A Study on Pharmacological Induction of Heat Shock Protein 70 as a Neuroprotective Strategy for the Treatment of Stroke

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **A Study on Pharmacological Induction of Heat Shock Protein 70 as a Neuroprotective Strategy for the Treatment of Stroke** and submitted by **Prashant. G. J** ID No **2006PHXF008P** for award of Ph.D. of the Institute embodies original work done by him/her under my supervision.

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Prashant G J

List of Tables

List of Figures

List of Abbreviations and Symbols

Abbreviation	Description
2-VO	Two-Vessel Occlusion
4V	Fourth Ventricle
4-VO	Four-Vessel Occlusion
5HT	5-hydroxy tryptamine
ACA	Anterior Cerebral Artery
ACEI	Angiotensin Converting Enzyme Inhibitor
ADC	Apparent Diffusion Coefficient
ADL	Activities of Daily Living
ADP	Adenosine Diphosphate
AF	Atrial Fibrillation
AIF	Apoptosis Inducing Factor
ALB	Albumin
ALIAS	Albumin in Acute Stroke
AMPA	3-hydroxy-5-methyl-4-isoxazole proprionic acid
ARBs	Angiotensin Receptor Blockers
ARTIST	AMPA Receptor Antagonist Treatment in Ischemic Stroke
ASTIN Trial	Acute Stroke Therapy by Inhibition of Neutrophils Trial
ATP	Adenosine Triphosphate
AUC	Area under the curve
BA	benzoquinone ansamycin antibiotics
BBB	blood-brain barrier
BDI	Beck Depression Inventory
BP	Blood pressure
BRAINS	Bayer Randomized Acute Ischemia Neuroprotectant Study
C0	Calculated zero time concentration
CB	Cerebellum
CBF	Cerebral Blood Flow
CCA	Common Carotid Artery
CCF	Congestive Cardiac Failure
CES-D	Center for Epidemiologic Studies Depression
cFN	cellular Fibronectin

CHHIPS trial	Controlling Hypertension and Hypotension Immediately
CHIP	*C*arboxyl-terminus of *HSP70 I*nteracting *P*rotein
Cmax	Maximum plasma concentration
CMV	Cytomegalovirus
CNS	Central Nervous System Collaborative Study
COPD	Chronic Obstructive Pulmonary Disease
COSSACS	Continue or Stop post-Stroke Antihypertensives
COX-1	Cyclooxygenase-1
CPu	Caudate Putamen
CSF	Cerebrospinal Fluid
CT	Computed Tomography
DALYs	Disability-Adjusted Life Years
dcb	dichlorobenzoyloxopentanoic acid
DWI	Diffusion Weight Image
ECA	External Carotid Artery
ECASS	European Cooperative Acute Stroke Studies
ECG	Electrocardiogram
ELAM-1	E-selectin
ENOS trial	Efficacy of Nitric Oxide in Stroke Trial
eNOS	endothelium Nitric Oxide Synthase
ET-1	Endothelin-1
FAD	Family Assessment Device
FASTMAG	Field Administration of Stroke Therapy — Magnesium
FIM	Functional Independence Measure
fmk	fluoromethylketone
FOV	Field Of View
GABA	Gamma Amino Butyric Acid
GDS	Geriatric Depression Scale
GIST-UK trial	Glucose Insulin in Stroke-United Kingdom trial
GLUT-1	Glucose Transporter-1
HC	Hippocampus
HIF	Hypoxia-Inducible transcription factor
HMG-CoA	Hydroxy Methyl Glutarate Co-enzyme A
HR	Heart Rate

HSE	Heat Shock Elements
HSF	Heat Shock Factor
HSP	Heat Shock Proteins
i.p	Intraperitoneal
i.v	Intravenous
ICA	Internal Carotid Artery
ICAM-1	Intercellular Adhesion Molecule-1
IHD	Ischemic Heart Disease
IL	Interleukin
IMAGES Trial	Intravenous Magnesium Efficacy in Stroke Trial
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
LV	Lateral Ventricles
MCA	Middle Cerebral Artery
MCAo	Middle Cerebral Artery occlusion
MCP-1	Monocyte Chemoattractant Protein-1
MERCI	Mechanical Embolus Removal in Cerebral Ischemia
MI	Myocardial Infarction
MIP-2	Macrophage Inflammatory Protein-2
MOS	Medical Outcomes Study
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR imaging	Magnetic Resonance imaging
MRI	Magnetic Resonance Imaging
mRS	modified Rankin Scale
NC	Neocortex
NCSE	Neurobehavioral Cognition Status Exam
NF-κB	Nuclear Factor kappa B
NHS	National Health Service
NIBP	Non-invasive blood pressure
NINDS	National Institute of Neurological Disorders and Stroke
NMDA	N-methyl-D-aspartate
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level

OB	Olfactory Bulb
PBN	α-phenyl-N-tertbutyl nitrone
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PICA	Porch Index of Communicative Ability
PK	Pharmacokinetic
PMA	Phorbol Merystyl ester
	Post-Stroke Trial
ROI	Region of Interest
RR	Respiration Rate
RRR	Relative Risk Reduction
rtPA	recombinant tissue Plasminogen Activator
SCA-1	Spinocerebellar Ataxia Type-1
SD	Sprague Dawley
SD	Standard Deviation
SEM	Standard Error of Mean
SEP	Somatosensory Evoked Potential
SHR	Spontaneously Hypertensive Rats
sHSP	small Heat Shock Protein
SIP	Sickness Impact Profile
SNR	Signal to Noise Ratio
STAIR	Stroke Therapy Academic Industry Roundtable
STZ	Streptozotocin
T	Thalami
T1/2	half life
TE	Echo time
TLR	Toll like Receptors
TNFα	Tumour Necrosis Factor-α
tPA	tissue Plasminogen Activator
TR	Repetition time
TTC	2,3,5-triphenyltetrazolium chloride
VCAM1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Derived Growth Factor

WHO	World Health Organization

Symbols	**Description**
α	alpha
ß	beta
∞	infinity
µ	micro

Abstract

Alterations in homeostatic mechanisms are implicated for several disease conditions and Ischemic stroke is one such disorder which encompass both acute and chronic neuronal death component. Ischemic stroke is a common clinical problem with increasing incidence, serious consequences, unsatisfactory therapeutic options and an enormous financial burden to society. Several neuroprotective strategies have been successfully evaluated in experimental models but ultimately failed in the clinical trials owing to lack of efficacy, limited time window for treatment initiation, design issues and serious toxicities. There exists a large unmet medical need for stroke and effective neuroprotective agents that have potential to modulate multiple injurious pathways are urgently needed.

Induction of HSPs is a natural response mounted by a stressed cell that has been shown to provide essential cellular maintenance, protection and repair functions. But studies employing a pharmacological agent that induce HSP are lacking. We hypothesized that an augmentation of the endogenous protective responses, such as induction of HSP70, employing a pharmacological agent could serve as a neuroprotective strategy for the treatment of stroke. To test this hypothesis we used TRC051384, a novel compound that was designed and synthesized and is a potent inducer of heat shock protein 70. Studies were designed with the aim to investigate the ability of TRC051384 in reducing neuronal injury and disability upon delayed treatments (4 and 8 hours post ischemia onset) in a rat model of transient, permanent cerebral ischemia. We further have evaluated TRC051384 for its efficacy in animal models of stroke present along with co-morbidities.

Here we show for the first time that treatment with TRC051384 significantly reduces stroke associated neuronal injury and disability in a rat model of transient ischemic stroke even when administered 8 hours post onset of ischemia. Significant improvement in survival was observed with TRC051384 treatment initiated at 4 hours after ischemia onset. TRC051384 was able to offer stroke protection even in permanent models and animal models of stroke with co-morbidities. Induction of HSP70 by TRC051384 involves HSF1 activation and results in elevated chaperone and anti-inflammatory activity. These results show, in specific, that TRC051384 has the potential to be developed as a novel pharmacological agent for the treatment of ischemic stroke and in general that induction of HSP70 offers as a promising strategy for the treatment of stroke and other such neurological disorders.

Table of Contents

Introduction

1. Introduction

Cellular homeostasis refers to the cells ability to regulate physiologically its inner environment to ensure its stability in response to fluctuations in the outside environment. An advantage of homeostatic regulation is that it allows the cell to function effectively in a broad range of environmental conditions which could be either physiological or pathological (Prahlad and Morimoto, 2009; Marder and Goaillard, 2006). Many diseases are a result of disturbance of homeostasis, a condition known as homeostatic imbalance or altered homeostasis. This imbalance could be due to physiologic or pathologic reasons. There exists majority of diseases which are a result of homeostatic imbalance encompassing vital organ systems (Prahlad and Morimoto, 2009; Díez-Tejedor and Fuentes, 2005),

Ischemic stroke is one such homeostatic disorder which encompass both acute as well as delayed neuronal death component (Xing, et al., 2012; Woodruff, et al., 2011; Díez-Tejedor and Fuentes, 2005; Lipton P, 1999). Stroke is defined by the World Health Organization (WHO) as “rapidly developed clinical signs of focal or global disturbance of cerebral function, lasting more than 24 hours or until death, with no apparent non-vascular cause”. This definition includes ischemic stroke, subarachnoid and intracerebral haemorrhage. Stroke is clearly a devastating disease that affects millions of people worldwide, and contributes to high morbidity and mortality. It is a common clinical problem with increasing prevalence, serious consequences, unsatisfactory therapeutic options and an enormous financial burden to the society (Ankolekar, et al., 2012; Zille, et al., 2012; Mukherjee and Patil, 2011; Feigin, et al., 2009).

Current management of ischemic stroke encompasses medical management, use of surgery devices/techniques to reperfuse occluded arteries and large dependence on preventive strategies. Thrombolysis using recombinant tissue plasminogen activator (tPA) is the only approved therapy for the treatment of stroke available till date. The National Institute of Neurological Disorders and Stroke (NINDS) recombinant tissue plasminogen activator (rtPA) investigators examined the use of thrombolytics for acute ischemic stroke. They found that administration of 0.9 mg/Kg body weight of rt-PA within 3 hours of stroke onset did not confer a benefit on 24 hour outcome measures, but a significantly better functional outcome was seen at 90 days (Marler, 1995). However many patients do not reach hospital soon enough for thrombolysis to be safely administered (Fagan, et al., 1998). The time window, risk of haemorrhage and necessity

for neuroimaging is only realistically met in less than 5% of ischemic stroke patients, and this further narrows the field of patients who are eligible to receive this treatment (Fisher and Bogousslavsky, 1998). High prevalence rates, mortality and limited therapeutics for stroke highlights large unmet need in the field of stroke therapy and necessitates investigation of new therapies with clinically relevant time window which can effectively treat ischemic stroke.

