ACE activity (Rademaker, et al., 2004) and renin release (Rubanyi and Botelho, 1991). The increased renin release and activation of ACE may further result in elevated Ang II levels (Rubanyi and Botelho, 1991).

Addition of ET-1 to cultured endothelial cells has been shown to double the conversion of Ang I to Ang II, the effect which was suppressed ACE inhibitors enalapril (Kawaguchi, et al., 1990) and captopril (Moroi, et al., 1996), suggesting role of ET in modulating the conversion of Ang I to Ang II. On same lines it was observed that long term treatment with ET_A receptor blocker, LU135252, completely prevented ACE activation, suggesting a role for ET_A receptors in ACE regulation, however, these effects probably require long term actions on ACE, because LU135252 had no effect on activity of purified human ACE (Barton, et al., 2000).

Evidence for interaction between RAS and ET system has been explored beyond their ability to influence mutual production and release. Ang II and ET-1 are known potent endogenous vasoconstricting and Na^+ retaining peptides which play key role in the regulation of renal function and arterial pressure and could lead to elevation in BP, renal vasoconstriction, Na^+ retention and end-organ damage. However, in most of these situations, a synergestic interaction between Ang II and ET-1 is postulated to contribute largely to the observed changes (Montanari, et al., 2003).

Monoclonal antibodies to ET-1, ECE inhibitors or ET_A receptor blockers have been shown to reduced or prevent the haemodynamic, hypertrophic and mitogenic effects of Ang II *in vitro* and *in vivo* (Boemke, et al., 2001). It has been demonstrated that rats infused chronically with subpressor dose Ang II show significant elevation in BP during combined infusion of ET-1 and Ang II, whereas ET-1 or Ang II alone failed to induce any significant changes in systolic BP (Yoshida, et al., 1992). On same line of thoughts, it has been demonstrated that hypertension in rats associated with chronic continuous infusion of ET-1 is prevented upon concomitant chronic administration of ACE inhibitor (Mortensen and Fink, 1992).

Interestingly, studies in rats have shown that such Ang II-ET-1 interaction may be Na^+ dependent, because under elevated Na^+ intake, ET_A blockade inhibited Ang II hypertension much more than under Na^+ restriction (Montanari, et al., 2003) and Ang II infusion which, at subpressor dose fail to affect arterial pressure in

animals on low or normal salt intake, cause a significant rise in BP of animals on higher salt intakes (Csiky and Simon, 1997;Kanagy, et al., 1990). Such results led researchers to conclude that ET-1 could participate in the mechanism of salt sensitivity in Ang II induced and several other salt-sensitive forms of hypertension (Ballew, et al., 2001).

Long term treatment with Ang II has been shown to increase tissue ET-1 content (d'Uscio, et al., 1998;Moreau, et al., 1997) and to induce vascular hypertrophy in small mesenteric and cerebral arteries, effects that were totally prevented by ET_A receptor blockade (Moreau, et al., 1997). The possibility of these structural changes being attenuated by antihypertensive activity of ET_A receptor blocker was ruled out in a study using the same Ang II regimen, where hydralazine lowered BP but did not modify Ang II induced vascular hypertrophy, suggesting that part of the proliferative effect of Ang II is pressure independent and is mediated through stimulation of vascular ET production (Moreau, et al., 1997).

Similarly, renal effects of Ang II-ET-1 interaction have been reported, where ET-1 and Ang II together were shown to stimulate matrix protein synthesis and mesangial cell mitogenesis through ET_A and AT_1 receptors, respectively, by complicated mechanisms (Gómez-Garre, et al., 1996). Fakhouri et al have shown that Ang II activates collagen type I gene in the renal cortex that can be blocked by the nonselective ET receptor blocker, bosentan, however, such effect was absent from group administered with ET-1 - candesartan combination suggesting that the pathway leading from Ang II to the collagen I gene requires an intermediate ET-1 action (Fakhouri, et al., 2001). In line with the haemodynamic, vascular and renal observations, attenuation in the development of cardiac hypertrophy resulting from chronic infusion of Ang II have been reported upon concomitant administration of the nonselective ET receptor blocker bosentan. Together, these data indicate that regulation of the ET system by Ang II may be an important factor in mediating the vascular, renal and cardiac effects of Ang II via stimulation of the ET_A receptor (Herizi, et al., 1998).

In some experimental scenarios, the vasoconstrictor actions of chronic Ang II appear to be less ET dependent, whereas the structural changes appear to be more ET mediated. Experiments conducted on rats transgenic for human renin and human angiotensinogen (hREN - hAGT transgenic rats) demonstrated that excess of Ang II generated via transgenic human renin was responsible for both the systemic hypertension and the end-organ damage observed in this animal model. Massive cardiac hypertrophy, progressive renovascular damage and nephrosclerosis were

observed as the characteristics of this transgenic model. ET was found to have no direct role in elevation of BP in this transgenic model; however, ET was suggested to play a supportive role because combination of AT_1 and ET_A receptor blockers, at a dose at which individual compound failed to attenuate hypertension, was successful in causing significant reduction in BP of this transgenic rat. Despite lack of antihypertensive effect of ET_A receptor blocker alone, ET apparently played an important role in end-organ damage in this model, because ET_A receptor blockade significantly reduced overall mortality rates. These results provided strong evidence for a direct participation of ET even in predominant Ang II dependent animal model (Bohlender, et al., 2000). Similarly, bosentan though failed to prevent hypertension in the transgenic (mRen2)27 rat (TGRen2) model of elevated endogenous Ang II, was successful in significantly attenuating cardiac hypertrophy and fibrosis (Schiffrin, 1999).

Thus important role of ET in scenarios like salt-sensitive hypertension, renal vasoconstriction and cardiovascular and renal fibrotic damage, produced by elevated Ang II levels, indicate that ET-1 is involved in mediating much of the Ang II actions which could be possible (Montanari, et al., 2003) because both ET and Ang II signal through multiple common intracellular pathways. Finally, it is possible that the novel, transmembrane receptor reported by Ruiz-Opazo and colleagues, which contains distinct ET and Ang II binding sites, is functionally active and requires both ET and Ang II ligands for activation (Ruiz-Opazo, et al., 1998).

In addition to crosstalk between RAS-ET systems, their crosstalk with other systems contributing to pathogenesis of hypertension has been established. Ang II, the key mediator of effects of RAS by acting on its receptors present on both adrenals stimulates the release of catecholamines from the adrenal medulla and aldosterone from the adrenal cortex (Dinh, et al., 2001). Stimulation of aldosterone synthesis and release results in consequent Na^+ and fluid retention by the kidney further contributing to volume overload and hypertension (Calhoun et al., 2004).

2.4.2. Crosstalk between renin-angiotensin and sympathetic nervous system

The interactions between the RAS and renal sympathetic nerves in the control of renal function and hypertension can be intrarenal, for example, the direct (by specific innervation) and indirect (by Ang II) contributions of increased renal sympathetic nerve activity (RSNA) to the regulation of renal function. These interactions can also be extrarenal, for example, in the central nervous system, where RSNA and its arterial baroreflex control are modulated by changes in activity

of the RAS. The renal sympathetic nerves innervate the tubules, the vessels and the juxtaglomerular granular cells of the kidney (DiBona, 2000;Le Fevre, et al., 2003). In this way, changes in RSNA directly influence the functions of these innervated renal effector units. Increases in RSNA decrease urinary Na^+ and water excretion by increasing renal tubular water and Na^+ reabsorption throughout the nephron, decrease renal blood flow and glomerular filtration rate by constricting the renal vasculature and increase activity of the RAS and thus Ang II by stimulating renin release from juxtaglomerular granular cells. Ang II, through direct actions on AT_1 receptors located on tubular and vascular segments, can further increase renal tubular Na^+, chloride and water reabsorption and constrict the renal vasculature contributing to increased volume overload and PR (DiBona, 2000).

The fact that presynaptic action of Ang II on renal sympathetic nerve terminals results in an enhanced norepinephrine release first came from the observation that intrarenal generation of Ang II facilitated renal venous outflow of norepinephrine during renal sympathetic nerve stimulation, an effect that was blocked by ARB. Subsequently, it was shown that a certain degree of RAS activity was necessary to optimize release of norepinephrine from renal sympathetic nerve terminals (presynaptic action) (Calhoun et al., 2004).

Ang II receptor binding sites which are found within discrete areas of the forebrain and the brain stem are suggested to have important involvement in the regulation of RSNA. A hormonal-sympathetic reflex model for the long-term control of arterial pressure has been proposed. A critical element of the model is that chronic increases in Ang II produce sustained increases in peripheral sympathetic nerve activity. There are a limited number of specialized central nervous system areas wherein the normal blood-brain barrier is lacking, thus enabling ready access to circulating Ang II. These are called circumventricular organs and consist (inter alia) of subfornical organ, organum vasculosum of the lamina terminalis, median eminence and area postrema (Calhoun et al., 2004;Coote, 2005). Of these, the area postrema is an important site at which circulating Ang II modulates peripheral sympathetic nerve activity. Ablation of the area postrema prevents hypertension caused by chronic intravenous (i.v.) administration of Ang II. The major established efferent connections of the area postrema are the nucleus tractus solitarius and the lateral parabrachial nucleus, both of which provide substantial input to sympathetic preganglionic neurons in the intermediolateral cell column of the spinal cord. Lesions of the lateral parabrachial nucleus also impair chronic Ang II induced hypertension,

thus suggesting area postrema as a link between systemic Ang II levels and resultant central stimulation of sympathetic activity (DiBona, 2000).

Peripherally, Ang II via AT_1 receptor is also demonstrated to facilitate the release of norepinephrine from cardiac sympathetic nerve terminals. In Ang II infused rats, surgical cardiac sympathectomy or treatment with atenolol, a β_1-adrenergic receptor blocker, significantly prevented cardiac myocyte necrosis, showing that Ang II-induced cardiac damage is at least in part mediated by catecholamine release from cardiac sympathetic neurons, suggesting that the activation of cardiac sympathetic neurons by Ang II also contributes to pathological cardiac hypertrophy. Similar results have been observed with SHR, where in contrast to Ca^{2+} channel blocker or an α_1-adrenergic blocker, treatment with both ARBs or ACE inhibitors at dose which caused only mild hypotension attenuated the increase in cardiac atrial natriuretic factor and collagen types I and III mRNAs and significantly normalized the decreased α-MHC mRNA (Kim and Iwao, 2000).

Thus, there are strong evidences to indicate that circulating Ang II can increase central and peripheral sympathetic nerve activity and that this can be influenced by physiological alterations in the level of activity of the endogenous RAS.

2.4.3. Crosstalk between endothelin and sympathetic nervous system

Similar to RAS, ET-SNS interaction had been reported. It is demonstrated that subthreshold concentrations of ET-1 can potentiate the contractile responses of human arteries in response to catecholamines by a Ca^{2+} dependent mechanism. ET-1 may therefore amplify vasoconstrictor reflexes and be of pathophysiological relevance even when plasma ET-1 concentrations are not clearly elevated (Cowburn and Cleland, 2001;Yang, et al., 1990).

2.4.4. Crosstalk between salt sensitivity and sympathetic nervous system

Another aspect of salt sensitivity is the ability of salt to potentiate SNS induced vascular reactivity. Obiefuna et al studied the vasoreactive response to several factors in the aortic rings obtained from salt induced hypertensive rats and demonstrated that hypertension induced by salt loading was associated with increased sensitivity to norepinephrine, enhanced Ca^{2+} entry through receptor-operated channels and impairment of adenosine triphosphatease activity (Obiefuna, et al., 1991).

Thus, crosstalk at various levels and between different systems contributing to pathogenesis of hypertension has been studied extensively. This indicates the

complexity which exists in the overall genesis and maintenance of hypertension. Such a scenario also points towards the need to target more than one mechanism involved in hypertension so as to have better control over the disease and resultant long term improvement in end-organ protection and better prognosis.

2.4.5. Molecules targeting multiple interrelated pathways of hypertension

The enormous benefits of inhibition of ACE demonstrate that manipulation of the metabolism of peptide hormones is a valuable therapeutic strategy for cardiovascular disease. Recent attempts to expand these benefits have combined ACE inhibition with inhibition of other peptidases such as NEP in a single molecule, ACE/NEP or triple ACE/NEP/ECE inhibitor, a strategy known as vasopeptidase inhibition (Daull, et al., 2007).

NEP is an endothelial membrane bound zinc metallopeptidase also present on vascular smooth muscle cells, cardiac myocytes, renal epithelial cells and fibroblasts (Graf, et al., 1995;Dussaule, et al., 1993). It is involved in the metabolism of natriuretic peptide(s) (NP)s, namely atrial natriuretic peptide, brain natriuretic peptide, C-type natriuretic peptide, substance P and the hypotensive hormone bradykinin (BK), as well as vasoconstrictor peptides such as ET-1 and Ang II (Stephenson and Kenny, 1987;Lang, et al., 1992;Kenny, et al., 1993). The effects of NEP inhibition are attributed in most part to the increased biological activity of NPs and BK that results from their reduced metabolism.

The primary rationale for addition of NEP inhibition to ACE inhibition is to improve on the benefits of ACE inhibition alone. The antihypertensive actions of ACE inhibitors are increased by increased levels of NPs, which have natriuretic, diuretic, antiproliferative and vasorelaxant properties (inhibit renin, aldosterone and ET secretion) that elicit a number of vascular, renal and endocrine effects that help to maintain BP and extracellular fluid volume (Stasch, et al., 1996;Dussaule, et al., 1991).

The inhibition of BK breakdown under NEP inhibition results in an increased endothelial NO production (Zhang, et al., 1999) that explains the cardioprotective effects of NEP inhibitors. However, NEP inhibition also resulted in increased ET-1 plasma concentrations that through sustained endothelial cells ET_B receptor activation produce NO too. Unfortunately, ET-1 is mainly a potent vasoconstrictor that possesses numerous deleterious effects on the vasculature.

Many studies in animal models demonstrate improved therapeutic efficacy from addition of NEP inhibition to ACE inhibition in hypertension and heart failure

(Pham, et al., 1993;Robl, et al., 1997). Addition of NEP inhibition to ACE inhibition has similarly shown reduction in BP more effectively in hypertensive patients than does ACE or NEP inhibition alone (Favrat, et al., 1995).

Several dual ACE/NEP inhibitors have been developed (Flynn, et al., 1993;Kirk and Wilkins, 1996;Fournie-Zaluski, et al., 1994). However, the most extensively investigated dual ACE/NEP inhibitor is omapatrilat, also known as BMS-186716. Omapatrilat, an effective hypotensive agent in experimental hypertension was shown to be more effective than the ACE inhibitor fosinopril in reducing BP and improving hemodynamics (Trippodo, et al., 1998;Maniu, et al., 2002). In patients with hypertension, omapatrilat produced greater decrease in both systolic, diastolic and pulse pressure than does ACE inhibition alone (Weber, 2001). Comparison with other antihypertensive agents, such as lisinopril, losartan and amlodipine, revealed more pronounced antihypertensive effects of omapatrilat (Corti, et al., 2001). In the Omapatrilat Cardiovascular Treatment Assessment Versus Enalapril (OCTAVE) study, compared with enalapril, omapatrilat reduced BP further, by 3 mm Hg systolic and 2 mm Hg diastolic. Angioedema was reported in 2.17% of patients receiving omapatrilat and in 0.68% of patients receiving enalapril and the individual episodes with omapatrilat were more severe and their timing was earlier, the majority occurring within the first few hours after the initial dose.

Angioedema is a well-documented, but rare, adverse event in patients taking ACE inhibitors. It occurs in 0·1% to 0·5% of patients taking these drugs and maybe even more commonly among black patients (Messerli and Nussberger, 2000;Agostoni, et al., 1999;Grossman, et al., 2000). ACE inhibitors alone have been shown to increase plasma kinin concentrations (Pellacani, et al., 1994). An increase in the levels of BK and its metabolite des-Arg^{9}-BK, as a result of the combined inhibition of ACE and NEP, has been implicated in this potentially life-threatening side effect (Cugno, et al., 2003;Molinaro, et al., 2002;Nussberger, et al., 1998). It has been hypothesized that the BK-induced NO vasodilatation and subsequent increase in vascular permeability was responsible for the occurrence of angioedema. Treatment with omapatrilat increases NO concentrations in the rat (Trippodo, et al., 1998). NEP inhibitors are also responsible for increased circulating concentration of ET-1. The subsequent ET-1 induced NO release and vasodilatation, through the activation of the ET_B receptor present on endothelial cells may be implicated in the worsening of the incidence of angioedema too. The fact that the incidence of angioedema is higher for black patients for whom the ETs is predominant than for the white patients is an argument in favor of a possible role of ET-1 in the development

of this complication. The concomitant increase in ET-1 induced NO production with the elevated levels of circulating des-Arg^9-BK, as a result of increased concentrations of BK and reduced activity of BK B1 receptor agonist degrading enzymes (aminopeptidase P and ACE), may be responsible of the high incidence of angioedema observed under omapatrilat treatment (Daull, et al., 2007).

Thus, increased incidence of angioedema appears to be associated with simultaneous inhibition of NEP and ACE. Correspondingly, omapatrilat was not approved for the treatment of hypertension by the US Food and Drug Administration's Cardiovascular and Renal Drug Advisory Committee in July 2002 because of its potential side effects (Armstrong, et al., 2002;Quaschning, 2005).

The development of dual NEP/ECE inhibitors was initiated in parallel or slightly after the dual ACE/NEP inhibitors entered into clinical studies (Jeng, et al., 2002). Amongst various NEP/ECE inhibitors developed SLV 306 reached Phase II clinical trials in year 2002 (Tabrizchi, 2003). Unfortunately dual NEP/ECE inhibitors including SLV 306 failed to affect systemic BP (Dickstein, et al., 2004). Thus it was realized that it is necessary to associate dual NEP/ECE inhibitors to RAS blockers (ACE inhibitors or ARB) or to use triple ACE/NEP/ECE inhibitors as a new approach. Presently, there are only three molecules from this class of agent that have been described in the literature (Trapani, et al., 2004;Daull, et al., 2005;Inguimbert, et al., 2002;Vemullapalli S, et al., 1997). The mechanism of action of the triple ACE/NEP/ECE inhibitor is by reducing the production of Ang II and ET-1 while increasing those of BK and NPs. Concomitantly, NO concentrations are expected to rise under triple vasopeptidase inhibitors treatment, similar to that observed for omapatrilat treatments. The ECE inhibitory ability of the triple vasopeptidase inhibition gives them a tremendous advantage over dual ACE/NEP inhibitors, for which the inhibition of NEP was responsible for an increase in ET-1 levels.

In year 2004, Trapani et al demonstrated CGS 35601 and its prodrug CGS 37808 to be orally active potent triple inhibitor of ECE-1, NEP and ACE in rats. By suppressing the biosyntheses of ET-1 and Ang II, two potent vasoconstrictors, while simultaneously potentiating the circulating levels of atrial natriuretic peptide, a vasorelaxant and diuretic, CGS 35601 and CGS 37808 were suggested to represent novel agents for the treatment of cardiovascular and renal diseases (Trapani, et al., 2004).

In chronic experiments, CGS 35601 was found to be well tolerated with a very good safety profile in healthy normotensive, hypertensive and type 2 diabetic rats (Battistini, et al., 2005;Daull, et al., 2006c;Daull, et al., 2006a). The side effects due

to treatment with the triple ACE/NEP/ECE inhibitors are not yet known. However they are expected to be those of selective ACE inhibitors (dry cough and angioedema) and dual ACE/NEP inhibitors (high incidence angioedema). In SHRs CGS 35601 significantly reduced BP without increasing the concentrations of NO. Thus by inhibiting ECE and subsequently avoiding the ET-1 induced endothelial ET_B receptor activation, triple vasopeptidase inhibitors may reduce their odds to induce angioedema. However, no experiments have yet been performed in rat models to specifically explore vascular permeability and edema upon treatment with CGS 35601. These experiments are important if the development of triple vasopeptidase inhibitors as therapeutics is to be pursued. The group developing these molecules was aware of angioedema as a concern with this class of molecules and therefore recommends need for long-term chronic experiments to assess possible angioedema and increases in vascular permeability (Daull, et al., 2006c); however no information on further development of these molecules was then published.

In line with the same basic concept, dual acting molecules targeting AT_1 and ET_A receptors simultaneously were also developed. These molecules are expected to be devoid of limitations of ACE inhibitors and vasopeptidase inhibitors as high incidence of angioedema and cough. Murugesan et al developed series of dual acting receptor blockers and demonstrated there activity and efficacy in *in vitro* and *in vivo* experimental models respectively. These molecules were shown to possess antihypertensive activity in various animal models of hypertension (Murugesan, et al., 2005;Kowala, et al., 2004). One of the molecules, PS433540, has been taken up by Pharmacopeia, Inc. for further clinical development (Pharmacopeia, 2009). Number of preclinical studies using PS433540 has resulted in positive outcomes in multiple disease models and a positive preclinical pharmacokinetic and safety profile. Results from Phase I clinical studies of PS433540 in normal subjects have indicated that the compound is well tolerated at all six doses administered ranging from 20 mg to 1000 mg for 14 days and that the compound has a half-life that is consistent with once daily administration. In the multiple ascending dose study, five dose levels from 50 mg to 1000 mg have not produced safety or tolerability issues. In an Ang II challenge study, PS433540 demonstrated its ability to block the increase in BP induced by administration of Ang II to healthy volunteers (Thomson Reuters, 2008).

Further randomized, double-blind, placebo-controlled, parallel-group Phase II clinical studies to evaluate the safety and efficacy of PS433540 in subjects with stage I and II hypertension were designed (Clinicaltrials, 2009). Four week treatment with PS433540 was found successful in producing statistically significant BP reductions in

a Phase IIa study in patients with mild to moderate hypertension. The molecule was found to be safe and well tolerated, with the side effect profile no different from placebo. Encouraging results of Phase IIa trails lead to initiation of Phase IIb study with PS433540 in 400 patients using the 200 mg, 500 mg and 800 mg doses and comparing the relative BP reductions against the ARB irbesartan (Neutel, et al., 2008;O'Riordan M, 2008). Results for this study are yet awaited.

Thus, molecules targeting multiple inter-related targets would address the multiple interacting pathways that contribute to the pathophysiology of multifactorial disease like hypertension. Future development in this therapeutic area suggests that this is going to be a preferred approach to address the unmet need that still exists in the management of hypertension.

2.5. Racial differences in pathophysiology of hypertension

Black population of sub-Saharan African descent (blacks) differs from Caucasians (white) persons and other populations in cultural, social and psychological roots, as well as in biological characteristics. Although the black population is heterogeneous, hypertension investigators have considered black persons a distinct biological entity when studying environmental and genetic factors that might explain the observed group differences in risk for hypertension and response to treatment (Brewster, et al., 2004).

Amongst one third of United States adults having hypertension, a disproportionate number of African Americans have hypertension, which is generally of greater severity, develops at a younger age, more resistant to treatment (Rosamond, et al., 2008) and is associated with more clinical squeal in this racial group than in age-matched non-blacks (Cooper and Rotimi, 1997;Wang and Wang, 2004). The most recent data for the United States estimated that the prevalence of hypertension is 41.4% in African-Americans compared with 28.1% in whites and the racial differences in BP have been shown to exist even before the age of 10 years in black female children. Consequently, blacks have a 1.3 times greater rate of nonfatal stroke, 1.8 times greater rate of fatal stroke, 1.5 times greater rate of heart disease death and 4.2 times greater rate of end-stage kidney disease. Overall, mortality due to hypertension and its consequences is 4 to 5 times more likely in African Americans than in whites (Rosamond, et al., 2008). It is evident, therefore, that normalizing BP in hypertensive African American patients continues to remain a challenge and a priority.

2.5.1. Pathways contributing to observed racial differences

The racial differences in the development and clinical course of hypertension have been attributed to environmental and physiological factors (Ergul, 2000). In addition to specific factors that may be associated with the increased risk of greater than optimal BP and hypertension in blacks compared with whites include a higher prevalence of obesity, type 2 diabetes**,** low birth weight and a strong family history of CVD (Ferdinand and Saunders, 2006). It is hypothesized that the development and progression of hypertension in blacks is related to abnormal hemodynamic reactivity characterized by increased peripheral vascular resistance and diminished vasodilatation in response to environmental stress. Although the mechanism for increased peripheral vascular resistance has not been elucidated, enhanced

sympathetic reactivity, salt sensitivity and an imbalance between vascular vasoactive substances as ET-1 and NO has been postulated (Ergul, 2000;Grubbs, et al., 2002).

2.5.1.1. Racial differences in sympathetic reactivity and salt sensitivity

An inherited heightened sympathetic reactivity has been demonstrated in blacks in response to social and environmental factors (Ray and Monahan, 2002;Thomas, et al., 2004). Consistent with this hypothesis, a number of studies have demonstrated that black Americans display greater cardiovascular reactivity to a number of physical and mental stressors. For example, black children and adolescents exhibited significantly higher BP increase during a video game challenge (Murphy, et al., 1986). Similarly, black children were found to have augmented pressor responses compared with white children during isometric hand-grip exercise and orthostatic testing (Voors, et al., 1980). Using microneurography, Calhoun et al by measuring the muscle sympathetic activity of the peroneal nerve at rest and during cold pressor test provided direct evidence that blacks exhibit increased peripheral SNS activity compared with whites (Calhoun, et al., 1993).

Both normotensive and hypertensive black individuals are known to be more salt sensitive than whites. Study conducted to identify racial differences in salt sensitivity revealed 37% blacks to be salt sensitive comparison to 18% whites. The retention of excess Na^+ and water causing volume overload has been suggested as a contributing factor to the development of sustained high BP in the black population. Another aspect of salt sensitivity is the ability of salt to potentiate SNS induced vascular reactivity, contributing further to genesis of hypertension (Ergul, 2000).

2.5.1.2. Racial differences in regulation of endothelin system

It is suggested that the regulation of the ET system in African Americans may be different than the regulation of the ET system in the white population. Differences at the level of ET-1 synthetic machinery, ET receptor expression and ET-1 levels have been documented. Study conducted on saphenous vein specimens obtained from normotensive and hypertensive African American and white patients undergoing coronary artery bypass grafting surgery demonstrated an increased expression of ECE-1a mRNA, the most commonly expressed subisoform in addition to ECE-1c. ECE-1 activity, an indirect determinant of ET-1 biosynthesis, was also increased in this patient population and was accompanied by elevated tissue prepro-ET-1 mRNA and ET-1 levels. This suggests that the molecular components of the ET system are upregulated at the transcriptional and translational levels in African American

hypertensive patients compared with white hypertensive as well as normotensive patients (Grubbs, et al., 2002).

In black American patients, who present an increased prevalence of hypertension, a modification of the ratio between endothelial and smooth muscle ET_B receptors has been described. The total ET receptor density has been reported to be higher in white patients and that they possessed only the ET_A subtype on vascular smooth muscle cells. Whereas, black patients had both receptor subtypes on vascular smooth muscle cells, yet lesser total ET_B receptors than in white patients; indicating a significantly lower ET_B receptor density on endothelial cells. This decrease in ET_B ratio of endothelial to smooth muscle cells suggested a shift in favor of vasoconstriction promoting receptors. These findings provided evidence that in addition to ET-1 expression, ET receptors are also differentially regulated in blacks. Thus, suggesting that an imbalance due to a change in the tissue distribution of receptors, between prodilatory ET_B receptor and vasoconstrictor ET_A receptors could result in a contribution of ET-1 in primary hypertension (Ergul, et al., 1999;Ergul, 2000;Grubbs, et al., 2002).

Significant difference in the plasma levels of ET-1 have been reported in healthy black and white men (Evans, et al., 1996), further this difference were established in both female and male black hypertensive when compared with white hypertensive patients. The study also revealed ET-1 levels in hypertensive blacks to be sevenfold to eightfold higher than in normotensive blacks and threefold to fourfold higher than in hypertensive whites (Ergul, et al., 1996).

Further, to assess the effect of rapid BP control on plasma ET-1 levels, black patients with uncontrolled hypertension upon being followed for 1 month after initiation of antihypertensive therapy presented dramatic reduction in plasma ET-1 levels which were parallel to the reduction in BP. This provided indirect evidence that ET-1 levels may rise as a consequence of hypertension (Ergul, 2000).

Circulating levels of ET-1, however, have always been questioned for being a true marker of enhanced ET system activity. This is because circulating levels of ET-1 could be related to variable spillover of the peptide from the vasculature into the bloodstream or secondary to its impaired renal clearance (Campia, et al., 2004;Grubbs, et al., 2002) and therefore may not accurately reflect its production or biological effects. The dilemma has been resolved by studies which demonstrated that though the vasoconstriction responses to exogenous ET-1 were not significantly different in both hypertensive subgroups, the selective ET_A receptor blockade with BQ123 results in a significantly higher forearm vasodilator response in black than in

white hypertensive patients. In contrast, BQ123 does not significantly affect forearm blood flow in either black or white healthy controls. Together these observations suggested that an increased production and/or a decreased clearance of ET-1 are the most likely mechanisms in play (Campia, et al., 2004).

Several mechanisms have been proposed that may potentially account for a selectively enhanced vascular production of ET-1 in blacks. Physiologically, ET-1 gene expression which is stimulated by numerous vasoconstrictor factors, including catecholamines and Ang II and is inhibited by vasodilators such as NO and prostacyclin (Haynes and Webb, 1998); it is possible that the amplified sympathetic reactivity to stressful environmental stimuli observed in blacks (Stein, et al., 2000) and the concomitant decreased sensitivity to NO dependent (Treiber, et al., 2000) and adrenergic vasodilation (Cardillo, et al., 1999), may lead to enhanced ET-1 gene transcription and ET-1 synthesis. This hypothesis is supported by findings which have shown higher baseline levels of plasma ET-1 and exaggerated plasma ET-1 responses to behavioral and physical challenges in adolescent blacks with a family history of primary hypertension (Treiber, et al., 2000). Further, ET-1 may amplify the contractile response to other vasoactive agents, thus enhancing hemodynamic reactivity and promoting a self-maintained vasoconstrictor cycle (Yang, et al., 1990).

Yet another potential mechanism that may contribute to the higher ET-1 plasma levels and activity observed in hypertensive black patients is a decreased clearance of ET-1. A significant proportion of ET-1 clearance is known to occur through endothelial cell ET_B receptor binding and internalization (Dupuis, et al., 1996a). *In vivo* animal studies have shown supporting results, that ET_B receptor blocking increases plasma ET-1 concentrations and prolongs its biological $t_{1/2}$ (Fukuroda, et al., 1994). It has been demonstrated that hypertensive black subjects have a decreased endothelial cell expression of ET_B receptors (Grubbs, et al., 2002), which may hinder ET-1 clearance, thereby increasing bioavailability of the peptide at the ET_A receptors.

Thus, augmented levels of ET-1 along with increased capacity for vascular ET-1 biosynthesis and an alteration in ET receptor subtypes may contribute to the increased incidence of hypertension and related complications in this high risk black patient population (Grubbs, et al., 2002). The increased plasma ET-1 levels have been suggested to predispose blacks to greater risk for cardiovascular diseases. According to Lerman et al, a twofold increase in plasma ET-1 levels significantly increases systemic vascular resistance and decreases both heart rate and CO. Progressive changes in these indexes could result in left ventricular hypertrophy and

myocardial dysfunction (Lerman, et al., 1991). Supporting evidence were provided in studies where strong association between black ethnicity and increased left ventricular mass and relative wall thickness in black hypertensive adults has been demonstrated after factoring for standard clinical and hemodynamic parameters (Evans, et al., 1996;Kizer, et al., 2004).

2.5.1.3. Racial differences in plasma renin activity

The possibility of differences in the RAS activity between blacks and whites has been recognised decades ago (Helmer and Judson, 1968) and has been reconfirmed in recent past. A much higher frequency of low PRA in black hypertensives (52%) was demonstrated compared with white hypertensives (31%) (Helmer and Judson, 1968). On average PRA in blacks is about 50% to that in whites and the differences are similar for men and women. However, wide variability observed in PRA of blacks indicates that the PRA in some proportion of blacks is likely to be in the range found in whites. Lower plasma PRA has also been reported in black children and neonates compared with whites (Sagnella, 2001).

That the lower PRA as a characteristic feature of blacks has been supported by population based studies of blacks and whites having there BP insignificantly different from each other. Further, statistically significant inverse association between PRA and both systolic and diastolic BP after adjusting for age and gender has also been established in these racial groups (He, et al., 1999b).

Studies relating PRA and plasma ET-1 levels have been performed which demonstrated that in patients with low renin primary hypertension, the plasma ET-1 concentrations were significantly increased with respect to those observed in the normal subjects and in hypertensive patient groups. Further, the pathophysiological concentration of ET-1 in presence of low PRA has been related to the development or maintenance of hypertension in such patient group (Letizia, et al., 1997).

It is suggested that, given the paramount importance of the RAS in the control of Na^+ balance, the lower renin may be a consequence of intrinsic differences in renal Na^+ handling between blacks and whites where the lower PRA could be part of the corrective mechanisms designed to maintain Na^+ balance in the presence of an increased tendency for Na^+ retention in black people. This increased tendency for Na^+ retention is suggested to be a reflection of an evolutionary adaptation for more efficient mechanisms to conserve Na^+ in people originally inhabiting semitropical regions where Na^+ intake was traditionally low and difficult to obtain (Sagnella, 2001).

2.5.1.4. Racial differences in response to inhibition of renin-angiotensin system

African American patients with hypertension have been shown to respond less than white patients to blockers of the RAS (Johnson, 2008). Although no conclusive explanation has been proposed, role of low-renin/high-volume hypertension has been implicated.

Several studies have shown poor response of African Americans to ACE inhibitors, whereas others suggested that African American patients require higher doses of ACE inhibitors (Materson, et al., 1993;Weir, et al., 1995). Further, studies with ARBs have shown BP response in African Americans that is less than that in whites, but this difference was eliminated when ARBs were combined with low dose diuretics. This has also been shown to be true for ACE inhibitors when given in combination with a diuretic (Papademetriou, et al., 2004).

Studies have been conducted to assess and compare the effect of the ACE inhibitor enalapril to the effect of the AT_1 receptor blocker candesartan on BP in African Americans with stage 1-2 hypertension. It was found that the changes in BP observed with either enalapril or candesartan were small and not significantly different from placebo at the low dose (10mg and 16 mg, respectively). At the higher dose (20mg and 32 mg, respectively), each monotherapy was only minimally effective in reducing systolic and diastolic BP. However, the reduction of diastolic BP was somewhat greater with candesartan than with enalapril and more patients were controlled to target with candesartan than with enalapril. Further, 41% patients who failed to respond to either monotherapy, upon treatment with combination of enalapril and candesartan demonstrated no further improvement in BP response at either the low or high dose. Overall in synergy with other trials the study demonstrated African Americans to be poor responders to BP lowering effect of therapies targeting RAS and that higher doses are required to achieve some effect on BP in this group of patients. However, within therapies intervening RAS, ARBs were found to be superior to ACE inhibition in this group of patients. Further, black hypertensives had shown less influence of RAS on there overall hypertensive status as either (ARB or ACE inhibitor) at low dose failed to significantly influence PRA; though high dose increased PRA as expected, but the effect was less than is usually seen in white patients (Papademetriou, et al., 2004).

Existing data also suggest that there may be a race- or ethnicity-related difference in the prevalence RAS inhibition mediated side effects (Elliott, 1996). Use of ACE inhibitors and ARBs to lesser extent, has been shown to be associated with incidences of cough and angioedema. The prevalence of cough requiring

discontinuation of ACE inhibitor therapy has been estimated ≈10% in blacks in comparison to ≈2% observed in whites (Ferdinand and Saunders, 2006). Similarly, black Americans have a substantially increased risk of ACE inhibitor associated angioedema compared with white subjects, a risk which has been found to be independent of specific ACE inhibitor, its dose or concurrent medications (Brown, et al., 1996). Although the mechanism of increased incidences of angioedema in blacks is not certain, racial differences in the kallikrein-kinin system has been implicated in its pathogenesis (Gainer, et al., 1996).

Despite substantial clinical and laboratory investigation, the exact reason or reasons for the increased prevalence of hypertension in blacks remain unknown. There clearly are environmental and psychosocial factors involved. There also is accumulating evidence that the pathophysiological basis of hypertension in blacks is different than that in whites. Hypertension is characterized with an abnormal hemodynamic reactivity and increased salt sensitivity. Plasma levels of potent vasoactive peptide ET-1 are significantly higher in blacks in response to acute stress and in the hypertensive states than in whites. Given the fact that ET-1 induces long-lasting vasoconstriction and modulates the sympathetic system-mediated contractility, it is likely that ET-1 contributes to the abnormal vascular reactivity in blacks. In the peripheral venous circulation, black patients have significantly lower numbers of ET_B receptors; all indicating toward a substantial overall contribution of ET system in the genesis, sustenance and complications of hypertension in black population.

2.6. Unmet need

Effective medical treatment has been available to lower BP for almost 50 years. Indeed, treatment of hypertension has been credited with the decline in stroke and heart attack rates over the past few decades. Awareness of hypertension and the percentage of hypertensive people who are receiving treatment have increased. Nevertheless, the control rates are unacceptable (Table2), indicating towards existence of an alarming situation (Muller and Luft, 2006).

Table 2: Hypertension prevalence, treatment and control in various regions of world (BP control based on "normal" systolic/diastolic BP of 140/90 mmHg) (Gu, et al., 2002;Israili, et al., 2007;Reddy, et al., 2006;Rosamond, et al., 2008;Wolf-Maier, et al., 2003).

Regions	Prevalence %	Hypertensives Taking Medication %	Control %
North America			
United States	27.8	52.5	34
Canada	27.4	36.3	19
Europe			
Italy	37.7	32.0	9
Sweden	38.4	26.2	6
England	41.7	24.8	6
Spain	46.8	26.8	5
Germany	55.3	26.0	8
Asia			
India	27.7	30.3	9
China	27.2	28.2	8

As per current standards, for BP measurement >140 mmHg systolic or 90 mmHg diastolic, ≈1 in 4 adults, worldwide, would be classified as hypertensive. Currently, this equates to ≈1 billion individuals and this number is expected to grow to ≈1.5 billion (≈30% of the global population) by year 2025 (Table 3). In developed economies, the number of hypertensive individuals is predicted to grow by 70 million people from year 2000 to 2025, whereas in still developing economies, the number is predicted to grow by >500 million over the same period. In China and India alone, the total number of hypertensives is expected to increase to >500 million by year 2025 (Perkovic, et al., 2007).

Table 3: Number of people with hypertension aged 20 years and older by world region in year 2000 and predictions for year 2025 (Perkovic, et al., 2007).

Regions	Prevalence 2000 (Millions)	Predicted Prevalence 2025 (Millions)	Increase (Millions)
Established market economies (USA, Canada, Spain, England, Germany, Greece, Italy, Sweden, Australia, Japan)	239.5	309.7	70.2
Latin America and the Caribbean (Mexico, Paraguay, Venezuela)	114.3	200.6	86.3
Former socialist economies (Slovakia)	93.1	103.7	10.6
Middle East crescent (Egypt, Turkey)	73.8	152.6	78.8
China	181.6	299.2	117.6
India	118.2	213.5	95.3
Other Asian and islands (Korea, Thailand, Taiwan)	71.4	129.4	58.0
Sub-Saharan Africa (South Africa, Cameroon, Tanzania, Zimbabwe)	79.8	150.7	70.9
Total	971.7	1559.4	587.7

Hypertension is one of the most important modifiable risk factors for CVD and renal disease in Western and Asian populations. CVD is the leading cause of mortality worldwide and is estimated to account for ≈14.3 million deaths in year 1990. The majority of those deaths (≈9.1 million) occurred in economically developing countries (Gu, et al., 2002), with no exception to India. It was reported that of a total of 9.4 million deaths in India in year 1990, CVD caused 2.3 million deaths (25%). A total of 1.2 million deaths were due to coronary heart disease and 0.5 million due to stroke. It has been predicted that by year 2020, there would be a 111% increase in cardiovascular deaths in India. This increase is much more than 77% for China, 106% for other Asian countries and 15% for economically developed countries (Gupta, 2004).

In spite of the availability of more than 75 antihypertensive agents in 9 different classes, BP control in the general population is yet inadequate. Various explanations have been suggested, for example poor compliance on the part of the

patients; factors related to antihypertensive medication itself; possibility that the awareness of prevention strategies and their implementation by primary physicians may not always be optimal; non responders to existing therapy; multifactorial nature of disease, etc. (Israili, et al., 2007). It is estimated that 30 to 50% of hypertensive patients do not comply with their treatment regimens. Factors associated with noncompliance include, side effects of the medication, age, apathy about the consequences of missed doses, use of home remedies, employment and cost. Failure to take medications regularly may account for end-organ damage in many patients. In addition, the duration of action of antihypertensive medications is an issue in intermittently compliant patients. Drugs with a rapid onset and short duration of action are suboptimal because this profile may be associated with sudden decrease in BP with the onset of the drug's action, followed by rapid disappearance of the antihypertensive effect when doses are missed. Also, missed doses of some short duration agents (example, short-acting β blockers) may be accompanied by excessive sympathetic discharge and the possibility of potentially fatal arrhythmias. Many currently available antihypertensive drugs have notable side effects. For example; diuretics can produce various biochemical abnormalities, such as hypokalemia and can also decrease magnesium. Peripheral adrenergic inhibitors can cause diarrhea. Patients treated with β blockers are at risk for fatigue, bradycardia, heart failure, insomnia, impaired peripheral circulation and asthma. Although ACE inhibitors have many benefits, cough is an irritating side effect (Carson, et al., 2001).

Recent clinical trials have demonstrated that, though effective BP control can be achieved in patients with hypertension, but the majority will require 2 or more antihypertensive drugs. The importance of administering more than one drug to achieve the goal of 'normalizing' BP in a large number of hypertensive patients is acknowledged by guidelines on the treatment of hypertension. Further, according to the JNC7 report about one third of patients undergoing treatment for hypertension still remain in the category of uncontrolled hypertension (Chobanian, et al., 2003). Although our understanding of the pathophysiology of elevated arterial pressure has increased, in most of cases the precise etiology (and thus potentially the means of prevention or cure) is still largely unknown (Fisher and Williams, 2005). Since hypertension is a major portent of future cardiovascular disease, there exists a significant medical need for an antihypertensive drug, which while acting through multiple mechanisms, is effective across a wide variety of patients as a monotherapy. Therefore, there exists a need to identify, validate and explore the usefulness of newer targets alone and in conjunction with existing well established targets for

treatment of hypertension with aim to develop better drugs for patients with primary hypertension (Murugesan, et al., 2005).

Of the more recent avenues explored for treating hypertension, disruption of the effects of either Ang II or ET-1 has shown promise (Murugesan, et al., 2002). The endogenous peptides Ang II and ET-1 are powerful vasoconstrictors and mitogens and both peptides have been implicated in the pathogenesis of hypertension and CVD (Murugesan, et al., 2005). Ang II is the effector molecule of the RAS and several AT_1 receptor blockers have been developed for treating hypertension (Fisher and Williams, 2005). The ETs exert diverse biological effects through their two distinct G-protein coupled ET_A and ET_B receptors (Remuzzi, et al., 2002). A number of selective, as well as nonselective, ET receptor blockers have been developed and recent studies have shown that selective blockade of the ET_A receptor may be beneficial in the management of hypertension (Dhaun, et al., 2008;Epstein, 2008). ET_A receptor blockers are also suggested as an alternative and effective treatment for hypertension, particularly in patients where RAS blockade has had little therapeutic effect (Moreau and Dao, 2001) and those who present resistant hypertension (Dhaun, et al., 2008;Epstein, 2008). However, use of ET receptor blocker in the treatment of hypertension is still in the stage of infancy. The fact that these two systems are upregulated in hypertensive patients has been clearly shown by elevated plasma levels of ET-1 (Ergul, et al., 1996) and PRA (Laragh, et al., 1980).