With the advancement in understanding the pathophysiology of stroke over the last couple of decade sufficient knowledge about the critical processes which lead to brain damage have been gathered. Brain injury that follows from a cerebral ischemic attack occurs as a result of a series of complex pathobiological events. The effects of ischemic stroke can be separated into two sequential processes: 1) vascular and hematological events that cause a local reduction in Cerebral Blood Flow (CBF), and 2) ischemia-induced alterations in the cellular environment (Zille, et al., 2012; Woodruff, et al., 2011). With respect to this second process, the spatial and temporal characteristics of stroke can be described by the following events. Within minutes, neurons are damaged and killed due to the over activation of excitatory neurotransmitters, a process known as excitotoxicity. Next, inflammation begins on the order of hours, followed by changes in gene expression, such as apoptosis, occurring on the order of days (Dirnagl, et al., 1999). Within the first six hours, the function of energy-dependent Na^+/K^+ ATPase membrane pumps will begin to fail (Broughton, et al., 2009; Majno and Joris, 1995). This disruption in the balance of ions will lead to edema. The region of the brain that has undergone irreversible damage, i.e. cell death, is called the ischemic core. Surrounding this core is brain tissue that may have compromised function because of the decrease in CBF. This tissue is damaged reversibly and is called the ischemic penumbra (Pulsinelli, 1992).

There are several parallel pathways in which ischemia can lead to tissue damage (Woodruff, et al., 2011; Lipton, 1999). Subsequent extension of the damage in the penumbra is contributed by free radical injury, inflammation and apoptosis which are further potentiated by reperfusion occurring several hours to days post ischemia (Lipton P, 1999; Pulsinelli, 1992). Hence interventional modalities which target free radical generation, inflammation and apoptosis are expected to reduce the delayed extension of brain damage post ischemic insult. In this regard many of the putative neuroprotective agents have been shown to protect extension of infarct to the penumbra in various animal models when administered soon after the initiation of ischemic insult. Such

therapies which have shown significant protection of penumbra include antioxidants, free radical scavengers, and antiapoptotic agents (Minnerup, et al., 2012; Takasago et al., 1997; Lees, et al., 2006; Amemiya, et al., 2005) amongst others. Even after they have protected penumbra in these animal models, these agents have not been translated into effective bedside therapies. The reason for this being all of them had very short window for efficacy, thus limiting their clinical utility.

Heat shock protein(s) (HSP)s are a group of highly conserved proteins, ranging in size from approximately 15 kDa to 110 kDa in molecular weight (Hageman, et al., 2011; Stetler, et al., 2010). Under physiological conditions, these proteins function as molecular chaperones. Some of these proteins are constitutive, while others are found to be induced in response to a variety of cellular stresses (Ma Y, et al., 2012; Fulda, et al., 2010; Nollen and Morimoto, 2002; Benjamin and McMillan, 1998). This induction of HSP is a natural response mounted by a stressed cell, which in turn would provide essential cellular maintenance, protection and repair functions. HSP70 is the one best studied in this class. Functionally, the 70 kDa HSP (HSP70) family is a group of chaperones that assist in the folding, transport, and assembly of proteins in the cytoplasm, mitochondria, and endoplasmic reticulum (Franklin et al., 2005; Nollen and Morimoto, 2002). Cerebral ischemia causes severe depletion of blood supply to the brain tissues, as a result of which the cells gradually proceed to death due to lack of oxygen. In such a situation, there is increased expression of HSP in the brain tissue. Transient ischemia induces HSP in the brain and the ability of neuronal population to survive an ischemic trauma is correlated with increased expression of HSP70. HSP70 mRNA was induced in neurons at the periphery of ischemia. It is proposed that the peripheral-zone of ischemia, penumbra can be rescued by pharmacological agents. It was in this zone that HSP70 protein was found to be localized primarily in neurons (van der weed et al 2005; Rajdev, et al., 2000). There is accumulating evidence that HSP70 (the inducible form, HSP72) protects neuronal cells from a variety of stimuli, both in-vitro and in-vivo (Qi D, et al., 2012; Barreto, et al., 2012). It is shown experimentally that HSP70 is induced in penumbral regions and amplification of such a response using HSP70 over expressing transgenic models or viral delivery of HSP70 protein has offered stroke protection (Rajdev et al., 2000; Zhan et al., 2010). Additionally HSP70 expressed in penumbra is reported to have an anti-inflammatory and anti-apoptotic activity in animal models of stroke (Kim N, et al., 2012; Kokubo et al., 2003; Pratt and Toft, 2003). Thus induction of HSP70 offers a potential target for the treatment of stroke (Mehta et al., 2007).

With these collective evidences we hypothesized that an augmentation of the endogenous protective responses employing a pharmacological agent at an appropriate time and at desirable magnitude could provide benefit by reducing the neuronal loss and associated deleterious cascade of events in the intra and extracellular environment. Further, we aimed at whether delaying intervention with a pharmacologic agent with the potential to induce HSP70 to demonstrate stroke protection possibly mediated through chaperonic action and mitigation of late events such as inflammation and apoptosis. MR imaging was incorporated in our study as it offers advantage of non invasiveness in order to examine the progression of neuronal damage along with the possibility to capture treatment related benefit which can be directly applied and compared to clinical setup.

Review of Literature

2. Review of Literature

2.1. Ischemic stroke epidemiology and pathophysiology

- 2.1.1. Stroke incidence and prevalence
- 2.1.2. Stroke etiology and pathophysiology
- 2.1.3. Stroke mortality
- 2.1.4. Financial burden of stroke
- 2.1.5. Stroke in co-morbid conditions

2.2. Current management of ischemic stroke

- 2.2.1. Medical management of stroke
- 2.2.2. Surgical treatment of stroke
- 2.2.3. Preventive treatment of stroke
- 2.2.4. Lifestyle modifications

2.3. Neuroprotective strategies for stroke

- 2.3.1. Prevention of early ischemic injury; excitotoxicity
- 2.3.2. Preventing reperfusion injury
  - 2.3.2.1. Reduction of oxidative stress
  - 2.3.2.2. Reduction of neuronal inflammation: Leukocyte Inhibition
  - 2.3.2.3. Inhibition of apoptosis
- 2.3.3. Miscellaneous other strategies
- 2.3.4. Possible reasons for the failure of different strategies
- 2.3.5. Possible reasons for the observed success in few strategies
- 2.3.6. Promising and new strategies

2.4. HSPs and neuroprotection

- 2.4.1. HSP family
- 2.4.2. HSPs relevant for neuroprotection
- 2.4.3. HSP70 expression and stroke protection
- 2.4.4. Postulated mechanisms of HSP70 mediated neuroprotection
  - 2.4.4.1. HSP70 and chaperone action
  - 2.4.4.2. Antiapoptotic effect
  - 2.4.4.3. Anti-inflammatory action

2.4.5. Evidence of HSP70 mediated neuroprotection in chronic neurological diseases

2.5. Clinical, experimental assessment of stroke outcome and relevant animal models.

2.5.1. STAIR recommendations for the evaluation of neuroprotective agents

2.5.2. Clinical stroke assessment methods

2.5.3. Non-invasive brain imaging strategies for stroke

2.5.4. Animal models of stroke

2.5.5. Measurement of ischemic stroke damage in animal models

2.5.6. Experimental assessment of neurological impairment

2.5.7. Behavioral tests to assess neurological deficit following MCAo

2.6. Unmet need

2.1. Stroke epidemiology and pathophysiology

Stroke is defined by the World Health Organization (WHO) as “rapidly developed clinical signs of focal or global disturbance of cerebral function, lasting more than 24 hours or until death, with no apparent non-vascular cause” (Hatano, 1976). This definition includes ischemic stroke, subarachnoid and intracerebral haemorrhage (ICH)

2.1.1. Stroke incidence and prevalence

Stroke is a leading cause of disease and death throughout the world (Kulshreshtha, et al., 2012; Diaz, 2012; World Health Report, 2011; Bonita, 1992; Adams and Gordon, 1991). In a systematic review of population-based epidemiologic studies, Feigin et al showed that in 2000-2008 in high-income countries ischemic strokes constituted 82% and 11% were caused by intracerebral hemorrhage (Feigin, et al., 2009).

Stroke was reported to affect 5.8 million Americans in 2005, killing 150,100 in 2004 (Heart Disease and Stroke Statistics, 2004). In Europe it is estimated that over 1 million acute ischemic strokes occur per year (Wolfe, et al., 1995). In the United Kingdom, stroke remains the single biggest cause of major disability (Bamford, et al., 1988). Annually in the UK, between 101,000 and 130,000 people experience a stroke, with the majority occurring in people aged over 65 years (Bogousslavsky, et al., 2000). Of these, a significant proportion is comprised of first- ever strokes. Within the UK, the crude annual prevalence for first -ever stroke is about 2 per 1000 people (Warlow, 1998; Sudlow, et al., 1997). It is estimated that this incidence will increase as a result of the ageing population. Stroke incidence in Asia is generally higher than in the USA and Europe (Kulshreshtha, et al., 2012; Wong, et al., 2001).

For India, the overall age adjusted prevalence rate for stroke is estimated to lie between 84-262/100,000 in rural and between 334-424/100,000 in urban areas (Gupta, et al., 2008; Banarjee and Das, 2006). Within India although the prevalence and incidence reports for stroke are less, few studies have reported high prevalence rates ranging 420-630 per 1000,000 (Sridharan, et al., 2009; Dalal, et al., 2008).

Stroke is, of course, a global problem and that the incidence and impact of stroke differs among ethnic groups (British Heart Foundation Statistics Database, 2008). Black, Chinese, South Asian and Japanese populations have a higher incidence of stroke compared with Caucasians (Mackay and Mensah, 2007). In a worldwide review on sex differences in stroke epidemiology, the mean age at first-ever stroke was 68.6 years

among men, and 72.9 years among women. Male stroke incidence rate was 33% higher and stroke prevalence was 41% higher than the female (Appelros, et al., 2009). Projections for the period 2006-2025 estimate new cases of ischemic stroke at 1.5 and 1.9 million cases in men and women respectively, highlighting the increasing profile of stroke in the future (Truelsen, et al., 2006; Kolominsky-Rabas, et al., 2006).

2.1.2. Stroke etiology and pathophysiology

Brain injury from cerebral ischemic attack occurs as a result of a series of complex pathobiological events. In the ischemic case, a stroke occurs when CBF is reduced below a threshold that will sustain cell survival. For example, this CBF threshold in the primate brain is 10 – 15 mL/100 g of brain tissue per minute (Krafft, et al., 2012; Pulsinelli, 1992). An ischemic stroke may be caused by a large artery atherosclerosis, a cardioembolism or a small-artery occlusion (Diaz, 2012).