Emerging experimental evidence suggests crosstalk between the Ang II and ET systems which has a synergistic effect in the pathophysiology of hypertension (Murugesan, et al., 2002). Elevated levels of Ang II promote the synthesis and vasoconstrictive action of ET-1 and elevated levels of ET-1 increase the synthesis vasoconstrictive action of Ang II, thus creating a positive dual-feedback mechanism and an excellent target for treating hypertension (Murugesan, et al., 2005). The possibility of additional benefits from simultaneous blockade of both AT_1 and ET_A receptors have been explored and a considerable number of preclinical studies have demonstrated that simultaneous blockade of both the RAS (primarily mediated by Ang II via AT_1 receptors) and the ET system (primarily mediated by ET-1 via ET_A receptors) can produce a greater reduction in BP and added end-organ protection than blocking either system alone (Bohlender, et al., 2000;Massart, et al., 1998;Murugesan, et al., 2002).

Monotherapies targeting RAS and ET system with aim to reduce the burden of BP and related diseases therefore require active research that will provide

evidence about the effects of novel interventions for disease prevention that may be of special relevance to particular populations and hypertensive population as whole. Novel antihypertensive therapies targeting hypertension at multiple levels and providing protection from secondary end-organ damage would be of great value.

Aims and Objectives

3. Aims and objectives

The aim of these studies was pre-clinical evaluation of selected newer dual action angiotensin and endothelin receptor blockers for treatment of hypertension.

The objectives of these studies were:

- To evaluate the hypothesis whether it is possible to achieve potentiation in antihypertensive effect of AT_1 receptor blockers by adding small doses of ET_A receptor blocker at concentrations which are potentially devoid of safety concerns.
- To study whether the potentiation in antihypertensive action achieved by combining an ET_A receptor blocker to AT_1 receptor blocker is pharmacological.
- To screen new chemical entitie(s) (NCE)s for their AT_1 and ET_A receptor blocking activity.
- To evaluate antihypertensive efficacy of selected NCEs in relevant animal model of hypertension.
- To identify potential molecule with desirable activity and antihypertensive efficacy for further development.

Materials and Methods

4. Materials and methods

4.1. Materials

- 4.1.1. New chemical entities and reference compounds
- 4.1.2. Experimental animals
- 4.1.3. Equipments
- 4.1.4. Softwares
- 4.1.5. Chemicals, drugs and disposables
- 4.1.6. Solutions and buffers
- 4.1.7. Formulation vehicles

4.2. *In vitro studies*

- 4.2.1. *In vitro* screening of new chemical entities for determination of their IC_{50} value for AT_1, ET_A and ET_B receptors
- 4.2.2. *In vitro* physico-chemical properties and drug metabolism studies of new chemical entities
  - 4.2.2.1. Solubility determination by nephelometery
  - 4.2.2.2. Drug permeability study using parallel artificial membrane permeation assay
  - 4.2.2.3. Determination of plasma protein binding of new chemical entities by ultrafiltration-centrifugation technique
  - 4.2.2.4. Drug metabolism study using rat liver microsomes

4.3. *In vivo* studies

- 4.3.1. Development of conscious telemetered disease model (for proof of concept studies: evaluating the hypothesis of potentiation in antihypertensive efficacy by combining two targets and screening new chemical entities for their antihypertensive efficacy)
  - 4.3.1.1. Rationale
  - 4.3.1.2. Animal model
  - 4.3.1.3. Surgical implantation of telemetry transmitter in rat
  - 4.3.1.4. Salt load tolerability and its effect on mean blood pressure of rat
- 4.3.2. Proof of concept study: To study whether there is potentiation of antihypertensive activity of AT_1 receptor blocker by addition of small dose of ET_A receptor blocker in conscious telemetered salt loaded SHRsp model

4.3.2.1. To determine effect on mean blood pressure upon adding small dose of ET_A receptor blocker ZD1611 to sub-maximally effective dose of AT_1 receptor blocker losartan

4.3.2.2. To determine effect on mean blood pressure upon adding small dose of ET_A receptor blocker ZD4054 to maximally effective dose of AT_1 receptor blocker candesartan

4.3.3. Development of anesthetized rat model for screening new chemical entities for their AT_1 and ET_A receptor blocking activity

4.3.3.1. To standardize type and dose of anesthetic to be used for arterial blood pressure monitoring

4.3.3.2. To standardize infusion dose of Ang II and big ET-1 to obtain a stable pressor effect

4.3.3.3. Blocking endogenous AT_1 receptors for isolating ET_A receptor blocker activity and blocking endogenous ET_A receptors for isolating AT_1 receptor blocker activity

4.3.3.4. Validation of anesthetized rat model for screening AT_1 and ET_A receptor blocking activity of new chemical entities

4.3.4. Screening of new chemical entities for pharmacological activity in anesthetized rat model

4.3.5. Pharmacokinetic profiling of new chemical entities in rat

4.3.6. Efficacy screening of new chemical entities in conscious telemetered salt loaded SHRsp model

4.3.7. *In vivo* safety pharmacology study

4.3.7.1. Central nervous system safety study

4.3.7.2. Cardiovascular and respiratory safety study

4.1. Materials

4.1.1. New chemical entities and reference compounds

All the NCEs studied were synthesized by the Medicinal Chemistry Department of Torrent Research Centre. This involved designing various potential dual AT_1 and ET_A receptor blockers as antihypertensive agents using the Computer Aided Drug Design (CADD) facility of Torrent Research Centre. Subsequently the novelty of the molecules designed was confirmed by searching appropriate databases. Then the synthetic feasibility of the designed NCEs was explored and molecules were finalized for synthesis. The NCEs synthesized were numbered from TRC23001 to TRC23050.

Losartan potassium (losartan) and the reference molecule BMS346567 were synthesized by Medicinal Chemistry Department of Torrent Research Centre whereas candesartan cilexetil (candesartan), ZD1611 and ZD4054 were gift samples obtained from AstraZeneca, Sweden.

4.1.2. Experimental animals

Sprague Dawley rat:

Sprague Dawley rats used in the study were obtained from National Institute of Nutrition (NIN) Hyderabad, India and bred in the Animal House facility of Torrent Research Centre. They were housed in polypropylene cages containing paddy husk as bedding with two rats of same sex in a cage.

Spontaneously hypertensive rat:

SHR, 4-6 weeks old were procured from Taconic Farms, Germantown, U.S.A. and inbred in the Animal House facility of Torrent Research Centre. They were housed in polypropylene cages containing paddy husk as bedding with two rats of same sex in a cage.

Spontaneously hypertensive rat stroke prone:

Male SHRsp, 4-6 weeks old were procured from Charles River Laboratories, Wilmington, U.S.A. After quarantine period of two weeks, animals were housed in the Animal House facility of Torrent Research Centre. They were housed individually in polypropylene cages containing paddy husk as bedding material.

Swiss albino mice:

Swiss albino mice 4-5 weeks old were procured from Charles River Laboratories, U.S.A. and bred in the Animal House facility of Torrent Research Centre. They were housed in polypropylene cages containing paddy husk as bedding with five mice of same sex in a cage.

Guinea pig:

Duncan Hartley guinea pigs used in the study were procured from National Institute of Nutrition (NIN) Hyderabad, India. After quarantine period of two weeks, animals were housed in the Animal House facility of Torrent Research Centre. Guinea pigs were housed in stainless steel cages with grilled floor with two animals of same sex in a cage.

The animals used for this work were housed in Good Laboratory Practice (GLP) - accredited Animal House facility of Torrent Research Centre. The animal house environmental conditions were as follows: temperature maintained at 22±3°C and relative humidity of 30-70%. High efficiency particulate air filters (0.2 micron; 99.9% efficiency) were installed at air inlet of animal holding room. The air circulation was maintained at 20-24 (100% fresh) air changes per hour in all the animal rooms. The animal rooms were maintained at positive pressure as compared to the corridors. Lighting schedule was 12 hr artificial light: in each 24-hour period. The rats and mice were fed with standard rodent pelleted diet (Pranav Agro, Sangli, India) and purified water (Aquaguard) *ad libitum,* whereas guinea pigs were fed with standard guinea pig diet (Pranav Agro, Sangli, India) and purified water (Aquaguard) containing 0.1% ascorbic acid *ad libitum.*

Animal ethics

All animal experimental protocols were approved by the Institutional Animal Ethics Committee. Every care was taken to adhere to highest standards of animal ethics in all procedures carried out.

4.1.3. Equipments

96 well microplate (Nunc, U.S.A.)

AM frequency radio transistor (Coby Electronics Corp., U.S.A.)

Ambient pressure reference (APR-1, Data Sciences International, U.S.A.)

Anesthesia induction chamber (Locally fabricated)

Animal anesthesia station (SurgiVet, U.S.A.)

Animal hair clipper (Oster, U.S.A.)

Applying forceps for micro clips (Biomedical Research Instruments, U.S.A.)

Autoclave (OT32, Nuve, Turkey)

BIO Amp (AD Instruments, Australia)

Data exchange matrix (Data Sciences International, U.S.A.)

Electric room heater (Omax, India)

Fume hood (Labexcel, India)

High performance liquid chromatography (HPLC, Shimadzu, Japan)

Homoeothermic blanket (Harvard Apparatus, U.S.A.)

Infusion pump (11 plus, Harvard Apparatus, U.S.A.)

Isoflurane vaporiser (Drager Vapor 2000, Drager Medical, Germany)

Liquid chromatography tandem mass spectrometry (LC-MS/MS, Thermo Finnigan, U.S.A.)

MacLab 8/s (AD Instruments, Australia)

Magnet (Data Sciences International, U.S.A.)

Micro vessel clip (Biomedical Research Instruments, U.S.A.)

Micropipette (Eppendorf, Germany)

Nephelometer (Nephelostar Galaxy, BMG Labtech, Germany)

Plexiglass open field arena (90 x 90 x 30 cm) (Locally fabricated)

Orbital shaker (Labline, Barnstead, U.S.A.)

pH meter (Eutech, Singapore)

Pressure transducer (SensoNor 840, SensoNor AS, Norway)

QUAD Bridge (AD Instruments, Australia)

Receiver (RPC-1, Data Sciences International, U.S.A.)

Refrigerated bench top centrifuge (Eppendorf, Germany)

Small animal ventilator (Columbus, U.S.A.)

Surgical microscope (Sethi Surgicals, India)

Surgical instruments (Locally sourced)

TA11-PA-C40 telemetery transmitter (Data Sciences International, U.S.A.)

Temperature controlled water bath (Pharmacia Biotech, Sweden)

Centrifuge (Heraeus Multifuge 3S-R, Thermo Scientific, U.S.A.)

Vortex mixer (Maxi mix-II, Barnstead International, U.S.A.)

Water bath (Julabo, Germany)

Weighing balance (Mettler Toledo, U.S.A.)

4.1.4. Softwares

Chart (version 5) (AD Instruments, Australia)

Chart Pro (version 5.4.2) (AD Instruments, Australia)

Dataquest A.R.T. analysis software (version 3.01) (Data Sciences International, U.S.A.)

Dataquest A.R.T. Gold (version 3.01) (Data Sciences International, U.S.A.)

GraphPad Prism (version 3.0) (GraphPad Software, U.S.A.)

SAS (version 9.1) (SAS, U.S.A.)

WinNonlin (version 5.1) (Pharsight Corporation, U.S.A.)

4.1.5. Chemicals, drugs and disposables

Acetic acid (E. Merck, India)

Acetonitrile (Qualigen, India)

Angiotensin II acetate human (Ang II, Sigma, U.S.A.)

Atropine sulphate (Loba Chemie, India)

Benzyl penicillin (Alembic, India)

Big endothelin 39 Rat (big ET-1, Sigma, U.S.A.)

Boric acid (Ranbaxy, India)

Braided silk suture 2-0 (Diamond, India)

Buprenorphine (Buprigesic, Neon Lab, India)

Cellulose patch (Data Sciences International, U.S.A.)

Centricon tubes (Millipore, U.S.A.)

Cotton buds (Rainbow, India)

Dextrose (Merck, India)

Dimethyl sulphoxide (DMSO, Sigma, U.S.A.)

Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4, Qualigen, India)

Disodium tetra borate (Ranbaxy, India)

Ethylene diamine tetra acetic acid disodium salt dihydrate (EDTA, Sigma, U.S.A.)

Gauge (Real Care, India)

Glycerine (Ranbaxy, India)

Hand disinfectant (Levermed, Qualigens, India)

Heparin sodium (Biological E, India)

Hexadecane (Aldrich, Germany)

Hexane (Aldrich, Germany)

Hydrochloric acid (HCl, Qualigens, India)

Hypodermic needle (BD, Singapore)

Hypodermic syringe (BD, Singapore)

Isoflurane (Forane, Abbott Laboratories, India)

Isopropyl alcohol (S D Fine Chem, India)

Ketamine (Aneket, Neon, India)

Liver microsomes (B.D. Gentest, U.S.A.)

Magnesium chloride hexahydrate ($MgCl_2$, Sigma, Germany)

Mecamylamine hydrochloride (Sigma, U.S.A.)

Medical oxygen (Locally sourced)

Methanol (Qualigen, India)

Microtubes (Axygen, U.S.A)

Multiscreen permeability assay plates (Millipore, U.S.A.)

Nicotinamide adenine dinucleotide phosphate reduced form (NADPH, SRL, India)

N-methyl-2-pyrrolidone (NMP, ISP, U.S.A.)

Normal saline (NS, Claris Lifesciences, India)

Ortho-phosphoric acid (H_3PO_4, Qualigen, India)

Polyethylene glycol 400 (PEG-400, Clarient, Germany)

Perchloric acid (Merck, India)

Potassium chloride (KCl, Qualigen, India)

Potassium dihydrogen phosphate (KH_2PO_4, Qualigen, India)

Povidone-iodine scrub (Betadine Scrub, Win Medicare, India)

PTFE acceptor plate (Millipore, U.S.A.)

Room disinfectant (Virosil Pharma, Sanosil Biotech, India)

Sodium carbonate (Na_2CO_3, S D Fine Chem, India)

Sodium chloride (NaCl, S D Fine Chem, India)

Sodium hydroxide (NaOH, SRL, India)

Sodium phosphate monobasic anhydrous (NaH_2PO_4, SRL, India)

Sterile surgical gloves (Kemwell, India)

Sterile water for injection (Sterilock, Marck Parenterals, India)

Surgical suture 2-0 (Mersilk, Ethicon, India)

Surgical suture 3-0 (Mersilk, Ethicon, India)

Tissue adhesive (Vetbond, 3M, U.S.A.)

Tween-80 (Sisco Research Laboratories, India)

Urethane (Sigma, U.S.A.)

Xylazine (Izine, Intas, India)

4.1.6. Solutions and buffers

1N NaOH solution

NaOH	4 gm
Milli-Q water q.s.	100 ml

1N Na_2CO_3 solution

Na_2CO_3	5.3 gm
Milli-Q water q.s.	100 ml

1% NaH_2PO_4 solution

NaH_2PO_4	1 gm
Milli-Q water q.s.	100 ml

10 mM EDTA solution

EDTA	372.2 mg
Milli-Q water q.s.	100 ml

200 mM Phosphate buffer pH 7.4

NaH_2PO_4	0.6 gm
Na_2HPO_4	2.3 gm
1% NaH_2PO_4 solution	for pH adjustment
Milli-Q water q.s.	100 ml

50 mM $MgCl_2$ solution

$MgCl_2$	476.05 mg
Milli-Q water q.s.	100 ml

Assay buffer for *in vitro* drug metabolism study

200 mM phosphate buffer pH -7.4	5 ml
50 mM $MgCl_2$ solution	1 ml
10 mM EDTA solution	1 ml
Milli-Q water	3 ml

Borate buffer standard solutions

Solution A: 0.2 M Boric acid and potassium chloride solution

Boric acid	12.366 gm
KCl	14.911 gm
Milli-Q water q.s.	1000 ml

Solution B: 0.2 M Sodium hydroxide solution

NaOH	8 gm
Milli-Q water q.s.	1000 ml

Borate buffer composition	*pH 9.2*	*pH9.4*
Solution A	50 ml	50 ml
Solution B	26.4 ml	32.1 ml
Milli-Q water q.s.	200 ml	200 ml

Borax buffer pH 8.0

KH_2PO_4	231 mg
Disodium tetra borate	1.639 gm
H_3PO_4	for pH adjustment
1N NaOH solution	for pH adjustment
Milli-Q water q.s.	100 ml

Phosphate buffer pH 6.8

KH_2PO_4	342.9 mg
Na_2HPO_4	163.8 mg
H_3PO_4	for pH adjustment
Milli-Q water q.s.	200 ml

Phosphate buffer saline of pH 7.4

NaCl	8.71 gm
KH_2PO_4	0.26 gm
Na_2HPO_4	2.71 gm
1N NaOH solution	for pH adjustment
Milli-Q water q.s.	100 ml

4.1.7. Formulation vehicles

BMS346567 (i.v., 0.010, 0.015, 0.020 mg/ml)

TRC23003 (i.v., 0.010, 0.015, 0.020, mg/ml)

TRC23012 (i.v., 0.006, 0.009, 0.012 mg/ml)

TRC23029 (i.v., 0.022, 0.033, 0.044 mg/ml)

TRC23030 (i.v., 0.015, 0.025, 0.035 mg/ml)

Glycerine	10% v/v
1N Na_2CO_3 solution	10% v/v
Borate buffer (pH 9.2)	q.s.

BMS346567 (i.v., 2.368, 7.100, 23.680 mg/ml)

TRC23029 (i.v., 2.000, 6.000, 20.000 mg/ml)

TRC23030 (i.v., 2.000, 6.000, 20.000 mg/ml)

NMP	3.3% w/v
PEG-400	6.6% w/v
Borate buffer (pH 9.2)	q.s.

BMS346567 (per oral (p.o.), 0.20, 1.73, 5.20, 17.28 mg/ml)

TRC23029 (p.o., 0.41, 3.41, 10.23, 34.08, 102.23 mg/ml)

TRC23030 (p.o., 0.40, 3.37, 10.11, 33.66, 100.99 mg/ml)

TRC23035 (p.o., 0.20, 1.73, 5.20, 17.28 mg/ml)

Tween- 80	1% w/v
NaCl	0.5 % w/v
Borate buffer (pH 9.2)	q.s.

Candesartan (p.o., 4 mg/ml)

Candesartan + ZD4054 (p.o., 4+0.4, 4+1.2 mg/ml respectively)

Tween-80	1% w/v
NaCl	0.5 % w/v
Milli-Q water	q.s.

Losartan (p.o., 0.045, 0.091, 0.45, 0.91, 2.27 mg/ml; i.v., 0.03, 0.045, 0.06, 30 mg/ml)

NaCl	0.9 % w/v
Milli-Q water	q.s.

TRC23003 (i.v., 2.368, 7.100, 23.680 mg/ml)

TRC23017 (i.v., 0.200, 0.300, 0.400,12.500, 25.000 mg/ml)

Glycerine	10% v/v
1N Na_2CO_3 solution	15% v/v
Borate buffer (pH 9.2)	q.s.

TRC23012 (i.v., 2.368, 7.100, 23.680 mg/ml)

TRC23031 (i.v., 2.368, 7.100, 23.680 mg/ml)

TRC23035 (i.v., 0.800, 2.400, 8.000 mg/ml)

NMP	3.3% w/v
PEG-400	6.6% w/v
Tween-80	1% w/v
Borate buffer (pH 9.2)	q.s.

TRC23031 (i.v., 0.025, 0.034, 0.045 mg/ml)

TRC23035 (i.v., 0.015, 0.023, 0.030 mg/ml)

Glycerine	10% v/v
1N Na_2CO_3 solution	15% v/v
Borate buffer (pH 9.4)	q.s.

ZD1611 (p.o., 0.0091, 0.027, 0.27 mg/ml; i.v., 0.03, 0.1, 0.3, 1.5 mg/ml)

NMP	3.3% w/v
PEG-400	6.6% w/v
Borate buffer (pH 9.2)	q.s.

ZD4054 (p.o., 0.12, 0.4, 1.2 mg/ml)

Tween-80	1% w/v
NaCl	0.5 % w/v
Milli-Q water	q.s.

All formulations were freshly prepared at the time of administration.

4.2. *In vitro* studies

4.2.1. *In vitro* screening of new chemical entities for determination of their IC_{50} value for AT_1, ET_A and ET_B receptors

In vitro assays of NCEs for determination of their inhibitory concentration to block 50% of initial response $(IC)_{50}$ value for AT_1, ET_A and ET_B receptors were performed by AstraZeneca at their Research Facility in Sweden. Method for same has been described in *Appendix-I.* Briefly, the assays were performed using Chinese hamster ovary cells over-expressing the human AT_1, ET_A and ET_B receptors. Ca^{2+} transit in response to challenge with Ang II and ET-1 in absence and presence of different concentrations of NCEs were measured by Flourometric Imaging Plate Reader (FLIPR, Molecular Devices Corp.) and the results were used to determine AT_1, ET_A and ET_B receptor IC_{50} value.

4.2.2. *In vitro* physico-chemical properties and drug metabolism studies of new chemical entities

4.2.2.1. Solubility determination by nephelometery

Principle

The nephelometer detects particles in liquid samples by measuring forward scattered light when a laser beam is directed through the solution. More the particles there are in the solution, greater the amount of forward scattered light being measured by nephelometer.

Methodology

A 50 mg/ml stock 1 for test compound was prepared in 100% Dimethyl sulfoxide (DMSO). Using stock 1, sample 1 and stock 2 were prepared by aliquoting 5 µl and 40 µl of stock 1 in 495 µl phosphate buffer saline (PBS) (pH 7.4) and 10 µl DMSO respectively to prepare sample 1 containing 0.5 mg/ml test compound in 1% DMSO and stock 2 containing 40 mg/ml test compound in 100% DMSO. Similarly, using stock 2, sample 2 and stock 3 were prepared and the dilution process was continued to produce 10 serially diluted samples for each test compound.

200 µl of blank solution (1% DMSO in PBS) and serially diluted samples of test compounds were loaded into predefined wells of 96 well microplate and readings in form of unit/concentration curve were obtained using Nephelostar Galaxy Nephelometer. From the curve, the inflection point was identified and was recorded as solubility point of the test compound.

4.2.2.2. Drug permeability study using parallel artificial membrane permeation assay

Principle

Parallel artificial membrane permeation assay (PAMPA) is a non-cell based assay designed to predict passive transcellular permeability of compounds (Wohnsland and Faller, 2001). The assay is carried out in a 96 well multiscreen permeability plate and measures the ability of the compounds to diffuse from donor to acceptor compartment separated by a hexadecane artificial membrane on a polycarbonate membrane support. The hexadecane forms a membrane on the polycarbonate filter which mimics the lipid bilayer. The ability of compounds to diffuse from donor compartment into acceptor compartment through hexadecane membrane is evaluated.

Methodology

Hexadecane artificial membrane was prepared by aliquoting 20 µl of 10% hexadecane solution (in hexane) into the test wells of donor plate, the filter surface of which was pre-wetted with 15 µl of 50% v/v methanol. Equal numbers of well in donor plate were left without treatment with hexadecane solution to be used as equilibrium wells. The plate was allowed to dry for an hour in fume hood to ensure complete evaporation of hexane resulting in a uniform layer of hexadecane.

250 µl phosphate buffer (pH 6.8) containing 5% DMSO was added to wells of PTFE acceptor plate with hexadecane treated and equilibrium wells, was placed over the acceptor plate ensuring contact of the donor membrane with the buffer present in the acceptor plate. 125 µl of test compound dilution (500 µmol in phosphate buffer (pH 6.8) containing 5% DMSO) was added to each of the test and equilibrium well of donor plate, the plate lid was closed and incubated at room temperature for 19 hr on orbital shaker. After incubation, the solutions from the acceptor plate were analyzed for test compound using High performance liquid chromatography (HPLC) in Bioanalytical Department, Torrent Research Centre. HPLC peak area of equilibrium acceptor well was considered as control (100%) and this was used for calculating test compound concentration from other samples.

The results obtained were used to calculate log Pe value using the equation below:

$$\log Pe = \text{Log}[C \times -\text{In}\,(1-[\text{Mean Area}_{acceptor} / \text{Mean Area}_{equilibrium}])]$$

$$Pe = -\,[\text{ln}\,(1-\text{Mean Area}_{acceptor} / \text{Mean Area}_{equilibrium}) / (A \times (1/ V_D+1/ V_A) \times T)]$$

Where:

A = Effective area of the membrane (that is 0.048 cm^2)

C = [V_D x V_A / (V_D + V_A) Area x time]

Mean $Area_{acceptor}$ = Area of HPLC peak of sample from the acceptor well of hexadecane treated donor plate

Mean $Area_{equilibrium}$ = Area of HPLC peak of sample from the acceptor well of non hexadecane treated donor plate

T = Time of incubation in sec

V_D= Volume of donor well (0.125 ml)

V_A= Volume of acceptor well (0.25 ml)

4.2.2.3. Determination of plasma protein binding of new chemical entities by ultrafiltration-centrifugation technique

Principle

When drug containing plasma is filtered through an ultrafiltration membrane, plasma water and low molecular weight compounds pass through, while large molecules, such as plasma proteins are retained. The ultrafiltrate obtained by this process is analyzed for drug content which represents the fraction of drug that remains unbound to plasma proteins.

Methodology

Centricon tubes (2 ml capacity) which consist of two reservoirs separated by 10 KDa filter were used as filtration assembly. The tubes were conditioned by filling the upper reservoir with 2 ml PBS and centrifuging at 3500 rpm for 10 min.

Spiked plasma sample was prepared by aliquoting 10 µl of test compound stock (500 µg/ml in 100% DMSO) in 990 µl of rat plasma. Similarly, externally spiked sample and aqueous equivalent standards were prepared by aliquoting 10 µl of test compound stock to 990 µl filtrate obtained by ultrafiltration of blank rat plasma through 10 KDa filter and PBS respectively. 1 ml of spiked sample, 1 ml of externally spiked sample and 2 ml of aqueous equivalent standards were loaded to the upper reservoir of separate centricone tubes which were than centrifuged at 3500 rpm for 40 min at 37°C. The filtrate obtained in the lower reservoir of centricon tubes was collected and analyzed to determine the test drug concentration using LC-MS/MS in Bioanalytical Department, Torrent Research Centre.

Externally spiked standard was used as standard (100%) and also to account for any interference caused by the matrix (rat plasma) during test compound

estimation in plasma filtrate by LC-MS/MS, whereas aqueous equivalent standard was used to account for test compound adsorption by the filter itself.

The unbound drug which appeared in the filtrate was calculated as percent of unbound fraction (%Fu) using formula mentioned below. Upon subtracting %Fu from 100, the fraction of test compound bound to plasma proteins was obtained.

%Fu = ((Af / Ar) x 100) x (100 / % recovery)

% recovery = (Aa / Ab) x 100

Where:

Af: The peak area response of drug in spiked rat plasma filtrate

Ar: The peak area response of drug in externally spiked standard

Aa: The peak area response of drug in aqueous equivalent standard after ultrafiltration

Ab: The peak area response of drug in aqueous equivalent standard before ultrafiltration

4.2.2.4. Drug metabolism study using rat liver microsomes

Principle

Microsome is the name given to the endoplasmic reticulum membrane fraction which can be isolated from whole cell homogenate. The cytochrome P450 enzymes, also known as the microsomal mixed function oxidase system are membrane bound proteins localized predominantly in smooth endoplasmic reticulum of liver and other tissues (Hrycay and Bandiera 2008). Cytochrome P450 enzymes comprise 4% to 6% of total microsomal protein (Hrycay and Bandiera 2008) and forms main family of enzymes responsible for approximately 70% to 80% of the rate limiting phase-I metabolism reaction which includes oxidation, reduction and enzymatic hydrolysis. Such biotransformation reaction aims at converting xenobiotics to more polar metabolite for further biotransformation or subsequent excretion from body (Pelkonen et al., 2008;Rollas, 2008).

Methodology

The test compound was dissolved to make 20 mM stock in 100% DMSO. This stock was serially diluted in ratio of 1:9 in 100% DMSO and then to 1:19 in pre warmed (37°C) assay buffer for *in vitro* drug metabolism study (composition mentioned under section 4.1.6.) to obtain 100 µM test compound in 5% DMSO as working solution. Working solution of 10 mg/ml NADPH was also prepared in pre warmed assay buffer. Rat microsomes which are commercially available at a

concentration of 20 mg/ml were stored at -70°C and before use were rapidly thawed at 37°C in water bath and maintained on ice until added to reaction mixture in microtube. The reaction was setup in three sets. The incubation mixture for the test compound consisted of liver microsomes 5µl, test compound 10 µl, NADPH 10 µl from 10 mg/ml stock and sufficient quantity of assay buffer to make final volume 100 µl. Reaction was commenced with the addition of NADPH and shaken in a water bath at 37°C. Six such reaction tubes were made with aim to arrest the reaction and study unmetabolised test compound at 0, 5, 10, 20, 30 and 60 min.

Similarly, control and plain reactions were setup which lacked test compound and microsomes respectively. Control reaction was arrested at 60 min of incubation and plain reaction was arrested at 0, 30 and 60 min of incubation. Reactions were arrested by adding 50 µl of 100% acetonitrile and the reaction mixture was centrifuged at 14000 rpm for 10 min at 4°C. Supernatant recovered after centrifugation was analyzed using HPLC in Bioanalytical Department, Torrent Research Centre. The HPLC peak area obtained from 0 min sample was considered as control and was used to estimate test compound concentration from samples of later time points.

The results obtained were used to calculate the *in vitro* intrinsic clearance value (Cl_{int} µl/min/mg protein) as described by Obach (Obach, 1999), using the following equation:

$$Cl_{int} = \frac{0.693}{\textit{in vitro}\ t_{1/2}} \times \frac{\mu l\ \text{incubation}}{\text{mg protein}}$$

To estimate $t_{1/2}$, nonlinear regression was used based on the equation described by Di et al (Di, et al., 2004).

$y = 100\ e^{(-t \ln 2 / t1/2)}$

4.3. *In vivo* studies

4.3.1. Development of conscious telemetered disease model (for proof of concept studies: evaluating the hypothesis of potentiation in antihypertensive efficacy by combining two targets and screening new chemical entities for their antihypertensive efficacy)

4.3.1.1. Rationale

The contribution of RAS and ET system in development of hypertension is now well established (Epstein, 2008;Ergul, 2000;Ergul, et al., 1998;Ibrahim, 2006;Kim and Iwao, 2000;Pinto-Sietsma and Paul, 1998). That the RAS and ET system are up-regulated in hypertensive patients has been clearly shown by elevated plasma levels of ET-1 and PRA (Ergul, 2000;Ibrahim, 2006;Pinto-Sietsma and Paul, 1998). ARBs are well established and safe antihypertensives, similarly, ET receptor blockers have also been shown to have antihypertensive effects in hypertensive patients (Dhaun, et al., 2008;Epstein, 2008;Kohan, 2008). Emerging experimental evidences from various studies suggest that Ang II and ET participate in a manner involving closely interwoven pathways in increasing BP (Boemke, et al., 2001;Gómez-Garre, et al., 1996;Herizi, et al., 1998;Montanari, et al., 2003;Yoshida, et al., 1992), hence concomitant blockade of both Ang and ET pathways may lead to enhanced BP reductions. Thus, we wanted to evaluate experimentally the extent of BP reduction by addition of ET_A receptor blocker over AT_1 receptor blockade.

Though, ET_A receptor blockade may provide substantial reduction in BP, ET receptor blockers are reported, at higher doses, to have liver toxicity, teratogenic effect and potential to cause testicular atrophy (Battistini, et al., 2006;Treinen, et al., 1999). Hence we also evaluated the hypothesis whether a non toxic minimal dose of ET_A receptor blockade can potentiate the antihypertensive effects of AT_1 receptor blockade. For these studies, two well established AT_1 receptor blockers losartan and candesartan alone and in combination with two selective ET_A receptor blockers, ZD1611 (Bialecki, et al., 1998;Wilson, et al., 1999) and ZD4054 (Morris, et al., 2005; Rosano, et al., 2006), respectively, were used. Plasma concentrations of AT_1 and ET_A receptor blockers were also monitored when administered alone and in combination to monitor for potential interaction.

Toxicity profile of ZD1611 and ZD4054 has been established by AstraZeneca (unpublished data on file: AstraZeneca). In study to establish teratogenic potential, female rats were dosed with ZD1611 from gestation day 7 to day 16, it was observed that achieving maximum plasma concentration (C_{max}) of 80000 ng/ml and beyond in

pregnant female rats is associated with malformed fetuses. Similarly, 28 days rat multiple dose toxicity studies with ZD4054 has revealed moderate seminiferous tubular atrophy with C_{max} of 8900 ng/ml and signs of liver toxicity as C_{max} of 83500 ng/ml was achieved.

4.3.1.2. Animal model

Genetically activated RAS in SHR's (Iyer, et al., 1996;Schiffrin, 2001;Tikellis, et al., 2006) and activation of ET system in salt dependent models of hypertension as DOCA-salt rats and Dahl salt-sensitive rats (Ikeda, et al., 1999;Ikeda, et al., 2000;Schiffrin, 2001;Schiffrin, 2005) is well established. However, screening NCEs for efficacy, which are dual action receptor blockers active against both AT_1 and ET_A receptors and for testing the concept that simultaneous blockade of AT_1 and ET_A receptors would produce further lowering of BP in comparison to AT_1 receptor blockade alone, an animal model which displays both activated RAS and ET system was required. SHRsp is one such rat model. These rats develop severe hypertension and cerebrovascular lesions which is further accelerated by salt (Krenek, et al., 2001). Hypertension in salt loaded SHRsp is now known to be contributed by activated ET system (Iglarz and Schiffrin, 2003;Schiffrin, 2001;Schiffrin, 2005) in addition to an activated RAS (Hubner, et al., 1995;Takai, et al., 2001). Hence, salt loaded SHRsp was selected as an animal model for efficacy screening of NCEs and for evaluating the concept that simultaneous blockade of AT_1 and ET_A receptors would produce further lowering of BP in comparison to AT_1 receptor blockade alone.

The most common techniques currently employed for monitoring BP in conscious rat employs use of either a tail-cuff device (Krege, et al., 1995) or an exteriorized, fluid-filled catheter that refers pressure to a transducer located nearby (Mattson, 1998;Mills, et al., 2000). The tail-cuff method has the advantage of being noninvasive. However, the accuracy of BP measurements with this method in rodents is known to be greatly affected by environmental factors, as well as by any physiological or pharmacological factor that influences blood flow in the tail (Mills, et al., 2000). Continuous measurement of pulsatile arterial pressure cannot be accomplished with the tail-cuff method but can be obtained with exteriorized catheters. When used with caution, reasonably accurate measurements of systolic, diastolic and pulse pressure can be made for several days to up to 1-2 weeks. Decreasing catheter patency is the usual problem that limits the duration of reliable recordings with exteriorized catheters (Mattson, 1998).

Monitoring BP using a telemetry device circumvents many of the problems associated with conventional methods of BP monitoring. Telemetry represents the most humane method for monitoring of physiological parameters in conscious, freely moving laboratory animals and eliminates need to anesthetize or restrain the animal which can alleviate a potential source of experimental artifact and inter-animal variability (Kramer and Kinter, 2003). This implantable device is known to provide accurate and reliable measurements of arterial BP in continuous or sem-icontinuous manner for period of weeks up to several months. The technique has been extensively validated and considered to be one amongst best ways to capture arterial BP in conscious laboratory animals (Brooks, et al., 1996;Guiol, et al., 1992;Kramer and Kinter, 2003;Kurtz, et al., 2005;Mills, et al., 2000).

Principle of telemetry

Telemetry combines miniature sensors and transmitters to detect and broadcast biological signals in animals to the receiver. The receiver converts the analog frequency signal into a digital signal to be imputed into a computerized data acquisition system (Kramer and Kinter, 2003). The acquisition system can store, format, tabulate and output the data in accord with the instructions of the user. The components of DSI telemetry hardware and its setup are described in *Appendix-II*.

4.3.1.3. Surgical implantation of telemetry transmitter in rat

Preoperative preparation

The surgery for implantation of telemetry transmitter was performed in surgery room prefumigated with room disinfectant. Before the transmitters were implanted, their calibrations were verified to be accurate within ±3 mmHg (the details of procedure used for calibration check is described in *Appendix-III*) and were then soaked in sterile saline for about 5 min before implantation. Male SHRsp weighing 180 to 210 gm were used for surgical implantation of telemetery transmitter. Anesthesia was induced in induction chamber using 6% mixture of isoflurane in medical oxygen; anesthetized rat was shifted to preparation table where they were maintained under anesthesia using 1.5-2% isoflurane in medical oxygen and then using a hair clipper, hair from the abdominal area of the anesthetized rat were removed. Any remaining hairs were also removed using a razor. The clipped abdominal area of rat was disinfected using Betadine Scrub and cleaned using 70% isopropyl alcohol. Rats were also administered benzyle penicillin (30,000 IU/rat, i.m.) and buprenorphine (0.75 mg/kg, s.c.) as a part of preoperative preparation.

Surgical procedure

After preoperative preparations, rats were shifted to the surgery table over the homoeothermic blanket where body temperature, measured rectally, was maintained at 37°C and were maintained under surgical anesthesia using 1.5-2% isoflurane in medical oxygen throughout the surgical procedure. All possible care was taken to perform this surgery under aseptic condition. A 4-5 cm long midline incision in the abdominal skin, immediately caudal to the xiphoid process was made. Subsequently, the abdominal wall was opened and retracted using small artery forceps. The intestine was lifted from the abdominal cavity using cotton buds, wrapped in sterile gauge moistened with sterile saline preheated to 37°C and were kept aside. This allowed good visualization of the descending aorta on the dorsal body wall of rat. The intestine was kept warm and moist by pouring ≈0.6 ml warm, sterile saline every 4-5 min. Under the surgical microscope, using cotton buds, abdominal aorta was carefully separated from surrounding fat and connective tissues and was carefully separated from vena cava just caudal to the point where the left renal vein crosses over the aorta. A 2-0 braided silk occlusion suture was inserted between the abdominal aorta and the vena cava just caudal to the left renal vein. Once the occlusion suture was in place, transmitter's catheter protective tip cover was carefully removed by applying gentle traction. By applying tension to occlusion suture perpendicular to aorta, very small portion of aorta could be seen separated from the dorsal body wall, through which, with the help of applying forceps, micro vessel clip was applied on aorta to temporarily occlude the blood flow in it. While the aorta was clamped, a small puncture hole was made in the aorta, 2-3 mm cranial to the iliac bifurcation, with a 23 gauge hypodermic injection needle bent at an angle of 90° at the beveled end. Using a pair of curved blunt forceps to hold the catheter and the concave surface of the bevel as catheter introducer, the catheter was inserted into the aorta and pushed cranial until the entire thin walled tip was inserted inside. After insertion, the catheter entry point was dried with a cotton bud and a small drop of tissue adhesive, using blunt end 27 gauge hypodermic needle, was applied at the catheter entry site. Immediately a cellulose patch (5 mm x 5 mm) was placed on the tissue adhesive over the catheter entry site. Another drop of tissue adhesive was applied at this point, if necessary, to achieve good coverage. Tissue adhesive was allowed to dry for 10-12 sec and the micro vessel clip was slowly removed, while the catheter entry site was observed for leakage upon restoration of blood flow in aorta. While inserting the catheter, an effort to minimize the total duration of aortic occlusion, by quick and efficient catheter insertion, was made in order to prevent

ischemia related hind limb paralysis to rat. Once hemostasis was ensured, the occlusion silk suture was removed and correct catheter placement was verified by bringing an AM radio transistor (adjusted to the frequency of 550 kHz) near to transmitter. A tone fluctuating corresponding to the cardiac cycle ensured proper placement of catheter in the aorta. The peritoneal cavity was then flooded with sterile-warm saline and the intestine was placed back in position. The transmitter body was placed over the intestine in the peritoneal cavity, parallel to the long axis of the body with the catheter looped caudally. The transmitter was secured by closing the abdominal incision and incorporating the tabs on the implant into the closure using 3-0 non-absorbable silk suture in a simple interrupted pattern. To complete the surgery, the skin incision was also closed using 2-0 non-absorbable silk suture in a simple interrupted pattern. Figure 2 shows rat implanted with telemetry transmitter.

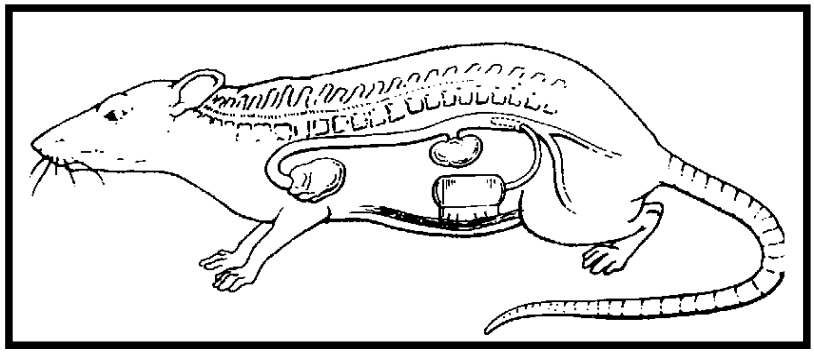


Figure 2: TA11-PA-C40 telemetery transmitter implanted intraperitonially in a rat.

Postoperative care

Immediately after completion of surgical procedure, rats were transferred to autoclaved cage lined with two layers of blotting paper in clean recovery room maintained at temperature of 26-28°C with help of room heaters to provide warm and dry environment. Animals recovering from anesthesia were closely attended until regaining the ability to maintain sternal recumbency. After recovery from anesthesia and acclimatization to new home cage for 30 min, food was presented in glass petri dish within the cage and water was provided in bottle having long nozzle to facilitate food and water intake. Blotting paper soaked with urine was gently removed or the rats were transferred to new cage as and when required. Rats in their recovery cage were frequently monitored for their normal well being. Body weight of rats was recorded daily and those showing continuous fall in body weight beyond two days of surgery were provided with 10% dextrose in drinking water. Post surgical pain was

minimized by a pre-emptive administration of buprenorphine (0.75 mg/kg, s.c.) and was continued twice a day for total 6 doses. Additional doses of buprenorphine were given for another day if post-operative pain/distress was evident. Rats were also administered a prophylactic dose of benzyl penicillin (30,000 IU/rat, i.m.) and were continued on it twice a day for total 14 doses. Factors as gain in body weight, food intake, dry and healed suture line, normal movement were taken as indicator of rat's recovery from surgery, which usually took 3-4 days post surgery. Rats were then transferred to individual cages containing normal paddy husk bedding and shifted to standard housing facility of animal house.

Data acquisition

Data acquisition was carried out in a dedicated telemetery recording room. On the recording day transmitters were switched on 2 hr before recording the data. Turning on of the magnetic switch was ensured by bringing an AM radio transistor (adjusted to the frequency of 550 kHz) near the rat cage. A tone fluctuating corresponding to the cardiac cycle ensures switching on of the transmitter. Scheduled rat cages were placed on the top of corresponding receiver for acquisition of BP wave. Data were collected with a computer driven data acquisition system (Dataquest A.R.T. Gold); the acquisition software was appropriately configured to record parameters, 10 sec every 10 min for 24 hr. The signals from receivers were consolidated by the Data Exchange Matrix and were stored and analyzed by a personal computer using Dataquest A.R.T. 3.01 analysis software. The telemetered pressure signals (the absolute pressure) were corrected automatically for changes in atmospheric pressure as measured by an ambient pressure monitor by the software.

Data analysis

Systolic BP and diastolic BP data were extracted from the pressure waveform using the same Dataquest A.R.T. 3.01 analysis software. For each 10 sec of pressure wave acquired, the software calculated systole and diastole of each pulse and presented a single mean value as systolic BP and diastolic BP. This mean value was considered as representative value for the entire 10 min duration before new pressure wave was acquired by system for another 10 sec interval. MBP values were derived from these raw systolic BP and diastolic BP data using the following formula (Berne and Levy 2002):

MBP = DBP + 1/3 (SBP-DBP)

Where:

MBP: Mean blood pressure

SBP: Systolic blood pressure

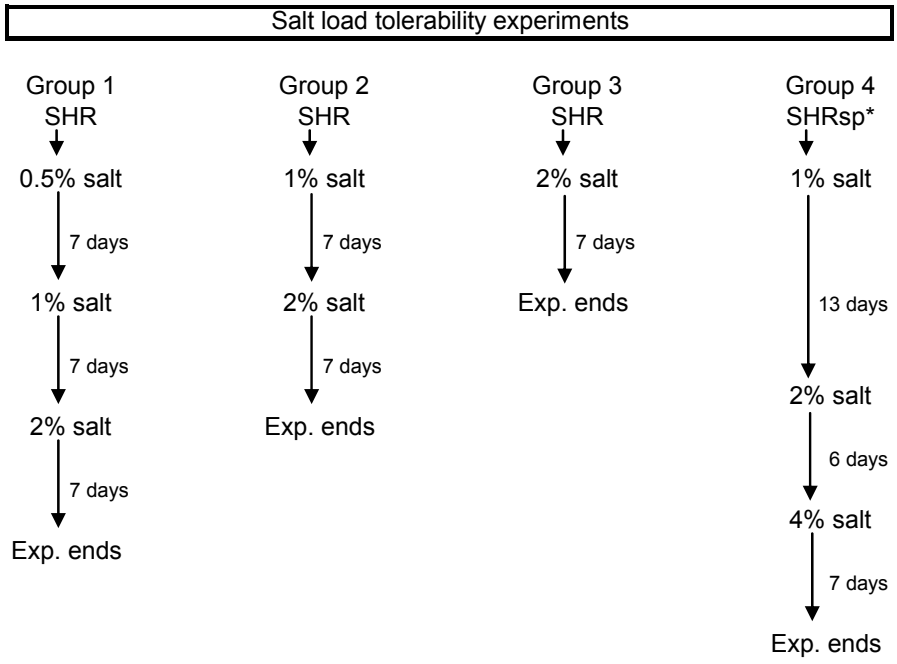
DBP: Diastolic blood pressure

Upon averaging 6 such MBP values, MBP values for each hour was calculated. The data acquired by the system immediately after switching on the lights of room (approximately 2 hr), while daily room cleaning and cage changing (approximately 1 hr 10 min), another 1 hr 10 min for stabilization and acclimatization period in new cage, during the period of dosing the rats (approximately 20 min) and 20 min post drug administration was excluded from the final analysis. This provided effective 19 hr BP data from telemetered rats (from 0 hr to 18 hr). The average of one hour MBP recording before dosing was taken as the basal MBP (0 hr) for the animal for the day. The antihypertensive effect of the drugs was determined after accounting for the effect of vehicle on MBP response.

4.3.1.4. Salt load tolerability and its effect on mean blood pressure of rat

Appropriate salt load (in form of sodium chloride) is an important component of SHRsp model because the contribution of ET to hypertension is greater in salt sensitive hypertensive models than in normal diet fed SHR and SHRsp (Ikeda, et al., 2000) as exemplified by prepro-ET levels, which elevates to twice in the aorta of salt loaded rats (Krenek, et al., 2001).

Different concentrations of salt load in different forms (saline or high salt containing diet) have been administered to SHRsp (Camargo, et al., 1993;Rocha, et al., 1998;Takai, et al., 2001). Therefore, after initial exploratory experiments on SHR for salt load tolerability, experiments on telemetered SHRsp were performed to estimate the tolerability and effect on MBP upon loading them with salt in drinking water.



* Surgically implanted with BP monitoring telemetry transmitters

Figure 3: Experimental protocol followed to establish salt load tolerability and its effect on MBP of rat. Exp.: experiment.

To determine tolerability to salt load, male SHR weighing 225-275 gm were randomly chosen from the colony to form 3 groups each having 3-4 rats. Rats from group 1 to 3 were provided with drinking water containing 0.5%, 1% and 2% salt respectively continuously for 7 days. Rats in group 1 and 2 were further continued with escalating concentrations of salt in drinking water. Group 1 rats were continued with 1% and then with 2% saline for 7 days each whereas group 2 rats were continued with 2% saline for 7 days (Figure 3). For entire period of salt being administered in drinking water, rats were monitored for their physical well being twice daily.

Based on our experience with SHR animals, experiment with SHRsp was designed to explore salt load regimen that could elevate their MBP by 40-60 mmHg. Therefore, in group 4, two male SHRsp weighing 180-210 gm were surgically implanted with telemetery transmitters as described under *section 4.3.1.3.* After recovery from surgery, rats were provided with 1% saline for drinking for 13 days followed by 2% saline for another 6 days and eventually 4% saline for 7 days (Figure 3).

4.3.2. Proof of concept study: To study whether there is potentiation of antihypertensive activity of AT_1 receptor blocker by addition of small dose of ET_A receptor blocker in conscious telemetered salt loaded SHRsp model

4.3.2.1. To determine effect on mean blood pressure upon adding small dose of ET_A receptor blocker ZD1611 to sub-maximally effective dose of AT_1 receptor blocker losartan

Male SHRsp rats after a quarantine period of 2 weeks (around 7.5 weeks of age) were surgically implanted with TA11-PA-C40 telemetry transmitters and at 10.5 weeks of age given 1% salt in drinking water for one week and then switched over to 2% salt in drinking water for rest of the study. Around 14 weeks of age, rats with their MBP above 150 mmHg were randomized into various treatment groups, with matching baseline MBP values.

Determination of ED_{75} dose of losartan and minimal effective dose of ZD1611

Losartan and ZD1611 were administered after 6 hr fasting and food was provided 2 hr post dosing. Losartan, ZD1611 and corresponding control groups received single oral doses of 0.5, 1, 5, 10 and 25 mg/11ml/kg ; 0.1, 0.3 and 3 mg/11ml/kg or vehicle 11ml/kg, respectively. Rats were randomized for their MBP before each day dosing and a time interval of minimum 48 hr was given between doses so as to allow sufficient time for washout of the drug administered earlier.

A dose response curve (DRC) for reduction in MBP brought about by different doses of losartan was obtained by plotting percentage reduction in MBP against the corresponding test dose and the effective dose 75 (ED_{75}) dose was derived. A best fit line was constructed and the theoretical dose for 75% reduction in MBP was derived from equation of best fit line. This ED_{75} dose of losartan was considered as its sub-maximal effective dose for further potentiation studies with minimal effective dose of ZD1611. There were 4-7 rats in each treatment group.

The minimum dose of ZD1611 which produce antihypertensive effect was identified and was considered as minimal effective antihypertensive dose for further potentiation studies with sub-maximal effective dose of losartan.

Potentiation study by combining ED_{75} dose of losartan and minimally effective antihypertensive dose of ZD1611

Sub-maximally effective dose of losartan, alone or in combination with minimally effective dose of ZD1611 or vehicle in equal volume were administered to different groups of rats and their antihypertensive effect was recorded.

At the end of various dosing regimes and 72 hr of wash out, the rats were redistributed and dosed orally with either sub-maximal dose of losartan alone or combination of sub-maximal dose of losartan and minimally effective dose of ZD1611. Blood samples were collected before dosing and at 0.25, 0.5, 1, 2, 4, 6 and 8 hr post drug administration and plasma was separated as described in *Appendix-IV*.

The plasma samples were analyzed using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) in Bioanalytical Department, Torrent Research Centre. Pharmacokinetic (PK) parameters were calculated using non compartmental model analysis module of WinNonlin (version 5.1). The calibration curve range for losartan was 5 ng/ml to 1000 ng/ml with r^2 = 0.9970. Similarly, the calibration curve range for ZD1611 was 1 ng/ml to 200 ng/ml with r^2 = 0.9975.

4.3.2.2. To determine effect on mean blood pressure upon adding small dose of ET_A receptor blocker ZD4054 to maximally effective dose of AT_1 receptor blocker candesartan

Male SHRsp rats were used in the study. Rats were prepared for treatment as described under *section 4.3.2.1*. Rats were treated orally in non fasted condition at uniform dosing volume of 2.5 ml/kg, once daily. Treatment groups had a common separate vehicle control group being treated parallel to them.

Treatment with candesartan and ZD4054

At 15 weeks of age, rats were treated with candesartan 10 mg/kg for eight consecutive days. 10 mg/kg is the maximum reported dose of candesartan in rats (Inada, et al., 1997;Kim, et al., 1994b;Kim, et al., 1994c;Kim-Mitsuyama, et al., 2005). Repeat dosing with drug was performed to saturate the rat with effect of this AT_1 receptor blocker.

Rats were treated with three escalating doses of ZD4054 0.3, 1 and 3 mg/kg for three consecutive days at an age of 15, 17 and 16 weeks respectively. The response from the third day of dosing was considered for computation of results. There were 5 to 7 rats in each treatment group.

Potentiation study by combining maximum effective antihypertensive dose of candesartan with minimally effective antihypertensive doses of ZD4054

Potentiation study with combination of candesartan and ZD4054 was carried out only at last two dose levels of ZD4054 (1 and 3 mg/kg), as ZD4054 alone at its lowest tested dose failed to produce any fall in rat MBP in comparison to vehicle. At 15 and 18 weeks of age rats were treated with candesartan 10mg/kg for eight consecutive days and from day 9 to day 11 ZD4054 was added at dose of 1 or 3 mg/kg to candesartan. There were 5 to 7 rats in each treatment group.

Plasma drug profiling was performed in blood samples collected from separate parallel groups of salt loaded SHRsp being treated with drug regimen similar to one received by corresponding rat groups in efficacy study. Method of blood collection and plasma separation is described in *Appendix-IV*.

Plasma samples from rats treated with candesartan 10 mg/kg were collected on day one and day eight of treatment. On day one, staggered sampling was performed so as to have 3 plasma samples at each time point. Samples were collected before drug administration and at 0.5, 1, 1.5, 2, 4, 6, 8, 12, 16, 20 and 24 hr post drug administration. However, on day 8 all seven rats were bled for all the time points and sampling were continued for another 30 and 36 hr post last dose administration.

Similarly, plasma samples were collected from rats treated with different doses of ZD4054 alone and with combination of candesartan and ZD4054. Samples were collected either after three days of dosing with of ZD4054 (1 or 3 mg/kg) alone or on day 11 after combining ZD4054 with candesartan 10 mg/kg from day 9 to day 11, in case of combination treated groups. Samples were collected on scheduled days before dosing and at 1, 3, 6, 12, 18, 24, 30 and 36 hr post drug administration.

The plasma samples were analyzed using LC-MS/MS in Bioanalytical Department, Torrent Research Centre. PK parameters were calculated using non compartmental model analysis module of WinNonlin (version 5.1). The calibration curve range for candesartan was 50 ng/ml to 6000 ng/ml with r^2 = 0.9982. Similarly, the calibration curve range for ZD4054 was 50 ng/ml to 10000 ng/ml with r^2 = 0.9992.

4.3.3. Development of anesthetized rat model for screening new chemical entities for their AT_1 and ET_A receptor blocking activity

4.3.3.1. To standardize type and dose of anesthetic to be used for arterial blood pressure monitoring

Ketamine-xylazine cocktail infusion and isoflurane inhalation were explored as anesthetics which could provide stable anesthesia for arterial BP monitoring in anesthetized rat continuously for 6 hr.

Three groups of Sprague Dawley rat (n=6-8), weighing 300-350 gm were used in the study. Anesthesia was induced either with intraperitoneal injection of ketamine–xylazine combination (60 mg/kg and 7.5 mg/kg, respectively) or in induction chamber with 6% isoflurane diluted in medical oxygen. While anesthetized, body temperature was measured rectally and was maintained at 37°C using homoeothermic blanket. Rats were tracheostomized and were artificially ventilated either with room air or with 1.5% isoflurane in medical oxygen, using a rodent ventilator adjusted to deliver volume of 10 ml/kg body weight at a respiration rate of 70 strokes/min (Hoshino, et al., 1998). The right jugular vein and left femoral artery were isolated and cannulated with heparin saline (heparin, 50 IU/ml) filled polyethylene cannula for the administration of drugs and the measurement of arterial BP, respectively.

After completion of the surgical procedure, atropine (0.2 mg/kg, i.v.) and mecamylamine (3 mg/kg, i.v.) were administered to inhibit autonomic nervous reflexes (Hoshino, et al., 1998) and then isoflurane inhalation was readjusted to either 0.8% or 1% or ketamine-xylazine cocktail infusion at dose of 1250 µg + 40 µg/kg/min was initiated after 45 min of initial induction dose of ketamine-xylazine combination. The decision to start ketamine-xylazine cocktail infusion 45 min after the induction dose was based on our previous observation that ketamine-xylazine combination at dose of 60 mg/kg and 7.5 mg/kg, respectively, comfortably produce surgical anesthesia for a period of 45 min. The ketamine-xylazine cocktail was prepared in 5% dextrose in Milli-Q water as vehicle and was infused using an infusion pump adjusted to deliver the cocktail at the rate of 33.33 µl/min/kg. While under maintenance anesthesia with either anesthetic infusion or inhalation, BP was recorded continuously for 6 hr.

During the experiment, BP signals from the transducer were amplified using a bridge amplifier and the BP was recorded using the Chart software of MacLab 8/s. Using analysis module of the Chart software the systolic and diastolic BP was

determined and MBP was computed using the formula described in *section 4.3.1.3.*

4.3.3.2. To standardize infusion dose of Ang II and big ET-1 to obtain a stable pressor effect

The model was developed to screen NCEs having dual AT_1 and ET_A receptor blocking activity. Both components of activity, AT_1 and ET_A receptor blocking were evaluated separately in separate sets of experiments.

AT_1 receptor blocking activity was assessed in terms of ability of NCE to suppress the pressor effect of Ang II infused at supra-physiological concentrations while endogenous ET_A receptors were blocked. Ang II infusion was essential, as in normotensive rat, any antihypertensive activity of NCE mediated through AT_1 receptor blockade would go unnoticed in absence of elevated arterial BP contributed by Ang II. Endogenous ET_A receptors were blocked while assessing NCEs for their AT_1 receptor blocking activity to prevent interference in response caused by activation of ET system during elevated *in vivo* Ang II levels. Further, since NCE were designed to be dual AT_1 and ET_A receptor blockers, ET_A receptor blocking activity of NCE would contaminate the effect produced by AT_1 receptor blocking component of NCE.

Similarly, ET_A receptor blocking activity of NCEs was assessed in terms of their ability to suppress the pressor effect of big ET-1 infused at supra-physiological concentrations while endogenous AT_1 receptors were blocked.

Exogenously administered Ang II produce pressor effect after binding to AT_1 receptors (Gossmann, et al., 2001;Kobori, et al., 2004;Prieto-Carrasquero, et al., 2005;Zhao and Navar, 2008).Various doses of Ang II i.v. bolus in dose range of 0.1 to 1.2 µg/kg have been used by researchers (Kowala, et al., 2004;Matys, et al., 2000;Murugesan, et al., 2002;Murugesan, et al., 2000) with AT_1 receptor blockers. Taking this as a reference, a DRC with log doses of Ang II was created to estimate infusion dose of Ang II which could provide a stable rise of 40-60 mmHg in MBP.

Male Sprague Dawley rat (n=4), weighing 300-350 gm were anesthetized with isoflurane and blood vessels were cannulated as described above in *section 4.3.3.1.* In addition to right jugular and left femoral vein, left jugular vein was also cannulated for administering the pressor agents. Rats were challenged with ascending log doses of Ang II (10, 30, 100, 300, 1000 and 3000 ng/kg; at volume of 250 µl/kg) administered as slow i.v. bolus (across 30 sec) with help of infusion pump. 30 min were allowed between challenges to allow BP to return to baseline.

On the basis of experience from above experiment, rats were infused with Ang II at ascending doses of 10, 15 and 20 ng/kg/min, each for duration of 30 to 120 min. Recovery of MBP to basal was ensured before infusing subsequent doses. In separate sets of rat (n=3), Ang II infusion at dose of 20, 25 and 35 ng/kg/min was tested. The infusion rate was adjusted to 2 ml/kg/hr to maintain euvolemia (Matys, et al., 2000).

Big ET-1 infusion was used for elevating circulating ET-1 levels *in vivo*. Exogenously administered big ET-1 is converted to the biologically active peptide ET-1 *in vivo* (Hemsen, et al., 1991) via a phosphoramidon-sensitive ECE (Fukuroda, et al., 1990). In the present study, the use of big ET-1 *in vivo* was preferred because this compound does not elicit the initial depressor response associated with i.v. administered ET-1 (Yanagisawa, et al., 1988) and yields a greater maximum response than that to ET-1 itself (Wilson, et al., 1999). Big ET-1 at infusion dose of 219.4 ng/kg/min (Wilson, et al., 1999) was explored and was found to be successful in elevating BP to desired level and provided stable MBP in 90 min.

4.3.3.3. Blocking endogenous AT_1 receptors for isolating ET_A receptor blocker activity and blocking endogenous ET_A receptors for isolating AT_1 receptor blocker activity

Losartan at the dose of 30 mg/kg, i.v has been reported to completely block AT_1 receptors (Kaminska, et al., 2005;Matys, et al., 2000). However, the duration of its blockade action was determined in group of 3 Sprague Dawley rats, which after surgery and stabilization were challenged with normal saline 1 ml/kg (volume load) followed by Ang II 300 ng/ml/kg, i.v. slow bolus (across 30 sec). BP was allowed to come back to basal and losartan (30 mg/ml/kg, i.v. slow bolus) was administered. Thereafter at an interval of every 30 min rats were challenged with Ang II (300 ng/kg, i.v. slow bolus) for next 5.5 hr.

ZD1611, a selective ET_A receptor blocker was selected at a dose of 1.5 mg/kg, i.v. to block all *in vivo* ET_A receptors of rats based on report by Wilson et al who have shown that ZD1611 at this dose was active for more than 7 hr (Wilson, et al., 1999).

4.3.3.4. Validation of anesthetized rat model for screening AT_1 and ET_A receptor blocking activity of new chemical entities

Validation of animal model was performed to ensure sensitivity of model to capture AT_1 or ET_A receptor blocking activity of test compounds. A DRC at three

doses of AT_1 receptor blocker losartan and ET_A receptor blocker ZD1611 was constructed in Ang II or big ET-1 infused Sprague Dawley rats respectively. Doses of blockers were selected to provide graded response with third dose capable of causing more than 50 percent fall in MBP so that effective dose 50 (ED_{50}) for the test compound could be computed.

Male Sprague Dawley rats weighing 250-350 gm were used in two separate groups (n=7 and 5). As described in sections above, rats were anesthetized with isoflurane, blood vessels were cannulated and rats were stabilized for one hour. For screening AT_1 receptor blocker for activity, Ang II was infused at the dose of 35 ng/kg/min and an i.v. bolus of ZD1611 1.5 mg/kg was administered to block endogenous ET_A receptors at 15^{th} min of Ang II infusion. ZD1611 administration at 15^{th} min of Ang II infusion was decided on the bases of observation made in *section 4.3.3.2* that the pressor effect of Ang II gets stabilized by 15^{th} min of its continuous infusion. Having administered ZD1611 for 30 min, any fall caused in MBP was compensated by increasing infusion dose of Ang II to 50-75 ng/kg/min. Similarly, for screening ET_A receptor blocker for activity, losartan 30 mg/kg, i.v. bolus was administered prior to initiation of big ET-1 infusion at dose of 219.4 ng/kg/min. In this model, DRC with losartan at dose of 0.030, 0.045 and 0.060 mg/kg/30min and with ZD1611 at dose of 0.03, 0.1 and 0.3 mg/kg/30min was created. A 15 min drug infusion free period was maintained before shifting to next dose of receptor blocker. All infusions were performed at volume of 2 ml/kg/hr to maintain euvolumia (Matys, et al., 2000). Rats which failed to show a pressor window of 40-60 mmHg with 70 ng/kg/min infusion of Ang II in 15 min or with 219.4 ng/kg/min infusion of big ET-1 within 90 min were not included.

Both losartan and ZD1611 at the doses used provided graded fall in MBP. Thus this animal model was validated to screen for AT_1 and ET_A receptor blocking activity of test compounds. The fall in MBP in response to different doses of receptor blockers was established by determining MBP before initiation and at the end of 30 min infusion.

The gap of 15 min before infusing next higher dose of blocker was kept to achieve recovery in MBP back to pre-infusion levels. However, 15 min drug infusion free period was found insufficient for MBP to recover back and therefore in subsequent experiments no such drug free infusion periods were kept and decision to take cumulative DRC with the test compound was taken.

4.3.4. Screening of new chemical entities for pharmacological activity in anesthetized rat model

After standardization and validation, the animal model was used to screen activity of reference molecule BMS346567 and seven NCEs namely, TRC23003, TRC23012, TRC23017, TRC23029, TRC23030, TRC23031 and TRC23035 designed to act as dual blockers of AT_1 and ET_A receptors.

Selection of 7 NCEs from group of total 50 NCEs was performed based on their potency and selectivity from *in vitro* assays. Low IC_{50} value for AT_1 and ET_A receptor; high IC_{50} value for ET_B receptor and ratio for ET_A : AT_1 IC_{50} value were considered. This was to ensure high selectivity of molecule for AT_1 and ET_A receptor in comparison to ET_B receptor. Since, no literature is available on the ideal ET_A : AT_1 ratio for class of dual action receptor blockers active against both AT_1 and ET_A receptor, molecules with low IC_{50} value for AT_1 and ET_A receptor and with ET_A : AT_1 IC_{50} ratio across the range, were selected for primary screening of activity in this animal model.

Each short listed molecule was screened for its AT_1 and ET_A receptor blocking activity in anesthetized animal model (validation described in *section 4.3.3.4.*) The doses at which the test compounds were screened for activities are mentioned in Table 4 and 5.

For ED_{50} calculation, from the reduction in MBP at each dose of test compounds a cumulative DRC was created by plotting percentage reduction in MBP against the corresponding test dose. A best fit line was constructed and the theoretical dose for 50% reduction in MBP was derived from this equation.

Table 4: Doses of NCEs and reference molecule screened in anesthetized rat model to assess their AT_1 receptor blocking activity.

Sr. No.	Test Compound	Dose 1	Dose 2	Dose 3
		mg/kg/30min		
1	TRC23003	0.010	0.015	0.020
2	TRC23012	0.006	0.009	0.012
3	TRC23017	0.200	0.300	0.400
4	TRC23029	0.022	0.033	0.044
5	TRC23030	0.015	0.025	0.035
6	TRC23031	0.023	0.034	0.045
7	TRC23035	0.015	0.023	0.030
8	BMS346567	0.010	0.015	0.020

Table 5: Doses of NCEs and reference molecule screened in anesthetized rat model to assess their ET_A receptor blocking activity.

Sr. No.	Test Compound	Dose 1	Dose 2	Dose 3
		mg/kg/30min		
1	TRC23003	12.500	25.000	37.500
2	TRC23012	2.368	7.100	23.680
3	TRC23017	12.500	25.000	37.500
4	TRC23029	2.000	6.000	20.000
5	TRC23030	2.000	6.000	20.000
6	TRC23031	2.368	7.100	23.680
7	TRC23035	0.800	2.400	8.000
8	BMS346567	2.368	7.100	23.680

4.3.5. Pharmacokinetic profiling of new chemical entities in rat

Male Sprague Dawley rats of age ranging from 8-10 weeks were used in the study. Rats were divided into total eight groups, each having 3 to 5 rats. Reference compound and NCEs selected for secondary efficacy study, that is, BMS346567, TRC23029, TRC23030 and TRC23035 were screened for their i.v. and oral PK profile in separate groups of animals. For oral PK screening, rats were fasted overnight with free access to water and in morning, rats in first four groups were administered one of the four test compounds at dose of 6 mg/kg by oral gavage. Food was presented to rats three hours after drug administration.

For i.v. PK study, right external jugular vein of rats was cannulated and the cannula was exteriorized subcutaneously from back of neck, which was used for i.v. drug administration and blood sample collection. Rats in different groups (group five to group eight) were administered one of the four test compounds at the dose of 2 mg/kg.

Blood samples were collected sublingually and via cannulated jugular vein from oral and i.v. drug administered groups, respectively. Plasma was separated from samples collected before dosing (0 hr) and at 0.25, 0.5, 1, 2, 4, 6 and 8 hr post drug administration. An additional sample at 0.125 hr was also collected from rats administered drug by i.v. route.

The plasma samples were analyzed using LC-MS/MS in Bioanalytical Department, Torrent Research Centre. PK parameters were calculated using non compartmental model analysis module of WinNonlin (version 5.1). The calibration curve range for TRC23029, TRC23030, TRC23035 and BMS346567 were 5 ng/ml to 2000 ng/ml, 10 ng/ml to 2000 ng/ml, 10 ng/ml to 2000 ng/ml and 25 ng/ml to 10000 ng/ml with $r^2 > 0.99$ respectively.

4.3.6. Efficacy screening of new chemical entities in conscious telemetered salt loaded SHRsp model

Amongst the NCEs screened for activity in primary anesthetized model of Sprague Dawley rats, most potent compounds identified were TRC23029, TRC23030 and TRC23035. Hence these compounds along with reference BMS346567 were chosen for secondary screening for efficacy in conscious telemetered salt loaded SHRsp model.

Male SHRsp rats were used in the study. Rats were prepared for treatment as described under *section 4.3.2.1.* All treatments were carried out in salt loaded SHRsp at dosing volume of 2.5 ml/kg by oral route, once daily. The study followed paired design in which rats were treated with vehicle on day 0 followed by treatment with escalating doses of NCEs every 24 hr without any drug free washout period in between. There were 3-8 rats at each treatment dose.

After treatment with vehicle on day 0, rats were treated with different doses of TRC23029 and TRC23030. After wash out period of around a week, rats were again treated with vehicle followed by TRC23035 and BMS346567. Test compounds TRC23029, TRC23030 and TRC23035 were screened at equimolar doses of 1.55, 13.00, 39.00, 130.00 and 390.00 µmol/kg whereas BMS346567 was screened at dose of 0.85, 7.30, 21.90 and 73.00 µmol/kg. The doses selected for screening were the log incremental doses (second dose onwards); however the first dose was randomly selected to be around 8.6 times less than the second dose with aim to capture even minimal effect, if any, with such a small dose.

Determination of ED_{50} of new chemical entities and reference standard for reduction in mean blood pressure

A cumulative DRC for reduction in MBP for different doses of TRC23029, TRC23030, TRC23035 and BMS346567 was created by plotting percentage reduction in MBP against the corresponding test dose and the ED_{50} dose was derived. A best fit line was constructed and the theoretical doses for 50% reduction in MBP were derived from these equations of best fit line.

4.3.7. *In vivo* safety pharmacology study

S7A guidelines from International Conference on Harmonisation (ICH) suggests requirement to investigate the effects of the test substance on vital functions. In this regard, the cardiovascular, respiratory and central nervous systems

have been considered the vital organ systems and therefore safety of TRC23029 (the most efficacious NCE identified), on these systems has been studied.

The ED_{50} dose for reduction in MBP in the conscious, telemetered salt loaded SHRsp served as a point of reference for various doses used in the safety pharmacology studies. Before administration of compound, appropriate conversion factor based on difference in body surface area (Ghosh, 2005) was applied to achieve rat equivalent dose for guinea pig and mouse.

4.3.7.1. Central nervous system safety study

TRC23029 was screened for its neuropharmacological toxicity potential after oral administration to male Swiss albino mice weighing between 28-32 gm. The experiment was carried out on four groups of which three were test drug groups and the fourth was vehicle control group. Each group comprised of 5 mice. TRC23029 was administered by oral gavage at dose of 6.5, 19.5 and 65 mg/10ml/kg representing rat equivalent ED_{50}, $3xED_{50}$ and $10xED_{50}$ dose for mice. Each mouse, before drug or vehicle administration (0 hr) and at 0.5, 1, 2, 4, 6, 10 and 24 hr after dosing was observed for various neuropharmacological symptoms as described in *Appendix-V* and was scored for the severity or suppression of various neuropharmacological symptoms in the proforma, as shown in *Appendix-VI*.

The scoring was performed on the scale of 0 to 8. The base score for normal signs or effects was 4, scores below 4 were assigned for subnormal responses and those scored above 4 were indicator of supernormal response. Similarly, the base score of 0 was assigned to signs normally absent. Depending upon the severity of sign, score ranging from 1 to 8 were assigned. The final value for each treatment group was finally arrived by averaging values from individual mouse of that group.

4.3.7.2. Cardiovascular and respiratory safety study

Male Duncan Hartley guinea pigs weighing 340-360 gm were used to study the cardiovascular and respiratory effects of TRC23029. The experiment was carried out on four groups, of which three were test drug groups and the fourth was vehicle control group. Each group comprised of 5 guinea pigs.

TRC23029 was administered by oral gavage at dose of 4, 12 and 40 mg/5ml/kg representing rat equivalent ED_{50}, $3xED_{50}$ and $10xED_{50}$ dose for guinea pig. 5 min after drug administration, guinea pigs were anesthetized with intraperitoneal injection of urethane (1.25 gm/kg) and standard limb lead II ECG was recorded continuously in these animals for 8 hr using BIO Amp connected to MacLab

8/s. The heart rate was calculated as a derived parameter from the lead-II ECG. Respiration rate was also captured by positioning pulse transducer beneath the guinea pig. The pulse transducer attached to MacLab 8/s acquired the rhythmic thoracic movement resulting due to inspiration-expiration during respiratory cycle. The ECG and respiration data was analyzed using Chart Pro (version 5.4.2) software to obtain heart rate, corrected QT (QTc) interval and respiration rate. QTc interval was calculated by Bazett's method as it is the most appropriate method suggested for calculating QTc interval from anesthetized guinea pig (Hamlin, et al., 2003). All the parameters were calculated at 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7 and 8 hr post administration of TRC23029.

Statistical Analysis

Data in text, tabulated data and data expressed graphically has been expressed as Mean ± SEM. Between groups comparison for repeat observations over the period of time has been done using repeated measures analysis of variance (RMANOVA). Difference between means of any two groups for data has been assessed by student's *t*-test. Statistical analysis has been performed using statistical analysis system (SAS, Version-9.1) and GraphPad Prism (version 3.0). Values exceeding the 95% critical limits ($p \leq 0.05\%$) are considered to be statistically significant. For determining the ED_{50} or ED_{75} dose of a compound, vehicle effect was subtracted from the effect of test compound to obtain net drug effect. This data was submitted to regression analysis to obtain the ED_{50} or ED_{75} of test compounds.

Results

5. Results

5.1. *In vitro* studies

5.1.1. *In vitro* screening of new chemical entities for determination of their IC_{50} value for AT_1, ET_A and ET_B receptors

5.1.2. *In vitro* physico-chemical properties and drug metabolism studies of new chemical entities

5.2. *In vivo* studies

5.2.1. Development of conscious telemetered disease model (for proof of concept studies: evaluating the hypothesis of potentiation in antihypertensive efficacy by combining two targets and screening new chemical entities for their antihypertensive efficacy)

5.2.1.1. Surgical implantation of telemetry transmitter in rat

5.2.1.2. Salt load tolerability and its effect on mean blood pressure of rat

5.2.2. Proof of concept study: To study whether there is potentiation of antihypertensive activity of AT_1 receptor blocker by addition of small dose of ET_A receptor blocker in conscious telemetered salt loaded SHRsp model

5.2.2.1. To determine effect on mean blood pressure upon adding small dose of ET_A receptor blocker ZD1611 to sub-maximally effective dose of AT_1 receptor blocker losartan

5.2.2.2. To determine effect on mean blood pressure upon adding small dose of ET_A receptor blocker ZD4054 to maximally effective dose of AT_1 receptor blocker candesartan

5.2.3. Development of anesthetized rat model for screening new chemical entities for their AT_1 and ET_A receptor blocking activity

5.2.3.1. To standardize type and dose of anesthetic to be used for arterial blood pressure monitoring

5.2.3.2. To standardize infusion dose of Ang II to obtain a stable pressor effect

5.2.3.3. Blocking endogenous AT_1 receptors for isolating ET_A receptor blocker activity

5.2.3.4. Validation of anesthetized rat model for screening AT_1 and ET_A receptor blocking activity of new chemical entities

5.2.4. Screening of new chemical entities for pharmacological activity in anesthetized rat model

5.2.5. Pharmacokinetic profiling of new chemical entities in rat

5.2.6. Efficacy screening of new chemical entities in conscious telemetered salt loaded SHRsp model

5.2.7. *In vivo* safety pharmacology study

5.2.7.1. Central nervous system safety study

5.2.7.2. Cardiovascular and respiratory safety study

5.1. *In vitro* studies

5.1.1. *In vitro* screening of new chemical entities for determination of their IC_{50} value for AT_1, ET_A and ET_B receptors

All the 50 molecules synthesized by the Medicinal Chemistry Department of Torrent Research Centre including the reference molecule BMS346567, were screened *in vitro* for their ability to inhibit AT_1, ET_A and ET_B receptors. Results of this *in vitro* assay established the IC_{50} value of these NCEs and reference molecule. These assays were performed by AstraZeneca at their Research Facility in Sweden. Results of these assays are presented in the Table 6.

IC_{50} values for NCEs ranged from 0.003 - >40 nM, 0.158 - >40 nM and 0.007 - >40 nM for AT_1, ET_A and ET_B receptors respectively. A ratio of IC_{50} values for ET_A: AT_1 receptor was computed to assess relative ET_A to AT_1 receptor blocking component in the molecules (Table 6).

Table 6: IC_{50} values for NCEs and reference molecule obtained from *in vitro* assay.

Sr. No.	Test Compound	AT_1 receptor IC_{50} (nM)	ET_A receptor IC_{50} (nM)	ET_B receptor IC_{50} (nM)	ET_A:AT_1 IC_{50}
1	TRC23001	0.042	0.421	22.700	9.953
2	TRC23002	1.740	0.381	8.970	0.219
3	TRC23003	0.019	2.650	16.600	140.212
4	TRC23004	0.125	2.920	16.700	23.360
5	TRC23005	0.032	48.600	17.300	1500.000
6	TRC23006	0.202	35.400	23.200	175.248
7	TRC23007	3.150	10.100	14.800	3.206
8	TRC23008	13.700	0.462	0.007	0.034
9	TRC23009	0.143	> 40	> 40	NA
10	TRC23010	0.029	6.200	14.200	210.884
11	TRC23011	0.007	6.480	> 40	920.455
12	TRC23012	0.023	0.562	25.300	24.541
13	TRC23013	0.025	0.814	11.500	33.089
14	TRC23014	0.109	> 40	> 40	NA
15	TRC23015	0.099	10.300	10.200	104.569
16	TRC23016	0.021	2.870	4.440	137.981
17	TRC23017	0.013	0.487	6.700	36.617
18	TRC23018	21.800	> 40	> 40	NA
19	TRC23019	> 40	0.500	19.400	NA
20	TRC23020	39.600	> 40	> 40	NA
21	TRC23021	0.763	19.200	> 40	25.164
22	TRC23022	> 40	27.800	> 40	NA
23	TRC23023	0.010	1.780	> 40	182.377
24	TRC23024	11.900	1.230	> 40	0.103
25	TRC23025	0.034	0.740	> 40	21.958
26	TRC23026	15.200	7.080	> 40	0.466
27	TRC23027	1.160	2.720	> 40	2.345
28	TRC23028	> 40	14.800	> 40	NA
29	TRC23029	0.006	0.173	18.000	26.947
30	TRC23030	0.003	0.158	11.500	53.378
31	TRC23031	0.011	0.503	37.100	44.123
32	TRC23032	0.006	2.920	> 40	530.909
33	TRC23033	0.004	1.330	> 40	367.403
34	TRC23034	0.008	1.430	5.870	179.198
35	TRC23035	0.003	0.287	8.030	93.182
36	TRC23036	16.600	36.300	> 40	2.187
37	TRC23037	> 40	> 40	> 40	NA
38	TRC23038	0.061	30.300	> 40	495.908
39	TRC23039	> 40	> 40	> 40	NA
40	TRC23040	9.570	> 40	> 40	NA
41	TRC23041	12.800	> 40	> 40	NA
42	TRC23042	1.350	39.300	26.500	29.111
43	TRC23043	1.170	22.400	> 40	19.145
44	TRC23044	0.300	> 40	> 40	NA
45	TRC23045	6.424	12.048	> 40	1.875
46	TRC23046	0.097	20.026	> 40	206.031
47	TRC23047	0.040	> 40	> 40	NA
48	TRC23048	1.409	13.403	> 40	9.513
49	TRC23049	0.067	> 40	> 40	NA
50	TRC23050	0.255	22.026	> 40	86.282
51	BMS346567	0.013	0.016	25.400	1.195

NA, not applicable

5.1.2. *In vitro* physico-chemical properties and drug metabolism studies of new chemical entities

Seven NCEs along with reference molecule BMS346567 were identified for further *in vitro* screening of physico-chemical properties. This selection was based on their low IC_{50} value for AT_1 and ET_A receptor; high IC_{50} value for ET_B receptor and ratio for ET_A : AT_1 IC_{50} values. All the seven NCEs were found to be soluble in 1% DMSO in range of 13.42 to 102.40 µg/ml, highly permeable through PAMPA, highly plasma protein bound (Table 7) and moderate to highly metabolizing (Di et al, 2004) in male rat liver microsomes (Table 8).

Table 7: *In vitro* solubility, permeability and plasma protein binding profile of NCEs and reference molecule.

Sr. No.	Test Compound	Solubility (µg/ml)	Permeability		Plasma protein binding (%)
			Log P_e	Grade	
1	TRC23003	102.40	-3.39	High	99.85
2	TRC23012	42.95	-4.54	High	98.90
3	TRC23017	13.42	-4.35	High	99.81
4	TRC23029	17.18	-3.91	High	99.85
5	TRC23030	41.94	-4.05	High	99.84
6	TRC23031	49.00	-5.09	High	99.85
7	TRC23035	89.75	-4.17	High	99.50
8	BMS346567	400.00	-4.31	High	99.57

Permeability classification; high >-5.5; moderate -5.5 to -7.0; low <-7.0

Table 8: *In vitro* metabolism profile of NCEs and reference molecule.

Sr. No.	Test Compound	*in vitro* drug metabolism		
		Cl_{int} (µl/min/mg protein)	$t_{1/2}$ (mins)	Grade
1	TRC23003	8.47	81.83	Moderate
2	TRC23012	139.66	4.96	High
3	TRC23017	17.52	39.54	Moderate
4	TRC23029	26.99	25.68	Moderate
5	TRC23030	27.65	25.06	Moderate
6	TRC23031	24.84	27.89	Moderate
7	TRC23035	16.57	41.81	Moderate
8	BMS346567	15.50	44.71	Moderate

Clearance classification; high >70; moderate 7-70; low <7

5.2. *In vivo* studies

5.2.1 Development of conscious telemetered disease model (for proof of concept studies: evaluating the hypothesis of potentiation in antihypertensive efficacy by combining two targets and screening new chemical entities for their antihypertensive efficacy)

5.2.1.1. Surgical implantation of telemetry transmitter in rat

The technique of telemetry transmitter implantation and data acquisition was successfully standardized. The surgical implantation technique was practiced to result in 70 – 90% success in telemetry transmitter implanted rats recovering from surgery. The data acquisition and analysis software was adapted for acquiring and analyzing data from forthcoming telemetry studies.

5.2.1.2. Salt load tolerability and its effect on mean blood pressure of rat

Male SHR in group 1 and 2 were able to tolerate salt load of 0.5% and 1% in drinking water respectively, with no signs of ill health. However, group 3 rats who were initiated with 2% salt in drinking water presented symptoms of intolerability as piloerection, decreased activity and sick appearance. Rats were able to tolerate gradual increment in salt load from 0.5% to 1% and then to 2%, each for 7 days, in group 1 and from 1% to 2% for 7 days in group 2 and showed no symptoms of ill health.

Based on the observation that SHR could tolerate gradual increment in salt load when increased from 1% to 2% over a week's duration, 1% salt was selected to initiate salt load in SHRsp implanted with telemetry transmitters. Continuous salt load of 1% for 13 days produced 21 mmHg rise in MBP (from 109.5 ± 0.5 mmHg to 130.5 ± 4 mmHg). Upon increasing salt load to 2% for next 6 days caused further rise of 27 mmHg (from 130.5 ± 4 mmHg to 157.5 ± 7.1 mmHg) (Figure 4). Though, the animals attained desired MBP while on 2% salt load, effect of 4% salt load on MBP was further explored. MBP of animals increased with increase in salt load to 4%; however their health started deteriorating. One rat was sacrificed after two days of 4% salt initiation and the other died on seventh day of 4% salt load.

Thus for all further telemetered SHRsp animal studies, it was decided to initiate salt load with 1% salt in drinking water and shift animals to 2% salt load after a weeks duration for rest of the study.

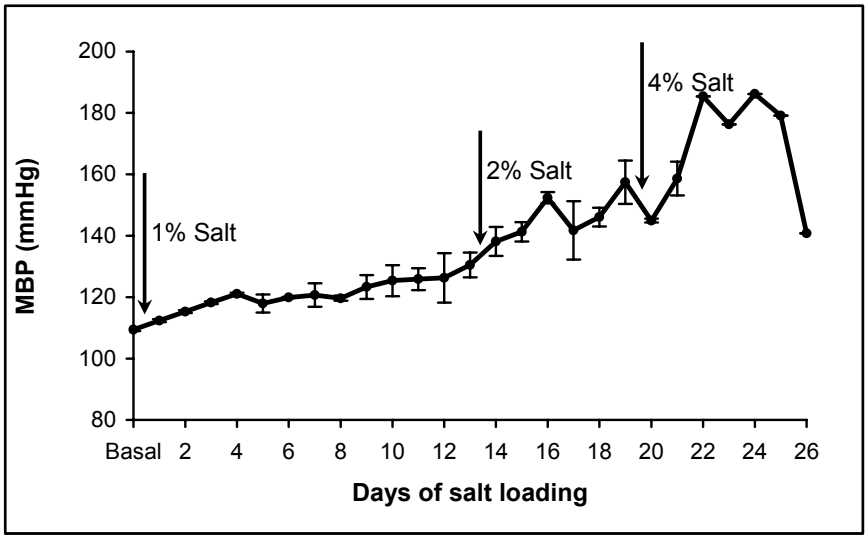


Figure 4: Effect of salt load on MBP profile of SHRsp. Value represented is mean of 2 observations up to day 21. Values from day 22 are from single animal. The error bars represent ±SEM. Salt, sodium chloride; MBP, mean blood pressure.

5.2.2. Proof of concept study: To study whether there is potentiation of antihypertensive activity of AT_1 receptor blocker by addition of small dose of ET_A receptor blocker in conscious telemetered salt loaded SHRsp model

5.2.2.1. To determine effect on mean blood pressure upon adding small dose of ET_A receptor blocker ZD1611 to sub-maximally effective dose of AT_1 receptor blocker losartan

Determination of ED_{75} dose of losartan and minimal effective dose of ZD1611

The maximum percent fall in MBP from baseline was increased dose dependently with losartan after taking into account the effect of vehicle. The duration of antihypertensive effect as reflected by the area under curve (AUC) of the fall in MBP from baseline for 18 hr, showed a similar trend (Figure 5 and 6). The highest dose of 25 mg/kg did not show further increase in the response as compared to 10 mg/kg and was not considered for computing the ED_{75}. The ED_{75} for losartan was found to be 5 mg/kg.

ZD1611 administered alone also showed a dose dependent increase in the maximum percent fall from baseline in MBP as well as AUC of the fall in MBP from baseline for 18 hr after taking into account the effect of vehicle (Figure 7 and 8). Though ZD1611 at dose of 0.1 and 0.3 mg/kg produced no significant difference in percentage change in MBP in comparison to vehicle (by RMANOVA), effect of ZD1611 at dose of 0.3 mg/kg produced more prominent fall with more time points

showing significant difference from vehicle as calculated by Student's *t* test. Thus, ZD1611 at dose of 0.3 mg/kg was selected as minimal effective dose of ZD1611 to be used in combination with losartan ED_{75} dose (5 mg/kg) in potentiation study.