The pathophysiology of stroke is complex and involves numerous processes which grossly lead to loss of cellular homeostasis resulting into brain cell death. These processes are discussed in depth in an excellent review by Woodruff and they include: energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium levels, excitotoxicity, free radical-mediated toxicity, generation of arachidonic acid products, cytokine mediated cytotoxicity, complement activation, disruption of the blood-brain barrier (BBB), activation of glial cells, and infiltration of leukocytes (Boltze, et al., 2012; Xing, et al., 2012; Magnus, et al., 2012; Woodruff, et al., 2011). These interrelated and coordinated events can lead to ischemic necrosis, which occurs in the severely affected ischemic core regions. Within a few minutes of a cerebral ischemia, the core of brain tissue due to severe blood flow reduction, is mortally injured, and subsequently undergoes necrotic cell death. Necrosis is morphologically characterized by initial cellular and organelle swelling, subsequent disruption of nuclear, organelle, and plasma membranes, disintegration of nuclear structure and cytoplasmic organelles with extrusion of cell contents into the extracellular space (Majno and Joris, 1995; Broughton, et al., 2009). This necrotic core is surrounded by a zone of less severely affected tissue which is rendered functionally silent by reduced blood flow but remains metabolically active (Majno and Joris, 1995; Broughton, et al., 2009). The region bordering the infarct core, known as the ischemic penumbra, comprises as much as half of the total lesion volume during the initial stages of ischemia, and represents the region in which there is opportunity for salvage via post-stroke therapy (Ginsberg, 1997). Less severe ischemia,

as occurs in the penumbra region of a focal ischemic infarct, evolves more slowly, and depends on the activation of specific genes and may ultimately result in apoptosis (Dirnagl, et al., 1999; Lipton, 1999; Zheng and Yenari, 2004). Recent research has revealed that many neurons in the ischemic penumbra may undergo apoptosis only after several hours or days, and thus they are potentially recoverable for some time after the onset of stroke. There are two general pathways for activation of apoptosis: the intrinsic and extrinsic pathways. Over the last decade, experimental studies have provided considerable new information characterizing apoptotic processes occurring after ischemic stroke. Recent experimental studies have shown several novel mechanisms to also play a role in ischemic stroke induced brain injury. Involvement of toll like receptors (TLR) (Tang, et al., 2007; Arumugam, et al., 2009), gamma secretase, contribution of NOTCH-1 (Arumugam, et al., 2006) and adiponectin receptors (Thundyil, et al., 2010; Choi, et al., 2010) leading to ischemic brain injury are reported. Collectively, these results demonstrate that there are multiple pathways influencing stroke outcome, and that there are correspondingly multiple pathways yet to be explored as new potential therapeutic targets for stroke therapy.

2.1.3. Stroke mortality

Worldwide 6.15 million people die of stroke every year contributing to 10.8% of total number of deaths by different causes (Sarti, et al., 2000; Mas and Zuber, 1993). Stroke related mortality increases with patient age: mortality rates range from 7 per 100,000 patients aged between 35 and 44 years, to 1400 per 100,000 patients aged over 75 years (Sarti, et al., 2000). Stroke mortality is also five times higher in Eastern Europe compared with Western Europe (Mackay and Mensah, 2004; Stegmayr, et al., 2000; Asplund, 1996; Uemura and Pisa, 1988). There are limited data available on stroke related mortality in India. However, it was estimated that stroke represented 1.2 % of the total deaths in the country, when all ages were included. The proportion of stroke death increased with age, and in the oldest group (> 70 years of age) stroke contributed to 2.4% of all deaths. (Banarjee and Das, 2006)

2.1.4. Financial burden of stroke

The financial burden of stoke impacts both patients and society as a whole in terms of premature death, long term disability, restricted social functioning, cost of treatment and loss of productivity (Caro, et al., 2000). In the USA alone, the direct and

indirect cost of stroke was estimated at $58bn in 2006 (Reeves, et al., 2005). The estimated cost of stroke to the health care and social services in the UK is £2.3bn per annum, more than double the cost of coronary heart disease management (Rothwel, 2001). The huge economic and social burden of stroke is attributed to its high prevalence, hospitalization rates, morbidity and mortality, and its association with long term disability in survivors (O'Brien, et al., 1996).

2.1.5. Stroke in co-morbid conditions

There are many studies of incidence and outcome data regarding stroke but relatively few of these consider the impact of co-morbidity on outcome. Hypertension and diabetes are two independent risk factors for all types of stroke. High prevalence of these two co-morbidities is reported in large scale stroke studies.

Hypertension is the most potent risk factor for all types of stroke (Shimizu, et al., 1984), this having been demonstrated in the large scale studies (Framingham study) (Wolf, 1985; Kannel, et al., 1981) and many other studies, having shown a prevalence of over 50% (Wolf, 1985).

Hypertension can cause stroke through many mechanism. The increased stress on the endothelium leads to structural alterations and can increase permeability over the BBB and local or multifocal brain edema. Endothelial damage and altered blood cell–endothelium interaction can lead to local thrombi formation and ischemic lesions. Furthermore, hypertension accelerates the arteriosclerotic process, thus increasing the probability for cerebral lesions related to stenosis and embolism originating from large extracranial vessels, the aortic arch and from the heart (Hu and Sarti, 2005; Amenta, et al., 2003). Adaptive structural changes in the resistance vessels, while having the positive effect of reducing the vessel wall tension, have the negative consequence of increased peripheral vascular resistance that may compromise the collateral circulation and enhance the risk for ischemic events in connection with episodes of hypotension or distal to a stenosis(Brayden, et al.,1983).

Diabetes mellitus is also a well-known vascular risk factor with a two-fold increase in the risk of stroke within the first five years of diagnosis when compared to non-diabetics (Lee, et al., 2012; Jeerakathil, et al., 2007). Large scale stroke prevalence studies report about 25-36% prevalence of diabetes as a co-morbid condition along with stroke (Rothwell, et al., 2004; Arboix, et al., 2006; Goldstein, et al., 2004). Several clinical trials have confirmed conclusively that even slight elevations of sugar following

stroke are associated with larger strokes which often grow larger in size. The increase in glucose following stroke cause significantly more stress and is directly toxic to nerve cells in the brain (Rizk, et al., 2005). Hyperglycemia in acute stroke is well known to be associated with a worse outcome. However, rapid lowering of glucose with insulin does not reduce infarct size and does not improve recovery of patients with acute stroke (McCormick, et al., 2010).

Stroke when occurs in combined conditions of hypertension and diabetes is greatly deleterious. A clinical study evaluated prospectively the joint association of history of hypertension and diabetes on the incidence of stroke and stroke mortality. The highest risk of an incident stroke event and, in particular, of stroke death, was found among subjects who had both hypertension and diabetes. This study confirmed that hypertension and diabetes increased the risk of stroke independently, and, having both together, the risk increased dramatically (Gisselsson, et al., 1999).

Although important, other less prevalent co-morbidities from large scale stroke epidemiological studies include ischemic heart disease (IHD), atrial fibrillation (AF), congestive cardiac failure (CCF) and chronic obstructive pulmonary disease (COPD). Prevalence rates ranging from 10-12% for IHD (Carter, et al., 2007), 3-5% for AF (Carandang, et al., 2006), 5-6% for CCF (Arboix, et al., 2000) and 5% for COPD (Arboix, et al., 2000) are reported in various studies.

2.2. Current management of ischemic stroke

2.2.1. Medical management of stroke

Control of physiological variables

Control of physiological variables such as blood pressure, hyperglycemia and fever are important in a clinical setup and their control is implicated with less severe stroke outcome. Arterial hypertension occurs in as many as 80% of patients following acute stroke (Leonardi-Bee, et al., 2002). Current guidelines suggest lowering blood pressure (European Stroke Organisation, 2008). Presently there exists a great deal of uncertainty in the majority of patient and it is unclear whether prior antihypertensive therapy should be discontinued in the acute phase and at what thresholds of blood pressure one should intervene and the treatment targets that one should aim for. Fortunately, several large clinical trials are now underway and may address these issues. These studies include Controlling Hypertension and Hypotension Immediately Post-Stroke Trial [CHHIPS trial] (Potter, et al., 2005), Continue or Stop post-Stroke Antihypertensives Collaborative Study [COSSACS] (Robinson, 2005) and Efficacy of Nitric Oxide in Stroke Trial [ENOS trial] (Thomas, et al., 2006). From the recently concluded CHHIPS trial early lowering of blood pressure with antihypertensive medication after acute stroke seems to be a promising approach to reduce mortality and potential disability.

Elevated blood glucose is common in the acute phase following ischemic stroke (Capes, et al., 2001) and is associated with a poor outcome (Weir, et al., 1997), regardless of the presence of pre-existing diabetes. This may be because elevated blood glucose increases brain lactate production, which is associated with increased infarct size (Parsons, et al., 2002), may reduce the efficacy of thrombolytic therapy (Ribo, et al., 2007; Ribo, et al., 2005) and may increase the risk of haemorrhagic transformation of infracted tissue. However, whether routine lowering of hyperglycemia with insulin improves outcome after acute stroke is as yet unproven. The Glucose Insulin in Stroke-United Kingdom (GIST-UK) trial (Gray, et al., 2007) enrolled 933 patients and found no evidence of benefit for insulin therapy (although lower blood glucose levels were seen) in terms of mortality or secondary outcomes. At present, current guidelines suggest insulin titration in those with serum glucose of > 10 mmol/l (European Stroke Organisation, 2008).

Fever has also been associated with a poor outcome following acute stroke (Hajat, et al., 2000), possibly because of a detrimental effect on intracerebral metabolism, increased free radical production (Globus, et al., 1995) or changes in BBB function (Dietrich, et al., 1991). Guidelines suggest the use of fanning and paracetamol if temperature rises above 37.5°C and that this should prompt a search for infection (European Stroke Organisation, 2008). It is again unclear whether this improves clinical outcome.

Reperfusion strategies using recombinant tissue plasminogen activator (rt-PA)

One of the few breakthroughs in acute ischemic stroke clinical trial has been the investigation of thrombolytics. These agents aim to dissolve the blood clots that cause blockage of arteries. In 1994 The National Institute of Neurological Disorders and Stroke (NINDS) recombinant tissue plasminogen activator (rtPA) investigators examined the use of thrombolytics for acute ischemic stroke. They found that administration of 0.9 mg/Kg body weight of rtPA within 3 hours of index ischemic stroke did not confer a benefit on 24 hour outcome measures, but a significantly better functional outcome was seen at 90 days (Marler, 1995). Currently this is one of the only interventions that have been approved for marketing.

However many patients do not reach hospital soon enough for thrombolytics to be safely administered (Fagan, et al., 1998). The time window, risk of haemorrhage and necessity for neuroimaging is only realistically met in less than 5% of ischemic stroke patients, and this further narrows the field of patients who are eligible to receive this treatment (Fisher and Bogousslavsky, 1998). Several trials were conducted in order to increase the time window for treatment with rtPA, however conclusion from these studies imply that there was only a marginal increase in time window. The European Cooperative Acute Stroke Studies (ECASS I and ECASS II) in 1994 and 1998 respectively, utilized a later therapeutic time window for rtPA administration (up to 6 hours). The investigators found that administration within 6 hours of onset did not confer any beneficial outcome (Hacke, et al., 1998; Hacke, et al., 1995). Hacke et al. (2004) conducted a pooled analysis of rtPA trials and reported a potential therapeutic benefit for rtPA administration up to 4.5 hours after index stroke (Hacke, et al., 2004). Two trials (ECASS III and IST-3) investigated the potential for broadening the treatment window for rtPA administration beyond 3 hours (Hacke, et al., 2008). The median time for the

administration of alteplase was 3 hours 59 minutes in ECASS III study as compared with placebo. Intravenous alteplase administered between 3 and 4.5 hours after the onset of symptoms significantly improved clinical outcomes in patients with acute ischemic stroke; alteplase was more frequently associated with symptomatic intracranial haemorrhage (Hacke, et al., 2008).