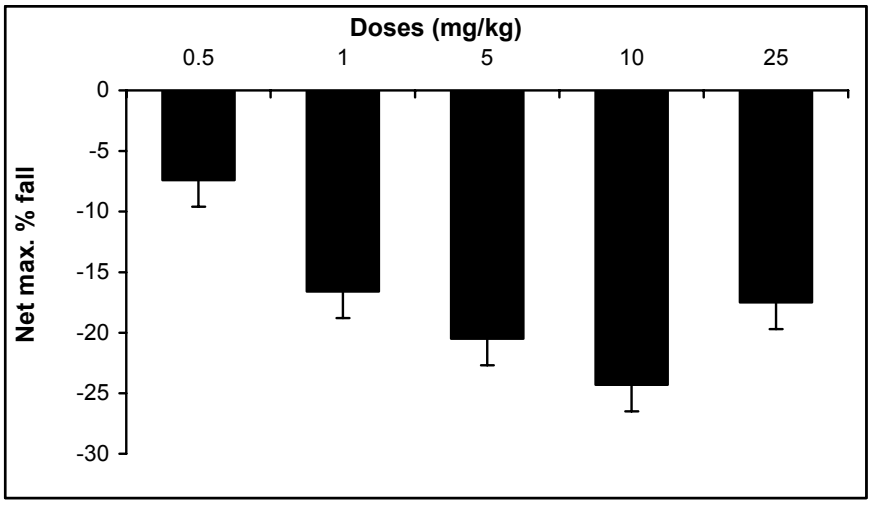


Figure 5: Net maximum percent fall in MBP by different doses of losartan (0.5, 1, 5, 10 and 25 mg/kg, p.o.) in salt loaded SHRsp. Values represented are mean of 6-7 observations. The error bars represent SEM. Max., maximum; MBP, mean blood pressure.

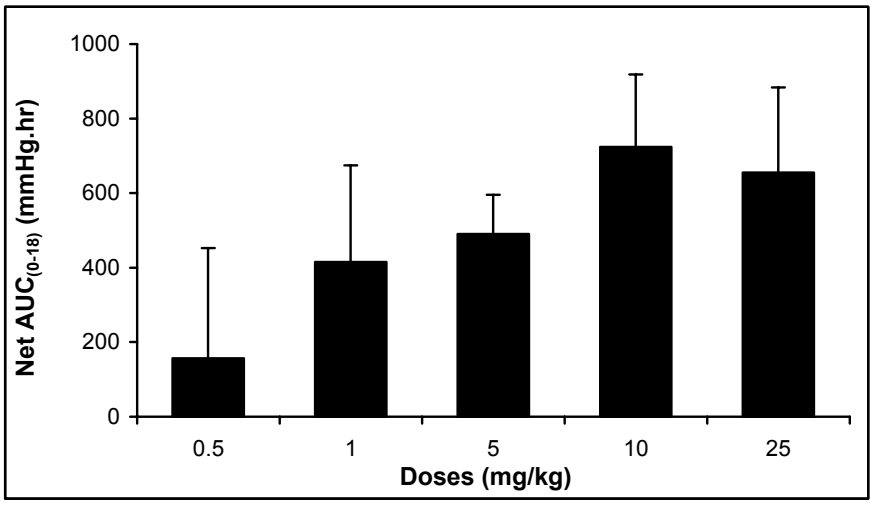


Figure 6: Net AUC of MBP calculated for 18 hr post oral administration of different doses of losartan (0.5, 1, 5, 10 and 25 mg/kg) in salt loaded SHRsp. Values represented are mean of 6-7 observations. The error bars represent SEM. AUC, area under the curve; MBP, mean blood pressure.

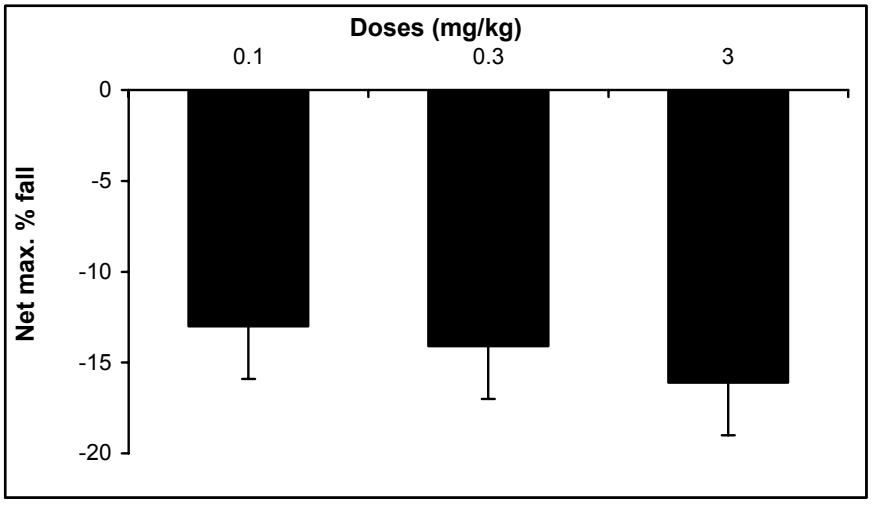


Figure 7: Net maximum percent fall in MBP by different doses of ZD1611 (0.1, 0.3 and 3 mg/kg, p.o) in salt loaded SHRsp. Values represented are mean of 6-7 observations. The error bars represent SEM. Max., maximum; MBP, mean blood pressure.

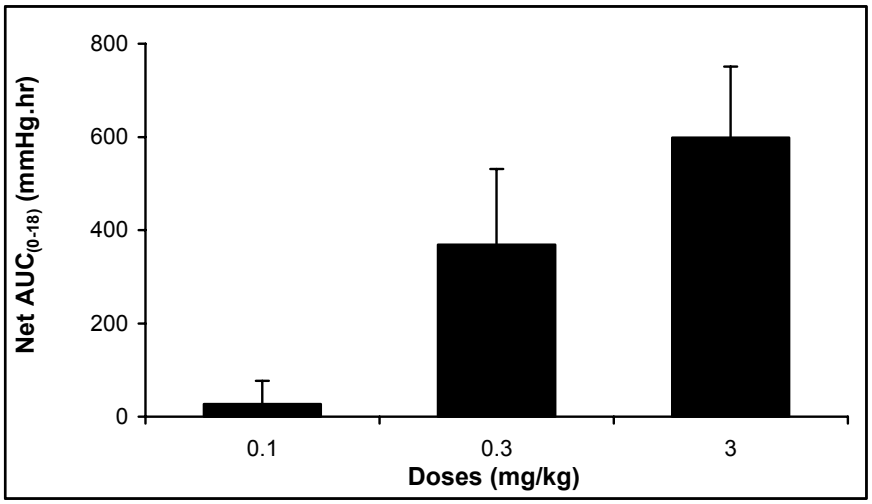


Figure 8: Net AUC of MBP calculated for 18 hr post oral administration of different doses of ZD1611 (0.1, 0.3 and 3 mg/kg) in salt loaded SHRsp. Values represented are mean of 6-7 observations. The error bars represent SEM. AUC, area under the curve; MBP, mean blood pressure.

Potentiation study by combining ED_{75} dose of losartan and minimally effective antihypertensive dose of ZD1611

In the disease model, the evaluation of proof of concept revealed that addition of the minimally effective dose of ZD1611 (0.3 mg/kg) to losartan at ED_{75} dose (5 mg/kg) produced a further fall in MBP, which was significantly more compared to either drugs administered alone (Figure 9). This reduction was in both the maximum effect and in duration of effect as exemplified from the AUC for the net fall in MBP for 18 hr of the combination, compared to either losartan or ZD1611 administered alone (Figure 10).

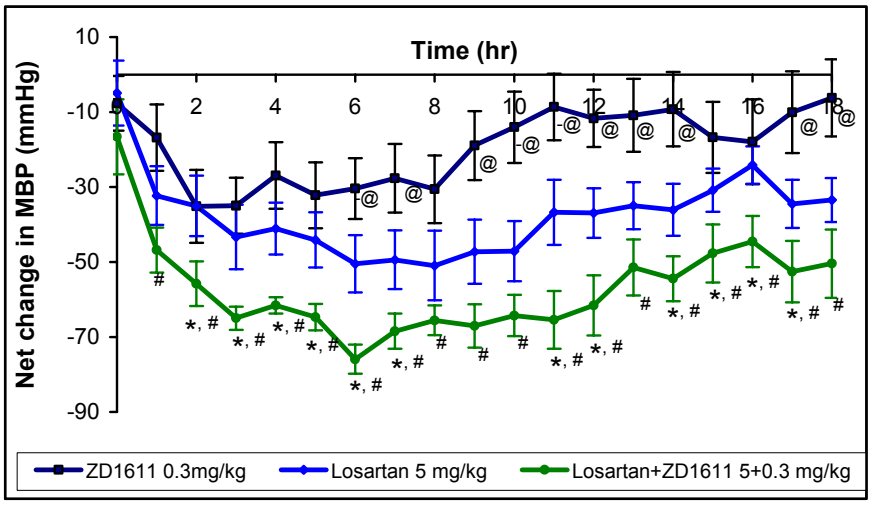


Figure 9: Net change in MBP produced by oral administration of losartan 5 mg/kg, ZD1611 0.3 mg/kg alone and by combination dose of losartan and ZD1611 (5 and 0.3 mg/kg, respectively) in salt loaded SHRsp. Values represented are mean of 6-10 observations. The error bars represents ±SEM. *p<0.05 combination compared to losartan 5 mg/kg; #p<0.05 combination compared to ZD1611 0.3 mg/kg alone; @p<0.05 losartan 5 mg/kg compared to ZD1611 0.3 mg/kg by Student's *t* test. RMANOVA over 18 hr is significantly different for all three treatments. MBP, mean blood pressure.

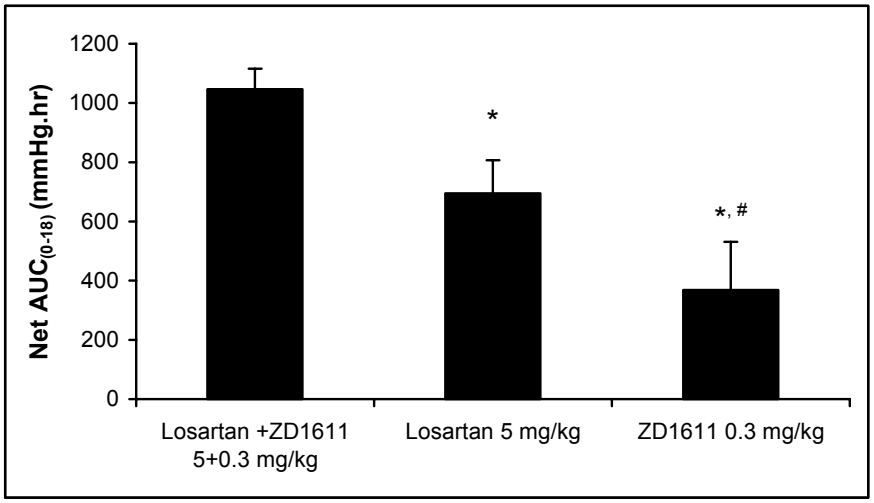


Figure 10: Net AUC of MBP calculated for 18 hr post oral administration of ZD1611 0.3 mg/kg, losartan 5 mg/kg alone or combination dose of losartan and ZD1611 (5 and 0.3 mg/kg, respectively) in salt loaded SHRsp. Values represented are mean of 6-10 observations. The error bars represent SEM. *p<0.05 combination compared to of losartan and ZD1611 alone (5 and 0.3 mg/kg, respectively); #p=0.05 losartan 5 mg/kg compared to ZD1611 0.3 mg/kg by Student's *t* test. AUC, area under the curve; MBP, mean blood pressure.

Pharmacokinetic evaluation of losartan administered alone and of losartan and ZD1611 when administered in combination

The PK studies were undertaken to support our hypothesis that combination of an AT_1 and ET_A receptor blocker would have antihypertensive effect which would be greater than that produced by sub-maximal dose of AT_1 receptor blocker alone. We intended to examine whether the observed potentiation in MBP lowering is result of pharmacological potentiation and not due to increased availability of AT_1 receptor blocker in presence of ET_A receptor blockers. Further, with available evidences demonstrating teratogenic potential of ET_A receptor blockers at high plasma concentration; we wanted to ascertain whether the plasma concentration of ET_A receptor blocker, when administered at minimally effective antihypertensive dose, is still below the levels capable of causing teratogenic effect of such therapy while still imparting a potentiating effect on AT_1 receptor blocker's antihypertensive effect.

The plasma drug concentration-time profile of losartan and ZD1611 administered in combination and when losartan was administered alone is shown in Figure 11. The PK parameters computed from plasma drug concentration-time data are shown in Table 9. Losartan showed slight increase in C_{max} and $AUC_{(0-8)}$ when co-administered with ZD1611 as compared to that observed when given alone. However, the differences were not statistically significant. Given that magnitude of

potentiation observed, it is unlikely to be the reason underlying potentiation in the fall in MBP observed when given in combination with ZD1611. The maximum concentration of ZD1611 at the potentiation dose of 0.3 mg/kg was substantially below the C_{max} of 80000 ng/ml reported to be associated with teratogenic potential in rats (unpublished data on file: AstraZeneca).

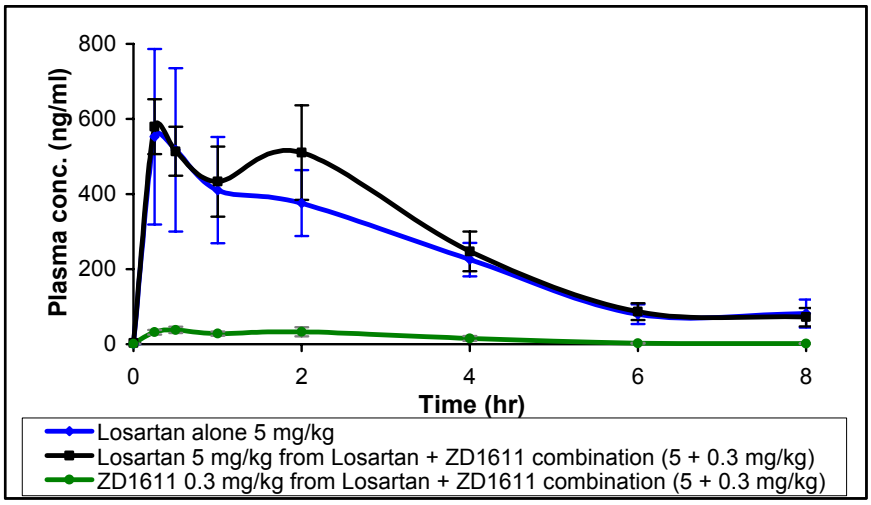


Figure 11: Plasma drug concentration-time curve upon oral administration of losartan 5 mg/kg alone and of losartan 5 mg/kg and ZD1611 0.3 mg/kg when administered in combination in salt loaded SHRsp. Values represented are mean of 6-7 observations. The error bars represent ±SEM. Conc., concentration.

Table 9: Pharmacokinetic parameters of losartan administered alone and of losartan and ZD1611 when administered in combination in salt loaded SHRsp.

Pharmacokinetic parameters	Losartan 5 mg/kg	Losartan 5 mg/kg from Losartan + ZD1611 combination	ZD1611 0.3 mg/kg from Losartan + ZD1611 combination
C_{max} (ng/ml)	603.83 ± 221.26	661.63 ± 102.91	44.84 ± 9.87
T_{max} (hr)	0.58 ± 0.29	0.79 ± 0.32	0.82 ± 0.22
$t_{1/2}$ (hr)	3.97 ± 1.72	3.05 ± 1.19	2.09 ± 0.43
$AUC_{(0-8)}$ (ng.hr/ml)	1827.50 ±404.88	2082.15 ± 414.00	117.58 ± 31.64
$AUC_{(0-\infty)}$ (ng.hr/ml)	2757.12 ± 796.50	2479.81 ± 495.64	137.09 ± 33.75

Values represented are mean ±SEM for 6-7 observations in each group. $p>0.05$ for all parameters of losartan when administered in combination compared to losartan 5 mg/kg administered alone by Student's *t* test.

AUC, area under the curve; C_{max}, maximum plasma concentration; $t_{1/2}$, half life; T_{max}, time to reach C_{max}

5.2.2.2. To determine effect on mean blood pressure upon adding small dose of ET_A receptor blocker ZD4054 to maximally effective dose of AT_1 receptor blocker candesartan

Effect on MBP upon repeated administration of candesartan

Salt loaded SHRsp were administered candesartan orally, at dose of 10 mg/kg (the maximally effective dose), once daily for 8 days with aim to achieve maximum possible reduction in MBP due to AT_1 receptor blockade. Candesartan at dose of 10 mg/kg resulted into fall in MBP on day 1 which was not different from fall observed after 8 days of repeated administration after considering the effect of vehicle (Figure 12).

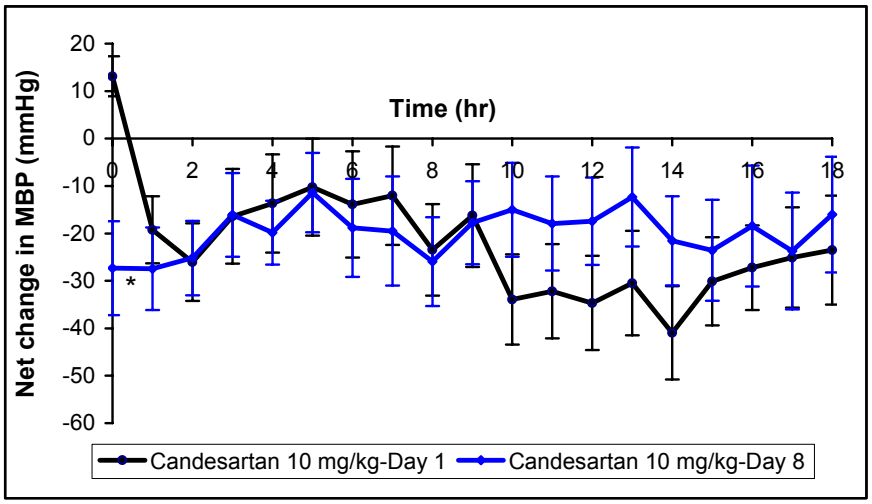


Figure 12: Net change in MBP produced upon oral administration of candesartan 10 mg/kg on day 1 and after repeat administration on day 8 in salt loaded SHRsp. Values represented are mean of 8 observations. The error bars represent ±SEM. *p<0.05 compared to day 1 losartan treatment by Student's *t* test. MBP, mean blood pressure.

Effect on MBP upon repeated administration of ZD4054

After oral administration of 0.3 mg/kg ZD4054 alone for 3 days, no reduction in MBP was observed in comparison to vehicle treatment. However, after administration of 1 mg/kg ZD4054, reduction in MBP was observed which persisted till 12 hr after dosing. ZD4054 at dose of 3 mg/kg demonstrated MBP lowering activity which persisted for even longer duration than observed with ZD4054 1 mg/kg. This reduction was evident in both the maximum effect and in duration of effect as exemplified from the AUC of the net fall in MBP for 18 hr (Figure 13 and 14).

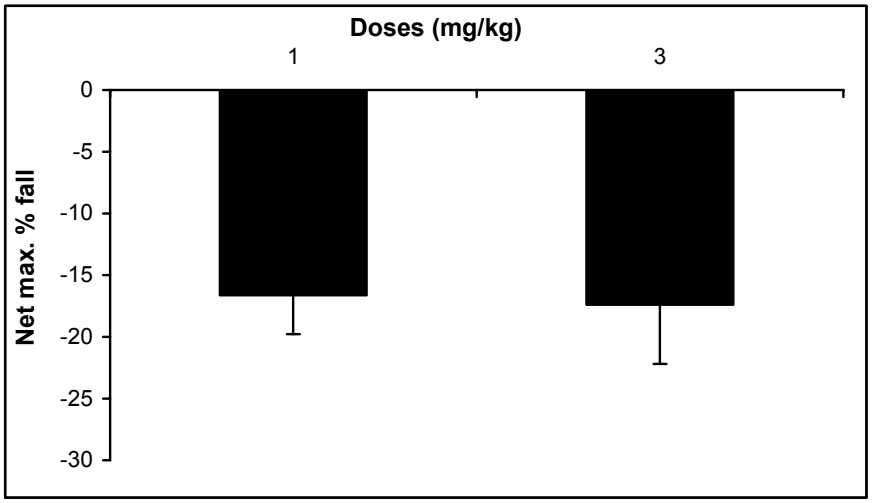


Figure 13: Net maximum percent fall in MBP by different doses of ZD4054 (1 and 3 mg/kg, p.o) in salt loaded SHRsp. Values represented are mean of 4-5 observations. The error bars represent SEM. Max., maximum; MBP, mean blood pressure.

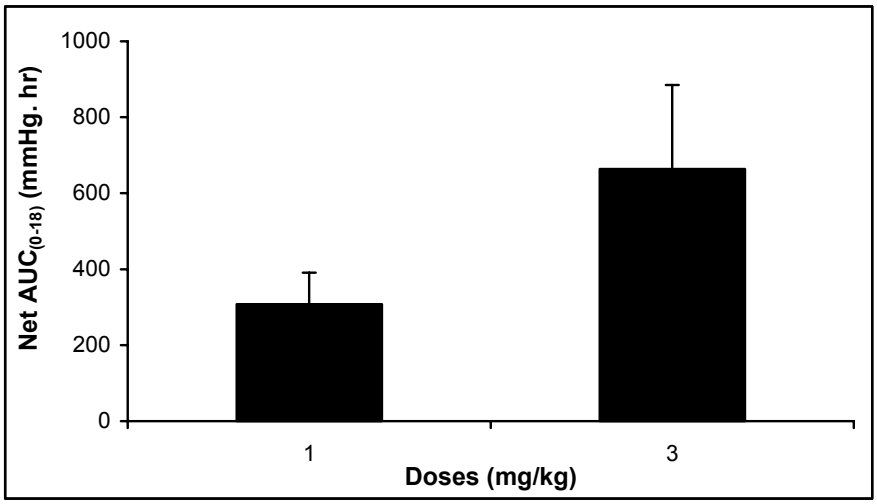


Figure 14: Net AUC of MBP calculated for 18 hr post oral administration of different doses of ZD4054 (1 and 3 mg/kg) in salt loaded SHRsp. Values represented are mean of 4-5 observations. The error bars represent SEM. AUC, area under the curve; MBP, mean blood pressure.

Potentiation study by combining maximum effective antihypertensive dose of candesartan with minimally effective antihypertensive doses of ZD4054

Salt loaded SHRsp were administered candesartan alone at dose of 10 mg/kg, p.o., which is the maximally effective antihypertensive dose, once daily for 8 days with aim to achieve maximum possible reduction of MBP due to AT_1 receptor blockade. Adding ZD4054 at dose of either 1 mg/kg or 3 mg/kg to candesartan 10 mg/kg from day 9 to day 11 potentiated the maximal antihypertensive effect of candesartan as evaluated on the third day of combination administration (Figure 15 and 17). There was a potentiation in maximum fall as well as in duration of effect by combination, as exemplified from AUC of the net fall in MBP for 18 hr (Figure 16 and 18).

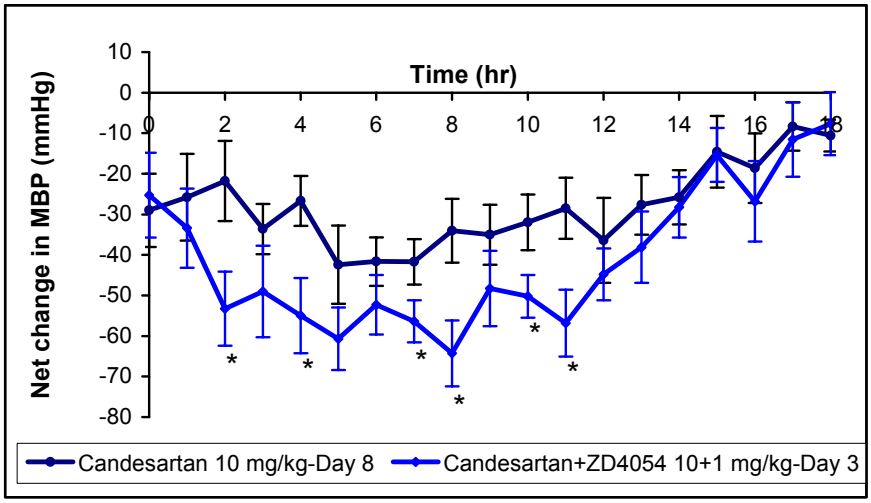


Figure 15: Net change in MBP produced on day 8, upon repeated once daily oral administration of candesartan 10 mg/kg and net change in MBP observed on day 3 of combining ZD4054 at a dose of 1 mg/kg to candesartan 10 mg/kg from day 9 to day 11 in salt loaded SHRsp. Values represented are mean of 7 observations. The error bars represent ±SEM. *p<0.05 combination compared to candesartan 10 mg/kg by Student's *t* test. MBP, mean blood pressure.

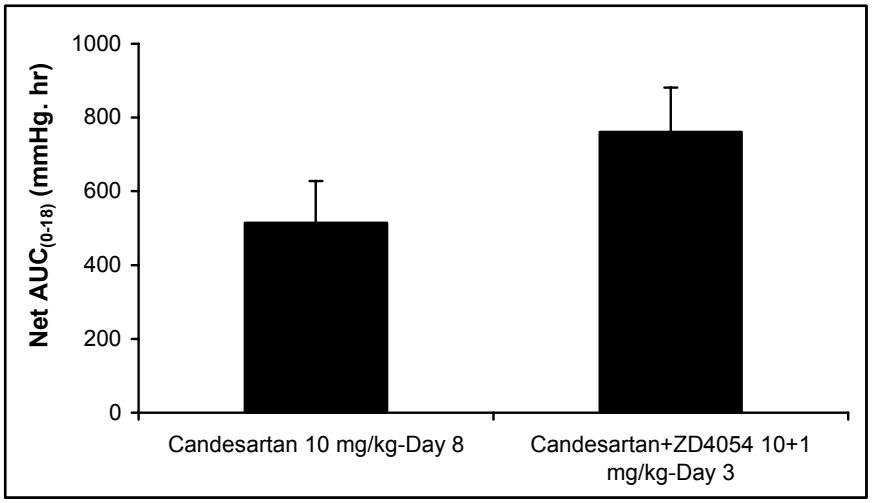


Figure 16: Net AUC of MBP calculated for 18 hr on day 8 upon repeated once daily oral administration of candesartan 10 mg/kg and net AUC of MBP calculated for 18 hr on day 3 of combining ZD4054 at a dose of 1 mg/kg to candesartan 10 mg/kg from day 9 to day 11 in salt loaded SHRsp. Values represented are mean of 7 observations. The error bars represent SEM. p>0.05 combination compared to candesartan 10 mg/kg by Student's *t* test. AUC, area under the curve; MBP, mean blood pressure.

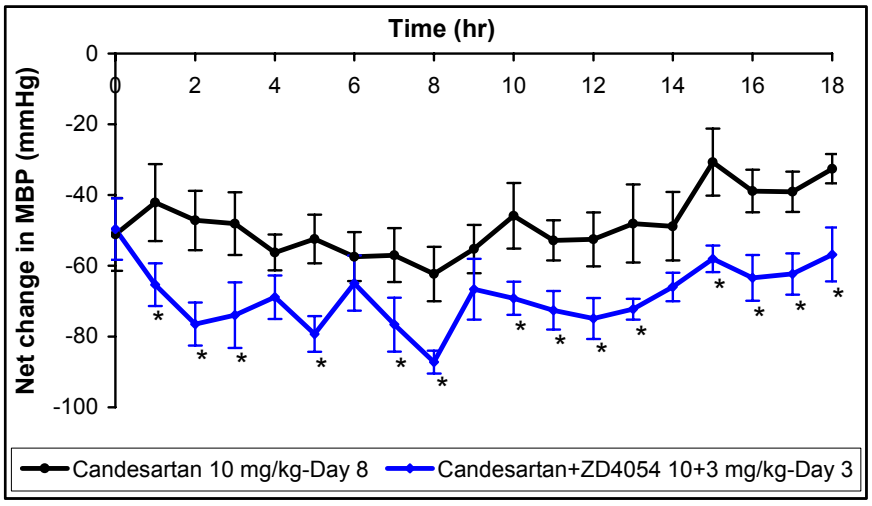


Figure 17: Net change in MBP produced on day 8 upon repeated once daily oral administration of candesartan 10 mg/kg and net change in MBP observed on day 3 of combining ZD4054 at a dose of 3 mg/kg to candesartan 10 mg/kg from day 9 to day 11 in salt loaded SHRsp. Values represented are mean of 5 observations. The error bars represent ±SEM. *p<0.05 combination compared to candesartan 10 mg/kg by Student's *t* test. RMANOVA over 18 hr is significantly different. MBP, mean blood pressure.

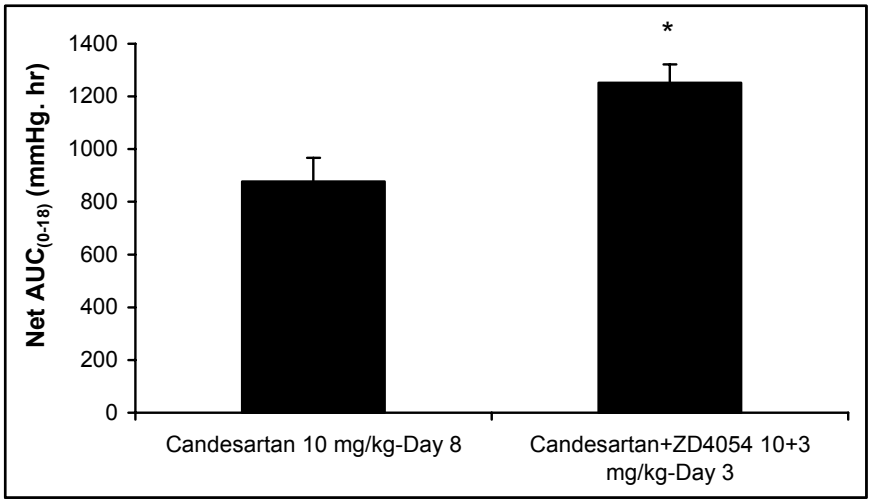


Figure 18: Net AUC of MBP calculated for 18 hr on day 8 upon repeated once daily oral administration of candesartan 10 mg/kg and net AUC of MBP calculated for 18 hr on day 3 of combining ZD4054 at a dose of 3 mg/kg to candesartan 10 mg/kg from day 9 to day 11 in salt loaded SHRsp. Values represented are mean of 5 observations. The error bars represent SEM. *p<0.05 combination compared to candesartan 10 mg/kg alone by Student's *t* test. AUC, area under the curve; MBP, mean blood pressure.

Pharmacokinetic evaluation of candesartan and ZD4054 administered alone and in combination

The PK studies were undertaken to support our hypothesis that combination of an AT_1 and ET_A receptor blocker would have antihypertensive effect which would be greater than that produced by even the maximum effective dose of AT_1 receptor blocker alone. We wanted to examine whether the observed potentiation in MBP lowering is result of pharmacological potentiation and not due to increased availability of AT_1 receptor blocker in presence of ET_A receptor blockers. Further, with available evidences demonstrating toxic potential of ET_A receptor blockers at high plasma concentration; we wanted to ascertain whether the plasma concentration of ET_A receptor blocker, when administered at minimally effective antihypertensive dose, is still below the levels capable of eliciting toxic effect of such therapy while still imparting a potentiating effect on AT_1 receptor blocker's antihypertensive effect.

With the aim to explore if repeated administration of candesartan 10 mg/kg for eight days causes any changes in its plasma drug concentration-time profile, plasma drug profiling for candesartan was performed after its first and eighth day administration in salt loaded SHRsp. Candesartan did not show any difference in exposure levels achieved between single and eight day repeated dosing (Figure 19; Table 10).

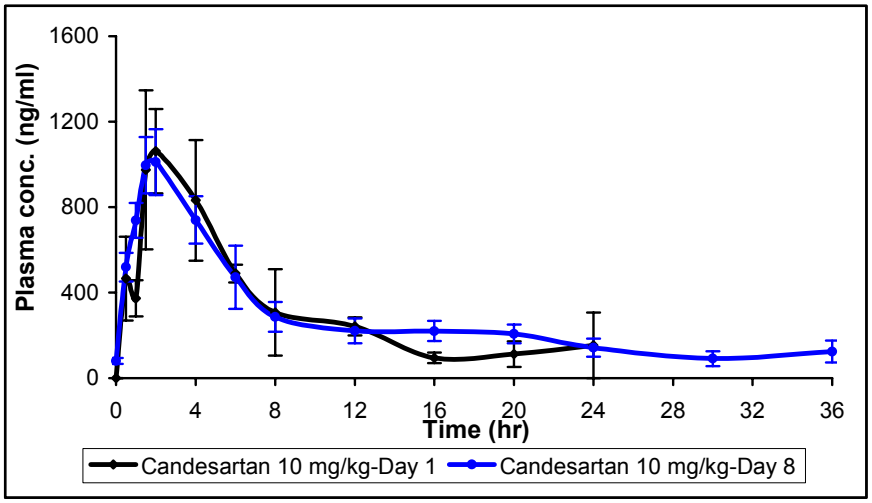


Figure 19: Plasma concentration-time curve upon oral administration of candesartan 10 mg/kg on day 1 and after repeat administration on day 8 in salt loaded SHRsp. Values represented are mean of 3-7 observations. The error bars represent ±SEM. Conc., concentration.

The plasma drug concentration-time profile of candesartan on day 8 of its repeat once daily oral administration and its plasma drug concentration profile on day 11 of repeated administration, when, ZD4054 at dose of either 1 or 3 mg/kg was added to it from day 9 to day11, is shown in Figure 20. The PK parameters computed from plasma concentration-time profile are shown in Table 10. No change in exposure of candesartan was observed when administered alone or in combination with different doses of ZD4054.

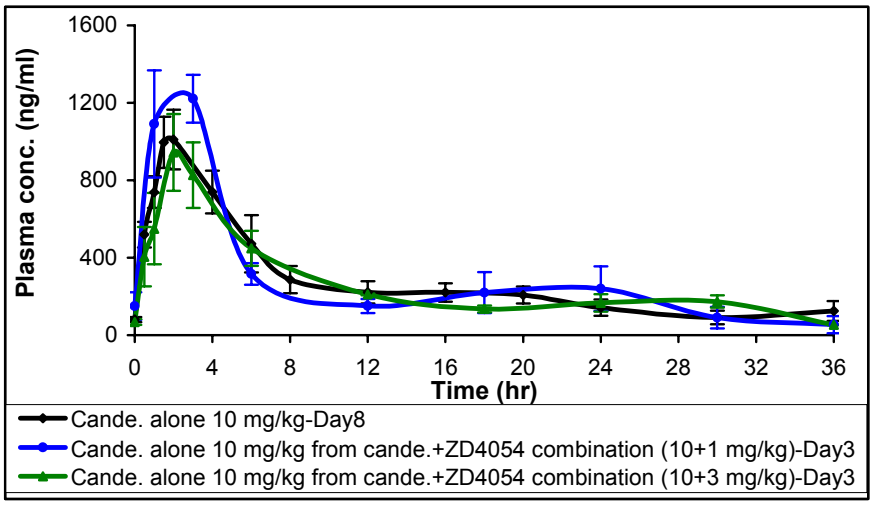


Figure 20: Plasma concentration-time curve on day 8 of repeated once daily oral administration of candesartan 10 mg/kg and plasma concentration-time curve observed on day 3 of combining ZD4054 at a dose of either 1 mg/kg or 3 mg/kg to candesartan 10 mg/kg from day 9 to day 11 in salt loaded SHRsp. Values represented are mean of 3-7 observations. The error bars represent ±SEM. Conc., concentration.

The PK profile of ZD4054 1 mg/kg and 3 mg/kg were computed from its third day plasma drug concentration-time profile when administered alone for three subsequent days. The PK profile of ZD4054 1 mg/kg and 3 mg/kg was also computed on day 3 of its repeat once daily administration in combination with candesartan 10 mg/kg, from rats being previously treated with candesartan 10 mg/kg alone, once daily, for last 8 days. (Figure 21, 22; Table 11). The results revealed a dose related increase in plasma exposure of ZD4054 at dose of 1 mg/kg and 3 mg/kg. Also, the maximum concentration of ZD4054 at the potentiation dose of 1 mg/kg as well as the next higher dose tested (3 mg/kg) were substantially below the C_{max} of 8900 ng/ml reported to be associated with moderate seminiferous tubular atrophy in rats (unpublished data on file: AstraZeneca) thus endorsing the safety of this combination at doses explored. However, the total exposure to ZD4054 was reduced when administered in combination and a significant reduction in C_{max} and $AUC_{(0-\infty)}$ was observed with ZD4054 1 mg/kg when administered in combination with candesartan 10 mg/kg. Since the two drugs do not share common metabolic pathways and candesartan is not known to induce any metabolic enzymes and is not significantly metabolized by the cytochrome P450 system, a metabolic interaction is unlikely with candesartan (AstraZeneca, 2007). One possibility could be candesartan mediated vasodilatation in renal arterioles (Duke, et al., 2005; Lansang, et al., 2000) facilitating excretion of ZD4054.

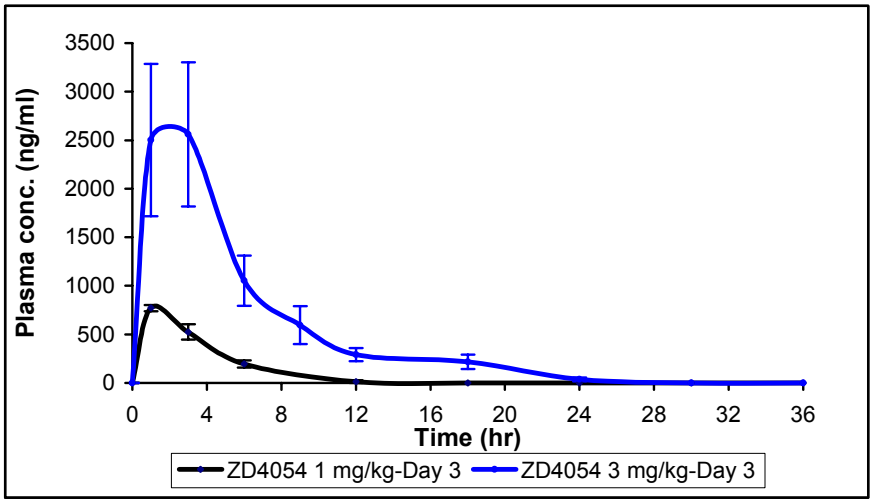


Figure 21: Plasma concentration-time curve on day 3 of daily single oral administration of ZD4054 at dose of 1 mg/kg or 3 mg/kg in salt loaded SHRsp. Values represented are mean of 4-5 observations. The error bars represent ±SEM. Conc., concentration.

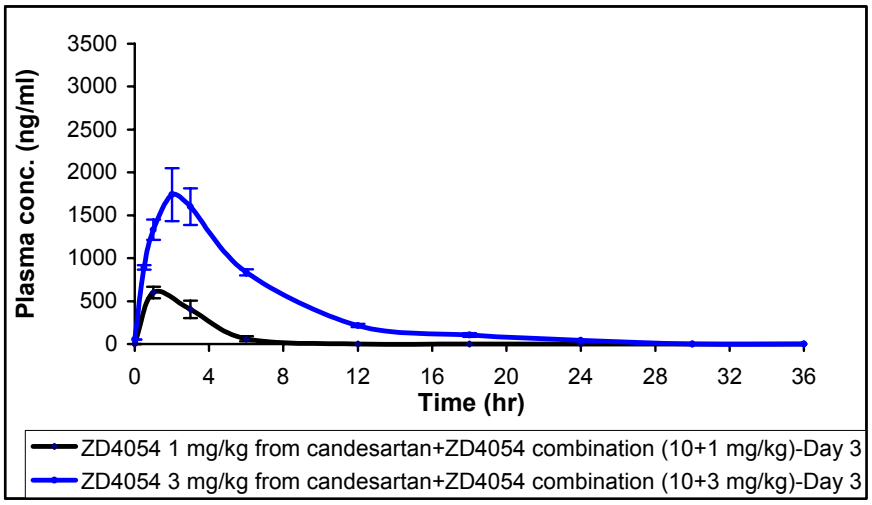


Figure 22: Plasma concentration-time curve on day 3 for ZD4054 administered at dose of 1 mg/kg or 3 mg/kg in combination with candesartan 10 mg/kg, to salt loaded SHRsp being previously treated with candesartan 10 mg/kg alone daily for last eight days. Values represented are mean of 3-5 observation. The error bars represents ±SEM. Conc., concentration.

Table 10: Pharmacokinetic parameters of candesartan administered alone and in combination with different doses of ZD4054 in salt loaded SHRsp.

Pharmacokinetic parameters	Candesartan 10 mg/kg alone - Day1	Candesartan 10 mg/kg alone - Day8	Candesartan 10 mg/kg from candesartan+ZD4054 combination (10+1 mg/kg) - Day3	Candesartan 10 mg/kg from candesartan+ZD4054 combination (10+3 mg/kg) - Day3
$t_{1/2}$ (hr)	6.26±1.40	8.45±1.25	6.43±1.08	9.01±1.91
T_{max} (hr)	1.83±0.17	2.00±0.35	2.20±0.49	2.00±0.00
C_{max} (ng/ml)	1378.00±208.45	1123.43±0.147.58	1334.00±171.86	943.67±198.04
$AUC_{(0-\infty)}$ (ng.hr/ml)	9843.47±3186.38	11622.00±2142.6	10799.62±1865.86	9236.87±984.96

Values represented are mean ±SEM for 3-7 observations in each group. p>0.05 for all parameters of candesartan when administered alone on day 1 or day 8 or in combination with different doses of ZD4054 by Student's t test.

AUC, area under the curve; C_{max}, maximum plasma concentration; $t_{1/2}$, half life; T_{max}, time to reach C_{max}

Table 11: Pharmacokinetic parameters of ZD4054 administered in different doses alone and in combination with candesartan in salt loaded SHRsp.

Pharmacokinetic parameters	ZD4054 1 mg/kg alone - Day3	ZD4054 3 mg/kg alone - Day3	ZD4054 1 mg/kg from candesartan+ZD4054 combination (10+1 mg/kg) - Day3	ZD4054 3 mg/kg from candesartan+ZD4054 combination (10+3 mg/kg) - Day3
$t_{1/2}$ (hr)	2.60±0.33	4.54±0.55	1.96±0.43	3.91±0.25
T_{max} (hr)	1.00±0.00	2.20±0.49	1.40±0.40	2.33±0.33
C_{max} (ng/ml)	770.86±32.62	2673.54±771.43	612.65±65.74*	1810.39±302.88
$AUC_{(0-\infty)}$ (ng.hr/ml)	3464.40±519.44	15136.51±2807.47	1891.45±327.53*	11758.54±689.12

Values represented are mean ±SEM for 3-5 observations in each group. *p<0.05 in comparison to ZD4054 1 mg/kg alone - Day3 by Student's *t* test.

AUC, area under the curve; C_{max}, maximum plasma concentration; $t_{1/2}$, half life; T_{max}, time to reach C_{max}

5.2.3. Development of anesthetized rat model for screening new chemical entities for their AT_1 and ET_A receptor blocking activity

5.2.3.1. To standardize type and dose of anesthetic to be used for arterial blood pressure monitoring

Ketamine and xylazine cocktail's continuous infusion at dose of 1250 µg + 40 µg/kg/min, following 45 min of initial induction of anesthesia with ketamine and xylazine combination at dose of 60 mg/kg and 7.5 mg/kg, i.p., though found successful in maintaining continuous surgical plain of anesthesia caused gradual fall in MBP beyond 4 hr of continuous infusion (Figure 23).

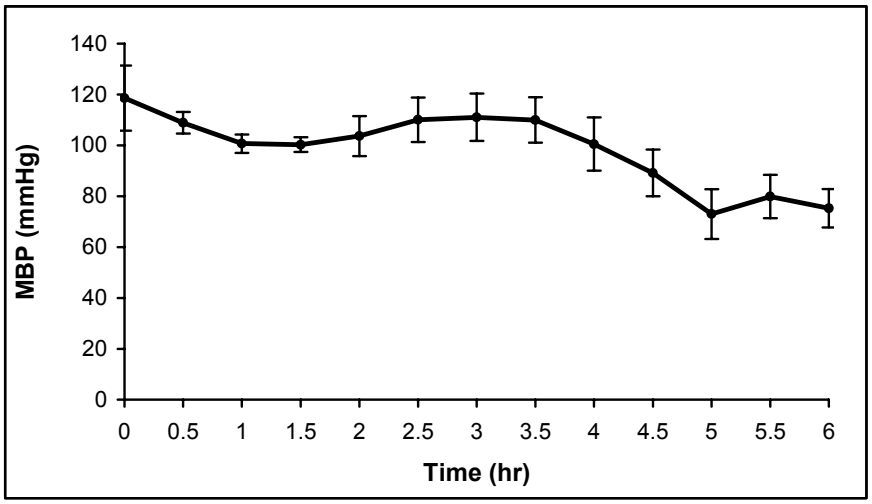


Figure 23: Effect of ketamine and xylazine cocktail's continuous infusion on MBP of Sprague Dawley rats at dose of 1250 µg + 40 µg/kg/min respectively. Values represented are mean of 7 observations. The error bars represent ±SEM. MBP, mean blood pressure.

Isoflurane at concentration of 0.8% and 1%, when delivered as an inhalation anesthetic to Sprague Dawley rat produced anesthesia with stable MBP (Figure 24 and 25). However, isoflurane at 0.8% concentration did not produce continuous stable surgical plane of anesthesia as rat frequently presented positive pedal reflex when tested for. Thus isoflurane inhalation at 1% was selected as anesthetic for further experiments as it was found successful in producing desired depth of anesthesia with minimum effect on stability of MBP. However, it was determined that in this model, it takes nearly one hour for MBP to get stabilized with 1% isoflurane anesthesia and therefore a stabilization period of an hour was maintained in all further experiments before initiating treatment with any test compound.

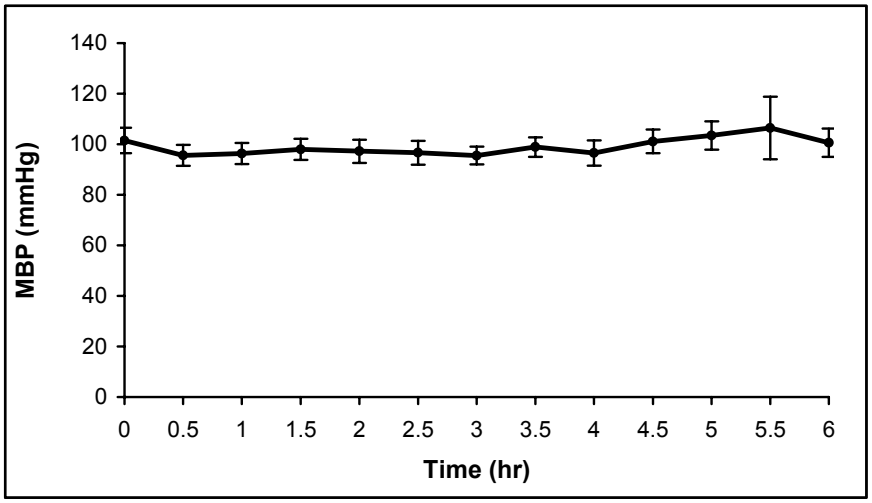


Figure 24: Effect of isoflurane inhalation anesthesia on MBP of Sprague Dawley rats when inhaled at concentration of 0.8% in medical oxygen. Values represented are mean of 8 observations. The error bars represent ±SEM. MBP, mean blood pressure.

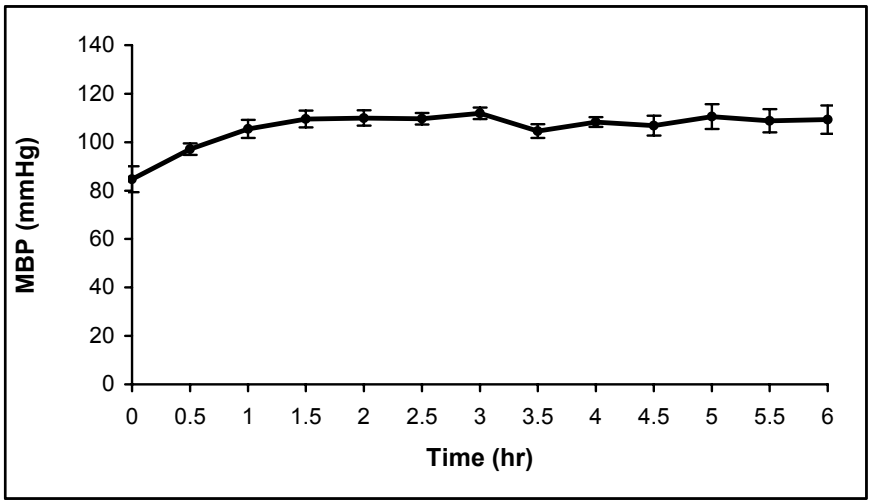


Figure 25: Effect of isoflurane inhalation anesthesia on MBP of Sprague Dawley rats when inhaled at concentration of 1% in medical oxygen. Values represented are mean of 6 observations. The error bars represent ±SEM. MBP, mean blood pressure.

5.2.3.2. To standardize infusion dose of Ang II to obtain a stable pressor effect

Increasing doses of Ang II administered as i.v. bolus caused increase in the MBP with maximum effect reaching with 1000 ng/kg. Increasing dose of Ang II beyond 1000 ng/kg did not produced further increment in MBP (Figure 26).

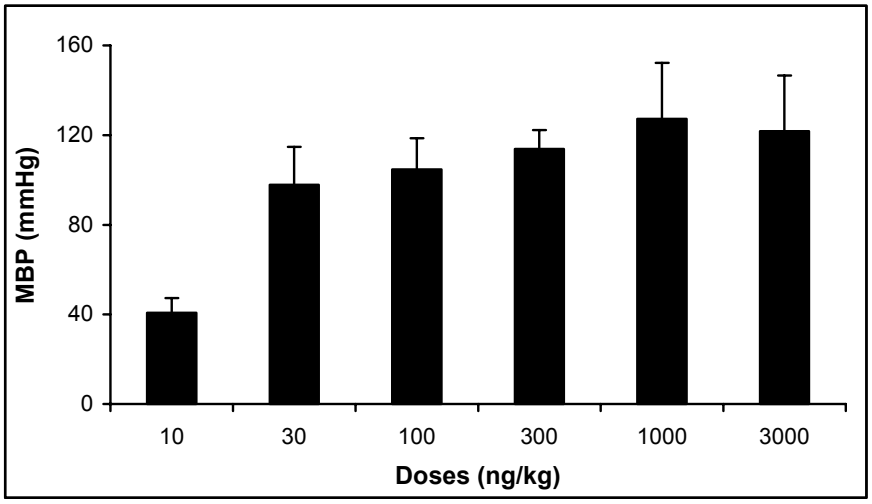


Figure 26: Increase in MBP upon challenging Sprague Dawley rats with escalating log doses of Ang II administered as i.v. bolus. Values represented are mean of 6 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

Challenge with 10 ng/kg, i.v. bolus of Ang II produced maximal rise of 40.7 mmHg in MBP and therefore this dose was selected as the first dose to be explored for continuous infusion. Rise in MBP after infusing Ang II at dose of 10 and 15 ng/kg/min was minimal, in range of 9-15 mmHg, therefore these doses were not explored further. Ang II infusion at dose of 20 ng/kg/min produced continuous increase in MBP but still failed to provide 40 mmHg rise over period of 2 hr. Ang II infusion at dose of 25 ng/kg/min produced a rise of 35 mmHg in MBP within 45 min of infusion and was maintained with little variation up to 2 hr. It was Ang II infusion at dose of 35 ng/kg/min which was found successful in raising and stabilizing MBP by 40-60 mmHg within 15 min and was thus selected as initial infusion dose of Ang II for subsequent experiments.

5.2.3.3. Blocking endogenous AT_1 receptors for isolating ET_A receptor blocker activity

Duration of AT_1 receptor blockade by losartan at dose of 30 mg/kg

Volume load of saline as slow i.v. bolus (across 30 sec) at a dosing volume of 1 ml/kg produced negligible rise of 2.2±0.2 mmHg in MBP of rats. However, subsequently upon challenging rats with Ang II slow i.v. bolus of 300 ng/kg (dose producing near saturation pressor effect) produced rapid massive rise in MBP up to 75.9±11.3 mmHg. After recovery of MBP to basal following Ang II bolus; administration of losartan 30 mg/kg, i.v. slow bolus was found successful in blocking AT_1 receptors of Sprague Dawley rats for a period of 5 hr. This is evident from negligible rise in MBP of rats challenged with Ang II (300 ng/kg, i.v. slow bolus) every 30 min following losartan administration. Beyond period of 5 hr post losartan administration a trend towards increase in MBP was observed (Table 12). Therefore, losartan 30 mg/kg was selected as a dose to block AT_1 receptors in successive experiments.

Table 12: Change in MBP observed upon challenging Sprague Dawley rats with Ang II 300 ng/kg, i.v. bolus before and after treatment with losartan 30 mg/kg.

Time (hours)	Treatment (1 ml/kg)	Rise in MBP (mmHg)
	Saline	2.2±0.2
	Ang II 300 ng/kg	75.9±11.3*
0	Losartan 30 mg/kg	-10.4±3.2
0.5	Ang II 300 ng/kg	2.9±0.4
1.0	Ang II 300 ng/kg	2.7±0.5
1.5	Ang II 300 ng/kg	3.1±0.2*
2.0	Ang II 300 ng/kg	4.6±1.1
2.5	Ang II 300 ng/kg	5.0±3.1
3.0	Ang II 300 ng/kg	3.6±0.3*
3.5	Ang II 300 ng/kg	2.2±1.6
4.0	Ang II 300 ng/kg	3.8±1.0
4.5	Ang II 300 ng/kg	1.7±3.2
5.0	Ang II 300 ng/kg	4.4±1.7
5.5	Ang II 300 ng/kg	5.5±7.5

Values represented are mean ± SEM of 3 observations. *$p<0.05$ in comparison to saline administration by Student's *t* test. Ang II, angiotensin II; MBP, mean blood pressure.

5.2.3.4. Validation of anesthetized rat model for screening AT_1 and ET_A receptor blocking activity of new chemical entities

Graded dose dependent reduction in MBP was observed with losartan in an Ang II infused rat model of hypertension having ET_A receptors blocked with ZD1611 1.5 mg/kg (Figure 27). Similarly, big ET-1 infused rat model of hypertension having AT_1 receptor blocked with losartan 30 mg/kg, demonstrated graded dose dependent reduction in MBP with ZD1611 (Figure 28). This led us to conclude that this model would be sensitive for screening antihypertensive activities being mediated through either AT_1 or ET_A receptor blocking.

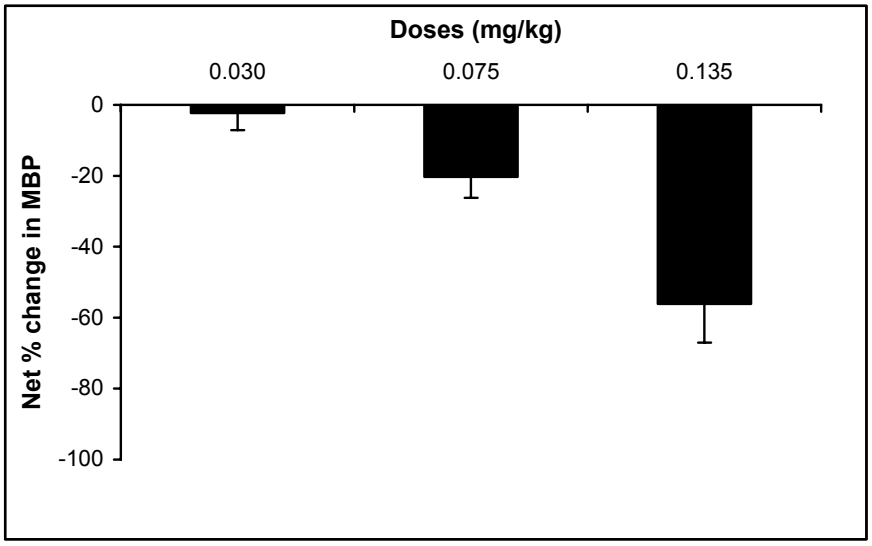


Figure 27: Net percentage reduction in MBP by i.v. infusion of losartan (0.030, 0.075 and 0.135 mg/kg), with an infusion free period of 15 min between consecutive doses, in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v. bolus. Values represented are mean of 5 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

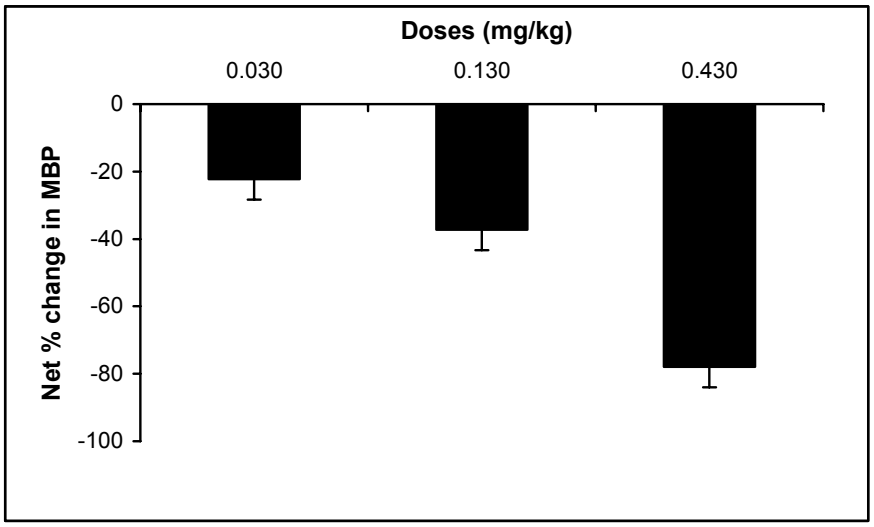


Figure 28: Net percentage reduction in MBP by i.v. infusion of ZD1611 (0.030, 0.130 and 0.430 mg/kg), with an infusion free period of 15 min between consecutive doses, in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v. bolus. Values represented are mean of 5 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

5.2.4. Screening of new chemical entities for pharmacological activity in anesthetized rat model

NCEs TRC23003, TRC23012, TRC23017, TRC23029, TRC23030, TRC23031 and TRC23035 and the reference molecule BMS346567 were screened for their activity in anesthetized rat model. Cumulative DRC was created for each test compound to capture separately their AT_1 and ET_A receptor blocking activity in anesthetized rat model. The percent reduction in MBP from respective dose baseline MBP after taking into account the effect of vehicle is shown in Figure 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43 and 44. For, limitations imposed by formulation, doses of TRC23003 and TRC23017 could not be escalated to those which could achieve 50% fall in MBP.

To evaluate comparative activity of various NCEs and reference molecule for their ability to block AT_1 and ET_A receptors, ED_{50} value for each test compound was calculated (Table 13). Of NCEs screened, TRC23012 was most potent in blocking AT_1 receptors whereas TRC23035 was most potent ET_A receptor blocker.

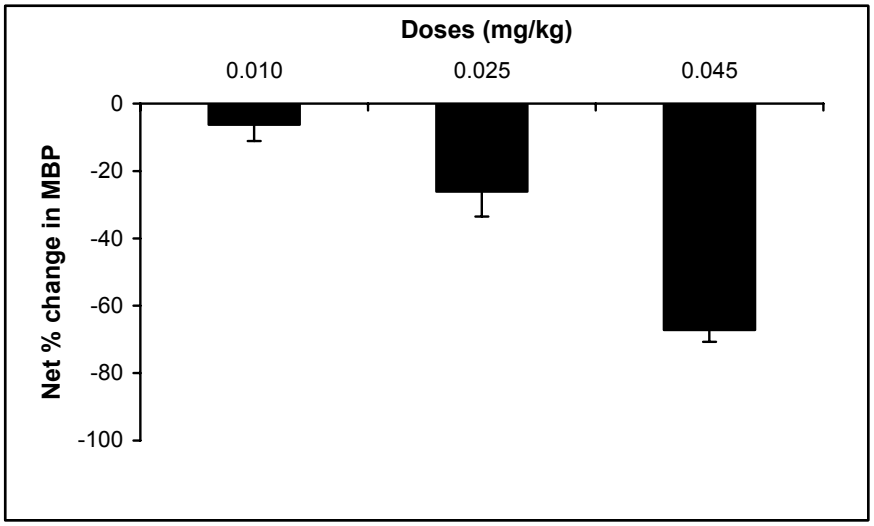


Figure 29: Net percentage reduction in MBP by i.v. infusion of TRC23003 (0.010, 0.025 and 0.045 mg/kg) in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

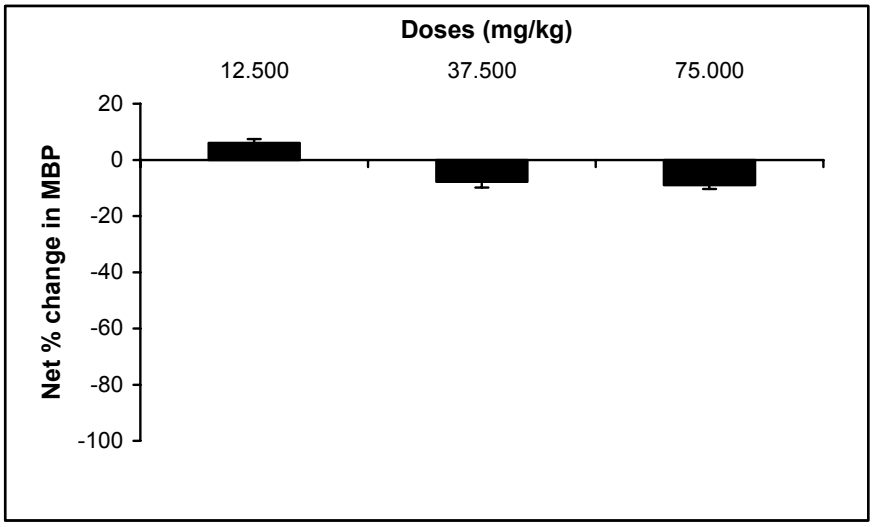


Figure 30: Net percentage reduction in MBP by i.v. infusion of TRC23003 (12.500, 37.500 and 75.000 mg/kg) in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v., bolus. Values represented are mean of 3 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

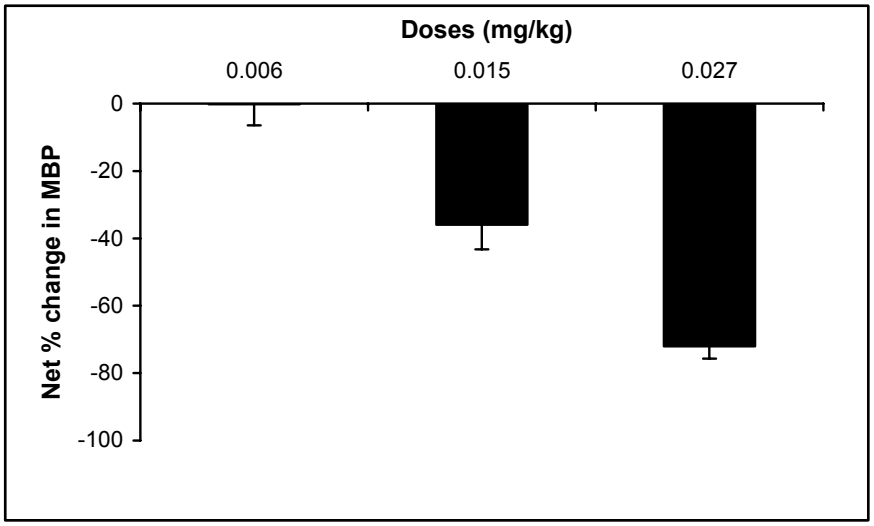


Figure 31: Net percentage reduction in MBP by i.v. infusion of TRC23012 (0.006, 0.015 and 0.027 mg/kg) in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v., bolus. Values represented are mean of 3 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

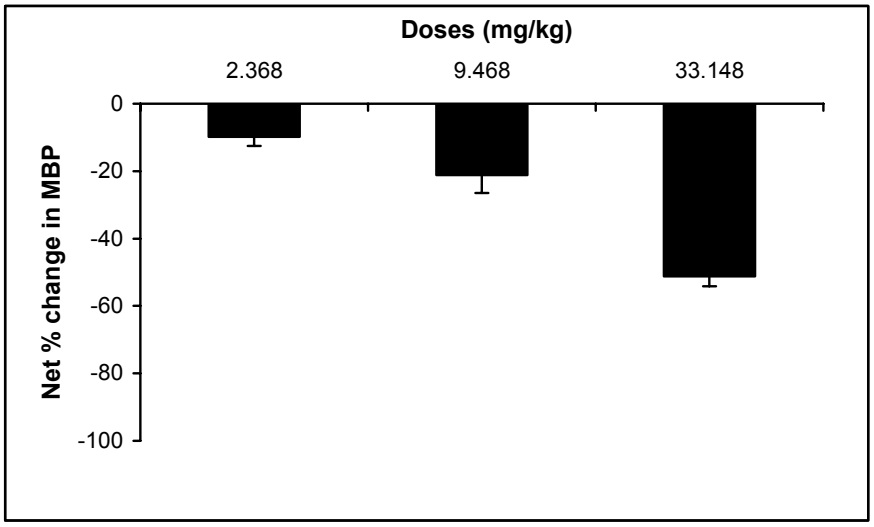


Figure 32: Net percentage reduction in MBP by i.v. infusion of TRC23012 (2.368, 9.468 and 33.148 mg/kg) in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v., bolus. Values represented are mean of 5 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

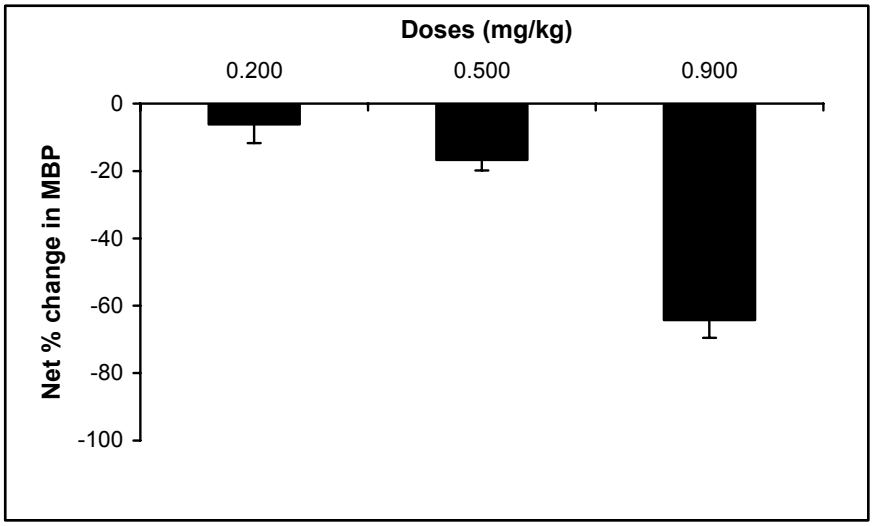


Figure 33: Net percentage reduction in MBP by i.v. infusion of TRC23017 (0.200, 0.500 and 0.900 mg/kg) in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

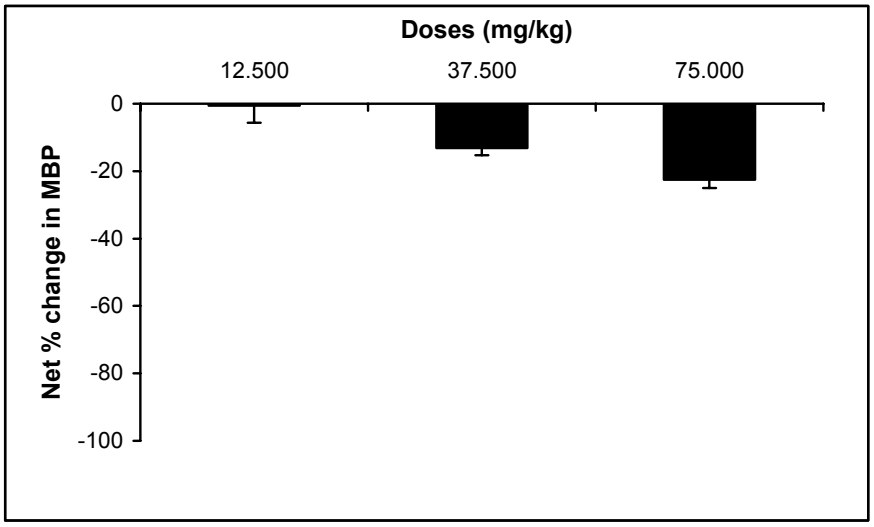


Figure 34: Net percentage reduction in MBP by i.v. infusion of TRC23017 (12.500, 37.500 and 75.000 mg/kg) in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

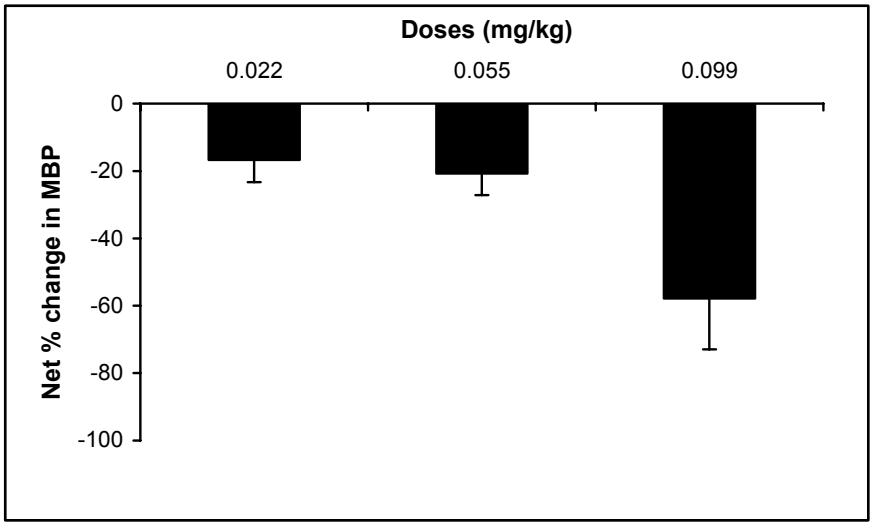


Figure 35: Net percentage reduction in MBP by i.v. infusion of TRC23029 (0.022, 0.055 and 0.099 mg/kg) in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

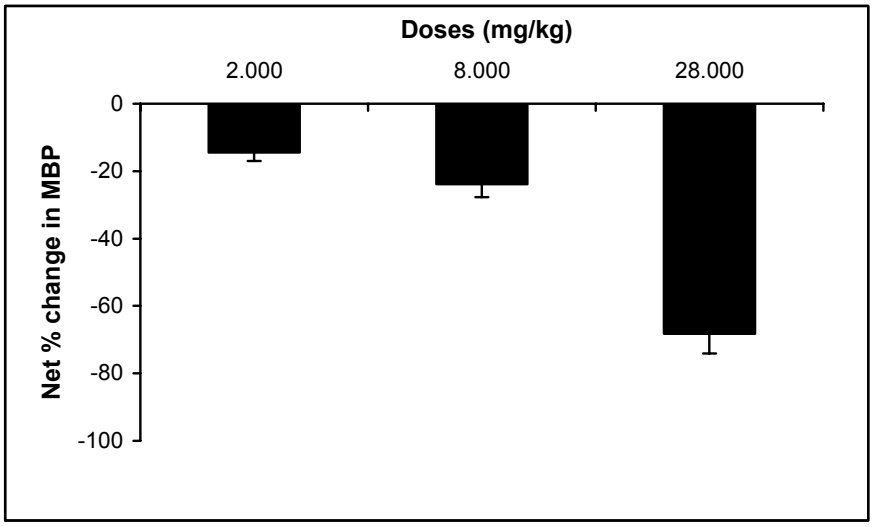


Figure 36: Net percentage reduction in MBP by i.v. infusion of TRC23029 (2.000, 8.000 and 28.000 mg/kg) in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

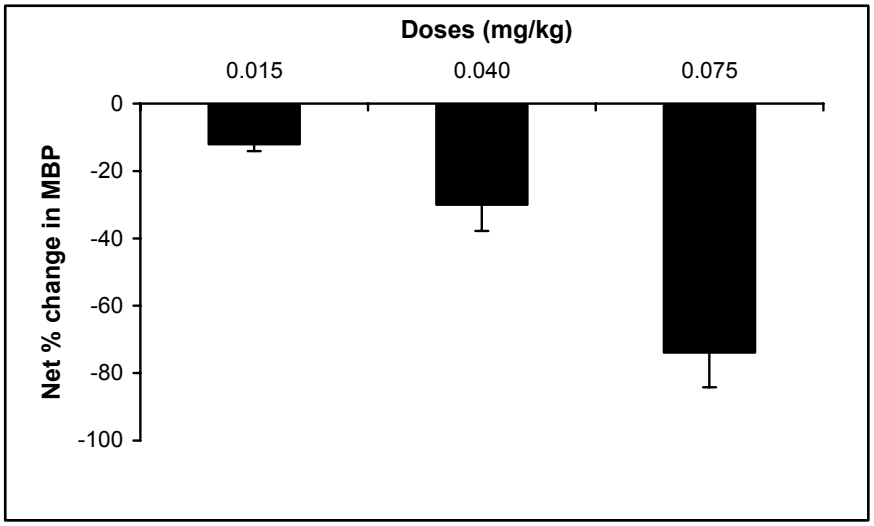


Figure 37: Net percentage reduction in MBP by i.v. infusion of TRC23030 (0.015, 0.040 and 0.075 mg/kg) in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

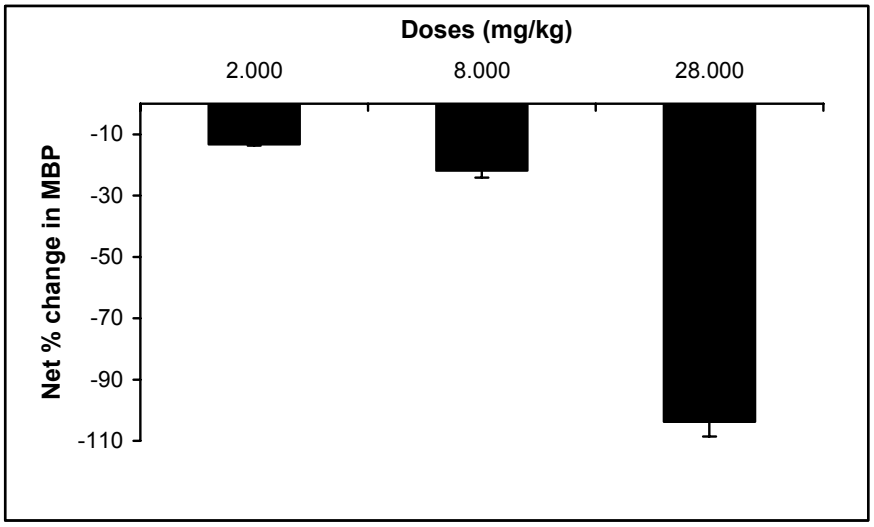


Figure 38: Net percentage reduction in MBP by i.v. infusion of TRC23030 (2.000, 8.000 and 28.000 mg/kg) in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

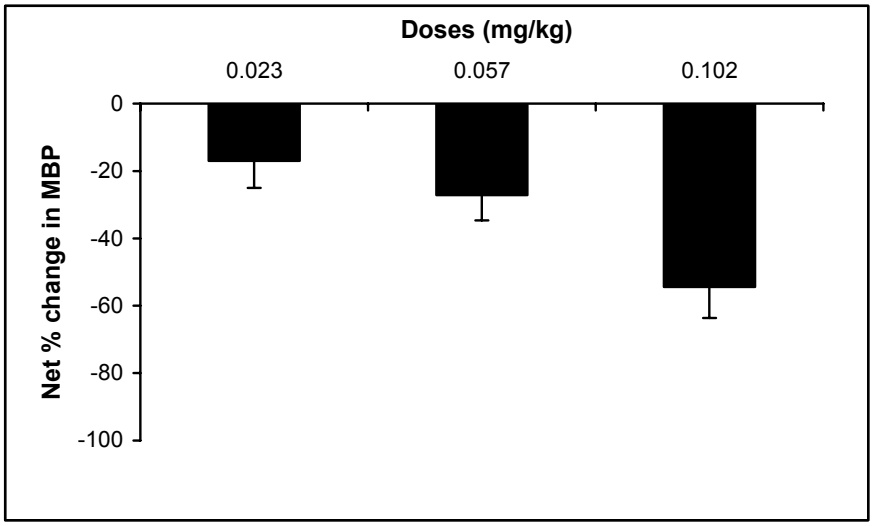


Figure 39: Net percentage reduction in MBP by i.v. infusion of TRC23031 (0.023, 0.057 and 0.102 mg/kg) in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

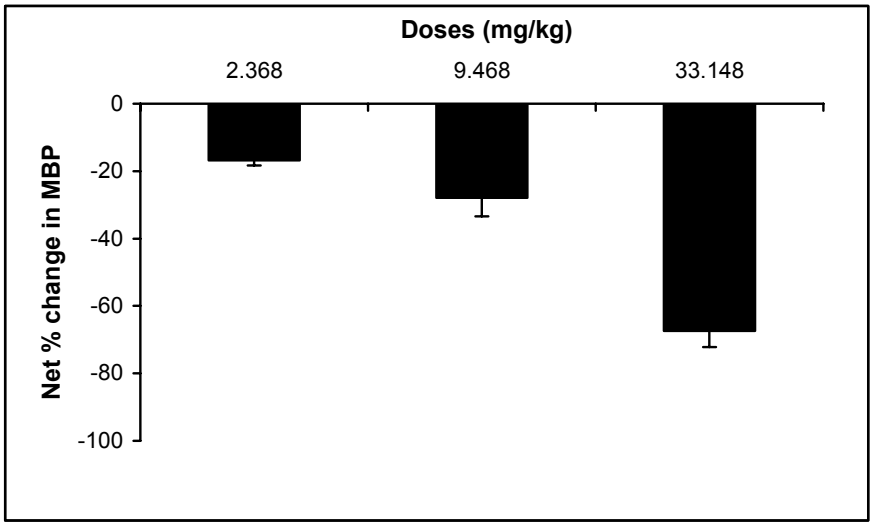


Figure 40: Net percentage reduction in MBP by i.v. infusion of TRC23031 (2.368, 9.468 and 33.148 mg/kg) in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

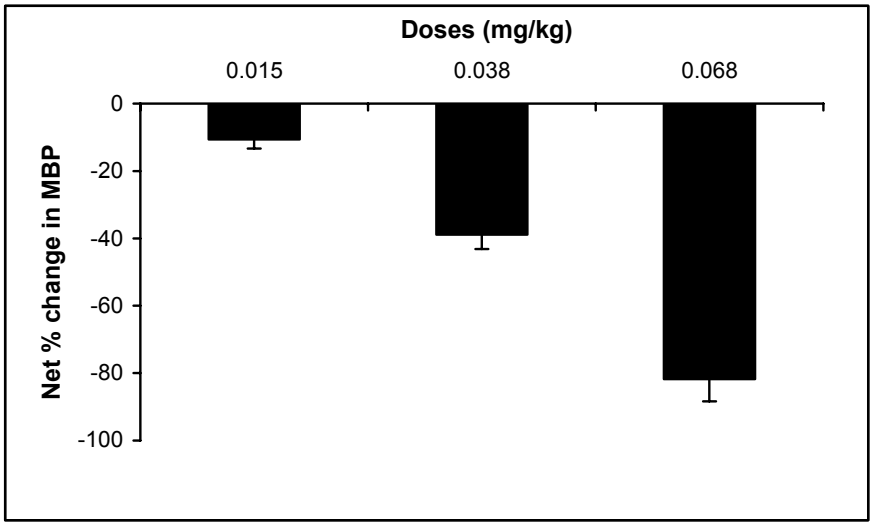


Figure 41: Net percentage reduction in MBP by i.v. infusion of TRC23035 (0.015, 0.038 and 0.068 mg/kg) in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

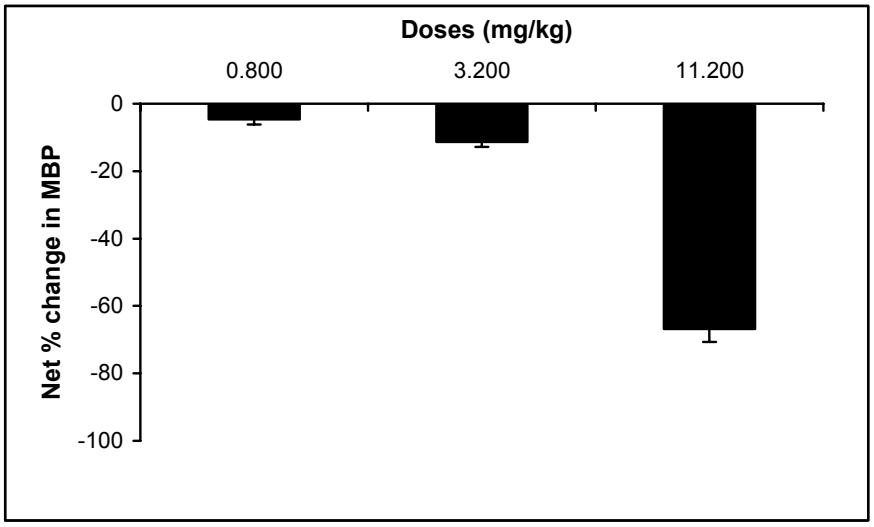


Figure 42: Net percentage reduction in MBP by i.v. infusion of TRC23035 (0.800, 3.200 and 11.200 mg/kg) in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v., bolus. Values represented are mean of 4 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

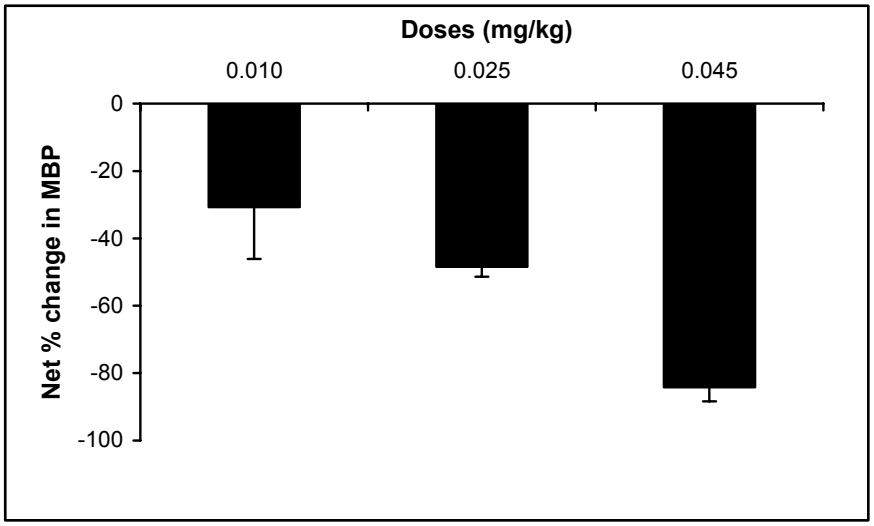


Figure 43: Net percentage reduction in MBP by i.v. infusion of BMS346567 (0.010, 0.025 and 0.045 mg/kg) in Ang II infused rat model of hypertension having ET_A receptors blocked by ZD1611 1.5 mg/kg, i.v., bolus. Values represented are mean of 3 observations. The error bars represent SEM. Ang II, angiotensin II; MBP, mean blood pressure.

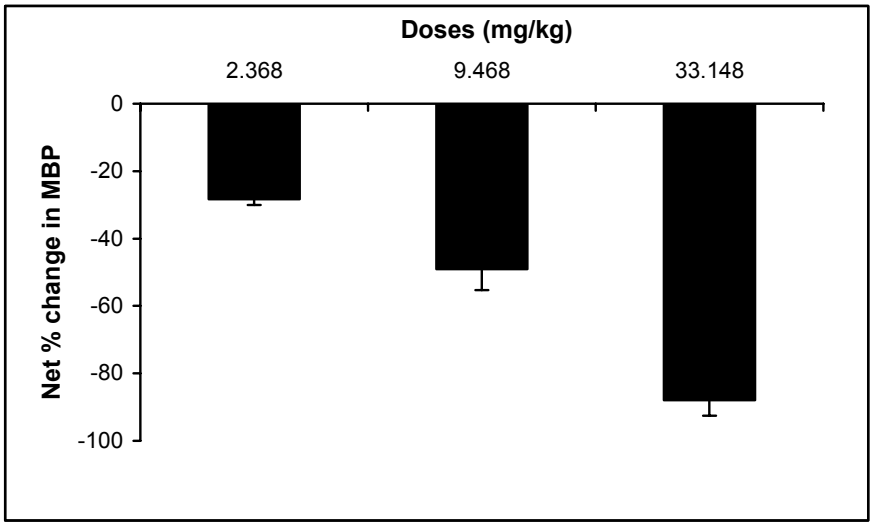


Figure 44: Net percentage reduction in MBP by i.v. infusion of BMS346567 (2.368, 9.468 and 33.148 mg/kg) in big ET-1 infused rat model of hypertension having AT_1 receptors blocked by losartan 30 mg/kg, i.v., bolus. Values represented are mean of 5 observations. The error bars represent SEM. Big ET-1, big endothelin-1; MBP, mean blood pressure.

Table 13: ED_{50} for NCEs and reference molecule calculated from DRC for reduction in MBP in Ang II or big ET-1 infused rats having their endogenous ET_A or AT_1 receptors blocked respectively.

Test Compound	ED_{50} for AT_1 receptor blocking (mg/kg)	ED_{50} for ET_A receptor blocking (mg/kg)
TRC23003	0.036	NA
TRC23012	0.020	32.050
TRC23017	0.780	NA
TRC23029	0.092	19.550
TRC23030	0.054	13.700
TRC23031	0.096	22.630
TRC23035	0.045	8.660
BMS346567	0.024	12.250

NA, not applicable

5.2.5. Pharmacokinetic profiling of new chemical entities in rat

The plasma drug concentration-time profile of TRC23029, TRC23030, TRC23035 and reference molecule BMS346567 when administered by i.v. and oral route are shown in Figure 45 and 46. The PK parameters computed from plasma concentration-time data are shown in Table 14 and 15.

Similar to reference molecule BMS346567; TRC23029, TRC23030 and TRC23035 (6 mg/kg) showed rapid oral absorption with C_{max} being reached within 0.38 to 0.63 hr of administration.

The volume of distribution and clearance of NCEs after i.v. administration (2 mg/kg) in Sprague Dawley rats was in range of 2289.06 to 5764.93 ml/kg and 1876.34 to 2710.51 ml/hr/kg respectively. High volume of distribution and clearance for NCEs suggest that they are widely distributed and rapidly cleared from the body. In contrast, though reference molecule BMS346567 show volume of distribution similar to NCEs it has much low clearance. The same is reflected in plasma $t_{1/2}$, where the reference molecule shows much longer $t_{1/2}$ in comparison to NCEs.

Though $t_{1/2}$ of NCEs was less than BMS346567, all NCEs demonstrated good oral bioavailability (F% $\geq$ 20%) in Sprague Dawley rats. The results are in consensus with *in vitro* permeability results, where all the NCEs were found to fall in high permeability class of compounds.

The oral bioavailability (F%) was calculated using the formula

$F\% = (AUC_{(0\text{-}t)}\ p.o./AUC_{(0\text{-}t)}\ i.v.) \times (dose\ i.v./dose\ p.o.) \times 100$

Where:

F = Oral bioavailability

$AUC_{(0\text{-}t)}$ = Area under curve from time zero to last sample collection time point

p.o. = per oral

i.v. = intravenous

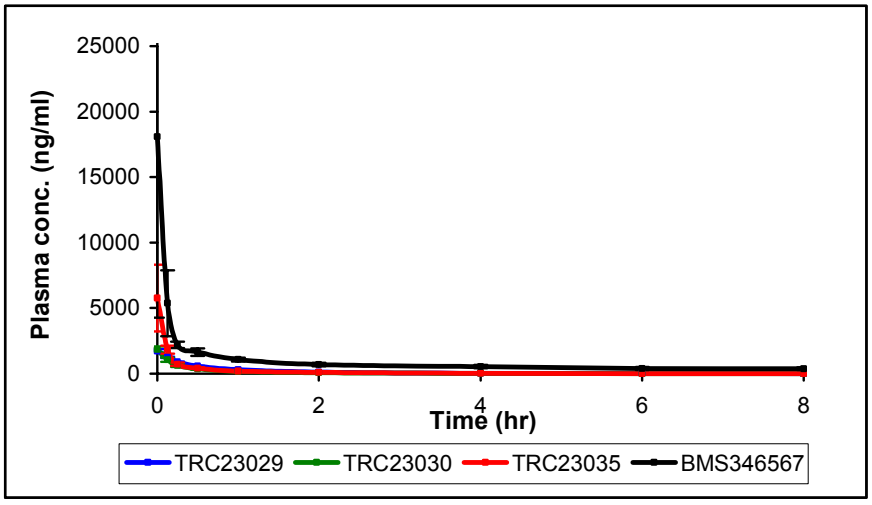


Figure 45: Plasma concentration-time curve upon i.v. administration of TRC23029, TRC23030, TRC23035 and BMS346567, each at dose of 2 mg/kg in Sprague Dawley rats. Values represented are mean of 3-6 observations. The error bars represent ±SEM. Conc., concentration.