Intra-arterial thrombolysis involves direct catheterization of an occluded artery and local administration of thrombolytic agents (rtPA or urokinase). This appears to be an effective therapy for confirmed middle cerebral artery (MCA) or basilar artery occlusion where treatment initiated within 6 hours of onset, following intra-arterial thrombolysis with urokinase and intravenous heparin, reperfusion rates in those with confirmed MCA occlusion (MCAo) were significantly higher (66% compared to 18% in heparin treated controls). Clinical outcomes were also significantly improved with more patients living independently at day 90 (40% compared to 25% of controls). However, a systematic analysis suggested reperfusion rates and clinical outcomes were similar with both intra-arterial and intravenous treatment emphasizing the need for administration of either form of thrombolysis in this devastating condition (Furlan, et al., 1999).

Aspirin, other antiplatelet and anticoagulant therapy

Though the effect size is modest but it is important to treat patients with ischemic stroke with aspirin; a combined analysis of the CAST (Chen, et al., 1997) and IST (Sandercock, et al., 1997) revealed 9 fewer deaths or fatal strokes for every 1000 patients treated with aspirin in the acute phase (Chen, et al., 2000). If patients receive thrombolytic treatment, it is generally advised that aspirin should be deferred for 24 hours. Early anticoagulation has not been shown to yield benefit early after stroke. In a meta analysis of 22 trials, anticoagulation within 24-48 hours after ischemic stroke onset led to a reduction in early recurrent stroke which was offset by an increase (of similar magnitude) in the number of symptomatic intracranial hemorrhages (Gubitz and Sandercock, 2004). Very early treatment with unfractionated heparin has been linked with better functional outcome and lower early recurrence rate (Chamorro, et al., 2005) but again at the cost of increased haemorrhagic complications. Early anticoagulation is therefore not recommended as a treatment for acute ischemic stroke (European Stroke Organisation, 2008) although is recommended by some for those with AF (Chamorro, 2006).

Therapeutic hypothermia

Therapeutic hypothermia is arguably the most promising novel therapy for acute stroke (Correia, et al., 2000). There are several mechanisms by which hypothermia may convey a neuroprotective mechanism. It will decrease cellular metabolism (Lanier, 1995), limit cytotoxic and excitatory cascades, reduce free radical formation and suppress BBB breakdown (Hammer and Krieger, 2002). Mechanisms of cooling include surface cooling which, while able to attain reductions in temperature (Kammersgaard, et al., 2000), has disadvantages of patient discomfort and may necessitate sedation and paralysis. The preferable, yet more invasive approach is to use intravascular cooling via a central venous heat exchange catheter and infusion of cold saline (Guluma, et al., 2006). It also allows greater control over rewarming, which may be associated with increases in intracranial pressure if performed rapidly (Schwab, et al., 2001). A wealth of data show therapeutic hypothermia is effective in animal models of cerebral ischemia, particularly with transient ischemia where cooling was initiated within one hour (Krieger and Yenari, 2004). Therefore, like thrombolytic therapy, it is likely that therapeutic hypothermia will only prove effective if initiated early after onset and in patients with reperfusion (either spontaneous or iatrogenic). Evidence of efficacy in humans is lacking, although a case report (Berger, et al., 2005) and some human feasibility studies (Krieger, et al., 2001) provide prima facie evidence of efficacy. Further research is required.

2.2.2. Surgical treatment of stroke

Blood clots within large arteries are relatively resistant to intravenous treatment with thrombolytics such as rtPA (Smith, et al., 2008). Mechanical removal of a thrombus using the Mechanical Embolus Removal in Cerebral Ischemia (MERCI) Retriever Device, Catch Device offer few options which are recently being successfully utilized (Gobin, et al., 2004). On the other hand decompressive surgery in malignant MCAo significantly improves survival (Juttler, et al., 2007; Vahedi, et al., 2007). However, despite these promising results, the issue of clinical benefit has yet to be formally tested in a randomized controlled trial, and as a result this intervention is not as widely available.

Despite the clear and significant burden of acute stroke, there are depressingly few effective treatments. For ischemic stroke, the only proven effective interventions are stroke unit care, aspirin and reperfusion therapy with intravenous thrombolytic therapy.

There are promising therapies in development but this lack of available treatment, the increasing number of strokes due to the ageing population and the escalating burden of stroke makes optimal and cheap preventative therapy vitally important.

2.2.3. Preventive treatment of stroke

In the absence of effective therapy for the treatment of stroke, effective management of stroke largely depends upon preventive strategies. There are several well proven effective strategies available for prevention of stroke. Following paragraphs would enumerate important strategies currently under practice for stroke prevention.

Antiplatelet therapy

Aspirin is the only licensed strategy for the primary prevention of stroke. Aspirin prevents platelet aggregation by irreversible inhibition of cyclooxygenase-1 (COX-1), thereby reducing production of prostaglandins and thromboxane A2 by platelets. Large scale clinical trails have concluded even for the primary prevention of stroke, aspirin appears to be of benefit perhaps only in women, whereas in men, most evidence suggests a reduction in risk of MI (Tognoni, et al., 2001; Hansson, et al., 1998 ; Meade, et al., 1998; Hennekens, 1989). There are no alternative anti-platelet agents with clear evidence to support primary prevention of stroke.

There are four licensed strategies for anti-platelet therapy to prevent recurrent stroke. These are aspirin, clopidogrel, dipyridamole and the combination of aspirin and extended release dipyridamole. Aspirin, clopidogrel and dipyridamole monotherapy appear to provide similar efficacy in the secondary prevention of stroke (Sacco, et al., 2008,), although clopidogrel may be slightly superior (CAPRIE trial (CAPRIE Steering Committee, 1992). The combination of aspirin and clopidogrel, aspirin and extended release dipyridamole are also in use. (Halkes, et al., 2006; Yusuf, et al., 2001).

Statins/Cholesterol lowering therapy

The link between serum cholesterol levels and coronary artery disease is well established and the evidence that cholesterol lowering therapy with HMG-CoA reductase inhibitors (statins) reduces coronary morbidity and mortality in those with and without established CHD is unequivocal (Larosa, et al., 1999). Debate however continues concerning the association between serum cholesterol levels and stroke.

However, statin therapy is now an accepted strategy following ischemic stroke (European Stroke Organisation, 2008). Further, in a large meta-analysis of over 90000 patients (mostly with manifest CHD) who were enrolled in statin trials, statin treatment significantly reduced the risk of incident stroke (Amarenco, et al., 2004). Each 10% reduction in LDL cholesterol appears to afford a 15.6% relative risk reduction (RRR) of stroke. Further compelling evidence arose from large scale studies in which simvastatin (Sever, et al., 2003) the risk of first stroke by 25% and atorvastatin (The SPARCL trial (Amarenco, et al., 2006) led to a 16% RRR of recurrent stroke.

Blood pressure lowering therapy

Hypertension is the most significant risk factor for stroke and the relationship between increasing blood pressure and increasing stroke risk even extends to within the normal blood pressure range (Lawes, et al., 2003; Lewington , et al., 2002). Treatment of hypertension is of unequivocal benefit in reducing stroke risk in the primary prevention (Collins and MacMahon, 1994) and evidence suggests that newer antihypertensive agents, such as angiotensin receptor blockers (ARBs), offer greater protection than “older” atenolol and diuretic based regimens. In a meta-analysis (Zhang, et al., 2006), calcium channel antagonist (except verapamil), angiotensin converting enzyme inhibitor (ACEI) and ARB based therapy was superior to diuretic or β- blocker therapy, affording 7% greater protection against stroke.

Treatment of hypertension in those who have stroke has now also been shown to reduce recurrent stroke risk. In a meta-analysis (Zhang, et al., 2006), odds of recurrent stroke were reduced by 24%, but interestingly, the strongest evidence exists for diuretic based therapies, as opposed to ACE inhibitor or β blocker based regimens. (Schrader, et al., 2005; The PROGRESS study group, 2001). Antihypertensive therapy is therefore recommended to reduce risk of first and recurrent stroke and risk of other vascular events following ischemic stroke, TIA and intra-parenchymal haemorrhage.

Diabetes management

Diabetes is a risk factor for stroke (Burchfiel, et al., 1994; Manson, et al., 1991; Kannel and McGee, 1979) and is an increasingly prevalent condition found in as many 33% of patients with ischemic stroke (Arauz, et al., 2003). Glycaemic control is effective in reducing the risk of microvascular complications, in both type1 and type2 diabetes (Reichard, et al., 1993). These include diabetic retinopathy, nephropathy and

neuropathy. Data concerning the impact of glycaemic control on risk of macrovascular complications including stroke are less convincing. Trends toward a reduction in cardiovascular event rates have been seen but this remains unproven (The ADVANCE group, 2008 & ACCORD group, 2008). European guidelines suggest those with type 2 diabetes who do not require insulin and have suffered stroke be commenced on pioglitazone therapy (European Stroke Organisation, 2008). However, this recommendation is based on data from subgroup analysis where pioglitazone has shown a trend toward a reduction in major vascular events and death and significant reduction in both fatal and non-fatal stroke and CV death, non-fatal MI and stroke (Dormandy, et al., 2005)

2.2.4. Lifestyle modifications

Cigarette smoking, alcohol consumption and obesity are independent risk factor for both stroke and recurrent stroke (Sherzai, et al., 2012; Kurth, et al., 2002; Robbins, et al., 1994; Gill, et al., 1986). Observational data suggest that stopping smoking affords a reduction in stroke risk and that risk returns to that of a non-smoker after 5 years (Robbins, et al., 1994). Guidelines suggest that those who are heavy drinkers should be advised to eliminate or reduce their consumption of alcohol. Weight management is encouraged through lifestyle measures for obese individuals.

There are thus a number of effective preventative therapies for both the primary and secondary prevention of stroke. Strategies include anti-platelet therapy and lipid lowering therapy to prevent ischemic stroke, anticoagulant therapy to prevent cardioembolic ischemic stroke and blood pressure reduction, treatment of prevalent diabetes, lifestyle therapy and behavioral modification to prevent all stroke subtypes.

2.3. Neuroprotective strategies for stroke

Neuroprotection for ischemic stroke refers to strategies, applied singly or in combination, that antagonize the injurious biochemical and molecular events that result in irreversible ischemic injury. Contribution of various neurotransmitters and neuromodulators have been implicated to the ischemic injury and neuronal death associated with stroke. Role of excitatory amino acid receptor activation, calcium overload, nitric oxide, and oxidative stress in the pathogenesis of ischemic brain damage is well established. Based on recent advances in understanding of molecular pathways that could be considered as potential therapeutic targets, several new strategies are currently emerging (Minnerup, et al., 2012). On this basis there has been a recent explosion of interest in this field. Following paragraphs would enumerate various neuroprotective strategies evaluated or being evaluated both in animal models and clinical trials. Broadly these strategies can be categorized as 1) Prevention of early ischemic injury 2) Preventing reperfusion injury 4) Miscellaneous other strategies 4) Promising and new strategies.