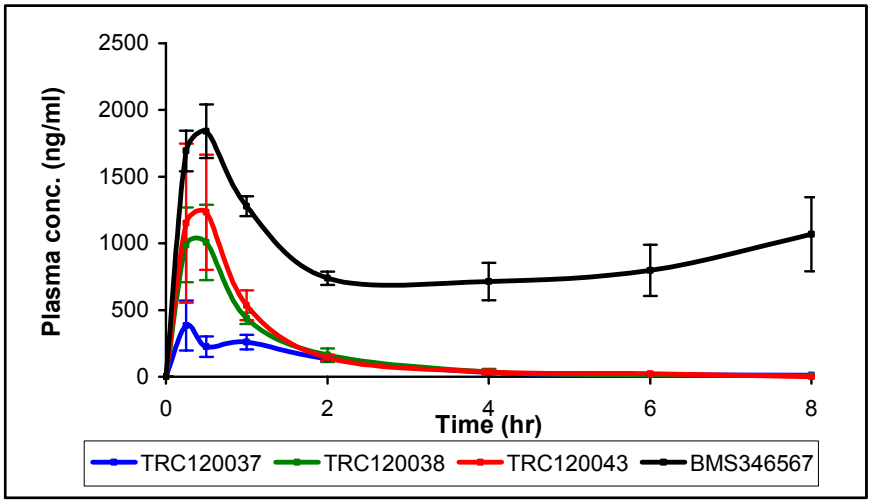


Figure 46: Plasma concentration-time curve upon oral administration of TRC23029, TRC23030, TRC23035 and BMS346567, each at dose of 6 mg/kg in Sprague Dawley rats. Values represented are mean of 4-5 observations. The error bars represent ±SEM. Conc., concentration.

Table 14: Pharmacokinetic parameters of TRC23029, TRC23030, TRC23035 and BMS346567 computed from mean plasma concentration-time curve following i.v. administration to Sprague Dawley rats at dose of 2 mg/kg.

Parameters	TRC120037	TRC120038	TRC120043	BMS346567
$t_{1/2}$ (hr)	1.13 ± 0.18*	1.51 ± 0.52*	0.84 ± 0.07*	7.46 ± 2.26
C_0 (ng/ml)	1726.75 ± 140.66	1847.34 ± 229.46	5761.38 ± 2546.39	18081.02 ± 13808.60
$AUC_{(0-t)}$ (ng/ml.hr)	1056.83 ± 72.01*	730.07 ± 52.91*	1061.63 ± 98.60*	6651.31 ± 1651.25
$AUC_{(0-\infty)}$	1074.69 ± 69.37*	746.03 ± 53.58*	1080.46 ± 96.58*	11147.98 ± 2986.68
Vz (ml/kg)	2996.6 ± 367.8	5764.93 ± 1739.45*	2289.06 ± 254.04	2095.15 ± 579.39
Cl (ml/hr/kg)	1876.34 ± 119.08*#	2710.51 ± 206.51*	1896.7 ± 170.26*#	245.18 ± 89.06

Values represented are mean ±SEM for 3-6 observations in each group. *p<0.05 in comparison to BMS346567; #p<0.05 in comparison to TRC23030 by one way ANOVA followed by Tukey's multiple comparison as post hoc test.

AUC, area under the curve; C_0, concentration at time 0; Cl, clearance; $t_{1/2}$, half life; Vz, volume of distribution

Table 15: Pharmacokinetic parameters of TRC23029, TRC23030, TRC23035 and BMS346567 computed from mean plasma concentration-time curve following oral administration to Sprague Dawley rats at dose of 6 mg/kg.

Parameters	TRC120037	TRC120038	TRC120043	BMS346567
$t_{1/2}$ (hr)	1.29 ± 0.25*	0.74 ± 0.13*	0.79 ± 0.19*	12.19 ±4.88
T_{max} (hr)	0.63 ± 0.22	0.50 ± 0.00	0.38 ± 0.07	0.44 ± 0.06
C_{max} (ng/ml)	478.00 ± 139.60*	1007.25 ± 281.45	1406.25 ± 531.87*	1959.39 ± 144.38
$AUC_{(0-t)}$ (ng/ml.hr)	626.44 ± 81.73*	1172.30 ± 144.30*	1323.70 ± 318.77*	7196.48 ± 957.26
$AUC_{(0-\infty)}$	648.55 ± 78.10*	1184.60 ± 144.08*	1347.42 ± 314.82*	27713.77 ± 11427.77
F (%)	20	53	42	36

Values represented are mean ±SEM for 3-6 observations in each group. *p<0.05 in comparison to BMS346567 by one way ANOVA followed by Tukey's multiple comparison as post hoc test.

AUC, area under the curve; C_{max}, maximum plasma concentration; F, oral bioavailability; $t_{1/2}$, half life; T_{max}, time to reach C_{max}.

5.2.6. Efficacy screening of new chemical entities in conscious telemetered salt loaded SHRsp model

Various *in vitro* and *in vivo* filtering criteria used to grade NCEs for further efficacy screening in conscious telemetered salt loaded SHRsp model are described in Table 16. The first criteria for the selection of NCEs was low *in vitro* IC_{50} values for AT_1 and ET_A receptors and at the same time having range of ET_A: AT_1 *in vitro* IC_{50} values. This criterion was qualified by seven NCEs TRC23003, TRC23012, TRC23017, TRC23029, TRC23030, TRC23031 and TRC23035.

The reference molecule and the seven NCEs were compared for their *in vitro* physicochemical properties as solubility, permeability, plasma protein binding and *in vitro* metabolism and were found similar except for TRC23012 which displayed high *in vitro* metabolism in rat liver microsomes. However, this molecule along with other six NCEs and the reference molecule were still considered for further *in vivo* activity screening in anesthetized rat model considering the concept of insurmountability, where the molecule continues to block the receptor even when its overall plasma concentration is reduced by virtue of its intrinsic property of very slow dissociation from receptor.

Out of seven NCEs screened for activity in anesthetized rat model, TRC23003 and TRC23017 were dropped from further efficacy evaluation because they had very low ET_A receptor blocking activity in comparison to other NCEs. Similarly, of remaining NCEs, though TRC23012 had good activity (low ED_{50}) for blocking AT_1 receptors it had poorest activity (highest ED_{50}) for blocking ET_A receptors. In same fashion TRC23029, TRC23030 and TRC23035 were found superior to TRC23031 (Table 16). Therefore TRC23029, TRC23030, TRC23035 and reference molecule BMS346567 were considered for further efficacy evaluation in telemetered salt loaded SHRsp.

TRC23029, TRC23030, TRC23035 and reference test compound BMS346567 produced a dose dependent fall in MBP across test doses, with maximum fall in MBP observed at 6.5 to 8 hr post dosing of NCEs and 8.25 hr after administration of BMS346567. However, plateauing of antihypertensive effect for TRC23030 and BMS346567 was observed at their penultimate dose itself (Figure 47 and 48). Similar trend was shown in maximum percent fall achieved in MBP and $AUC_{(0\text{-}18)}$ which reflects duration of antihypertensive effect, after considering effect of vehicle.(Figure 49, 50, 51 and 52; Table 17). From DRC for reduction in MBP for different test compounds, ED_{50} was calculated and amongst all TRC23029 showed highest and TRC23035 showed lowest ED_{50} (Figure 53).

Table 16: Filtering parameters considered for arriving at NCEs to be screened for efficacy in salt loaded SHRsp model.

Screen	NCEs	TRC23003	TRC23012	TRC23017	TRC23029	TRC23030	TRC23031	TRC23035	BMS346567
in vitro	AT_1 receptor IC_{50} (nM)	0.02	0.02	0.01	0.01	0.00	0.01	0.00	0.01
	ET_A receptor IC_{50} (nM)	2.65	0.56	0.49	0.17	0.16	0.50	0.29	0.02
	ET_B receptor IC_{50} (nM)	16.60	25.30	6.70	18.00	11.50	37.10	8.03	25.40
	ET_A:AT_1 IC_{50}	140.21	24.54	36.62	26.95	53.38	44.12	93.18	1.20
	Solubility (µg/ml)	102.40	42.95	13.42	17.18	41.94	49.00	89.75	400.00
	Permeability (grade)	High	High	High	High	High	High	High	High
	Plasma protein binding (%)	0.15	1.10	0.19	0.15	0.16	0.15	0.50	0.43
	IVM (grade)	Moderate	High	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
in vivo	ED_{50} for AT_1 receptor blocking (mg/kg)	0.04	0.02	0.78	0.09	0.05	0.10	0.05	0.02
	ED_{50} for ET_A receptor blocking (mg/kg)	NA	32.05	NA	19.55	13.70	22.63	8.66	12.25
Overall Grading	Efficacy Grading	6	5	7	3	2	4	1	Reference
	Selection for in vivo PK and efficacy screening	Dropped	Dropped	Dropped	3	2	Dropped	1	Reference

ED_{50}, Effective dose 50; IC_{50}, inhibitory concentration 50; IVM, *in vitro* metabolism; NA, not applicable; PK, pharmacokinetic.

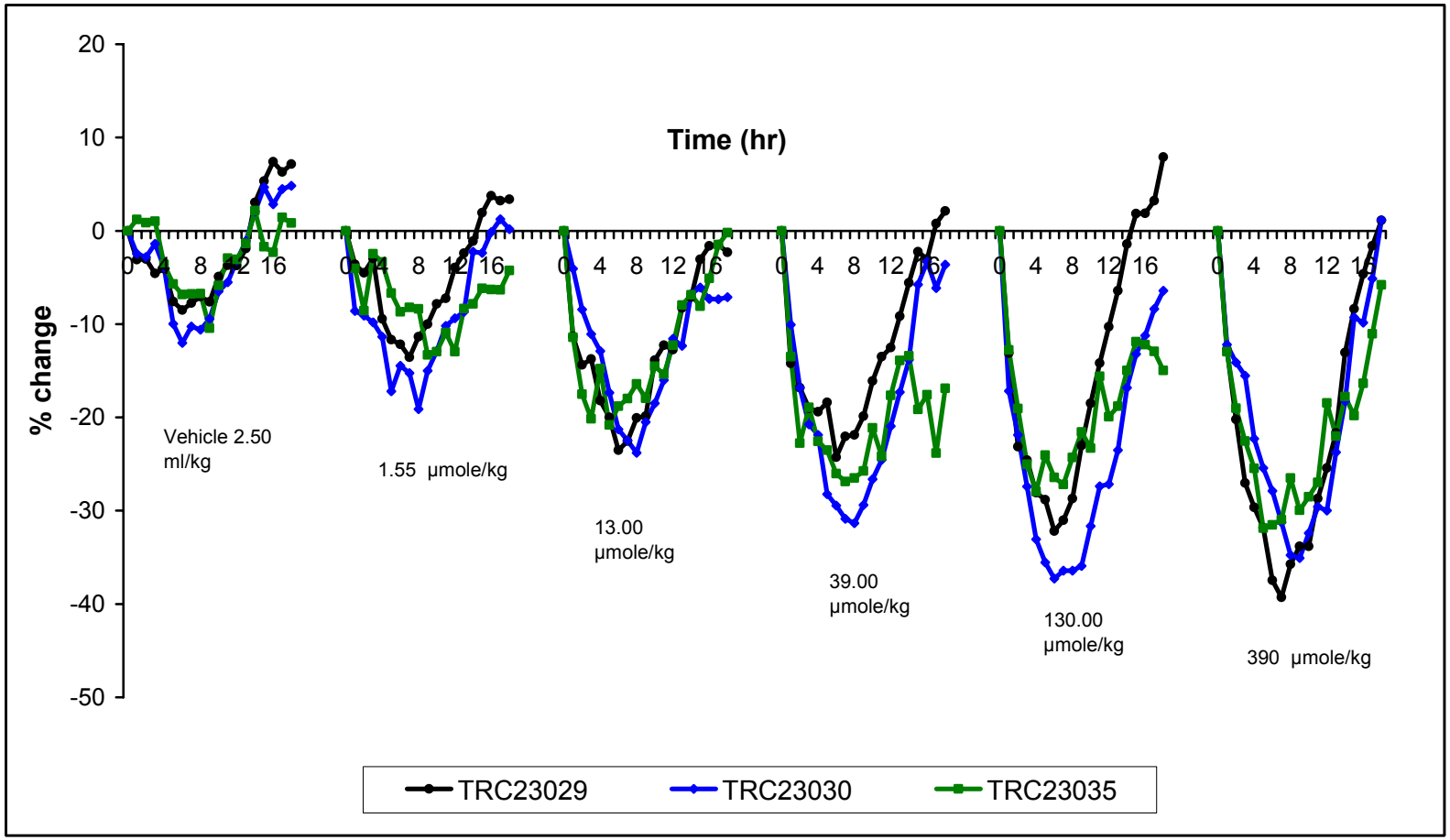


Figure 47: Profile of percent reduction in MBP upon oral administration of vehicle, TRC23029, TRC23030 and TRC23035 at dose levels 1.55, 13.00, 39.00, 130.00 and 390.00 µmole/kg over a period of 18 hr in salt loaded SHRsp. Values represented are mean of 3-8 observations. RMANOVA over 18 hr is significantly different for last four doses of all three NCEs in comparision to their respective vehicle. MBP, mean blood pressure.

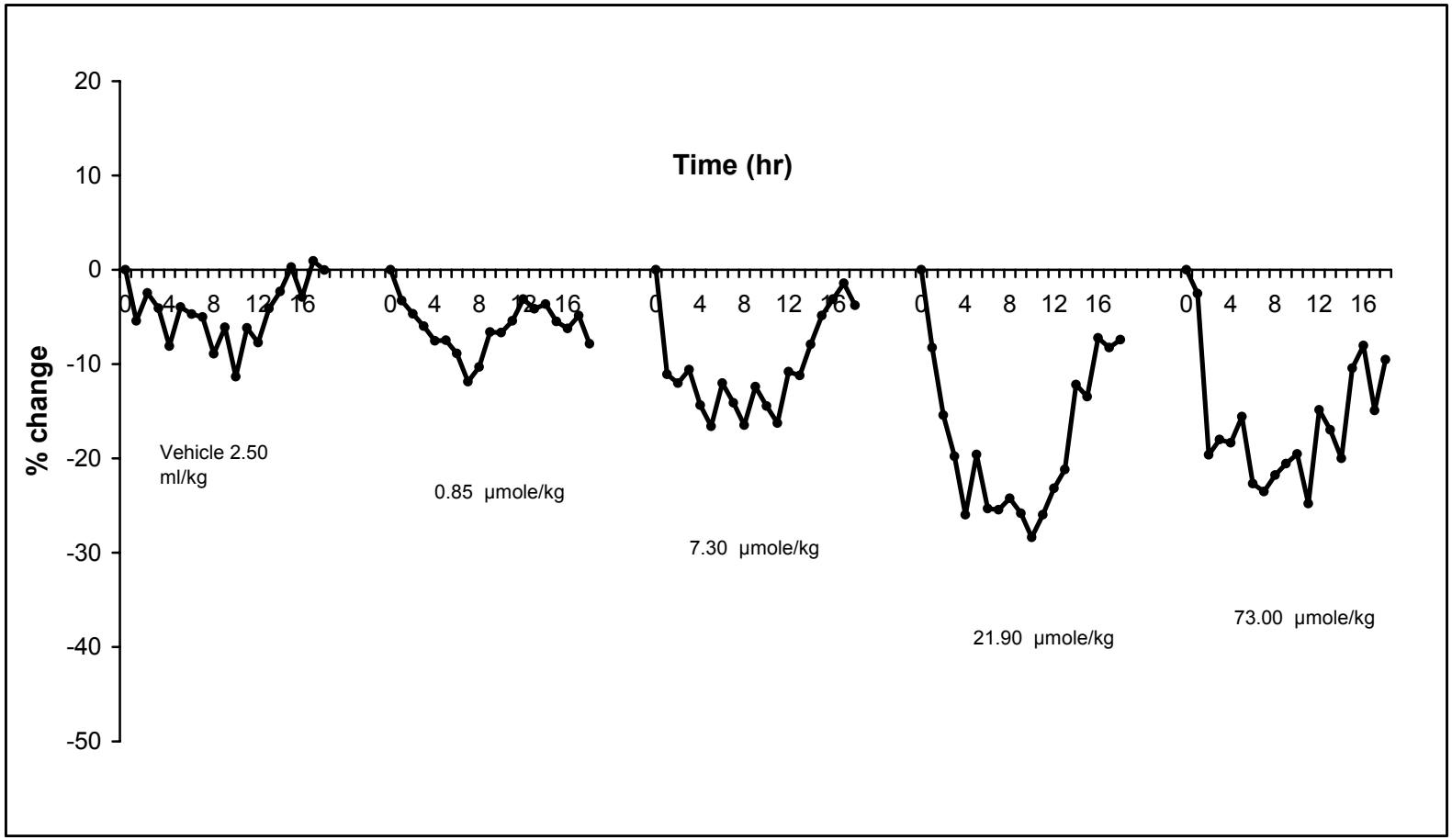


Figure 48: Profile of percent reduction in MBP upon oral administration of vehicle and BMS346567 at dose levels 0.85, 7.30, 21.90 and 73.00 µmole/kg over a period of 18 hr in salt loaded SHRsp. Values represented are mean of 2-4 observations. RMANOVA over 18 hr is significantly different for last three doses of BMS346567 in comparision to its vehicle. MBP, mean blood pressure.

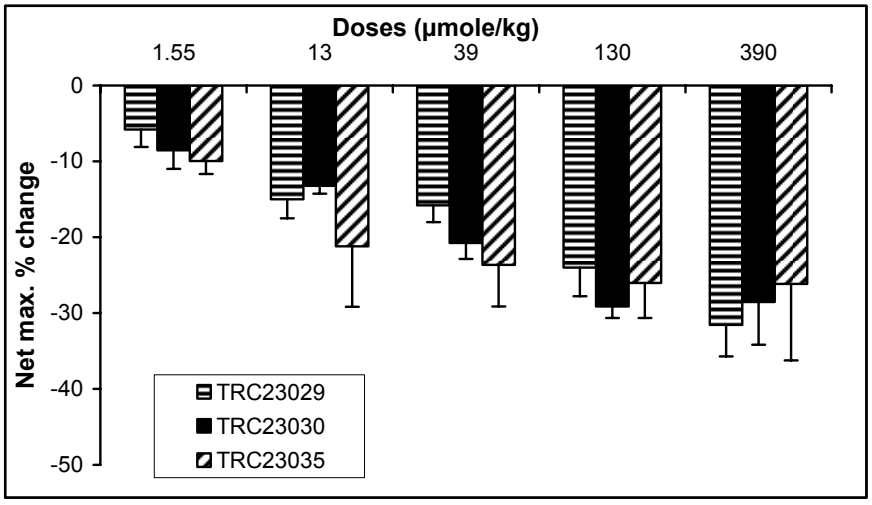


Figure 49: Net maximum percent fall in MBP upon oral administration of TRC23029, TRC23030 and TRC23035 at dose levels 1.55, 13.00, 39.00, 130.00 and 390.00 µmole/kg in salt loaded SHRsp. Values represented are mean of 3-8 observations. The error bars represent SEM. Max., maximum; MBP, mean blood pressure.

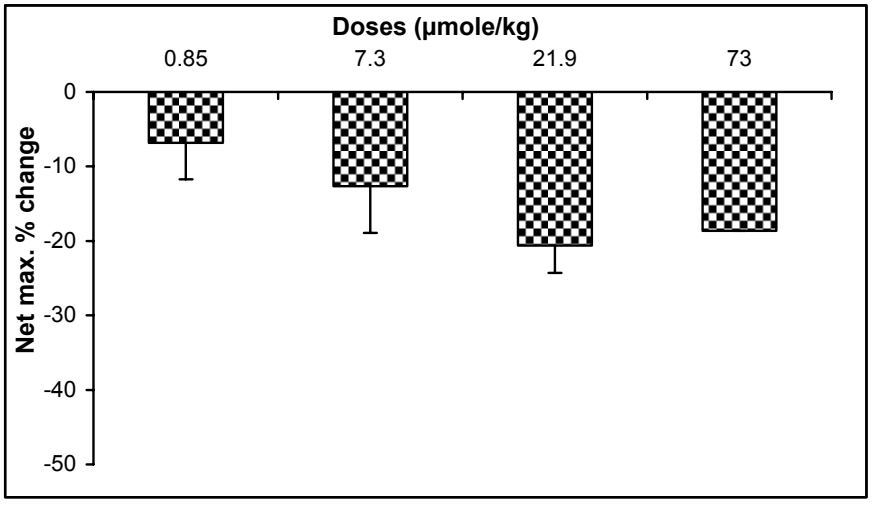


Figure 50: Net maximum percent fall in MBP upon oral administration of BMS346567 at dose levels 0.85, 7.30, 21.90 and 73.00 µmole/kg in salt loaded SHRsp. Values represented are mean of 2-4 observations. The error bars represent SEM. Max., maximum; MBP, mean blood pressure.

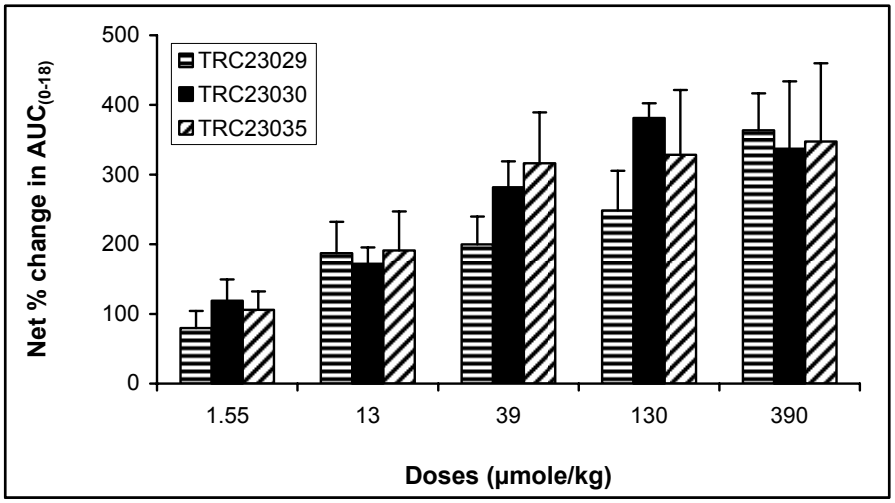


Figure 51: Net percentage change in AUC of MBP calculated for 18 hr post oral administration of TRC23029, TRC23030 and TRC23035 at dose levels 1.55, 13.00, 39.00, 130.00 and 390.00 µmole/kg in salt loaded SHRsp. Values represented are mean of 3-8 observations. The error bars represent SEM. AUC, area under the curve; MBP, mean blood pressure.

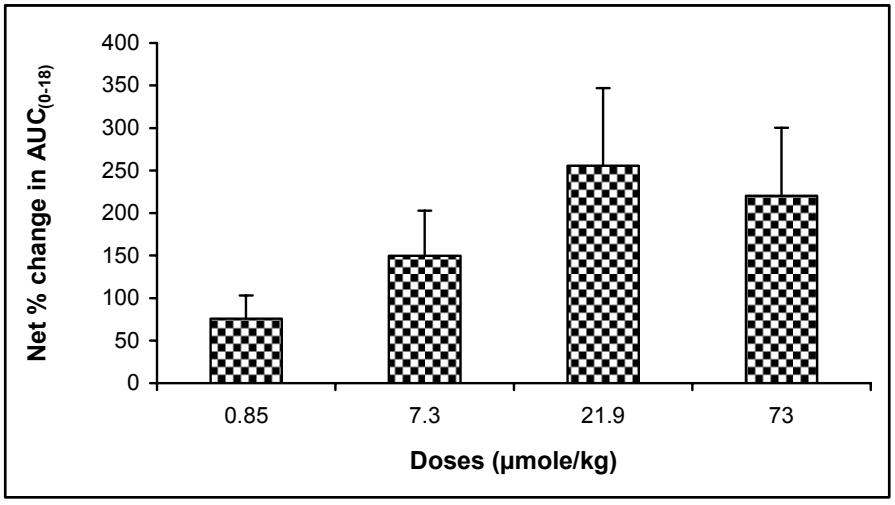


Figure 52: Net percentage change in AUC of MBP calculated for 18 hr post oral administration of BMS346567 at dose levels 0.85, 7.30, 21.90 and 73.00 µmole/kg in salt loaded SHRsp. Values represented are mean of 2-4 observations. The error bars represent SEM. AUC, area under the curve; MBP, mean blood pressure.

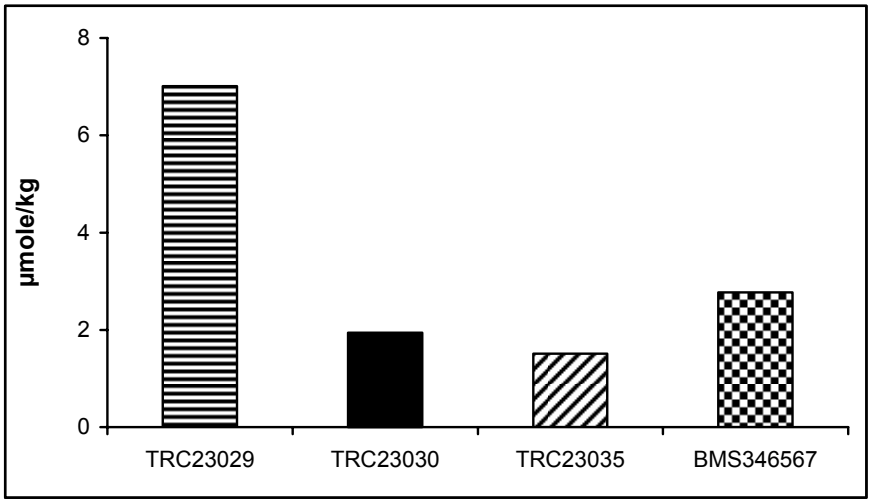


Figure 53: Effective dose of TRC23029, TRC23030, TRC23035 and BMS346567 capable of producing 50 percent of the maximum observed fall in MBP of salt loaded SHRsp. Values represented are derived from of 3-8 observations. MBP, mean blood pressure.

Table 17: Efficacy comparison of NCEs and reference molecule.

	Net max. % change in MBP		
	TRC23029	TRC23030	TRC23035
1.55 µmole/kg	-5.80 ± 2.31	-8.53 ± 2.46	-9.98 ± 1.68
13.00 µmole/kg	-15.02 ± 2.47	-13.20 ± 1.05	-21.19 ± 7.97
39.00 µmole/kg	-15.78 ± 2.25	-20.74 ± 2.13	-23.64 ± 5.48
130.00 µmole/kg	-24.00 ± 3.78	-29.13 ± 1.49	-26.04 ± 4.59
390.00 µmole/kg	-31.54 ± 4.16	-28.54 ± 5.63	-26.17 ± 10.08

	Net % change in AUC of $MBP_{(0-18)}$		
	TRC23029	TRC23030	TRC23035
1.55 µmole/kg	79.67 ± 24.45	118.79 ± 30.79	105.88 ± 26.26
13.00 µmole/kg	187.05 ± 44.84	171.91 ± 23.24	190.84 ± 56.37
39.00 µmole/kg	199.49 ± 40.38	281.68 ± 37.39	316.18 ± 73.04
130.00 µmole/kg	248.59 ± 57.13	381.07 ± 21.30	328.52 ± 92.86
390.00 µmole/kg	363.33 ± 53.43	337.033 ± 96.89	347.27 ± 112.55

	BMS346567	
	Net max. % fall in MBP	Net % change in AUC of $MBP_{(0-18)}$
0.85 µmole/kg	-6.85 ± 4.89	75.70 ± 27.30
7.30 µmole/kg	-12.66 ± 6.27	149.73 ± 53.19
21.90 µmole/kg	-20.6 ± 3.70	255.46 ± 91.49
73.00 µmole/kg	-18.61 ± 0.03	220.25 ± 79.95

AUC, area under curve; max., maximum; MBP, mean blood pressure

5.2.7. *In vivo* safety pharmacology study

Of the NCEs screened for efficacy, TRC23029 was found to be most efficacious in its antihypertensive effect. Though the $AUC_{(0-18)}$ achieved with highest dose of TRC23029 was less than the maximum $AUC_{(0-18)}$ achieved with TRC23030; TRC23029 was selected for further development. There was plateauing of antihypertensive effect for TRC23030; however TRC23029 did not showed saturation of its antihypertensive effect even up to the highest dose tested. Hence, the possibility of achieving further MBP lowering and increase in $AUC_{(0-18)}$ by increasing dose of TRC23029 yet exists. Hence, after evaluating potential of TRC23029 for development into a drug molecule, it was screened for neuropharmacological, cardiovascular and respiratory safety.

5.2.7.1. Central nervous system safety study

Swiss albino mice in all the four groups (three treatment groups and one vehicle treated control group; n=5 in each group) fed orally with either test compound or vehicle did not show any abnormal symptoms (Table 18). All the animals in TRC23029 low dose group ED_{50} (6.5 mg/kg), middle dose group $3xED_{50}$ (19.5 mg/kg) and the high dose group $10xED_{50}$ (65 mg/kg) retained their normal behaviour and reflexes throughout the study. On the basis of the observations made it could be concluded that the NCE TRC23029 dose not have any neuropharmacological safety concern over a wide range of doses. A representative sheet used for recording various neuropharmacological scoring is shown as Table 19.

Table 18: Irwin's test scores of conscious mouse treated with vehicle and different doses of TRC23029.

Time (hr)	Vehicle	TRC23029		
		6.5 mg/kg	19.5 mg/kg	65 mg/kg
0	72.0±0.0	72.0±0.0	72.0±0.0	72.0±0.0
0.5	72.0±0.0	72.0±0.0	72.0±0.0	72.0±0.0
1	72.0±0.0	72.0±0.0	72.0±0.0	72.0±0.0
2	72.0±0.0	72.0±0.0	72.0±0.0	72.0±0.0
4	72.0±0.0	72.0±0.0	72.0±0.0	72.0±0.0
6	72.0±0.0	72.0±0.0	72.0±0.0	72.0±0.0
10	72.0±0.0	72.0±0.0	72.0±0.0	72.0±0.0
24	72.0±0.0	72.0±0.0	72.0±0.0	72.0±0.0

Values represented are mean ± SEM for 5 observations in each group. $p>0.05$ for all observation in comparison to vehicle treatment by Student's *t* test.

Table 19: Neuropharmacological screening of TRC23029 in mice using Irwin scale

Test Compound:TRC23029
Date:29-Feb-08
Time of drug admin.:10:00 am
Route of admn: Oral
Dose: 19.5 mg/kg
Volume: 10 ml/kg

Animal Species: Mice
Strain: Swiss Albino
Sex: Male
Weight: 30 g
Time of death: -

Vehicle: 1% w/v Tween 80
0.5% w/v NaCl
Milli-Q water

		Normal score	0 hr	0.5 hr	1 hr	2 hr	4 hr	6 hr	10 hr	24 hr
Awareness	Alertness	4	4	4	4	4	4	4	4	4
	Visual placing	4	4	4	4	4	4	4	4	4
	Passivity	0	0	0	0	0	0	0	0	0
	Stereotypy	0	0	0	0	0	0	0	0	0
Mood	Grooming	4	4	4	4	4	4	4	4	4
	Vocalization	0	0	0	0	0	0	0	0	0
	Restlessness	0	0	0	0	0	0	0	0	0
	Irritability (aggression)	0	0	0	0	0	0	0	0	0
	Fearfullness	0	0	0	0	0	0	0	0	0
Motor activity	Spontaneous activity	4	4	4	4	4	4	4	4	4
	Reactivity	4	4	4	4	4	4	4	4	4
	Touch response	4	4	4	4	4	4	4	4	4
	Pain response	4	4	4	4	4	4	4	4	4
CNS excitation	Startle response	0	0	0	0	0	0	0	0	0
	Straub tail	0	0	0	0	0	0	0	0	0
	Tremors	0	0	0	0	0	0	0	0	0
	Twiches	0	0	0	0	0	0	0	0	0
	Convulsions	0	0	0	0	0	0	0	0	0
Posture	Body posture	4	4	4	4	4	4	4	4	4
	Limb position	4	4	4	4	4	4	4	4	4
Motor incordination	Staggering gait	0	0	0	0	0	0	0	0	0
	Abnormal gait	0	0	0	0	0	0	0	0	0
	Righting reflex	0	0	0	0	0	0	0	0	0
Muscle Tone	Limb tone	4	4	4	4	4	4	4	4	4
	Grip strength	4	4	4	4	4	4	4	4	4
	Body tone	4	4	4	4	4	4	4	4	4
	Abdominal tone	4	4	4	4	4	4	4	4	4
Reflexes	Pinna	4	4	4	4	4	4	4	4	4
	Corneal	4	4	4	4	4	4	4	4	4
	IFR	4	4	4	4	4	4	4	4	4
Autonomic	Exophthalmos	0	0	0	0	0	0	0	0	0
	Urination	0	0	0	0	0	0	0	0	0
	Salivation	0	0	0	0	0	0	0	0	0
General	Piloerection	0	0	0	0	0	0	0	0	0
	Skin color	4	4	4	4	4	4	4	4	4
	Writhing	0	0	0	0	0	0	0	0	0
	Respiration rate	4	4	4	4	4	4	4	4	4
	Lacrimation	0	0	0	0	0	0	0	0	0
Dead	Dead	0	0	0	0	0	0	0	0	0

IFR, Ipsilateral flexor reflex

5.2.7.2. Cardiovascular and respiratory safety study

At doses tested, low dose ED_{50} (4 mg/kg), middle dose $3xED_{50}$ (12 mg/kg) and the high dose $10xED_{50}$ (40 mg/kg) administered orally; TRC23029 did not have any major changes in the lead II ECG pattern and QTc interval, computed using Bazett's method in comparison to vehicle treated group (Table 20). Similarly, no effect of TRC23029 was observed at any of the test doses, on Guinea pig's heart rate and respiration rate (Table 21 and 22). Though QTc interval calculated by Bazett's formula at 2.5 hr for 40 mg/kg dose of TRC23029 showed significant decrease and at 7 hr for 4 mg/kg dose of TRC23029 showed significant increase in comparison to vehicle control, it could not be considered to be treatment related because such trend was not seen at subsequent time points and with higher dose in case of 4 mg/kg. Bazett's formula for calculating QTc interval is mentioned below:

$$QTc = QT/\sqrt{RR}$$

Where:

QTc = Corrected QT interval by Bazett's formula

QT = QT interval on ECG

RR = RR interval on ECG

Table 20: QTc interval derived using Bazett's formula from ECG of anesthetized Guinea pig treated with vehicle and different doses of TRC23029.

Time (hr)	Vehicle	TRC23029		
		4 mg/kg	12 mg/kg	40 mg/kg
0.5	0.282±0.022	0.326±0.022	0.287±0.020	0.251±0.031
0.75	0.306±0.019	0.322±0.020	0.290±0.016	0.264±0.033
1	0.288±0.023	0.323±0.028	0.311±0.025	0.234±0.023
1.5	0.299±0.016	0.341±0.036	0.311±0.027	0.271±0.017
2	0.310±0.014	0.298±0.023	0.313±0.027	0.279±0.036
2.5	0.296±0.020	0.304±0.021	0.332±0.022	0.228±0.0248*
3	0.291±0.015	0.298±0.021	0.331±0.025	0.265±0.018
4	0.304±0.014	0.332±0.038	0.348±0.027	0.252±0.023
5	0.295±0.014	0.276±0.041	0.311±0.025	0.301±0.026
6	0.280±0.011	0.306±0.021	0.333±0.026	0.271±0.007
7	0.264±0.013	0.365±0.033*	0.304±0.023	0.298±0.034
8	0.279±0.015	0.322±0.036	0.325±0.025	0.247±0.013

Values represented are mean ± SEM for 5 observations in each dose group. * $p<0.05$ in comparison to vehicle by Student's *t* test.

Table 21: Heart rate derived from ECG of anesthetized Guinea pig treated with vehicle and different doses of TRC23029.

Time (hr)	Vehicle	TRC23029		
		4 mg/kg	12 mg/kg	40 mg/kg
0.5	322.5±16.6	289.0±28.7	272.7±21.4	271.2±41.7
0.75	290.5±11.4	275.4±22.6	266.3±19.3	259.2±35.0
1	286.7±14.6	297.2±17.2	255.3±22.6	237.4±36.2
1.5	287.7±17.4	285.2±20.0	254.3±20.8	276.1±26.3
2	289.3±20.6	256.3±22.8	260.4±19.0	262.8±13.4
2.5	289.1±16.9	258.6±16.1	259.2±18.6	278.5±21.6
3	293.2±18.7	270.3±11.2	264.1±18.9	265.9±18.0
4	306.8±15.5	281.0±16.1	275.8±20.0	261.5±14.9
5	311.0±18.2	298.6±15.0	275.4±19.8	282.2±14.8
6	319.1±12.9	304.2±15.1	277.0±19.6	284.6±20.8
7	325.4±12.8	304.0±14.1	279.6±17.8	293.0±15.7
8	326.9±15.4	311.5±14.3	291.1±17.6	308.5±19.2

Values represented are mean ± SEM for 5 observations in each dose group. p>0.05 for all observation in comparison to vehicle treatment by Student's *t* test.

Table 22: Respiration rate of anesthetized Guinea pig treated with vehicle and different doses of TRC23029.

Time (hr)	Vehicle	TRC23029		
		4 mg/kg	12 mg/kg	40 mg/kg
0.5	75.4±5.0	67.0±6.1	69.0±8.0	79.2±5.8
0.75	73.7±6.2	65.0±4.2	66.0±5.4	73.2±7.9
1	69.0± 4.3	57.6±4.1	63.0±5.1	66.0±5.4
1.5	60.0±7.6	52.0±5.7	56.0±4.0	72.0±9.9
2	56.6±7.0	54.0±2.7	55.0±6.8	69.610.1
2.5	61.7±8.8	57.0±4.6	52.0±7.2	66.0±6.3
3	56.6±7.6	46.0±4.6	50.0±5.7	69.0±9.3
4	58.3±10.5	53.0±4.2	50.0±9.5	65.0±6.6
5	55.7±8.0	48.0±2.2	48.0±4.4	62.0±4.8
6	62.0±7.0	50.4±4.5	50.0±5.1	57.6±4.9
7	46.3±3.6	54.0±3.5	49.0±5.7	59.0±5.7
8	58.0±3.0	48.0±3.8	51.0±5.1	57.0±5.7

Values represented are mean ± SEM for 5 observations in each dose group. p>0.05 for all observation in comparison to vehicle treatment by Student's *t* test.

Discussion

6. Discussion

Hypertension is clearly a pervasive condition, with important health and economic implications for the future of both individuals and society. There is accumulating evidence from large outcome studies that support the need to move towards lower treatment targets in hypertensives, particularly for those with concomitant risk factors or evidence of established target organ damage (Chobanian, et al., 2003). While current BP control rates have greatly improved over the past decades (Kannel, 2002), the problem remains far from solved and further challenges are posed by facts that of those hypertensive patients who are on treatment, only 34% have their BP under control (Chobanian, et al., 2003) and an overall increase of about 50% in hypertensive adults is speculated to occur by year 2025 (Perkovic, et al., 2007).

Furthermore, consensus document generated by the Hypertension in African Americans Working Group (HAAW Group) emphasizes the disproportionate prevalence of hypertension and especially detrimental effect of hypertension on the incidence of stroke and end-stage renal disease in African Americans. Compounding the problem is the issue of decreased efficacy of β-blockers, ACE inhibitors and ARB's in groups of African Americans compared with groups of European Americans (Douglas, et al., 2003).

Given multifactorial nature of hypertension, targeting more than one physiologic mechanism has been looked at as a plausible strategy. With Ang II as an established target of pharmacological intervention that has made its way from bench to bedside (Fisher and Williams, 2005), there has been increasing interest in the biological effects of other vasoactive peptides like atrial natriuretic factor and ET. Although remarkable hemodynamic and cardioprotective effects were observed with endopeptidase modulators, life threatening adverse effects precluded their use (Battistini, et al., 2005;Messerli and Nussberger, 2000). Meanwhile there has been growing recognition that targeting specifically receptors, for example, AT_1, ET_A receptors rather than endopeptidase inhibition, would preserve the benefits accrued through additional effects mediated via other receptor subtypes (AT_2, ET_B) (Goddard, et al., 2004;Leslie, et al., 2005;Siragy, et al., 2000), at the same time it would minimize the attenuation of the effect of specific endopeptidase inhibition due to generation of the effectors as Ang II and ET-1 through alternate sources as chymase and other isoforms of ECE (Daull, et al., 2006b;Ferro, et al., 1998).

Emerging experimental evidence suggests interplay between the Ang II and ET systems. Ang II increases prepro-ET mRNA expression in endothelial cells (Imai, et al., 1992). Ang II stimulates the release of ET-1 from endothelial cells that involves AT_1 receptors, Ca^{2+} mobilization and activation of protein kinase C (Emori, et al., 1991). Ang II infusion into rats increases aortic ET-1 content 3-fold and this response is blocked by the AT_1 receptor blocker losartan (d'Uscio, et al., 1998). Conversely, ET-1 treatment of pulmonary endothelial cells stimulates the conversion of Ang I to Ang II (Kawaguchi, et al., 1990) by stimulating vascular ACE activity (Rademaker, et al., 2004). ET_A receptor blockers on the other hand prevent the hypertrophic, mitogenic, natriuretic and diuretic effects of Ang II (Moreau and Dao, 2001;Riggleman, et al., 2001). Pretreatment with nonselective ET_A/ET_B receptor blocker blunts the increase in BP evoked by an infusion of Ang II (Balakrishnan, et al., 1996;Ficai, et al., 2001).

As Ang II and ET-1 similarly cause hypertension and copromote each other's actions, it is plausible that concomitant blockade of both Ang and ET pathways may lead to enhanced BP reductions. Thus targeting RAS and ET system together presents attractive target if combined. Mixed AT_1 and ET_A receptor blockers are therefore expected not only to enhance the antihypertensive effect of AT_1 receptor blockade but also to attenuate the severity of end-organ damage. To avoid the recognized side effects of ET receptor blockade and take the advantage of a greater antihypertensive efficacy of AT_1 receptor blockade, a dual acting receptor blocker should be selective for the AT_1 receptor over the ET_A receptor. Selective and nonselective ET receptor blockade is associated with several toxicological lesions. There is little doubt that embryo-fetal, liver and testicular toxicity are directly related to ET receptor modulation (Epstein, 2008;Remuzzi, et al., 2002). This aspect alone has so far hindered the broader therapeutic use of ET receptor blocker. Any combination product or multi-potent modulator leading to ET receptor blockade and associated lesions can be expected to carry warning for its effects on reproduction, liver and possibly testicular toxicity; quite similar to the only marketed, nonselective ET_A/ET_B receptor blocker bosentan (Battistini, et al., 2006;Remuzzi, et al., 2002). The novelty in the current studies lies in the concept of combining the beneficial effects of AT_1 receptor blockade with a small, sub-toxic contribution of ET_A receptor inhibition. An ideal dual AT_1 and ET_A receptor blocker should be selective for the AT_1 receptor over ET_A receptor and should not elicit the typical toxicological profile associated with ET receptor blockade. According to our knowledge, we for the first time have explored this strategy in our antihypertensive program.