2.3.1. Prevention of early ischemic injury: excitotoxicity

Glutamate Antagonist

It is well established that glutamate, the major excitatory CNS neurotransmitter, is capable of inducing excitotoxic neural injury in cerebral ischemia and other disorders (Schurr, 2004; Arundine, et al., 2004; Aarts, et al., 2003 Bruno, et al., 2001; Billups,e al., 1998; Pellegrini-Giampietro, et al., 1997; Hansen, 1995; Ginsberg, 1995; Hossmann, 1994a; Benveniste, 1991; Choi, et al., 1990; Rothman, et al., 1986; ;). Glutamate and related excitatory amino acids interact with several receptor-classes, thus eliciting excitotoxic effect on the neurons. These include the N-methyl-D-aspartate (NMDA) and the 3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors. Thus, antagonists of these receptors are relevant to neuroprotection.

Non-competitive NMDA antagonism

The NMDA receptor complex, which mediates both calcium and sodium ion fluxes, contains an agonist-binding site, a glycine modulatory site, and binding sites within the ion channel, where magnesium exerts a voltage-dependent block (Ginsberg, 1995).

MK-801 (dizocilpine), dextromethorphan and aptiganel (CNS-1102, Cerestat) are most extensively studied non competitive NMDA antagonist in pre-clinical ischemia models, which binds to NMDA receptors with high affinity and produces a long-lasting blockade. MK-801 and dextromethorphan were capable of reducing infarct volume substantially in several models; typically, it was most effective with treatment initiated prior to ischemia or within the first 1–2 hours thereafter (Ginsberg, 1995). In pre-clinical reports, CNS-1102 treatment (~2000 micrograms/kg) begun 15 min after (MCAo) in rats yielded significant reductions in infarct volume (both gray and white matter) by diffusion weighted MR imaging and post mortem analysis (Schabitz, et al., 2000; Minematsu, et al., 1993). Aptiganel is the only non-competitive NMDA antagonist to have progressed to later-phase clinical trials.

Clinical stroke trials in the case of dextromethorphan and MK-801, was halted in the pilot phase due to variety of dose-related reversible neuropsychological side effects (Albers, et al., 1995; Olney, 1994). The maximal tolerated dose for aptiganel was ~30 mg/kg i.v. over 15 min (This dose-range, it should be noted, is several orders of magnitude below the effective neuroprotective doses shown in animals). A double-blind phase II/III trial (The Aptiganel Acute Stroke Trial), treatment initiated within 6 hours with high or low-dose aptiganel or placebo (Albers, et al., 2001) was undertaken. No improvement in the primary outcome was seen with either dose of aptiganel and the trial was aborted by the sponsor and by the independent data and safety monitoring board because of both a lack of efficacy and the imbalance in mortality.

Competitive NMDA antagonism

CGS 19755 (selfotel) was evaluated pre-clinically and progressed to a larger clinical trial. In limited preclinical studies, CGS 19755 administered within 5 min of the insult in permanent MCAo in rats reduced infarct size and reduced postischemic glucose hyper metabolism (Simon, et al., 1990). In cats with 90-min MCAo plus only 4 h of reperfusion, CGS 19755 in a dose of 40 mg/kg at 75 min led to ~50% infarct size reduction (Miyabe, et al., 1997). Two pivotal phase 3 prospective studies were then carried out, in which subjects were randomized to a single 1.5 mg/kg dose of selfotel within 6 hours of stroke onset (Davis, et al., 2000). The trials were suspended because of severe adverse effects (Grotta, et al., 1995) and a trend toward higher mortality in the selfotel-treated group (Davis, et al., 2000; Davis, et al., 1997).

NMDA receptor glycine-site antagonism

GV150526 (gavestinel) is a highly potent, highly selective antagonist of the glycine site. In rats with MCAo, GV150526, 3 mg/kg i.v., administered up to 6 h after occlusion yielded significant reduction of infarct volume measured at 24 h and protected somatosensory evoked potential (SEP) responses only when treatment was begun at 1 h (Bordi, et al., 1997). In an MRI study in rats with MCAo, when GV150526 was first administered at 6 h, ischemic volume was still reduced by ~45% (Reggiani, et al., 2001). At the same time GV150526 was shown to have much greater margin of safety than other NMDA antagonists (Bordi, et al., 1999).

Two pivotal phase III trials, the GAIN Americas Trial (Sacco, et al., 2001), and the GAIN International Trial (Lees, et al., 2000) were then conducted. Patients were stratified by age and stroke severity and were randomly assigned to treatment within 6 hours with either intravenous gavestinel (loading dose 800 mg, plus 5 doses of 200 mg each at 12-h intervals) or placebo. The primary outcome measure was the trichotomized Barthel index at 3 months. Each of these trials was entirely neutral – i.e., showed no difference between treatment groups for either the primary outcome or for mortality or any secondary outcome measures (Sacco, et al., 2001; Lees, et al., 2000). A small MRI sub study drawn from these two trials, designed to test whether gavestinel would attenuate lesion growth relative to placebo, was also negative (Warach, et al., 2006).

AMPA receptor antagonism

It is of interest that, while AMPA antagonists showed neuroprotective efficacy in certain preclinical settings of both focal and global cerebral ischemia (Takahashi, et al., 2002; Lees, 2000; Gill, 1994; Xue, et al., 1994;Li, et al., 1993;), the successful completion of larger clinical trials has not been reported. In a phase II double-blind multicenter trial (Elting, et al., 2002), the AMPA antagonist ZK200755, when administered in a higher dose, produced a marked transient worsening of the NIHSS score attributable to a depression of consciousness (stupor or coma) which lead to suspension of trial. The AMPA Receptor Antagonist Treatment in Ischemic Stroke Trial (ARTIST MRI) and a related ongoing trial, ARTIST +, with patients randomized within 3 hours to YM872 or placebo; all patients received i.v. tPA within 3 hours; were abandoned after failing an interim futility analysis (Internet Stroke Center, 2011).

GABA agonism

Clomethiazole is a GABA-A agonist; its mechanism of action involves potentiation of the activity of GABA, the brain's major inhibitory neurotransmitter (Wilby, et al., 2004). It has been widely used as a sedative. Clomethiazole was perhaps more extensively evaluated in animal models of transient global forebrain ischemia, focal ischemia and permanent MCAo models. Focal ischemia studies included a study of 1-h MCA suture occlusion in rats, followed by 24-h reperfusion, in which clomethiazole was given either 60 min prior to occlusion or at 10 min after the start of reperfusion (i.e., at 70 min after onset of ischemia) (Sydserff, et al., 1995b) and histological protection was observed. When studied in a rat model of permanent MCAo, clomethiazole reduced the volume of ischemic damage by 58% when administered at 1 h after occlusion but was ineffective when given at 3 h (Sydserff, et al., 1995a). Clomethiazole was also studied in marmosets (small primates) with permanent MCAo; treatment at 5 min reduced overall damage by 32% and improved use of the disabled arm (Marshall, et al., 1999). However, when treatment was initiated at 1 h after occlusion in this model, the agent improved spatial neglect but not the affected limb's severe motor impairment (Marshall, et al., 2000).

Two pilot efficacy and safety trial (the CLASS Trial, Clomethiazole vs. placebo and CLASS-T, clomethiazole+ tPA vs. placebo) were then conducted to explore the effect of clomethiazole treatment initiated within 12 hours. No difference was found in the proportions of patients achieving functional independence at 90 days (Wahlgren, et al., 1999;Lyden, et al., 2001). A subsequent large trial, CLASS-I (Lyden, et al., 2002) that studied patients with major acute ischemic stroke randomized subjects to clomethiazole or placebo beginning at 12 hours. The proportion of patients attaining a Barthel index score of 60 or above at 90 days did not differ between groups. Subgroup analysis of patients treated early (< 6h) versus 6–12 hours also failed to show a treatment effect.

Diazepam, a GABA-ergic drug, was studied in a trial of 880 stroke patients randomized within 12 hours of onset. The primary outcome - independence on the Rankin scale at 3 months - was not significantly different between groups (Lodder, et al., 2006).

Calcium channel antagonists

Calcium plays a central role in the pathophysiology of ischemic brain injury as well as cardiac disorders. Thus, calcium channel antagonists have found applications in the therapy of stroke (Tymianski, et al., 1996; Erecinska, et al., 1996; Choi, 1995; Siesjo, et al., 1989). In the brain, blockade of slowly activating (L-type) calcium channels reduces calcium entry into neurons, a potentially beneficial action in ischemia and other disorders. Nimodipine, a calcium channel antagonist is the most extensively studied. Over 250 animal studies of nimodipine in cerebral ischemia have been published. A careful survey of this literature, however, was able to identify only 20 controlled animal studies of focal cerebral ischemia in which nimodipine was administered after the induction of ischemia and its consequences assessed (Horn, et al., 2001b). Ten of these studies reported a positive outcome, but 10 did not; in the 7 studies in which exact infarct-size data were presented, the pooled effect size (standardized mean difference) favoring nimodipine was −1.2 (Horn, et al., 2001b). In several of the positive studies, nimodipine treatment was initiated within the first 15 minutes after onset of ischemia. Subgroup analysis of studies in which treatment was initiated at 1 hour or longer after induction of ischemia revealed overall non-significance (Horn, et al., 2001b). The general quality of the studies analyzed was judged to be methodologically weak.

Five larger randomized, double-blinded efficacy trials of nimodipine in acute ischemic were subsequently published (Internet Stroke Center, 2011). All these trials were negative for the primary end-point. (Kaste, et al., 1994; Wahlgren, et al., 1994; American Nimodipine Study Group, 1992; TRUST Study Group, 1990;). Meta-analysis published later, using data from these and other, smaller ischemic-stroke trials found no overall benefit of nimodipine (Horn, et al., 2000; Mohr, et al., 1994). The VENUS Trial, which followed upon these meta-analyses, was intended to test the efficacy of early nimodipine therapy. In this trial too the primary end point, defined as poor outcome -- death or dependency after 3 months – did not differ between treatment groups. (Horn, et al, 2001a).

Magnesium therapy

Magnesium may be viewed as an endogenous calcium antagonist that may protect via multiple mechanisms, including NMDA receptor blockade, inhibition of excitatory neurotransmitter release, blockade of calcium channels, as well as vascular

smooth muscle relaxation (Ovbiagele, et al., 2003). Studies of magnesium as a neuroprotectative in focal ischemia have been rather limited in scope. In one study, intraperitoneal MgCl2 administered just after MCAo in rats reduced infarct volume (Izumi, et al., 1991). In another study in rats with autologous MCA thromboemboli treated with 5% MgSO4 (90 mg/kg), improved neurological outcome was described with administration out to 6 h (Yang, et al., 2000). Another study employed pre-ischemic intracarotid infusion of MgSO4 prior to MCAo in rats; with higher Mg doses and shorter ischemia durations, infarct-volume reductions up to ~60% could be achieved (Marinov, et al., 1996). The overall impression of these trials was of mixed results, and concern that a possibly confounding (i.e., protective) influence of hypothermia could not be excluded in the positive studies (Meloni, et al., 2006).

A large multicenter efficacy trial was conducted (Intravenous Magnesium Efficacy in Stroke Trial; IMAGES) in 2,589 patients with acute stroke, in which subjects were randomized to intravenous MgSO4 or placebo with a 12-hour window to treatment. The primary outcome was a global endpoint incorporating death or disability at day 90; this outcome failed to be improved by magnesium, and mortality was slightly higher in the magnesium than in the placebo-group (Muir, et al., 2004).