The first challenge was to identify an animal model wherein the pathophysiology of hypertension is contributed by both Ang and ET components. SHR (Iyer, et al., 1996;Tikellis, et al., 2006), two-kidney, one-clip hypertensive rats (Imamura, et al., 1995;Okamura, et al., 1986;Ploth, 1983) and transgenic (mREN2) 27 rats (Bader, et al., 1992;Montgomery, et al., 1998;Peters, et al., 1993;Prieto-Carrasquero, et al., 2008;Rossi, et al., 2000;Veniant, et al., 1995) are rat models of hypertension in which overactivated RAS has been shown to be the cause for the observed hypertension. Similarly, it has been demonstrated that salt sensitive rat model of hypertension as Dahl salt sensitive rats, DOCA salt hypertensive rats have ET as major contributor to observed hypertensive state (d'Uscio, et al., 1997;Ikeda, et al., 1999;Lariviere, et al., 1993;Schiffrin, 2005). However, for establishing potentiation of antihypertensive activity caused by addition of ET_A receptor blocker to classical ARB therapy, a model in which both activated RAS and ET system contribute to pathogenesis of hypertension was required. Salt loaded SHRsp was evaluated for validating this hypothesis. Though salt loaded SHRsp have shown responsiveness towards treatment with ACE inhibitors and AT_1 receptor blockers, these drugs only temporarily delayed but did not prevent the development of severe hypertension (Abrahamsen, et al., 2002;Camargo, et al., 1993;Savage and Jeng, 2002). This suggests that, although inhibition of the RAS has great beneficial effects, other mechanisms may be involved in the establishment of pathological conditions in SHRsp. Elevated ET-1 levels which, once suggested is now well documented (Iglarz and Schiffrin, 2003;Savage and Jeng, 2002;Schiffrin, 2001;Schiffrin, 2005;Sharifi, et al., 1998) and proven (Blezer, et al., 1999) in studies using selective ET_A receptor blocker, as contributory factor responsible for elevated BP observed in salt loaded SHRsp in addition to overactivated RAS. The concept that ET-1 might be involved in salt sensitive form of hypertension originated from early observation where plasma and urinary ET-1 levels in salt sensitive hypertensive patients were found to be elevated (Ikeda, et al., 1999). Similar results were confirmed by Savage and Jeng who demonstrated upregulated tissue binding for ET-1 in salt loaded SHRsp in comparison to age-matched normotensive Wistar-Kyoto rats (Savage and Jeng, 2002). Therefore, salt loaded SHRsp was selected as animal model for our studies requiring screening of antihypertensive effect of AT_1 and ET_A receptor blocker combination or molecules having dual AT_1 and ET_A receptor blocking property.

Different investigators have used different sodium chloride concentration ranging from 1% to 8% either in food or water as salt load to SHRsp (Orth, et al., 2007;Quintas, et al., 2007;Takai, et al., 2001;Zhang, et al., 2008). However, to

establish optimal salt load which is tolerable to SHRsp in drinking water, as well as capable of augmenting hypertension to level required (40-60 mmHg rise in MBP), a pilot study was undertaken. Exploratory studies for tolerability were performed in SHR and the learning from SHR exploratory study were further tested on telemetered SHRsp. Similar to what is reported (Zhang, et al., 2008), salt loading resulted in increase in MBP in our SHRsp and this increase was found directly related to the salt load provided to SHRsp. A progressive increase in BP was observed upon initiating 1% salt load to SHRsp and upon shifting them to 2% salt after a week. This confirmed salt sensitivity of our model.

Salt loaded SHRsp showed a dose dependent fall in MBP upon treatment with selective AT_1 receptor blocker losartan (Kobayashi, et al., 2008;Polizio, et al., 2008) and also with selective ET_A receptor blocker ZD1611 (Jones, et al., 2003;Luscher and Barton, 2000;Stenman, et al., 2007;Wilson, et al., 1999). These results further established contribution of both Ang II and ET-1 in genesis of hypertension in our salt loaded SHRsp model.

Once validated for the contribution of Ang II and ET-1 in the pathogenesis of hypertension observed in salt loaded SHRsp, the model was used for testing our hypothesis whether addition of minimally effective (and potentially non toxic) dose of an ET_A receptor blocker could further potentiate antihypertensive effect of an AT_1 receptor blocker. The hypothesis was tested in two parts; first, whether potentiation in antihypertensive effect is possible by adding ET_A receptor blocker to sub-maximally effective dose (ED_{75}) of an AT_1 receptor blocker and second, whether potentiation in antihypertensive effect is possible by adding ET_A receptor blocker to maximally effective dose of an AT_1 receptor blocker.

ED_{75} for losartan in salt loaded SHRsp is not described in literature, however a clear dose response relationship has been established from studies performed in humans where plateau of losartan's antihypertensive effect was observed at dose ranging between 50-100 mg/day (Andersen, et al., 2002;Gradman, et al., 1995). In absence of any reference data for rat, complete DRC for losartan was created in our salt loaded SHRsp model so as to arrive at ED_{75} dose which could be used in further experiments as sub-maximally effective antihypertensive dose of losartan. Similar to human studies (Gradman, et al., 1995), a clear dose response of losartan for its antihypertensive action was established in our model of salt loaded SHRsp with maximal antihypertensive effect achieved at dose of 10 mg/kg. Though, others have reported same dose of losartan (10 mg/kg) as subpressor in rats that were transgenic for both the human renin and angiotensinogen genes (Bohlender, et al., 2000); the

reason for this discrepancy in results could be due to greater contribution of RAS activation in these rats by virtue of bearing human renin and angiotensinogen transgene in their genome, which is not the case with our animal model of salt loaded SHRsp. At the same time only partial DRC for antihypertensive activity of ZD1611 was created with aim to establish its minimal effective dose capable of eliciting an antihypertensive effect in this animal model.

A potentiation in the antihypertensive effect upon oral co-administration of ED_{75} of losartan (10 mg/kg) and minimally effective dose of ZD1611 (0.3 mg/kg), was observed. While achieving potentiation in the antihypertensive effect, we optimized dose of both losartan and ZD1611 and characterized antihypertensive potential of such a combination when minimally effective antihypertensive dose of an ET_A receptor blocker as ZD1611 is added to sub-maximal (ED_{75}) dose of AT_1 receptor blocker losartan. These set of studies provided confirmation of our hypothesis that with this combination, the maximum plasma levels of ZD1611 achieved were still less than what is known to be associated with teratogenic potential in rats (unpublished data on file: AstraZeneca), making such combination with titrated dose, safe for use even in child bearing females. Further, on the basis of drug plasma concentration-time profile for losartan administered alone and losartan administered in combination with ZD1611, we have demonstrated that the potentiation effect of such a combination is not incidental due to their interaction leading to increased availability of losartan but is pharmacological by targeting two contributory pathways, RAS and ET system, together in our hypertensive rat model.

A sub-maximal dose of losartan was selected in the initial study so as to give a window for the ET_A blocker to further enhance the antihypertensive action of AT_1 blocker, however on the basis of the outcome of this study the concept was further extended to examine whether a small dose of another selective ET_A blocker ZD4054 (Morris, et al., 2005;Rosano, et al., 2006), which has successfully completed phase-II clinical trial (Chustecka, 2007), can further potentiate antihypertensive activity of an AT_1 receptor blocker, candesartan beyond maximal attainable fall in MBP at its maximally effective dose. The rationale for this study was to establish whether the combination of AT_1 and ET_A receptor blocker would benefit the resistant case of hypertension the most. These patients would presumably be at the highest effective dose of candesartan and yet their BP not adequately controlled. Therefore, if the combination would be able to lower BP over and above that achieved with maximally effective dose of candesartan, would give it a distinct advantage in this patient population. Candesartan was selected instead of losartan in this study because it is

more potent AT_1 receptor blocker than losartan. Experiments in animals have shown that the affinity of candesartan for the AT_1 receptor is 10 and 80 times greater than that of EXP3174 (active metabolite of losartan) and losartan, respectively (Fabiani, et al., 2000;Fuchs, et al., 2000;Gradman, 2002;Inada, et al., 1999) and thus losartan is 10-30 fold less potent than candesartan in inhibiting Ang II receptor binding (Fabiani, et al., 2000). The high potency and long duration of action in candesartan is attributed to its insurmountable inhibition which is result of its tight binding and slow dissociation from AT_1 receptors. The dissociation rate of tritiated candesartan binding ($t_{1/2}$ = 66 min) is shown to be five times slower than that of radiolabelled Ang II binding ($t_{1/2}$ = 12 min) to AT_1 receptor (Fuchs, et al., 2000;Inada, et al., 1999). Most investigators in their studies with SHRsp have used candesartan at dose of 10 mg/kg or less as high dose or have escalated candesartan up to dose of 10 mg/kg for establishing its dose response in rats (Inada, et al., 1997;Kim, et al., 1994b;Kim, et al., 1994c;Kim-Mitsuyama, et al., 2005). Further, candesartan at a dose of 10 mg/kg/day in rats is approximately 2.8 times the maximum recommended daily human dose of 32 mg on a mg/m^2 basis (AstraZeneca, 2007). Antihypertensive effect of 10 mg/kg candesartan is not further augmented by combining it with an ACE inhibitor enalapril in SHR (Wada, et al., 1996). This suggests that maximal antihypertensive effect attainable by intervening RAS is achieved with candesartan at dose of 10 mg/kg in rats and thus candesartan at dose of 10 mg/kg was selected to achieve maximum possible antihypertensive effect through AT_1 receptor blockade in our animal model of salt loaded SHRsp.

Extending the finding from healthy humans that, repeated daily oral dose of 8 mg candesartan for seven days produce stronger blockade effect to exogenously administered Ang II than after single administration (Fuchs, et al., 2000), an eight day repeat daily oral dosing with candesartan (10 mg/kg) was decided in our salt loaded SHRsp. This was also to ensure that maximum possible antihypertensive effect attainable by blocking AT_1 receptors using candesartan is achieved. Results obtained in our study indicates no significant difference in the MBP lowering by candesartan 10 mg/kg administered on day one and day eight of repeated administration with no change in overall PK upon repeat administration.

Similar to ZD1611, minimum dose of ZD4054 capable of producing antihypertensive effect in salt loaded SHRsp was established by administering 0.3, 1.0 and 3.0 mg/kg of ZD4054 orally, once daily for three days. Though the minimal dose of ZD4054 (0.3 mg/kg) failed to cause any reduction in MBP, ZD4054 at dose of 1.0 mg/kg and 3.0 mg/kg were found to lower MBP. Thus, to establish whether or not

minimally effective dose of ET_A receptor blocker ZD4054 potentiates the MBP lowering effect of a maximally effective antihypertensive dose of AT_1 receptor blocker candesartan (10 mg/kg) and whether or not ZD4054 would dose dependently further potentiate the antihypertensive action of candesartan 10 mg/kg; combination studies with candesartan 10 mg/kg with either ZD4054 1 mg/kg or 3 mg/kg were carried out. With help of these studies, to our knowledge, we for the first time have shown that further potentiation in maximal antihypertensive effect of an AT_1 receptor blocker could be achieved by adding minimally effective dose of ET_A receptor blocker. The hypothesis is further strengthened by showing further potentiation of effect by increasing dose of ZD4054 to 3 mg/kg in a dose dependent manner. That the observed potentiation and dose response observed with different doses of ZD4054 is pharmacological was evident from the plasma drug concentration profile of candesartan 10 mg/kg which was not significantly altered when either administered alone or when administered in combination with different doses of ZD4054. This was evident despite reduction Cmax observed for ZD4054 in combination. Thus, the observed potentiation in antihypertensive effect of combination could be ascribed to a potentiation effect of ET_A receptor blocker ZD4054. Safety of this combination was also established by the fact that plasma concentration of ZD4054 at the lowest potentiation dose of 1.0 mg/kg was found to be lower than levels reported to be associated with moderate seminiferous tubular atrophy in rats (unpublished data on file: AstraZeneca).

Thus with help of our studies using well characterized AT_1 and ET_A receptor blockers, we validated the fundamental concept that addition of minimally effective antihypertensive dose of selective ET_A receptor blockers can potentiate antihypertensive action of AT_1 receptor blockers even beyond their maximal effect. We have also shown that the observed potentiation of antihypertensive effect is pharmacological in nature and that the combination is safe in terms of possibilities of known toxic effects associated with the use of ET_A receptor blockers in rats. This was a unique attempt to study the concept of a dual action target wherein, pharmacological blockade at two receptors, by adding individual blockers of both receptors was tested to determine if simultaneous blockade at two sites with measured contribution of each blockade would provide more effective control of BP.

Based on the proof for our hypothesis, new dual action receptor blockers which could selectively block AT_1 and ET_A receptors simultaneously and produce antihypertensive action were designed and synthesized by the Medicinal Chemistry

Department of Torrent Research Centre and screened for antihypertensive activity and efficacy.

Similar to the conventional approach in new drug discovery, NCEs, which were designed using rational drug design approach and subsequently synthesized were screened initially for their selectivity toward AT_1, and ET_A receptors. To ascertain selectivity of compounds towards target receptors, *in vitro* Ca^{2+} mobilization assay in cell lines over-expressing human AT_1, ET_A and ET_B receptor was performed by AstraZeneca at their Research Facility in Sweden and IC_{50} values of each NCE for each of human AT_1, ET_A and ET_B receptor was established. IC_{50} value is one of the most simplest and common way of expressing a receptor blocker quantitatively and allows comparison of molecules relative to each other in terms of their potency (Ghosh, 2005). Selection of NCEs from group of total 50 NCEs for further detailed activity and efficacy screening was performed considering their low IC_{50} value for AT_1 and ET_A receptor; high IC_{50} value for ET_B receptor and ratio for ET_A : AT_1 IC_{50} value. This was to ensure high selectivity of molecule for AT_1 and ET_A receptor in comparison to ET_B receptor. Since, no literature is available on the ideal ET_A : AT_1 IC_{50} ratio for class of dual action receptor blocker active as blocker for both AT_1 and ET_A receptor, molecules with low IC_{50} value for AT_1 and ET_A receptor and with range of ET_A : AT_1 IC_{50} ratio were selected for preliminary *in vitro* characterization and *in vivo* primary screening of activity in animal model.

In absence of any approved dual action receptor blocker for human use, which acts as antihypertensive drug by selectively blocking AT_1 and ET_A receptors, BMS346567, a dual action receptor blocker (having similar affinity for AT_1 and ET_A receptors which does not address the ET_A receptor blockade associated drawbacks) from Bristol-Myers Squibb (Murugesan, et al., 2005), being developed currently by Pharmacopeia, Inc. as PS433540 (O'Riordan, 2008) was taken as reference molecule for comparison with our newly synthesized NCEs.

Preliminary *in vitro* characterization of NCEs for their physicochemical and PK profiling allows rapid identification and elimination of compounds with properties not suitable for further development as drug candidates. Many investigative compounds with good therapeutic potential fail to progress beyond the early developmental stages, primarily because of poor biopharmaceutical properties (Hwang, et al., 2003). It is suggested that insufficient knowledge of metabolic and PK properties of NCEs was responsible for about 10% of failures during drug development in year 2000 (Baranczewski, et al., 2006). Thus, an effort to establish critical physicochemical parameters of selected NCEs was made *in vitro* by determining for selected NCEs,

their solubility, permeability, plasma protein binding and *in vitro* metabolism with rat microsomes. It is generally desirable for a drug molecule to have some level of solubility. Poor solubility can present problems from reliability and reproducibility of several *in vitro* tests to significant formulation issues (Adeniji and Adejare, 2008). A high throughput screening method for the determination of aqueous drug solubility using laser nephelometry in microtiter plates was used in our study. In the samples, the percent DMSO was kept constant allowing direct comparison of results. The nephelometric method has been shown to produce results equivalent to those produced by an HPLC method and to be largely unaffected by colour of solutions (Bevan and Lloyd, 2000). Drug solubility along with its permeation across biological membrane is essential for drug bioavailability (Hwang, et al., 2003). Methods as cell culture models of intestinal permeability such as Caco-2 monolayer and artificial membrane as PAMPA are commonly used *in vitro* methods to assess the oral absorption potential of NCEs (Kerns and Di, 2008). PAMPA is an alternative to Caco-2 cell culture model, which is used to predict passive permeability of NCEs. It has been suggested that artificial membrane method such as PAMPA could soon replace Caco-2 assays, because PAMPA is an excellent biomimetic model in terms of high throughput, reproducibility and cost (Ganta, et al., 2008). Permeability prediction using PAMPA has shown good correlation with drug fraction absorbed orally in humans (Hwang, et al., 2003) and is extensively used today for the evaluation of early drug candidates (Avdeef, et al., 2007;Masungi, et al., 2008). Though, PAMPA may not be sensitive enough to predict the absolute human fraction absorbed, it is considered accurate enough to rank compound permeability, which is solely transported by transcellular passive diffusion (Hwang, et al., 2003). Since, about 95% of commercially available drugs cross the gastrointestinal wall by passive diffusion (Idealp-Pharma, 2005), permeability estimation using PAMPA has become an ideal method for early preliminary permeability estimation of potential drug candidate.

The fact that only unbound drug is available for diffusion or transport across cell membrane and interaction with pharmacological target, makes plasma protein binding an important parameter to be assessed during early drug development. High drug-protein binding can both reduce the fraction of free drug available for target sites and prolong the duration of drug activity (Kratochwil, et al., 2002). The most common techniques used for estimation of plasma protein binding are equilibrium dialysis and ultrafiltration (Bridges and Wilson, 1976;Kerns and Di, 2008). Equilibrium dialysis is often regarded as the “reference method”, however it suffer from limitations of being time consuming where prolong incubation may cause bacterial

contamination and protein denaturation (Bridges and Wilson, 1976;Kerns and Di, 2008). Ultrafiltration on other hand is a rapid technique, suggested to be closely approximating *in vivo* situation and at the same time shown to produce results comparable to equilibrium dialysis technique (Barre, et al., 1985;BD Biosciences, 2008). Therefore, in current preliminary screen, we used ultrafiltration technique to establish plasma protein binding of our NCEs.

After entering the body, drug is eliminated either by excretion or by metabolism to one or more active or inactive metabolites. The stability of a drug candidate, which refers to the susceptibility of compounds to undergo biotransformation, can have important PK and clinical significance *in vivo*. High metabolic lability usually leads to poor bioavailability and high clearance. The primary site of metabolism for many drugs is the liver, where biotransformation reactions are mainly catalyzed by the cytochrome P450 family of enzymes located in the endoplasmic reticulum. Therefore, the most relevant information about a drug's metabolic clearance *in vivo* can often be derived from *in vitro* studies using *in vitro* cellular/subcellular fraction methods (Gerhold, et al., 2001). Liver contains the largest number and the highest levels of cytochrome P450 enzymes involved in drug biotransformation (Hrycay and Bandiera 2008); microsomal fraction from human liver contains a full complement of P450 enzymes which can metabolize (either oxidize or reduce) a large number of structurally different endogenous and exogenous compounds. Seven of the 57 known human isoforms of P450s are responsible for >90% of the metabolism of all pharmaceuticals in current clinical use (de Groot, 2006;Taavitsainen, 2001).Though the results obtained from *in vitro* microsomes metabolism studies are not adequate because it represents only endoplasmic fraction of metabolizing enzymes (Plant, 2004;Sun, et al., 2004), but still microsomal preparations are probably the most widely used *in vitro* systems for assessing initial metabolic profile of an NCE, mainly because of relative ease and throughput (Venkatakrishnan, et al., 2003).

NCEs and reference molecule BMS346567 screened through battery of *in vitro* screen were found to be soluble in range of 13.42-400 µg/ml in 1% DMSO, highly permeable through PAMPA, highly plasma protein bound and moderately metabolized in male rat liver microsomes except for TRC23012 which was found to be highly metabolized. Having shown almost similar characteristics *in vitro*, all NCEs and the reference molecule BMS346567 was further screened for activity in primary *in vivo* screen. TRC23012, though highly metabolized, was considered for further primary *in vivo* activity screening in anesthetized rat model considering the concept

of insurmountability, where the molecule continues to block the receptor even when its overall plasma concentration is reduced, by virtue of its intrinsic property of very slow dissociation from receptor.

For primary *in vivo* screen, the anesthetized rat model was developed with aim to dissect out and measure individually the AT_1 and ET_A receptor blocking activity of our NCEs.

First, selection of appropriate anesthetic which would maintain stable BP for extended duration in our anesthetized rat model was performed between ketamine-xylazine combination and isoflurane inhalation. Urethane, another commonly used anesthetic was not considered for evaluation as it has been shown to suppress pressor response to ET-1, big ET-1 and Ang II in rats (Gratton, et al., 1995). Though slow continuous infusion of ketamine-xylazine cocktail is reported to maintain rats under anesthesia with their MBP stabilized to around 90 mmHg for period of up to 12 hr (Simpson, 1997), for reason beyond explanation, similar results could not be reproduced in our lab. Isoflurane has been shown to maintain cardiovascular and respiratory functions better than other inhalant anesthetics of its class as halothane and enflurane (Brunson, 1997). Similar to published reports (Biddlestone, et al., 2007) we were successful in performing arterial and venous cannulation in rat maintained under 1.5-2% isoflurane anesthesia and found 1% isoflurane suitable for maintenance of anesthesia in cannulated rat (Hare, et al., 2006;Yang, et al., 2008). This was also first attempt wherein, using the same model, in order to be able to isolate the pharmacological action at one of the targets which interacts with the second target, the endogenous contribution of the second target was blocked and then the challenge to the target of interest was measured. A separate estimation of activity mediated through action of our NCEs on individual AT_1 and ET_A receptor was important in light of evidences demonstrating interaction between RAS and ET system. Thus, it was essential to estimate the activity of our NCEs mediated via blocking AT_1 receptors in animal model having its endogenous ET_A receptors blocked and vice versa. Antihypertensive action of our NCEs mediated through blocking of AT_1 and ET_A receptors could therefore be dissected in this model separately.

Effect of AT_1 receptor blockade was studied by measuring reversal of exogenously administered Ang II pressor response in presence of maximal blockade of ET_A receptors by ET_A receptor blocker ZD1611 in male Sprague Dawley rats by the test drug administered as infusion and a cumulative dose response was determined. Similarly, effect of ET_A receptor blockade was studied by measuring the reversal of exogenously administered big ET-1 pressor response in presence of

maximal blockade of AT_1 receptors by losartan in male Sprague Dawley rats, by the test drug administered as infusion and a cumulative dose response was obtained. With this model a valid comparison of the activity of various NCEs and the reference molecule by establishing ED_{50} for their AT_1 and ET_A receptor blocking activity could be derived at from cumulative DRC created in separate anesthetized rat infused with Ang II or big ET-1.

The infusion dose of pressor agent Ang II and big ET-1 was adjusted to achieve stable increase in MBP in range of 40-60 mmHg so that appropriate window of elevated MBP could be made available to NCEs over which they could show their antihypertensive activity by virtue of their AT_1 and ET_A receptor blocking activity respectively. Since supra normal levels of circulating Ang II stimulate ET-1 formation (Moreau, et al., 1997;Rajagopalan, et al., 1997) and ET-1 facilitates conversion of Ang I to Ang II (Kawaguchi, et al., 1990); overall antihypertensive performance of NCEs mediated through specific receptor blocking (AT_1 and ET_A receptor) could be confounded in model with exogenously administered Ang II or big ET-1. Therefore, selective blocker of ET_A receptor (ZD1611) and AT_1 receptor (losartan) were administered at doses capable of blocking respective endogenous receptors throughout the experiment duration while evaluating NCEs for their AT_1 or ET_A receptor blocking activity respectively. The precursor of ET-1, big ET-1, which gets converted to the biologically active peptide ET-1 *in vivo* via a phosphoramidon-sensitive ECE, was used for *in vivo* analysis of the effects of NCEs against their ET_A receptor blocking activity because big ET-1 *in vivo* does not elicit the initial depressor response associated with i.v. administered ET-1 and yields greater maximum response than that to ET-1 itself (Wilson, et al., 1999).

Since this anesthetized rat model having its endogenous AT_1 or ET_A receptors blocked was first developed by us, it was validated by capturing dose dependent antihypertensive activity of losartan by virtue of its AT_1 receptor blocking action in Ang II infused rat having its ET_A receptor blocked with ZD1611. Similarly, capability of this model to capture ET_A receptor blocking activity of NCEs was validated by capturing dose dependent antihypertensive activity of ZD1611 by virtue of its ET_A receptor blocking action in big ET-1 infused rat having its AT_1 receptor blocked with losartan. Validation of this model was followed by capturing antihypertensive activity of selected NCEs and reference molecule as a cumulative DRC to i.v. infused Ang II and big ET-1. Comparative activity of NCEs was calculated as dose capable of lowering the MBP to half from beginning of infusion of respective dose that is, ED_{50}. With this model a valid comparison of the activity of various NCEs

and the reference molecule by establishing ED_{50} for their AT_1 and ET_A receptor blocking activity could be derived at from cumulative DRC created in separate anesthetized rat infused with Ang II or big ET-1.

It has been found that many compounds which are both potent and efficacious *in vitro* fail to elicit desired effect *in vivo*. In many cases one of the most common reasons for this lack of *in vivo* activity is either poor absorption from site of administration or rapid metabolism and excretion. Therefore, it was essential to estimate the bioavailability and other PK parameters of NCE after administering them from desired route of administration *in vivo* (NoAb BioDiscoveries, 2008).

Although more resource consuming and labor intensive than *in vitro* experiments, animal studies are considered to be the most predictive of human PK (Mei, et al., 2006). In addition, obtaining acceptable bioavailability and PK in rats is considered to be important for proof-of-concept and for planning of further studies with chemical entity. Also, the process of characterizing the preclinical bioavailability and PK properties of NCE is an important component of lead compound selection and optimization in drug discovery (Ward, et al., 2001). Oral route of drug administration is most desirable because it is generally the most convenient method of administration for patients and the one most likely to result in high patient compliance. Also, for diseases like hypertension which requires chronic treatment, the preferred route of drug administration remains oral. Thus, oral administration is the most desirable route for NCEs being developed by us. Rats are most commonly used species for the purpose. The initial choice of the rat as the primary species for PK studies is also relevant to their use in pre-clinical pharmacology and toxicology studies (Logan, 2003). Oral bioavailability is assessed based on drug concentrations in the general circulation. Absolute oral bioavailability is a measure of systemic exposure of an orally dosed drug relative to an i.v. dose. Though, oral bioavailability lacks a predictive correlation between species, it is considered fair enough to suggest a trend in the bioavailability (Mandagere and Jones, 2003).

In our study too, we measured oral bioavailability of our NCEs and reference compound in rats. This was essential as no information about their oral absorption and bioavailability was available and earlier activity studies were performed by infusing chemical entity by i.v. route. In the process of oral bioavailability estimation, other important PK parameters as C_{max}, T_{max}, $t_{1/2}$ and AUC were also established to get a fair idea about PK profile of our NCEs in comparison to reference compound.

In line with published data we found almost similar oral bioavailability (36% versus 40%) and T_{max} (0.44 hr versus 0.6 hr) for BMS346567 in our rats (Murugesan,

et al., 2005). However, in our setup much higher $t_{1/2}$ (12.19 hr versus 4.1 hr) and much lower C_{max} (1959.39 ng/ml versus 12000 ng/ml) was observed for reference compound. No direct reason for such difference observed in some of the PK parameters could be identified; however difference could be probably due to difference in sampling time points, difference in formulation used or difference in strain of rat used in our study.

In comparison to reference compound BMS346567 our NCEs had shorter half life, smaller AUC and high clearance; though they demonstrated comparable or better absolute oral bioavailability, making them suitable candidates for oral administration. However, their further development for human use may not warrant multiple dose administration provided the drug candidate binding is of longer duration or irreversible.

Having identified TRC23029, TRC23030 and TRC23035 as most active NCEs in our primary *in vivo* screen of anesthetized rat and after establishing oral bioavailability, these NCEs and reference compound BMS346567 were evaluated in secondary *in vivo* efficacy model of telemetered salt loaded SHRsp.

To compare the efficacy of NCEs and reference molecule, DRC for all the four chemical entities was created. The dose for BMS346567 were referred from study published by Murugesan et al. in year 2005 where they have shown that BMS346567 at dose of 10 µmol/kg caused significant lowering of BP of SHR (Kowala, et al., 2004;Murugesan, et al., 2005). However, since activated RAS is the main contributor to elevated BP observed in SHR (Iyer, et al., 1996;Schiffrin, 2001;Tikellis, et al., 2006), we expected more potent antihypertensive effect of BMS346567 in our salt loaded SHRsp model where both activated RAS and ET system is known to contribute to hypertension (Hubner, et al., 1995;Iglarz and Schiffrin, 2003;Schiffrin, 2001;Schiffrin, 2005;Takai, et al., 2001). Hence, BMS346567 at dose of 7.3 µmol/kg (with 27% dose reduction) was selected as the first log dose for creating DRC and subsequent log doses were selected to be 21.9 and 73 µmol/kg. Further, since BMS346567 at dose of 7.3 µmol/kg was expected to cause significant antihypertensive effect, to initiate DRC, much smaller dose of 0.85 µmol/kg (8.6 times lower than 7.3 µmol/kg) was also identified for establishing dose response. As the total exposure (AUC) of our NCEs was much lower than BMS346567, our NCEs were administered at doses which were 1.8 times higher than that of BMS346567.

TRC23029 and TRC23035 demonstrated a dose dependent increase in their antihypertensive action right from first dose of 1.55 µmol/kg to tested maximal dose

of 390 µmol/kg, further increment in dose was not possible because of formulation limitation. TRC23030 and BMS346567 though produced their maximal antihypertensive effect at dose of 130 µmol/kg and 21.9 µmol/kg respectively; doses much less than that of TRC23029 and TRC23030; the maximal fall in net percent MBP (after considering fall in MBP brought about by vehicle treatment) observed with TRC23029 was 31.54%, in comparison to TRC23030, TRC23035 and BMS346567 which produced 29.1%, 26.2% and 20.6% fall respectively. AUC for fall in MBP, which incorporates factor of duration of effect along with the effect itself, demonstrated similar profile, where our NCEs were found to be superior to reference molecule BMS346567. Although, TRC23029 demonstrated highest efficacy amongst screened NCEs and reference molecule, it was found to be least potent of the group, having highest EC_{50} value. However, considering highest efficacy of TRC23029 amongst the group, it was selected for further development by performing core battery of safety pharmacology studies.

Safety pharmacology studies were performed in accordance with International Conference on Harmonisation Guideline S7A, which advocates performing of safety pharmacology core battery tests for NCEs being developed as therapeutic agents for human use. The purpose of the safety pharmacology study is to investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above (ICH, 2001). Accordingly, the ED_{50} dose of TRC23029 derived from conscious, telemetered salt loaded SHRsp was used as a starting point of reference for various doses used in the safety pharmacology studies (ED_{50}, $3xED_{50}$ and $10xED_{50}$), to be administered by oral route, the intended clinical route of administration.

The ICH S7A guidelines emphasize performing of the safety pharmacology core battery test to investigate the effects of the test substance on vital functions. In this regard, the cardiovascular, respiratory and central nervous systems have been considered as the vital organ systems (ICH, 2001). Irwin's test was performed to evaluate the effect of TRC23029 on central nervous system. Irwin's test is the most commonly used test for neuropharmacological screening of test compounds in mice (Mordvintsev, et al., 2007;Navarro, et al., 2005;Schwenk, et al., 2003). The Irwin test involving observations on behavioural, neurologic and autonomic symptom revealed that TRC23029 is a safe compound having no safety issues related to central nervous system.

Cardiovascular and respiratory system safety upon use of TRC23029 was assessed in anesthetized guinea pig. Heart rate, gross ECG pattern and QTc interval

were considered as checkpoints to evaluate cardiovascular safety of TRC23029 whereas respiratory rate was taken as an index to evaluate respiratory safety of TRC23029.

The QT interval (time from the beginning of the QRS complex to the end of the T wave) of the ECG is a measure of the duration of ventricular depolarization and repolarization (Kulmatycki, et al., 2001). Prolongation of the QT interval on an ECG has been linked to the rare (but potentially fatal) ventricular arrhythmia known as Torsade de Pointes. *In vitro* studies suggest that most QT-prolonging drugs block the delayed rectifier potassium current carried by the pore forming subunit encoded by human ether-a-go-go-related gene (hERG) in humans (McDermott, et al., 2002). Guinea pigs were used as a model to study cardiovascular safety because they possess specific ion channels similar to man (Hamlin, et al., 2003) whereas use of rats and mice was not considered because the ionic mechanisms of repolarization in adult rats and mice differ from larger species, including humans (the primary ion currents controlling repolarization in adult rats and mice is Transient Outward Potassium Current (I_{TO})) and rats ventricles have little (if any) functional delayed rectifier potassium current or hERG (ICH, 2001;Wymore, et al., 1997).

Since QT interval is strongly and inversely related to heart rate, change in heart rate exerts an effect on QT interval, which can confound the assessment of the effect of the test substance on ventricular repolarization and the QT interval. Therefore to compare the QT interval from different subjects with different heart rates, correction to QT interval is applied (Davey, 1999;ICH, 2001). Various QT interval heart rate correction formulae have been proposed (Chan, et al., 2007;Piotrovsky, 2005) however Bazett's method have been shown best for correcting QT interval in anesthetized Guinea pigs (Hamlin, et al., 2003) and thus same has been used in our anesthetized Guinea pig model for estimation of QTc interval. During 8 hours (duration of almost complete disappearance of TRC23029 from plasma) of post administration screening for cardiovascular and respiratory safety, TRC23029 was found to be safe on these vital systems.

TRC23029 which has evolved through the screening process as a potential drug candidate is expected to be a potential drug for treatment of hypertensive patients and especially for those presenting resistant form of hypertension. Resistant hypertension, defined as elevated BP despite treatment with 3 or more rationally combined agents, including a diuretic, is present in more than 3 million persons in the U.S.A. alone and may represent a niche for use of limited ET receptor blockade (Epstein, 2008). The usefulness of selective ET_A receptor blocker, darusentan, in

resistant hypertension has already been proven in randomized, double-blind, placebo controlled study (DAR-201) (Black, et al., 2007). Additional attribute of TRC23029 for having ET_A receptor blocker activity in addition to AT_1 receptor blockade is expected to provide an edge over an above existing antihypertensive therapy in terms of its dual mechanism of action which would provide an option where there is no effective line of therapy.

Studies have also suggested increased ET-1 activity as one of the contributors for increase incidence of hypertension in diabetics with insulin resistance (Sarafidis and Bakris, 2007). There are studies which have also shown that in human hypertension, increased body mass index is associated with enhanced ET_A dependent vasoconstrictor activity (Cardillo, et al., 2004). Some researchers also suggest that increased endogenous ET action contributes to insulin resistance in skeletal muscle of obese humans, through both vascular and tissue effects (Ferri, et al., 1996). Similarly most of the obese and diabetic hypertensives have shown to have overactive RAS (Boustany, et al., 2004;Chan, et al., 2005;Engeli and Sharma, 2001;Kumar and Winocour, 2005;Stevanovic, et al., 2007). All these evidences suggest abnormality in the RAS and ET system in hypertensive comorbid conditions as diabetes and obesity. Existence of comorbid conditions along with hypertension have been recommended as reason for more aggressive control of hypertension by JNC7 (Chobanian, et al., 2003) and have thus suggested even lower BP goals in such patient population. Such patient population is thus expected to be benefited by novel therapeutic strategy offered by TRC23029.

In addition to involvement of RAS and ET system in pathogenesis of hypertension and comorbidities, their direct role in end-organ damage has also been established. Increased activity of RAS and ET system has been shown in cardiovascular complications and nephropathy (Barton, 2008;Dhaun, et al., 2008;Kim and Iwao, 2000). Drugs targeting RAS are already part of standard therapy for hypertension resultant complications. Establishing the fact that ET is also involved in the pathogenesis of these complications, provides strong basis for use of dual AT_1 and ET_A receptor blocker as TRC23029 in such conditions (Boemke, et al., 2001;Gómez-Garre, et al., 1996;Herizi, et al., 1998;Montanari, et al., 2003).

The fact that this program considered evaluation of the effect of our NCEs in acute setting, though adequate enough to advance the molecule into the clinic, could be regarded as a limitation. The antihypertensive therapy program at Torrent was a collaborative effort with ideas shared between Torrent and AstraZeneca. Using our strengths and experience strategically, this program moved forward at an

unprecedented pace from conception to optimized lead and finally a drug candidate. The rationale was to use Torrent's strength in drug design, medicinal chemistry and *in vivo* pharmacology and AstraZeneca experience in cardiovascular domain to fast track the program into clinical development. We chose to use models which would capture and delineate the dual pharmacological activity of our NCEs and to allow an accurate selection of molecules at each stage. Different from the traditional approach to carry out long-term efficacy studies, our approach was to develop a model which would capture both the components of our NCEs for their ability to act on RAS and ET system simultaneously; the factors contributing to the disease pathology. Hence, instead of choosing common models like the SHR, extensive studies were undertaken to validate the animal model which would be predictive of the disease pathophysiology that would actually be encountered in the population this therapy is ultimately targeted for. For resistant cases like people of African origin and those with co-morbidities like diabetes, there is no therapy currently available which even with multiple drugs lowers their BP in the range which would retard end-organ damage. The importance of maintaining BP within normal range is emphasized by studies which suggested that even a modest decrease in BP could have significantly reduce hypertension related morbidity and mortality. It has been shown that lowering systolic BP by 2 mmHg would translate into approximately 7% lower mortality from ischemic heart disease or other vascular causes and 10% lower risk of stroke mortality in middle age (Ferdinand and Saunders, 2006). The concept that the two targets when combined would lead to additional benefit in terms of BP lowering to a level which has been shown to be predictive of improved long term outcomes in the clinical setting, was an additional exercise which was undertaken to establish that the acute effect in a disease model with severe and progressive hypertension would translate into long-term benefit in the clinic.

Though safety pharmacology studies on vital organs have been performed, effect of chronic treatment on safety parameters is not evaluated which could be of concern during repeated chronic administration of our NCEs for the indication they are developed for. The same would be evaluated in long-term toxicity studies at appropriate stage during the clinical development. Given the concerns over reproductive toxicity, though, the same has been addressed by ascertaining that the concentrations at which the ET_A blockers potentiate the effect of AT_1 blockade, are much below the threshold at which an effect on reproductive toxicity is expected, thorough evaluation of the reproductive safety needs to be done before administration to individuals of childbearing age.

Summary and Conclusions

7. Summary and Conclusions

As per current standards, individual presenting systolic BP greater than 140 mmHg and diastolic BP greater than 90 mmHg is classified as hypertensive (Perkovic, et al., 2007). Under such classification about 25% of the world's adult population (≈1 billion) would be classified as hypertensives (Epstein, 2008;Perkovic, et al., 2007). Further, based on the clinical evidence, the JNC7 have described that only about 34% of patients undergoing treatment for hypertension have their BP controlled to recommended limits (Chobanian, et al., 2003). This suggests inadequacy of available antihypertensive drugs to treat hypertension. The gravity of situation becomes more evident with expected growth in number of hypertensive subjects by another 50% by year 2025 (Perkovic, et al., 2007).

Factors related to antihypertensive medication itself, multifactorial nature of disease, non responders to existing therapy and non compliance on the part of the patients have been suggested as few of the possible reasons contributing to poor outcome from existing antihypertensive therapies (Israili, et al., 2007). Though combination antihypertensive therapy helps to achieve target BP levels, many still fail to respond adequately to even such combination therapies (Chobanian, et al., 2003). The African Americans constitute substantial part of this non-responding population characterized by salt sensitivity and low renin level (Douglas, et al., 2003;Ergul, 2000). Patients with hypertension therefore still present significant medical need for antihypertensive agents, which while acting through multiple mechanisms, is effective across a wide variety of patients and epidemiological presence as a monotherapy (Ergul, 2000;Murugesan, et al., 2005).

There are ample clinical evidences to show that angiotensin receptor blockade effectively reduces BP (de la, 2006;Julius, et al., 2006;Xi, et al., 2008). Similarly, studies with non-selective ET receptor blocker like bosentan (Attina, et al., 2005) and selective ET_A receptor blocker darusentan (Epstein, 2008) point to the fact that ET also plays a significant role in the pathophysiology of hypertension. That these two systems are upregulated in hypertensive patients has been clearly shown by elevated plasma levels of ET-1 (Ergul, et al., 1996) and PRA (Laragh, et al., 1980). Ang II and ET-1 by acting on their AT_1 and ET_A receptors respectively induces vascular smooth muscle cell contraction and the local action of these in heart, vasculature and kidney, are hypothesized to regulate local blood flow and have cell growth and proliferative action (Iglarz and Schiffrin, 2003). Ang II and ET-1 are

implicated in the pathophysiology of hypertension, CHF and renal insufficiency (Kedzierski and Yanagisawa, 2001). Emerging experimental evidence suggests crosstalk between the Ang II and ET systems which has a synergistic effect in the pathophysiology of hypertension. Ang II is reported to increase prepro-ET mRNA and stimulate ET-1 release in endothelial, vascular smooth muscle, cardiac and mesangial cells and this response is blocked by the AT_1 receptor blocker losartan (d'Uscio, et al., 1997;d'Uscio, et al., 1998;Emori, et al., 1991;Herizi, et al., 1998;Imai, et al., 1992). ET reciprocally stimulates the conversion of Ang I to Ang II in pulmonary endothelial cells (Kawaguchi, et al., 1990). ET-1 induced contraction of aorta in SHR is antagonized by a AT_1 receptor blocker (Maeso, et al., 1997). ET_A receptor blockers prevent the hypertrophic, mitogenic, natriuretic and diuretic effects of Ang II (Moreau, et al., 1997;Riggleman, et al., 2001) and nonselective ET_A/ET_B receptor blocker have shown to inhibit vasoconstriction and pressor effect of Ang II (Balakrishnan, et al., 1996;Ficai, et al., 2001;Webb, et al., 1992;Wenzel, et al., 2001).

Concomitant blockade of both Ang and ET pathways may thus lead not only to enhanced BP reductions but also retard the end-organ damage directly and indirectly. The physiological benefits of dual Ang II and ET-1 blockade was demonstrated in hypertensive transgenic rats that over express the human renin gene (Ren-2 rats) (Bohlender, et al., 2000;Gardiner, et al., 1995). Specifically, combination of losartan and SB290670 (nonselective ET_A/ET_B receptor blocker) produced an additive reduction in BP compared with either drug alone in transgenic rats (Gardiner, et al., 1995). A combination of losartan and nonselective ET_A/ET_B receptor blocker reduced the MBP more than monotherapy in SHR, SHRsp and Dahl salt sensitive hypertensive rats (Ikeda, et al., 2000).

On the basis of these experimental evidences it was conceptualized to develop new dual receptor blocker against AT_1 and ET_A receptors. While combining pharmacologically effective doses of AT_1 receptor blocker with ET_A receptor blockade may provide substantial reduction in BP; ET receptor blockers are reported at higher doses to have liver and testicular toxicities and have teratogenic potential. Hence concept studies were needed to confirm the hypothesis that a non toxic minimal dose of ET_A blockade can potentiate the antihypertensive effects of AT_1 receptor blockade. For these studies, two selective AT_1 and ET_A receptor blockers were used.

Telemetered salt loaded SHRsp were used as animal model for studies designed to test our hypothesis. Telemetry as a technique for recording BP from animals enables continuous, direct measurements of BP without the need for restraint or the use of tethering devices making it extremely sensitive technique for

recording BP in conscious animals. Salt loaded SHRsp on the other hand were selected as animal model because hypertension observed in these animals represents contribution of activated RAS as well as ET system.

To establish sub-maximally effective dose of losartan, a selective AT_1 receptor blocker, its DRC was created and ED_{75} dose was computed and was found to be 5 mg/kg. Minimally effective antihypertensive dose of ZD1611, a selective ET_A receptor blocker, was also derived experimentally and was found to be 0.3 mg/kg. Upon combining ED_{75} dose of losartan with minimally effective dose of ZD1611, fall in MBP was monitored which was found to be more than that observed with ED_{75} dose of losartan alone. That the potentiation in antihypertensive response achieved was because of pharmacological reasons was ascertained by establishing plasma drug concentration of losartan when administered alone and when administered in combination with ZD1611; which were found to be indifferent. Similarly, safety of this combination in relation to teratogenic potential of ET_A receptor blocker was also ascertained by establishing that C_{max} obtained by using ZD1611 at dose of 0.3 mg/kg is far below the C_{max} known to be associated with teratogenic potential of this ET_A receptor blocker.