2.3.2. Preventing reperfusion injury

2.3.2.1. Reduction of oxidative stress

Free radical scavengers

NXY-059, a proprietary nitrone spin-trap agent, is a bis-sulfonated derivative of PBN [α-phenyl-N-tertbutyl nitrone], a generic nitrone spin-trap agent that has been shown to protect in models of brain ischemia and trauma. In a transient (2-h) MCAo model in rats, NXY-059 treatment begun at 1 hour after recirculation had a marked dose-dependent effect on infarct volume, with the highest dose reducing infarct volume by 77% (Kuroda, et al., 1999). Even when started at 3 h of recirculation (i.e., 5 h after the onset of ischemia), neurologic deficits were still improved and infarct volume was still reduced by approximately two-thirds. Other workers confirmed these effects in similar models. In one such study, spontaneously hypertensive (SHR) rats led to a 36% reduction in cortical infarct volume (Zhao, et al., 2001). In another permanent-occlusion model in rats, 44% infarct reduction was observed even when treatment was initiated at 4 h after stroke onset (Sydserff, et al., 2002). Beneficial effects of NXY-059 were also reported in a small-clot embolus model in rabbits (Lapchak, et al., 2002b). In similar

studies employing large-clot embolism, NXY-059 used alone increased the brain-hemorrhage rate but, when used together with tPA, appeared to reduce tPA-induced hemorrhage (Lapchak, et al., 2002a).

This compound was then evaluated in non-human primate models. In marmosets pre-trained on behavioral tasks and subsequently given permanent MCAo, NXY-059 treatment begun at 5 min and continued for 48 hours led to improved function of the hemiparetic arm and a lessening of spatial perceptual neglect at 3 and 10 weeks, and to 51% reduction in overall brain damage (Marshall, et al., 2001). When treatment was begun at 4 hours after stroke onset, behavioral improvement and a 28% reduction in the overall infarct still resulted (Marshall, et al., 2003).

NXY-059 was studied in two large randomized, double-blind trials, which to some extent proceeded concurrently. The first trial, SAINT I, involved 1,722 patients (Lees, et al., 2006); SAINT II enrolled 3,306 subjects (Shuaib, et al., 2007). In both trials, subjects were randomly assigned to receive a 72-hour infusion of intravenous NXY-059 or placebo beginning within 6 hours of stroke onset; each study site was required to maintain an average time-to-treatment of 4 hours or less. In SAINT I, NXY-059 significantly improved the overall distribution of scores on the modified Rankin scale (mRS) as compared to placebo ($p=0.038$), with a common odds ratio of 1.20 (95% CI, 1.01–1.42) (Lees, et al., 2006). However, NXY-059 did not improve outcome on the NIHSS scale or the Barthel index. By contrast, the results of SAINT II were entirely negative: the distribution of mRS score was virtually identical in the NXY-059 and placebo groups, and there was no evidence of efficacy for the secondary end-points (Shuaib, et al., 2007). As regards safety, an excess of hypokalemia was noted in NXY-059-treated patients (Serebruany, 2006).

Other antioxidant agents

Three antioxidant/radical scavenging agents deserve mention because each has undergone clinical trials of over 200 subjects.

Edaravone is an oxygen radical scavenger and blocker of lipid peroxidation. Early pre-clinical reports described salutary effects in both global and focal cerebral ischemia (Watanabe, et al., 1994). In a rat model of transient MCAo, edaravone reduced infarct volume when administered prior to the insult (Nakajima, et al., 2005) and administered at reperfusion (Amemiya, et al., 2005). In mice with 60-min MCAo, edaravone appeared to reduce infarct volume and improve neurological score at 24 h

even when administered at 6 h after onset of ischemia (Zhang, et al., 2005a). Only a single randomized clinical trial of edaravone has been fully published: a phase II study of ischemic-stroke patients with a 72-hour window to treatment; this trial showed improved outcome on the modified Rankin scale at 3 months (Edaravone Acute Infarction Study, 2003).

Tirilazad mesylate (U-74006F) is a non-glucocorticoid 21-aminosteroid inhibitor of iron dependent lipid peroxidation. An extensive systematic pre-clinical review and meta analysis has been published, surveying animal models of focal ischemia involving tirilazad in which outcome was measured as infarct volume and/or neurological score (Sena, et al., 2007). The overall effect size was modest with efficacy higher in temporary occlusion than in either permanent or thrombosis models (Sena, et al., 2007). Maximum efficacy was seen when treatment was given before the onset of ischemia, with a trend for efficacy to decline thereafter (Sena, et al., 2007). Furthermore, tirilazad was effective over a very narrow dose range of 3–10 mg/kg. Prospective randomized trails conducted later were either terminated prematurely (The RANTTAS Investigators, 1996, RANTTAS II) for the reasons of lack of efficacy (van der Worp, et al., 2002) or questions regarding safety emerged (Internet Stroke Center, 2011). In comparing the pre-clinical and clinical tirilazad studies, it is apparent that the clinical trials a) used a much broader dose range and b) employed a much longer time to treatment (median ~5 h) than the animal trials (median, 10 min) (Tirilazad International Steering Committee, 2000).

Ebselen is a selenium compound with glutathione peroxidase-like activity; it also reacts with peroxynitrite and inhibits a variety of enzymes (Parnham, et al., 2000). In rodent models of MCAo ischemia, ebselen reduced ischemic damage when administered prior to ischemia (Namura, et al., 2001) and was modestly protective if begun 30 min after induction of ischemia (Takasago, et al., 1997). When treatment was begun at the onset of recirculation after 2-h MCAo in rats, ebselen reduced early brain injury and improved neurological deficits at 24 hours (Imai, et al., 2001), but this protection was not sustained at 7 days (Salom, et al., 2004). In a clinical trial of acute ischemic-stroke patients randomized to oral ebselen or placebo treatment begun within 48 hours and continued for 2 weeks, the intent-to-treat primary-outcome analysis was significant for ebselen at 1 month but not 3 months (Yamaguchi, et al., 1998). A secondary analysis suggested benefit in the ebselen-subgroup treated within 24 h. A

Phase III study of 394 patients incorporating a 24-hour window was scheduled to begin in 2001 (Internet Stroke Center, 2011), but no report has appeared.

2.3.2.2. Reduction of neuronal inflammation: leukocyte inhibition

Enlimomab is a murine intercellular adhesion molecule-1 (ICAM-1) antibody that reduces leukocyte adhesion. In rats with 2-h transient MCAo treated at 1 h of reperfusion, a reduction in size of the ischemic lesion was seen; there was no effect, however, in permanent occlusion (Zhang, et al., 1995). In the EAST multicenter clinical trial, 625 patients with acute ischemic stroke were randomized to enlimomab or placebo within 6 hours of onset. The primary efficacy endpoint was the mRS at 90 days. This trial showed a highly significant worsening of outcome in enlimomab-treated patients compared to placebo ($p=0.004$), and a higher death rate (EAST Trial, 2001). To elucidate the reasons for this negative clinical outcome, an experimental study was performed in which a murine anti-rat ICAM-1 antibody was administered to rats with focal ischemia (Furuya, et al., 2001). This treatment failed to reduce infarct size but elicited host antibody production and activated circulating neutrophils and complement; these mechanisms were offered as a possible explanation for the treatment-related deterioration in the clinical trial.

UK 279,276**,** a recombinant protein inhibitor of the CD11b/CD18 receptor, blocked neutrophil activation. In the ASTIN Trial (Acute Stroke Therapy by Inhibition of Neutrophils), 966 acute stroke patients were treated within 6 hours of onset with the agent or placebo, and neurological recovery was assessed at 90 days. The trial, which incorporated a Bayesian sequential design, was terminated early for futility (Krams, et al., 2003). A possible reason to explain lack of efficacy was a body of pre-clinical evidence that UK 279,276 conferred benefit in reperfused focal ischemia models but failed in the absence of reperfusion (Sughrue, et al., 2004).

Hu23F2G is a human antileukocytic antibody. This agent did not appear to produce the immune response seen with enlimomab. However, no clinical benefit was seen with Hu23F2G on any of the planned measures.

Another anti-adhesion monoclonal antibody strategy targets platelets. These antibodies inhibit platelet aggregation, potentially preventing additional ischemic injury during reperfusion, as well as promoting thrombolytic action. Such an antiplatelet drug, abciximab (ReoPro), was in phase III clinical stroke treatment trials, but an

increased rate of symptomatic and fatal intracranial hemorrhage and lack of efficacy led to discontinuation of the trial after 808 patients were enrolled.

2.3.2.3. Inhibition of apoptosis

Accumulating evidence strongly suggests that apoptosis contributes to neuronal cell death in stroke injury. Caspases, a family of cysteine-aspartate proteases that include at least 14 members divided into three groups (I, II, and III), are essential players in apoptotic neuronal cell death (Prunell, et al., 2005). Many groups have studied the effects of caspase inhibition on cerebral ischemia-induced neurodegeneration by using the broad spectrum caspase inhibitor z-VAD, either in the fluoromethylketone (fmk) or dichlorobenzoyloxopentanoic acid (dcb) form and z-DVED-fmk. Both inhibitors were neuroprotective in mouse models of transient cerebral ischemia and z-VAD was neuroprotective also in transient and permanent models in the rat (Hara, et al., 1997). Ac-YVAD-cmk (Ac-Tyr-Val- Ala-Asp-cmk), a caspase group I (caspase-1-like) inhibitor, also was shown to be neuroprotective in a mouse transient model of cerebral ischemia (Hara, et al., 1997). In addition, peptide-based caspase inhibitors have been shown to prevent neuronal loss in animal models of stroke (Robertson, et al., 2000). To date however, the efficacy of anti-apoptotic agents in human stroke patients has not yet been tested.

2.3.3. Miscellaneous other strategies

Phospholipid Precursor molecule

Orally administered citicoline is hydrolyzed in the gut to cytidine and choline, which are rapidly absorbed (Weiss, 1995), cross the BBB, and can be incorporated into the phospholipid fraction of neuronal membranes (Secades, et al., 1995). Several studies in rodent models of focal ischemia have been reported. In a rat model of transient MCAo, high-dose citicoline treatment begun at the time of reperfusion (2 h) reduced infarct volume by one-half, but the study was flawed by failure to control brain temperature and substantial premature mortality (Schabitz, et al., 1996). In another MCAo study, treatment was begun at 15 min after onset of ischemia; citicoline improved behavioral and morphological indices chiefly in rats with submaximal insults (ischemia of 30–75 min) (Aronowski, et al., 1996a). When ischemia was produced by clot embolus, one study showed that citicoline begun at 45 min failed to affect infarct size unless tPA was co-administered (Andersen, et al., 1999); and another showed that citicoline

administered prior to tPA failed to confer additional benefit but, if administered 10 min after tPA, led to less ischemic injury than with tPA alone (Alonso de Lecinana M, et al., 2006). An interesting report (Hurtado, et al., 2007) later provides evidence that citicoline treatment begun 24 hours after MCAo and maintained for 28 days improves functional outcome suggesting that chronic treatment may increase neuronal plasticity within non injured, functionally connected brain regions.

Four important trials and a pooled meta-analysis have been reported. Citicoline treatment was found positive in the first trial (Clark, et al., 1997), while remaining three were negative for the primary outcome (Clark, et al., 2001; Warach, et al., 2000; Clark, et al., 1999b) A pooled analysis of individual patient data from the 4 randomized trials of oral citicoline described above (N=1,372 with baseline NIHSS = >8), re-analyzed using a global 3-month outcome measure incorporating NIHSS, mRS and Rankin scales, revealed a statistically significant effect of citicoline on global recovery (25.2% vs. 20.2%, OR 1.33 (95% CI, 1.10 – 1.62, p=0.0034) (Davalos, et al., 2002). The highest effect was seen in the 2000 mg dose-group.

Agents modulating nitric oxide signal

Lubeluzole is thought to act by down regulating the glutamate-activated nitric oxide synthase pathway (Lesage, et al., 1996). Lubeluzole was studied most extensively in a photochemical model of parietal sensory motor cortical stroke in rats; in this model, lubeluzole rescued tactile/proprioceptive hindlimb placing reactions when administered 5 min after stroke onset and protected 60% of rats when administered at 6 h (De Ryck, et al., 1996). The optimal regimen begun at 5 min reduced infarction by 28% at 7 days (De Ryck, et al., 1996) and attenuated infarct growth as assessed by MRI (De Ryck, et al., 2000). Lubeluzole also blocked peri-infarct glutamate increases in this model (Scheller, et al., 1997). In rats with reversible MCA and carotid artery occlusions for 120 min or more, lubeluzole treatment started 15 or 30 min after onset of ischemia reduced infarct size by 50% and 34%, respectively (Aronowski, et al., 1996b). Other workers described infarct-volume reductions of 33% in rat MCAo with treatment begun at 3 h (Culmsee, et al., 1998). In rats with global cerebral ischemia, lubeluzole treatment was begun at 5 min protected hippocampal CA1 neurons (Haseldonckx, et al., 1997).

Three large scale clinical trails were conducted later with patients randomized to receive lubeluzole within 6 hrs of symptom onset. All three trails were negative for their primary outcome (Diener, et al., 2000; Grotta, 1997; Diener, et al.,

1996) A Cochrane Database meta-analysis, reviewing five trials and 3,510 patients, also found no effect on mortality or dependency but noted a significant increase of heart-conduction disorders (Q-T prolongation) in lubeluzole-treated subjects (Gandolfo, et al., 2002).

Agents acting on ion channels

BMS-204352, a fluoro-oxindole, is an activator of neuronal potassium channels. In rats with permanent occlusion, BMS-204352 showed ischemic protection with treatment at 2 hours (Gribkoff, et al., 2001). This compound was studied in two parallel clinical trials with subjects randomized to treatment within 6 hours of stroke onset. The agent failed to exhibit efficacy (Internet Stroke Center, 2011).

Flunarizine, this calcium channel blocker and Pentoxifylline, a vasodilator were studied in a multicenter randomized trial treated within 24 hours. There was no effect of flunarizine (Franke, et al., 1996) and pentoxifylline (Hsu, et al., 1988) treatment.

Fosphenytoin, a sodium channel blocker and anticonvulsant, was studied in a phase III trial of ischemic stroke randomized to treatment within 4 hours. The trial was stopped after an interim analysis that found no differences between treatment groups. The results were not published (Internet Stroke Center, 2011).

Basic fibroblast growth factor is thought to stabilize intracellular calcium ion homeostasis, induce antioxidant enzymes, and diminish glutamate-mediated excitotoxicity (Mattson, 1997b). A randomized multicenter trial, in subjects with a 6-hour treatment window, was halted intermittently. There was no significant difference in outcome between groups (Bogousslavsky, et al., 2002). Another similar trial was aborted after 302 subjects, and the results were not published.

Piracetam is an agent thought to act as a modifier of membrane fluidity (Muller et al., 1999), cognition- and microcirculation-enhancer, neuroprotectative, and anticonvulsant (Winblad, 2005). A phase III trial of piracetam was conducted in acute stroke patients randomized to treatment within 12 hours of onset. The primary outcome, neurological function at 4 weeks, was similar in the two groups, but post-hoc analyses suggested superiority of piracetam in the subgroup presenting within 7 h of onset (De Deyn, et al., 1997).

Agents acting on opioid and serotonin receptors

Two compounds which deserve mention under this category include Nalmefene and Repinotan.

Nalmefene (Cervene), an opioid antagonist with relative selectivity for kappa opiate receptors, received very little experimental testing in animal models of focal ischemia but was nonetheless brought to clinical trial. A phase II trial of 312 patients randomized to treatment within 6 hours revealed safety but no significant difference in functional outcome at 3 months (Clark, et al., 1999a). Similarly, a phase III trial of 368 subjects treated within 6 hours showed no difference in the primary outcome – Barthel index and Glasgow Outcome Scale at 12 weeks (Clark, et al., 2000).

Repinotan (BAY x3072), a serotonin 5HT1A receptor agonist, was studied in a phase II trial of 240 subjects with hemispheric ischemia treated within 6 hours (Bayer Randomized Acute Ischemia Neuroprotectant Study – BRAINS). The results, which are assumed to have been negative, were not published (Internet Stroke Center, 2011).

2.3.4. Possible reasons for the failure of different strategies

Several obvious factors emerge from the preclinical studies mentioned above. Virtually all pre-clinical studies suggested that NMDA antagonists protected in focal ischemia only with very early administration, which is least relevant clinically. For some investigational drugs it was impossible to approximate the pre-clinically protective doses in clinical trials because of dose-limiting neurobehavioral toxicity in humans. The possibility exists that NMDA antagonism, while potentially protective in focal ischemia, is also deleterious in terms of adversely affecting endogenous NMDA-receptor-mediated neuronal-survival mechanisms (Hoyte, et al., 2004). The demonstration of pre-clinical efficacy consisted of only a few published studies, mostly from one lab, and most often whose methodological quality was sub-optimal (van der Worp et al., 2005) and whose results were not independently replicated (Gorelick, 2000), i.e. preclinical studies with gavestinel. Some of these experimental agents were either less potent neuroprotective for example: NXY059 and Magnesium, for these the physicochemical properties were such that there were issues such as poor BBB penetration. For some experimental agents like citicoline failed to provide key translational data needed to assess the potential for clinical neuroprotection (particularly, defining the therapeutic window of efficacy). These pre-clinical results support mechanistically based neuroprotective

efficacy with very early administration but do not adequately substantiate whether robust neuroprotection is possible with longer, clinically relevant, treatment delays.

Most of the failures for neuroprotective agents observed from clinical studies can be attributed to design issues such as, most common design issue related to clinical trials is the initiation of treatment in major trials at well beyond the likely window of therapeutic efficacy. For example: 4 out of 5 clinical trials of nimodipine used time to initiate treatment at 24 or 48 h after stroke onset. This is much beyond the optimum therapeutic window what is shown from preclinical studies. Other design issues were inadequate sample size to detect a modest treatment effect. Most often for drugs like NMDA antagonists side effects precluded dosing to achieve adequate plasma drug concentrations. The study included a substantial percentage of lacunar stroke patients for a drug with no preclinical evidence of protection of the white matter. Statistical weaknesses of studies such as inadequate powering of study subgroups for analysis of primary outcome. For example: One curious design feature was the inclusion of both tPA-treated and non-tPA-treated subjects (tPA fraction, 29% in SAINT I, 44% in SAINT II) without adequately powering these subgroups for analysis of the primary outcome (Saver, 2007; Shuaib, et al., 2007; Lees, et al., 2006;,Koziol, et al., 2006). Inclusion of a substantial percentage of patients who received I.V. tPA in whom measuring an additional treatment effect is difficult or where the study agent adversely affected outcome with concomitant tPA use. Use of inappropriate end points is another common design issue associated with prospective stroke trials. For example: The use of mortality as a primary outcome measure is considered to be a weak design feature; in current trials, neurological outcome and functional recovery are preferred as primary end-points.

2.3.5. Possible reasons for the observed success in few strategies

Because cerebral ischemia involves a cascade of injury pathways, it may be preferable to focus on drugs/devices/treatments with multiple mechanisms of action and that target multiple pathways. Most of the putative neuroprotective agents evaluated earlier had the potential to address either single component or had limited capacity (interms of time window) to modulate the complex injury pathways. Historically, the simplest approach is to use a drug with multiple targets. The need for more novel approaches is presently felt and these should be evaluated, for example, combinations of single target agents within or across cell death/recovery pathways. An approach that takes into account stroke biocomplexity is to target processes within the dynamic

network that activate ensembles to promote maintenance of homeostasis under stress. In this regard the present promising agents that have shown preliminary clinical success such as high dose albumin therapy, hyperacute magnesium therapy which act on multiple pathways provide proof for the hypothesis stated above. Similarly strategies under experimental evaluation like up regulation of cell survival/adaptive pathways also hold promise for similar reasons.

2.3.6. Promising and new strategies

High-dose human albumin therapy

Moderate- to high-dose human albumin (ALB) therapy proved to be highly neuroprotective in animal models of both temporary (Belayev, et al., 2001; Belayev, et al., 1998; Belayev, et al., 1997a) and permanent (Liu, et al., 2001) focal cerebral ischemia as well as in global cerebral ischemia (Belayev, et al., 1999b) and traumatic brain injury (Belayev, et al., 1999a). In focal ischemia, albumin (dose, 1.25 g/kg i.v.) diminished total infarct volume by two-thirds and reduced brain edema by three quarters or more, with a therapeutic window of full efficacy extending to four hours (Belayev, et al., 2001). In a comprehensive meta-analysis of focal ischemia data, albumin-treated rats exhibited ~80% reductions in mean cortical infarct volume (Ginsberg, et al., 2004).

Subsequently a pilot clinical trial named ALIAS (Albumin in Acute Stroke) in 82 patients at two sites was begun (Palesch, et al., 2006; Ginsberg, et al., 2006a). This dose-escalation, safety trial treated acute ischemic-stroke subjects within 16 hours of stroke onset with 25% human albumin in doses ranging from 0.37 to 2.05 g/kg. One-half of the subjects also received standard-of-care i.v. tPA therapy. At 3 months, the NIHSS, mRS, and Barthel Index were measured. The 3- month outcome data were analyzed for suggestions of efficacy and found that tPA-treated subjects who received higher-dose ALB were 3 times more likely to achieve a good outcome than subjects receiving lower-dose ALB, suggesting a positive synergistic effect between ALB and tPA. Based on these encouraging results, a large randomized multicenter placebo-controlled efficacy trial – the ALIAS Phase III Trial is currently in progress. In this trial, subjects are randomized in a 1:1 ratio to either 25% ALB (2.0 g/kg i.v.) or saline as placebo; treatment is initiated within 5 hours of stroke onset. The primary efficacy end-point is either an NIHSS score of 0 or 1, or mRS of 0 or 1, or both, at 3 months (Ginsberg, et al., 2006b).