A sub-maximal dose of losartan was selected in the first study so as to give a window for the ET_A blocker to further enhance the antihypertensive action of AT_1 receptor blocker, however on the basis of the outcome of this study the concept was further extended to examine whether minimally effective antihypertensive dose of another selective ET_A blocker ZD4054, can further potentiate antihypertensive activity of more potent AT_1 receptor blocker, candesartan, beyond maximal attainable fall in MBP which could be achieved with its maximally effective dose of 10 mg/kg. Similar to previous study, the combination (candesartan 10 mg/kg + ZD4054 1mg/kg) was found to be more potent than candesartan alone and safe from toxic potentials of ET_A receptor blockade at the doses used.

Verification of our hypothesis led to designing and synthesis of a series of fifty NCEs. Molecules were designed and synthesized by the Medicinal Chemistry Department of Torrent Research Centre. To ascertain selectivity of compounds towards target receptors, *in vitro* Ca^{2+} mobilization assay in cell lines over-expressing human AT_1, ET_A and ET_B receptor was performed by AstraZeneca at their Research Facility in Sweden and IC_{50} values of each NCE for each of human AT_1, ET_A and ET_B receptor was established.

Selection of 7 NCEs, namely, TRC23003, TRC23012, TRC23017, TRC23029, TRC23030, TRC23031 and TRC23035, from group of total 50 NCEs was

performed considering their low IC_{50} value for AT_1 and ET_A receptor; high IC_{50} value for ET_B receptor and ratio for ET_A : AT_1 IC_{50} value. This was to ensure high selectivity of molecule for AT_1 and ET_A receptor in comparison to ET_B receptor. Since, no literature is available on the ideal ET_A : AT_1 ratio for class of dual action receptor blockers active against both AT_1 and ET_A receptor, molecules with low IC_{50} value for AT_1 and ET_A receptor and with range of ET_A : AT_1 IC_{50} ratio were selected for primary screening of activity in animal model. In absence of any approved dual AT_1 and ET_A receptor blockers for human use, BMS346567, a dual action receptor blocker (having similar affinity for AT_1 and ET_A receptors which does not address the ET_A receptor blockade associated drawbacks), currently undergoing clinical trials, was taken as reference molecule for comparison against our NCEs.

Preliminary *in vitro* characterization of selected 7 NCEs and reference molecule for their physicochemical and PK profiling was undertaken and their solubility, permeability through artificial membrane, plasma protein binding and extent of their metabolism in rat liver microsomes was established.

All the 7 NCEs and the reference molecule were found to have similar *in vitro* physicochemical and PK profile except for TRC23012, which was found to be highly metabolized by rat liver microsomes. However, all the 7 NCEs along with the reference molecule were screened for their primary activity against AT_1 and ET_A receptor. For purposes of separately measuring each component of the dual activity of our NCEs, a model was developed which allowed delineation of separate components of our NCEs in the same model. Separate *in vivo* anesthetized rat model infused with either Ang II or big ET-1 having their endogenous ET_A or AT_1 receptors blocked respectively, were standardized and used to establish antihypertensive activity of our NCEs mediated through AT_1 and ET_A receptor blockade respectively. Using this animal model a comparative activity profile was created for NCEs which was expressed in form of ED_{50} values of NCEs for their AT_1 and ET_A receptor blocking activity. Of all the 7 NCEs, TRC23012 was found to be most potent in blocking AT_1 receptors whereas TRC23035 was most potent ET_A receptor blocker.

Based on combined activity profile for AT_1 and ET_A receptors, 3 NCEs, namely TRC23029, TRC23030, TRC23035 and the reference molecule BMS346567 were selected for further efficacy screening in telemetered salt loaded SHRsp by oral route. Before screening NCEs for their efficacy by oral route in telemetered salt loaded SHRsp, oral bioavailability of these NCEs was established in rats. All the NCEs were found to be having reasonable oral bioavailability in range of 20% to 53%.

In efficacy screening, salt loaded SHRsp upon attaining MBP of approximately 150 mmHg, were treated with vehicle followed by log ascending doses of NCEs and reference molecule for maximum of 5 doses for NCEs and 4 doses for reference molecule. 4 instead of 5 doses were used for the reference molecule, as its maximal MBP lowering effect was achieved at third dose itself. No washout period was provided between dosing. The fall in MBP attained with each of the NCEs and reference molecule was evaluated in terms of percent maximum reduction in MBP from same day's basal response, maximum percent fall achieved in MBP and percent change in AUC of MBP for 0-18 hr post dosing; after factoring for the effect of vehicle.

TRC23029 was identified as most potential candidate on the basis of above parameters and was thus taken forward for safety pharmacology studies in animal model. Safety pharmacology studies with TRC23029 were carried in line with the ICH S7A guidelines which emphasizes on performing of the safety pharmacology core battery test to investigate the effects of the test substance on vital functions. In this regard, the cardiovascular, respiratory and central nervous systems have been considered as the vital organ systems. Irwin's test was performed to evaluate the effect of TRC23029 on central nervous system while using mouse as the animal model. Whereas cardiovascular and respiratory system safety, upon use of TRC23029, was accessed in anesthetized Guinea pig. Heart rate, gross ECG pattern and QTc interval were considered as checkpoints to evaluate cardiovascular safety of TRC23029 whereas respiratory rate was taken as an index to evaluate respiratory safety of TRC23029. Using ED_{50} dose for reduction in MBP in the conscious telemetered salt loaded SHRsp as reference, these safety studies were carried at rat equivalent ED_{50}, $3xED_{50}$ and $10xED_{50}$ doses. The Irwin test involving observations on behavioral, neurologic and autonomic symptom and results from cardiovascular and respiratory safety study reveal that TRC23029 is a safe compound having no safety issues related to any of these vital organ systems.

Thus, TRC23029, in addition to its efficacy, has wide safety margin. It is likely to have better antihypertensive effect than the classical ARBs by virtue of its mechanism of action through which it would be able to block both AT_1 and ET_A receptors simultaneously; thus interrupting two separate pathological pathways culminating in pathogenesis of hypertension. Hence the molecule is recommended to be taken up for detailed regular toxicity studies and further clinical development.

To conclude:

- It is possible to achieve potentiation in antihypertensive action of classical ARBs by adding minimal effective antihypertensive dose of an ET_A receptor blocker.
- The potentiation in antihypertensive action of an AT_1 receptor blocker is possible by adding ET_A receptor blocker in a dose which will not pose a toxic potential.
- The potentiation in antihypertensive action observed upon combining an AT_1 and ET_A receptor blocker is pharmacological potentiation.
- Salt loaded SHRsp model is suitable for efficacy screening of dual action AT_1 and ET_A receptor blockers
- TRC23029 is an efficacious dual action AT_1 and ET_A receptor blocker which has potential to be developed into an antihypertensive drug candidate.

Specific Contributions

8. Specific contribution

1. The hypothesis that simultaneous blockade of AT_1 and ET_A receptor would produce superior antihypertensive effect than that produced by AT_1 receptor blockade alone has been established by:
    a. Shown for the first time that, while using well characterized ET_A receptor blockers (ZD1611 and ZD4054) and established AT_1 receptor blockers (losartan and candesartan), potentiation in antihypertensive action of AT_1 receptor blockers is possible by addition of minimally effective antihypertensive dose of ET_A receptor blockers.
    b. Showing that such a combination is safe in terms of maximum plasma levels attained by ET_A receptor blockers which are far less than one known for liver and testicular toxicity and teratogenic effect in rats.
    c. Demonstrated for the first time that, such a potentiation in antihypertensive effect by addition of minimally effective antihypertensive dose of ET_A receptor blocker is possible over and above the maximum possible antihypertensive effect attainable by one of the most potent AT_1 receptor blocker candesartan.
2. Developed and validated a model for *in vivo* screening of dual action molecules targeting AT_1 and ET_A receptors, which allowed dissection of each component of the dual activity in the same model.
3. Established an animal model wherein the RAS and the ET system contribute to the development of progressively severe hypertension mimicking the treatment - resistant population targeted in the clinic.
4. Contributed to the preclinical development of novel dual action AT_1 and ET_A receptor blockers.
5. On the basis of activity and efficacy screening of series of NCEs, have identified TRC23029 as the potential novel dual action AT_1 and ET_A receptor blocker which could be developed further as drug molecule for treating clinical hypertension.
6. Performed safety pharmacology studies for TRC23029.

Future Scope of Work

9. Future scope of work

The program undertaken was designed with two major objectives. The first was with an aim to evaluate the hypothesis that combining an ET_A receptor blocker to an AT_1 receptor blocker would potentiate the antihypertensive action of AT_1 receptor blocker. The hypothesis was confirmed by us in two scenarios; first, that such a potentiation is possible upon combing minimal effective dose of an ET_A receptor blocker to sub maximal effective dose of AT_1 receptor blocker and second, that such a potentiation is also possible upon combing minimal effective dose of an ET_A receptor blocker to maximally effective dose of AT_1 receptor blocker. In both the scenarios we have also shown the safety of such combination in terms of known toxic potential of the ET_A receptor blockers used. Following from the proof of these studies the second part of the program was to design and synthesize, NCEs to be selective AT_1 and ET_A receptor blockers which were screened for activity and acute antihypertensive efficacy in relevant animal model. The study proved antihypertensive efficacy of NCEs and produced a clear DRC. Safety pharmacology studies were performed with TRC23029 and was found safe upon single administration for vital organ system as central nervous system, cardiovascular system and respiratory system. Thus, we established by safety pharmacological studies that TRC23029 is a safe molecule and can be evaluated as candidate drug.

This program has successfully achieved its milestones of new drug development from concept to a drug candidate. Going from identifying unmet needs in the area of hypertension, arriving at a proposed target based on the understanding of the pathophysiology of the disease condition, testing and confirming the hypothesis, rational targeted designing of molecules, developing screens fit to provide qualitative measurement and comparison of relative activity, evaluation in a validated animal model which recapitulates the clinical condition. Based on these a drug candidate has been identified and its pharmacological safety ascertained.

In order for a pre-clinical program to advance for clinical development, it is essential that safety issues if any with the target and/or drug candidate be addressed as the cost of clinical development escalates substantially as compared to the pre-clinical phase. For this program, given that ET_A receptor blocker as a class is known have embryo-fetal toxicity potential, further studies aimed to establish teratogenic potential of TRC23029 would need to be evaluated.

Another aspect that pharmaceutical drug development aims for is an early proof of concept in man. Regulatory approval of a new drug entails extensive clinical trials which are time and resource consuming. Therefore, before embarking on full-fledged clinical trial, the strategy that most programs adopt is to design a trial which is aimed at evaluating the efficacy in a restricted patient population where the drug if it were to translate its pre-clinical profile its man, is most likely to work and provide the proof of efficacy. Plan for subsequent studies are then based on the findings of these studies and extended into a larger population. The future clinical development plan for this program to establish therapeutic efficacy of TRC23029 as antihypertensive would be aimed at evaluation in elderly, obese hypertensive population of patients who are known to have all the underlying factors making them resistant to treatment and whose BP is not under control on existing line of antihypertensive drugs. Elderly and obese patients could be the target population as these are more likely to have higher levels/sensitivity to ET (Goodwin, et al., 1999;Parrinello, et al., 1996;Van Guilder, et al., 2007). Therefore, this population would represent the group most likely to benefit with treating the pathology at dual targets. End points for evaluation would be fall in BP in TRC23029 treated group, as compared to the pre-treatment level in a group not adequately controlled at maximum achievable dose of a AT_1 blocker. This would be compared to a control group who would continue on the standard line of therapy.

Usefulness of TRC23029 could be explored further in resistant hypertensive patients, those with uncontrolled hypertension despite being treated with three rationally combined antihypertensives, including a diuretic. Such a randomized blinded trial could include patients having their systolic BP greater than 140 mmHg or those with greater than 130 mmHg with comorbid condition as chronic kidney disease or diabetes. End points in this study too could be kept as for earlier study.

Further usefulness of TRC23029 could be established in African American population by including reasonable number of patients of this ethnicity in both the above mentioned trial designs.

The usefulness of selective ET_A receptor blocker, darusentan, in resistant hypertension has already been proven in randomized, double-blind, placebo controlled study (Black, et al., 2007). Additional attribute of TRC23029 for having ET_A receptor blocker activity in addition to AT_1 receptor blockade is expected to provide an edge over and above existing antihypertensive therapy in terms of its dual mechanism of action and improved compliance for administering lesser number of pills at the end of patients.

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Appendices

11. Appendices

Appendix- I: *In vitro* selectivity assay of NCEs for their $AT_{1,}$ ET_A and ET_B receptor blocking ability

Appendix- II: DSI telemetry hardware and its setup

Appendix- III: Checking the accuracy of pressure transmitters prior to implantation

Appendix- IV: Sublingual blood collection and plasma separation

Appendix- V: Neuropharmacological symptoms assessment

Appendix- VI: Proforma for recording scores for neuropharmacological symptoms

Appendix- I: *In vitro* selectivity assay of NCEs for their AT_1, ET_A and ET_B receptor blocking ability

Equipment

Plate washer (BioTek, U.S.A.)

Fluorometric imaging plate reader (FLIPR, Molecular Devices, U.S.A.)

Micropipette (Eppendorf, Germany)

Chemicals, drugs and disposables

(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES, Gibco, U.S.A.)

Angiotensin II (Ang II, Tocris, U.S.A.)

Bovine serum albumin (BSA, Sigma, U.S.A.)

Dimethyl sulfoxide (DMSO, Sigma, U.S.A.)

Endothelin I (ET-1, Tocris, U.S.A.)

Fetal bovine serum (FBS, Gibco, U.S.A.)

Fluo-4 AM (Tef Labs, U.S.A.)

Ham's F-12 (Gibco, U.S.A.)

Hanks balanced salt solution (HBSS, Gibco, U.S.A.)

Pluronic F-127 (Sigma, U.S.A.)

Probenecid (Sigma, U.S.A.)

Sodium hydroxide (NaOH, Sigma, U.S.A.)

Trypsin (Sigma, U.S.A.)

Nunc T-175 flasks (NUNC, U.S.A.)

Polystyrene plate (Greiner, Germany)

Reagents and buffers

Assay buffer

Wash buffer	100 ml
BSA	50 mg

Dye loading buffer

Fluo-4 AM stock solution	200 µl
Pluronic F-127 stock solution	200 µl
Probenecid stock solution	100 µl
Wash buffer	9.5 ml

Fluo-4 AM stock solution

Fluo-4 AM	1 mg
DMSO	460 µl

Growth medium

Ham's F-12	450 ml
FBS	50 ml

Pluronic F-127 stock solution

Pluronic F-127	1 g
DMSO	5 ml

Probenecid stock solution

Probenecid	0.71 g
NaOH 1M	5 ml
HBSS 1x	5 ml

Wash buffer

HBSS 10x	10 ml
20 mM HEPES	476.6 mg
Milli-Q water	90 ml

Methodology

Chinese Hamster ovary cells over-expressing the human AT_1, human ET_A and ET_B receptors (AstraZeneca, in-house) were used. Cells were grown in Nunc T-175 flasks at 37°C under 5% CO_2 in growth medium, until 90% confluent. Cells were trypsinized by 0.25% trypsin for 2 min at 37°C. Approximately 1x 10^4 cells suspended in 25 µl wash buffer were seeded in 384 well, black-sided, clear-bottomed polystyrene plates and incubated at 37°C overnight.

The growth medium was aspirated and cells were washed thrice with 100 µl wash buffer per well using plate washer. The cells were loaded with the dye loading buffer, 20 µl/well and incubated at 37°C for 1 hr. The plate was again washed with wash buffer as before.

Antagonism of AT_1, ET_A and ET_B receptor mediated Ca^{2+} transients was determined by adding NECs to Fluo-4 AM loaded Chinese Hamster ovary cells and incubating them at 37°C for 30 min. The assay plate was than transferred to the

flourmetric imaging plate reader (FLIPR) where 20 µl agonist (Ang II or ET-1) diluted in assay buffer to provide their effective concentration 80 (EC_{80}) (0.5 nM and 9.6 nM respectively) in each well was added. Test compounds were dissolved in 100% DMSO and added at volume of 0.6 µl at 7 log concentrations ranging from 1nM to 1000nM (1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM and 1000 nM) to cells in 9.4 µl of buffered media in all 384 wells and fluorescence was monitored under FLIPR for 2 min. Dilutions of NCEs were prepared such that final concentration of DMSO in each well was not exceeding 1% of total well volume. Results were calculated from relative fluorescent units obtained from FLIPR assay.

Appendix- II: DSI telemetry hardware and its setup

The DSI telemetry hardware setup consists of telemetry transmitter, telemetry signal receiver, ambient pressure reference monitor and data exchange matrix, which is connected to a computer.

Telemetery transmitter (TA11-PA-C40): The implantable rat BP transmitter used in this study is shown schematically in Figure 54. It contains a semiconductor strain gauge pressure sensor, mounted on Pyrex (thermal shock resistant glass) to safeguard it from thermal and mechanical stresses, thereby increasing stability of the pressure measurements. The sensor receives pressure fluctuations from the fluid filled catheter and sends the signal to the electronic module. Micropower electronics module located on a multilayer ceramic hybrid receives power from a silver cell and translates the pressure fluctuations into digitized signals and transmits them to the receiver in form of radio frequency signals.

Arterial pressure is referred to the sensor via a 0.6 mm diameter (outer diameter), 8 cm long fluid-filled catheter called pressure catheter. The catheter is composed of two distinct sections; the distal 8 mm is composed of thin-walled, highly compliant polyurethane with an antithrombogenic surface preparation. The function of this thin-walled tip is to efficiently transmit the high-frequency components of the pressure signal into the lumen of the catheter. The remainder of the catheter (referred to as the stem) is specially constructed of polyurethane to provide a combination of low compliance and kink resistance. The lumen of the catheter is filled with a low viscosity fluid, whereas the distal 3 mm of the thin-walled tip is filled with a blood compatible gel that prevents blood from entering the catheter lumen. The electronics, sensor and battery are packaged in a thermoplastic housing, coated with silicone elastomer to provide biocompatibility. A suture tab molded into the housing provides three sites for securing the device *in vivo* during implantation. Total weight of the implant is 9 gram and its volume displacement is 4.5 ml. The device has a continuous use battery life of 4 month. Transmitter power can be toggled off and on with the module *in situ* by passing a magnet near the transmitter, thus extending the useful battery life of the implant.

Receiver (RPC-1): The receiver is placed below the animal cage which detects the radiofrequency signal from the transmitter and converts the telemetry information to a form readily accessible for further processing by DSI's Dataquest A.R.T. acquisition software. It has two receiving antennae oriented at right angles to minimize dropouts due to directionality of the transmission pattern.

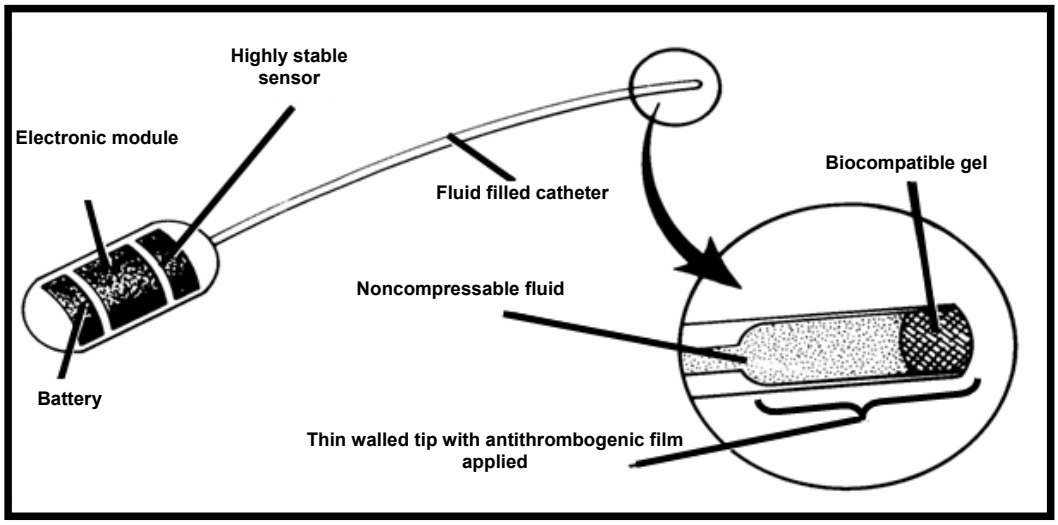

Figure 54: Schematic drawing of rat blood pressure telemetry transmitter.

Ambient Pressure Reference Monitor (APR-1): It measures the ambient barometric pressure of the room and subtracted from the telemetered pressure by data collection software to compensate for changes in atmospheric pressure.

Data exchange matrix: It acts as a junction box between peripheral telemetry hardware as receivers; ambient pressure reference located in the data acquisition room and the acquisition computer located in laboratory or a separate room. Each data exchange matrix connects a maximum number of 20 devices, including a combination of receivers, ambient pressure reference monitor and additional matrices to the acquisition computer. It automatically detects the connected devices; multiplexes the data from each device and sends the signal stream to the data acquisition system via a local area network type cable. The overall telemetry hardware setup is schematically shown in Figure 55.

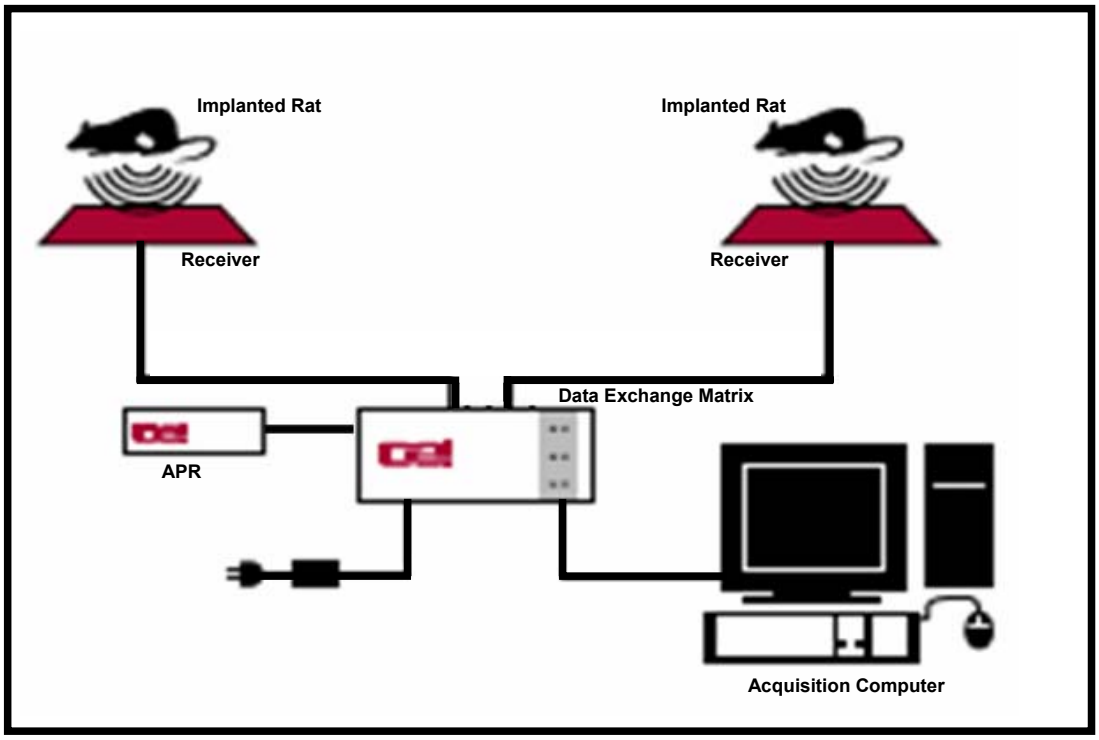

Figure 55: Telemetry hardware setup.

Appendix- III: Checking the accuracy of pressure transmitters prior to implantation

The accuracy of pressure transmitter is assured in terms of its pressure offset prior to implantation. A pressure offset of zero for a transmitter ready for implantation is considered to be optimal. To check the offset, transmitters were turned on 24 hr prior to surgery by bringing magnet in their proximity, thereby activating the magnetic switch present in the electronic circuitry of the transmitter. Switching transmitters on prior to offset recording allows the electronics circuitry of to stabilize. The factory calibration information for each transmitter was entered into the configuration module of the acquisition software and each transmitter was assigned to a receiver. Prior to surgery, the transmitters were placed on their assigned receiver while they were still in the sterile pack. The pressure value being measured by the transmitter were visualized and saved using the Save and Trace option in the acquisition software. The tracings were analyzed for there pressure value using the analysis software.

Transmitters showing an offset value beyond the range of ±3 mmHg were sent back to the manufacturer for replacement; however those showing an offset within the range of ±3 mmHg were recalibrated using the formula given below to arrive at new calibration values, which were entered in configuration of the transmitter.

750 Cal New = 750 Cal Old + (850 Cal Old - 750 Cal Old)/100 x Offset
850 Cal New = 850 Cal Old + (850 Cal Old - 750 Cal Old)/100 x Offset
950 Cal New = 950 Cal Old + (850 Cal Old - 750 Cal Old)/100 x Offset

Cal New: New calibration value
Cal Old: Factory provided calibration value
Offset: Drift observed in the pressure from value of 0 mmHg

Appendix- IV: Sublingual blood collection and plasma separation

Sublingual Blood Collection

Rats were anesthetized in induction chamber with 6% isoflurane in medical oxygen, removed from induction chamber and handled in supine position with head towards the investigator. The tongue was pulled out with blunt forceps and held at its tip. Using cotton bud, tongue was dried to remove saliva and to have clear view of two fairly large and superficial sublingual veins. One of the sublingual veins was punctured with the help of 26 gauge hypodermic needle and blood flowing out was collected in a microtube containing 20 IU of heparin sodium. After collecting required amount of blood, the puncture site was covered with a cotton swab which acts as a hemostat.

Plasma separation from blood

Blood collected in microtube was gently mixed with heparin and centrifuged at 4000 G for 15 min at 4°C. The straw coloured supernatant, plasma was aspirated using micropipette and was collected in another microtube.

Appendix- V: Neuropharmacological symptoms assessment

Behavioral profile

Awareness

1. Alertness: The alertness or stupor was recorded. Alertness is the state of paying close and continuous attention whereas stupor is an unresponsive state from which one can be aroused only briefly and with vigorous, repeated attempts. Mice were observed for these attributes in their home cage itself. This parameter provides indication about stimulation or depression of central nervous system. Alertness being a normal sign had a base score of 4.
2. Visual placing: It measures the animal response to being placed in different positions and its ability to orient itself without bumps or falls. An observed subnormal ability may indicate motor incoordination. Visual placing being a normal sign had a base score of 4.
3. Passivity: It measures the animal response to being placed in unaccustomed positions and indicates tranquillization, central depression, myorelaxation, paralysis or anesthesia. Passivity being an abnormal sign had a base score of 0.

   While performing this test (Figure 56), the mouse was grasped with the thumb and index finger, which hold the dorsal skin of the neck, while the mouse is in a walking position. An unaffected mouse moves its head and limbs in trying to escape (0). If the mouse, still grasped in the same manner, was held in a vertical position, it struggles (2). When the unaffected mouse was placed in the supine position on the back of the investigator's hand, held in a fist so that the thumb can support the mouse's head, it tries to escape (4). The unaffected mouse tries to escape when held vertically by one forepaw (6), or by one hind paw (8). Passivity in any of these positions was scored as given. Intermediate scores were used when the struggle is diminished but not abolished.
4. Stereotypy: Stereotypy is frequent, mechanical repetition of a movement. In mice it may involve searching movements of the head, circling, self-biting, walking backwards, licking the lips and tail-lashing. It indicates central stimulation. Stereotypy being an abnormal sign had a base score of 0.

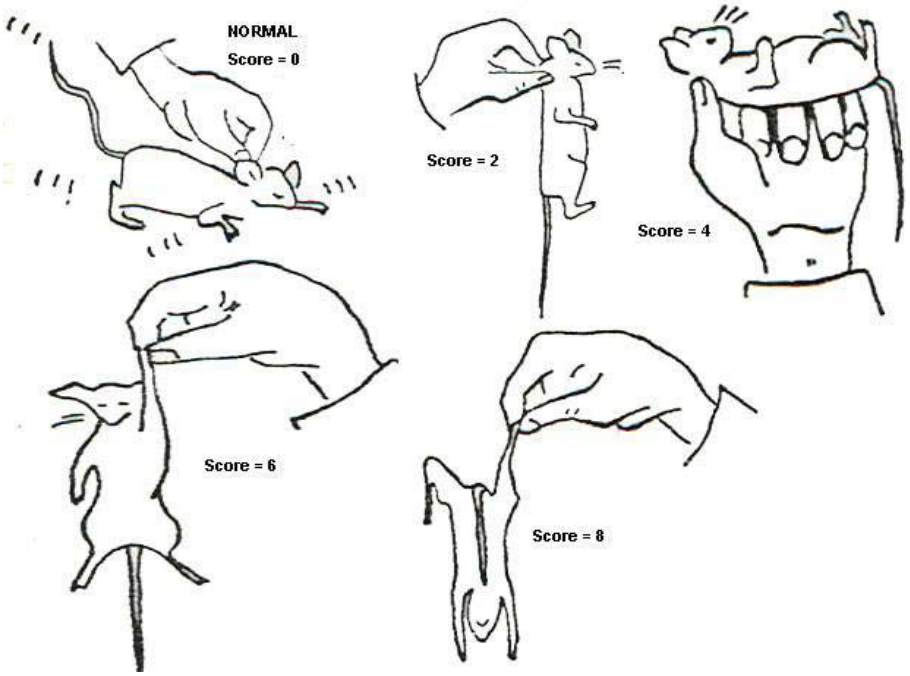

Figure 56: Scoring pattern adopted for possible results which could be observed while performing passivity test.

Mood

5. Grooming: The unaffected mouse grooms itself frequently. Excessive grooming may indicate central stimulation or sympathetic stimulation. Grooming being a normal sign had a base score of 4.
6. Vocalization: The mouse normally utters no sound. Vocalization therefore may be indicator of a noxious stimulus. Vocalization being normally absent had a base score of 0.
7. Restlessness: Restlessness which is normally absent in the unaffected mouse, may indicate central stimulation, discomfort, visceral changes or the approach of convulsions. Restlessness being normally absent had a base score of 0.
8. Irritability: Irritability is an extension of restlessness and in the higher degrees is demonstrated by aggressiveness. Irritability being normally absent had a base score of 0.
9. Fearfulness: Fearfulness depicts itself as endurance to gentle manipulation without much apprehension and had a base score of 0.

Motor activity

10. Spontaneous activity: Mice when placed in a bell jar usually show a moderate degree of inquisitive behaviour. Spontaneous activity has a base score of 4. Less activity was scored 3 or 2. If there was little motion the score was 1. If the animal slept, the score was 0. Excessive inquisitive activity/constant walking was scored 6; walking with some running, 7; and agitated spurts, 8.
11. Reactivity: A test similar to spontaneous activity was performed with the same scoring, when the animals were removed from the jar and placed in open arena. This was termed reactivity and had a base score of 4.
12. Touch response: Touch response was recorded when the animal was touched with a forceps at various parts, example, on the side of the neck, the abdomen, on the groin and had a base score of 4.
13. Pain response: Pain response was graded when the base of the tail was pressed with small forceps. Pain response being a normal sign had a base score of 4.

    Spontaneous activity and reactivity measure the stimulation of the central nervous system or its sedation and the stimulation of ganglia and neuromuscular junctions as well. The pain response measures analgesia, sedation and central depression. The touch response may indicate the presence of anesthetic activity.

Neurological profile

Central excitation

14. Startle response: Startle response is the response of mind and body to a sudden unexpected stimulus, such as a flash of light, a loud noise or a quick movement near the face. The startle response of the animal was recoded and had a base score of 0.
15. Straub tail: The degree of elevation of the mouse tail was recorded as the straub response and had a base score of 0.
16. Twitches, tremors and convulsions: The degree of twitches, tremors and convulsions were estimated, all had a base score of 0.

Motor incoordination

17. Body posture and limb position: Having base score 4, the body position and the limb position, if deviate much from the normal, may indicate neuromuscular blockade or central disturbance.

18. Staggering gait and abnormal gait: These may indicate muscular relaxation or ataxia induced by the test substance had a base score of 0.
19. Righting reflex: Righting reflex in this scenario was measured by somersault test where the mouse was picked up by the tail and tossed in the air so that a somersault of 2 or 3 turns was made before the animal falls upon a pad of sponge. This procedure was repeated 5 times. The scoring was scheduled as shown in Figure 57, according to the mouse landing position. When it touched the pad standing on four feet in all of the 5 trials: 5/5, score 0. Lying on one side: 1/5 or 2/5 score 1; 3/5 or 4/5, score 2; 5/5, score 3. Lying on the back: 1/5 or 2/5, score 4; 3/5 or 4/5, score 5; 5/5, score 6. Slowly regaining a normal position; from a supine or side position: score 7. Remaining on the back: score 8.

    High scores in this group of tests may indicate central nervous system depression, an ataractic agent, a myorelaxant, an anesthetic or an agent causing synaptic blockade in some part of the nervous system.

Muscle tone

20. Limb tone: The limb tone was estimated by grasping a forepaw of the mouse and noting the resistance to extension of the paw. A base score of 4 was assigned to this test.
21. Grip strength: The grip strength was measured by allowing the animal to grasp a metal grid and pulling it back to have an estimate of force required to overcome the mouse grip of grid. A base score of 4 was assigned to this test.
22. Body tone and abdominal tone: Having a base score 4; the body tone and the abdominal tone were estimated by noting the muscle-tension in comparison with the control animals. The tests in this group may indicate myorelaxant activity, neuromuscular blockade and central depression.

Reflexes

23. Pinna: Pinna reflex was tested by touching the center of the pinna with a hair. The unaffected mouse withdraws from the irritating hair. The test had a base score of 4.
24. Corneal: Upon touching cornea with a hair, causes the animal to withdraw. The test had a base score of 4.
25. Ipsilateral flexor reflex (IFR): In the IFR, a toe-pad is compressed with a forceps, which causes the animal to flex its leg in a retiring movement. The

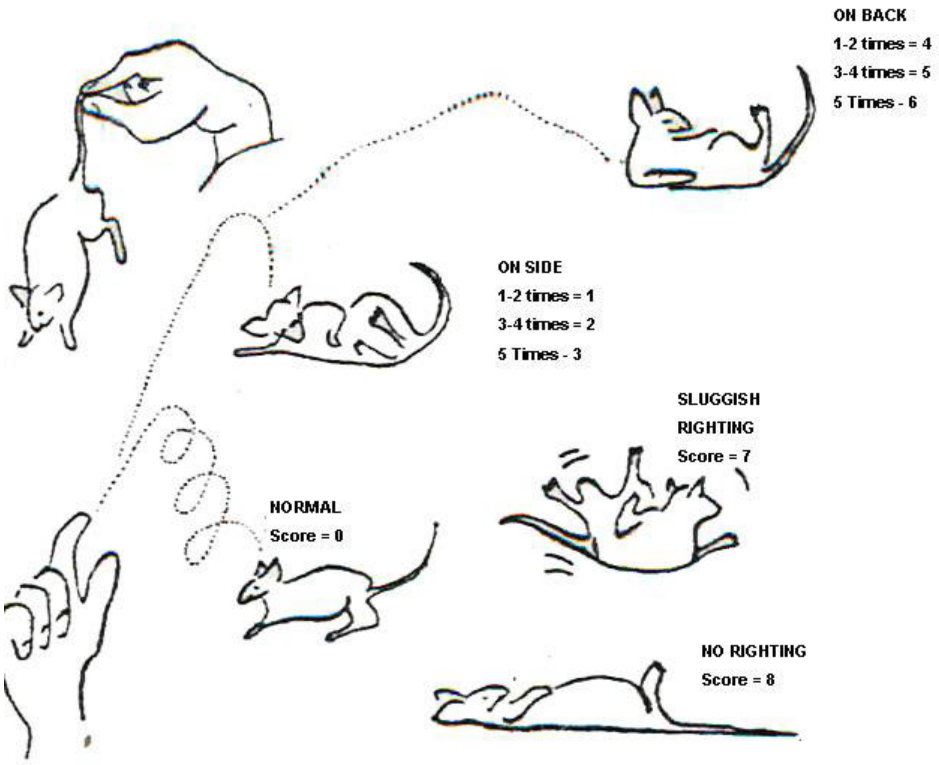

Figure 57: Scoring pattern adopted for possible results which could be observed while performing somersault test.

test had a base score of 4.

If the score shows an impairment of the reflexes, the test substance may be active in blocking some part of the sensory nerve, the spinal synapse or the efferent pathway.

Autonomic profile

Optical signs

26. Exophthalmos : Exophthalmos which is normally absent had a base score of 0. When present indicates sympathetic stimulation.

Secretory signs

27. Urination: Excessive urination of the animal may indicate muscarinic activity or the irritation of the urinary tract by the test substance or a metabolite. It had a base score of 0.

28. Salivation: Salivation an indicator of muscarinic activity had a base score of 0.

General signs

29. Writhing: Writhing indicates irritation of tissues or stimulation of sensory receptors and had a base score of 0.
30. Piloerection: Piloerection an indicator of compensation to a lower temperature or sympathomimetic activity. It had a base score of 0.
31. Skin color: Skin color especially that of the ear may change from pink to red or white was compared with control mice. A red colour indicates vasodilatative and possibly a sympatholytic activity. A white colour indicates vasoconstriction and possibly sympathomimetic activity and had a base score of 4.
32. Respiration rate: It may be accelerated by toxic substance or respiratory analeptics; it is decelerated by respiratory depressant and agents causing central depression especially in higher dose. It had a base score of 4.
33. Lacrimation: Lacrimation is usually absent and had a base score of 0.

Toxicity

34. Death: Acute and delayed death of animal following administration of test substance is indicator of potential toxic effect of the substance at that particular dose. It had a base score of 0.

Appendix- VI: Proforma for recording scores for neuropharmacological symptoms

Table x: – Neuropharmacological screening of TRC23029 in mice using Irwin scale

Test Compound:TRC23029
Date:
Time of drug admin.:
Route of admn: Oral
Dose:
Volume: 10 ml/kg

Animal Species: Mice
Strain: Swiss Albino
Sex: Male
Weight:
Time of death:

Vehicle: 1% w/v Tween 80
0.5% w/v NaCl
Milli-Q water

		Normal score	0 hr	0.5 hr	1 hr	2 hr	4 hr	6 hr	10 hr	24 hr
Awareness	Alertness	4								
	Visual placing	4								
	Passivity	0								
	Stereotypy	0								
Mood	Grooming	4								
	Vocalization	0								
	Restlessness	0								
	Irritability (aggression)	0								
	Fearfullness	0								
Motor activity	Spontaneous activity	4								
	Reactivity	4								
	Touch response	4								
	Pain response	4								
CNS excitation	Startle response	0								
	Straub tail	0								
	Tremors	0								
	Twiches	0								
	Convulsions	0								
Posture	Body posture	4								
	Limb position	4								
Motor incordination	Staggering gait	0								
	Abnormal gait	0								
	Righting reflex	0								
Muscle Tone	Limb tone	4								
	Grip strength	4								
	Body tone	4								
	Abdominal tone	4								
Reflexes	Pinna	4								
	Corneal	4								
	IFR	4								
Autonomic	Exophthalmos	0								
	Urination	0								
	Salivation	0								
General	Piloerection	0								
	Skin color	4								
	Writhing	0								
	Respiration rate	4								
	Lacrimation	0								
Dead	Dead	0								

IFR, Ipsilateral flexor reflex

List of Publications & Presentations

12. List of publications and presentations

1. Pathak P, **Gupta R,** Chaudhari A, Shiwalkar A, Dubey A, Mandhare A, Gupta RC, Joshi D and Chauthaiwale V (2008) TRC4149 a novel advanced glycation end product breaker improves hemodynamis status in diabetic spontaneously hypertensive rats. *European Journal of Medical Research* **13**:388-398.

2. **Gupta R,** Mohanan A and Joshi D. Development and Validation of Rat Model for Dissecting AT_1 and ET_A Receptor Blocking Activity of Dual Action AT_1 and ET_A Receptor Antagonist. Accepted for publication with minor revisions in *High Blood Pressure & Cardiovascular Prevention*.

3. Mohanan A, **Gupta R** and Joshi D (2005) Potentiation in antihypertensive efficacy of selective AT_1 receptor blocker upon combining small dose of selective ET_A receptor blocker. Presented at AstraZeneca, Gothenburg, Sweden.

4. **Gupta R**, Mathur A, Mohanan A and Joshi D (2008) Selection of anesthetic agent to achieve prolong stable blood pressure in rats. Presented at 41st annual conference of Indian Pharmacological Society and International Conference on Translational Pharmacology 2008. New Delhi.

5. Mathur A, **Gupta R**, Mohanan A and Joshi D (2008) Oral salt load tolerability and its effect on arterial blood pressure of conscious SHRsp implanted with radiotelemetry transmitter. Presented at 41st annual conference of Indian Pharmacological Society and International Conference on Translational Pharmacology 2008. New Delhi.

6. **Gupta R** and Joshi D (2009) Standardization of surgical technique and setup for rodent telemetry to monitor blood pressure in conscious, freely moving laboratory rats and to compare blood pressure profile from different rat models of hypertension. Presented at National Conference on Emerging Trends in Life Science Research. Pilani.

Biography

13. Biography

Brief biography of the supervisor

Dr. Deepa Joshi, is heading Early Development at Reliance Clinical Research Services, Pvt. Ltd., Bangalore.

Having a basic degree in medicine (M.B., B.S.,) form University of Rajasthan, her research aptitude took her to McGill University, Canada where she completed her Ph.D., in year 1994. Back in India, she joined Shri B.V. Patel Pharmaceutical Education and Research Development Centre, Ahmedabad and continued research activities for a period of four years as Head, Clinical Pharmacology.

She joined Torrent Research Centre, Gandhinagar in year 1998 as senior scientist and was subsequently promoted to the position of Assistant General Manager, Department of Pharmacology and then to General Manager, Discovery Biology. Her activities, in addition to core pharmacological research and early clinical development, involved monitoring the overall biological aspects of the New Drug Discovery program at Torrent Research Centre.

In addition to vast research experience of 20 years, Dr. Deepa Joshi had to her credit several awards and fellowship, has participated in many national and international conferences, published abstracts, participated in poster presentation and research publication in various journals of international repute. She is also amongst the reviewer panel for Indian Journal of Pharmaceutical Sciences.

Recently in year 2008, she moved from Torrent Research Centre, Gandhinagar to Reliance Clinical Research Services, Pvt. Ltd., Bangalore, to lead Early Development team in its endeavor to translate pre-clinical research into early clinical development phase.

Brief biography of the candidate

Mr. Ram Gupta, in addition to pursuing Ph.D., from Birla Institute of Technology and Science, Pilani is also working as scientist in Department of Pharmacology, Torrent Research Centre, Gandhinagar.

Having completed his schooling from Jaipur, he opted pharmacy as his profession and graduated B. Pharm from Lachoo Memorial Collage of Science and Technology affiliated to University of Rajasthan. He is University merit holder and has been awarded Gold Medal for securing maximum marks in B. Pharm course.

Having decided to pursue higher education, he qualified all India Graduate Aptitude Test in Engineering examination and selected Pharmacology as specialization subject for M. Pharm. He was guided through his M. Pharm by Dr. Manjeet Singh and Dr. Ajay Sharma at Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala. He was ranked second in University merit for Pharmacology discipline in M. Pharm.

Immediately after completing M. Pharm, Mr. Ram Gupta joined in Department of Pharmacology at Torrent Research Centre, Gandhinagar as Trainee Scientist and was subsequently confirmed and promoted to position of Research Associate in span of 4 years. While working as scientist in Torrent Research Centre, he got himself registered for Ph.D., with B.I.T.S., Pilani under supervision of Dr. Deepa Joshi who was then Head, Department of Pharmacology, Torrent Research Centre.

Mr. Ram Gupta has attended various conferences and workshops, have presented paper and participated in poster presentation at IPS and has two research publication in journals of international repute.