Hyperacute magnesium therapy

Jeffrey Saver and colleagues are continuing to assess magnesium, however, as a prototype of hyperacute neuroprotectative therapy for stroke, initiated prior to hospitalization and without the need for prior CT-scanning. They established the feasibility of this approach in the Field Administration of Stroke Therapy — Magnesium (FASTMAG Pilot Trial), where the median time-to-treatment initiation was an impressive 100 min. No serious adverse events were encountered (Saver, et al., 2006). A FAST-MAG phase III multicenter efficacy trial is now underway, for subjects with a 2-hour treatment window. Probable stroke patients (both infarction and hemorrhage) will be randomized to hyperacute treatment with either intravenous MgSO4 or placebo; one-half of subjects will be treated within 1 hour of stroke; and the other half within 1–2 hours. The primary end-point is functional outcome on the mRS at 90 days.

Augmenting endogenous cell survival pathways: Induction of HSP

Molecular chaperones are intracellular proteins which protect cellular proteins against environmental and physiologic stress e.g. hypoxia, oxidative damage, heat stress, heavy metals etc. (Soti and Csermely, 2002; Calabrese et al., 2007). HSP are one important class of molecular chaperones present in all cell types and in multiple cellular compartments like cytosol, mitochondria, endoplasmic reticulum and nucleus. Most of the recent studies indicate that, chaperones, which are mostly heat or stress induced proteins when over expressed, could confer cells and tissues stress tolerance and provide benefit on various pathological states associated with cellular stress (Morimoto and Santoro, 1998; Mehta et al., 2007). There is an experimental evidence in-vitro, on the role of HSP in causing neuroprotection (Magrane et al., 2005; Wagstaff et al., 1999; Batulan et al., 2006).

In-vivo experiments conducted mostly in mice overexpressing HSP have shown clinical benefit in many acute and chronic conditions associated with neuronal loss. Transgenic mice overexpressing HSP70 was shown to have significantly smaller lesion volume in stroke model of permanent cerebral ischemia (van der Weerd et al., 2005; Matsumori et al., 2006). Protein aggregation is a part of etiology of chronic neurodegenerative disease such as AD. Overexpression of HSP70 in hippocampal CA1 neurons reduces evidence of protein aggregation thereby increasing neuronal cell survival (Soti and Csermely, 2002; Soti et al., 2005). Administration of HSP70 in-vivo has also been shown to inhibit motor and sensory neuron degeneration after sciatic nerve axotomy in neonatal mouse (Tidwell et al., 2004). There are many experimental

evidences to show the improvement in clinical conditions in animal models when chaperonic activity was increased. HSP70 gene transfer by a virus vector reduced 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced apoptosis in the substantia nigra in mouse model of Parkinson's disease. Overexpression of HSP70 was also shown to suppress the neuropathology and improves motor function in spinocerebellar ataxia type-1(SCA-1) disease which is characterized by loss of motor coordination due to degeneration of cerebellar purkinje cells and brain stem neurons (Cummings et al., 2001). HSP 70 has shown to protect DNA damage both in-vitro and in-vivo against oxidative injury by interfering with mitochondrial apoptotic pathways shedding some light on the mechanistic details behind the action of HSP70 during oxidative stress (Chan et al., 2007; Doulias et al., 2007).The findings discussed above clearly indicate the ability of stress proteins to protect neuronal cells from a variety of stimuli, including those which induce apoptotic cell death. The protective effects of stress proteins indicate the potential of developing therapeutic approaches to treat conditions of acute and chronic neuronal loss.

Stabilization of hypoxia inducible factor

Hypoxia-Inducible transcription factor (HIF) activation is one of the prominent adaptive mechanisms associated with hypoxia/ischemia. Under normoxic conditions, HIF-α is degraded rapidly via the ubiquitin-proteasome pathway. To mark HIF-α for degradation, specific amino acid residues are hydroxylated by an enzyme family of HIF- hydroxylases which require dioxygen and 2-oxoglutarate as co-substrates (Schofield CJ, 2004). Reduced activity of these hydroxylases under hypoxic conditions, when oxygen is lacking as a substrate or after application of 2-oxoglutarate-analogues, results in HIF-α accumulation (Warnecke C, 2003). Subsequently, HIF-α forms dimer with HIF-β and binds to a specific DNA motif in the hypoxia response elements, thus transactivating target genes. HIF activation induces transcription of genes in a concerted manner which performs multiple functions to adapt and to recover from hypoxic/ischemic conditions (Maxwell P, 2003). HIF targets include genes responsible for vasomotor regulation (e.g. Adrenomedullin, eNOS, Heam Oxygenase), energy metabolism (e.g. GLUT-1, carbonic anhydrase-9), angiogenic signaling (e.g. VEGF, VEGF receptor-1) and cytoprotection (e.g. adrenomedullin, erythropoietin, VEGF). It is therefore plausible that activating HIF may improve the survival of ischemic cells and also promote beneficial adaptive changes.

In an experimental model of stroke in mice hypoxic post-conditioning was shown to be protective to brain (Claire Leconte, 2009). More delayed ischemic post-conditioning, starting 6 hours and 24 hours after focal and global cerebral ischemia, respectively, has also been described. (Claire Leconte, 2009). HIF activation by inhibition of upstream HIF hydroxylases has emerged as promising therapeutic strategy (Wanja M., Norma M, 2003). Thus small-molecule inhibitors of HIF hydroxylases offer a pharmacological means of HIF activation which has the potential for clinical application.

Toll like receptor (TLR) antagonists

Stroke triggers an intense inflammatory response that could be a consequence of Toll-like receptors (TLRs) activation. In a clinical study, the association between the expression of TLR2 and TLR4, inflammatory molecules and endogenous ligands, and clinical outcome of ischemic stroke patients was assessed. In this study involving 110 patients with ischemic stroke, it was found that TLR2 and TLR4 are independently associated to poor outcome and correlated with higher serum levels of interleukin (IL)1β, IL6, tumor necrosis factor α, and VCAM1, and that TLR4 was independently associated to lesion volume. In addition, Cultured cells (monocytes and human umbilical vein endothelial cells) were treated with serum from ischemic stroke patients, showing a strong inflammatory response that was blocked when TLR2/4 or cellular fibronectin (cFN) or HSP60 were blocked. In conclusion, TLR2 and TLR4 are associated to outcome in stroke patients and TLR2/4 or their endogenous ligands; cFN/HSP60 could be new therapeutic targets for ischemic stroke. (Brea et al., 2011; Marsh et al., 2009; Arumugam et al., 2009)

Histone Deacetylase (HDAC) inhibition

Class I and II HDAC inhibitors have been shown to upregulate HSP70 and found to be neuroprotective against glutamate excitotoxicity. Using rat neurons in vitro, both valproic acid (VPA) and sodium phenyl butyrate (HDAC inhibitors) caused the up-regulation of pro-survival and anti-apoptotic genes, including HSP70 (Marinova et al., 2009). VPA and other HDAC inhibitors all upregulated levels of the neuroprotective heat shock protein 70 (HSP70) in rat astrocytes (Leng Y et al., 2010). VPA treatment markedly up-regulated HSP70 protein levels and this was accompanied by increased HSP70 mRNA levels and promoter hyperacetylation and activity (Marinova et al., 2011). Various elegant experiments suggest that the phosphatidylinositol 3-kinase/Akt pathway and Sp1 are likely involved in HSP70 induction by HDAC inhibitors, and induction of HSP70 by VPA in cortical neurons may contribute to its neuroprotective and therapeutic effects (Ren et al., 2004; Marinova et al., 2009)

2.4. HSPs and neuroprotection

2.4.1. HSP family

HSP are a group of proteins whose expression is increased when the cells are exposed to elevated temperatures. This increase in expression is transcriptionally regulated. Such dramatic upregulation of the HSP induced mostly by Heat Shock Factor (HSF) is a key part of the heat shock response. Production of high levels of HSP can also be triggered by exposure to different kinds of environmental stress conditions such as infection, inflammation, exposure of the cell to toxins (ethanol, arsenic, trace metals and ultraviolet light and many others), starvation, ischemia/hypoxia (oxygen deprivation), nitrogen deficiency (in plants), or water deprivation (Hageman, et al., 2011; Stetler, et al., 2010). Consequently, the HSP are also referred to as stress proteins and their upregulation is sometimes described more generally as part of the stress response (Chen, et al., 1996).

HSP are present in all cells at all biological levels. They appear when the cell is under heat stress (or other stress). HSP also occur under non-stressful conditions, simply "monitoring" the cell's proteins. Some examples of their role as "monitors" are that they carry old proteins to the cell's "recycling bin" and they help newly synthesized proteins fold properly. These activities are part of a cell's own repair system, called the "cellular stress response" or the "heat-shock response" (Fulda, et al., 2010; Nollen and Morimoto, 2002; Benjamin and McMillan, 1998). The function of HSP is similar in virtually all-living organisms, from bacteria to humans.

HSP are molecular chaperones for protein molecules. They are usually cytoplasmic proteins and they perform functions in various intra-cellular processes. Some members of the HSP family are expressed at low to moderate levels in all organisms because of their essential role in protein maintenance (Clark and Muchowski, 2000; Jindal, 1996; Macario, 1995). HSP are named according to their molecular weights, for example HSP70 and HSP90 each define families of chaperones. The major classes of HSP and their functions are tabulated below.

Structure and functions of HSP

The principle HSP that have chaperone activity belong to five conserved classes: HSP100, HSP90, HSP70, HSP60 and the small heat-shock proteins (sHSP) (Latchman, 2004). Although the most important members of each family are tabulated

here, it should be noted that some species may express additional chaperones, co-chaperones, and HSP not listed. Additionally, many of these proteins may have multiple splice variants (HSP90α and HSP90β, for instance) or conflicts of nomenclature (Inducible form of HSP70 is sometimes called HSP72).

Table 1: Important members of HSP family

Approximate molecular weight (kDa)	Eukaryotic proteins	Basic Function
10	HSP10	General functions are thermotolerance *in vivo* , cytoprotection, and the support of cell survival under stress conditions
20-30	The HSPB group of HSP. Ten members in mammals including HSP27 or HSPB1	
40	HSP40	
60	HSP60	
70	The HSPA group of HSP including HSP70, HSP71, Hsc70, HSP72, Grp78, BiP, Hsx70 found only in primates	Protein folding and unfolding, provides thermotolerance to cell on exposure to heat stress
90	The HSPC group of HSP including HSP90, Grp94	Maintenance of steroid receptors and transcription factors
100	HSP104, HSP110	Tolerance of extreme temperature

HSP90

HSP90 is a molecular chaperone and is one of the most abundant proteins in unstressed cells. It is a ubiquitous molecular chaperone found in eukaryotes. In mammalian cells, there are two genes encoding cytosolic HSP90 homologues, with the human HSP90α showing 85% sequence identity to HSP90β (Neckers and Ivy, 2003). HSP90 is one of the HSP, and is upregulated in many cells in response to stress. HSP90 is among the most common heat related protein. The role of HSP90 covers many things, including: signaling, protein folding and tumor repression (Lee, et al., 2010; Harrison EM, et al., 2008; Balligand, 2002).

Structure

The structure of HSP90 is like every other protein and have all of the common structures associated with all proteins: alpha helixes, beta pleated sheets and random coils. HSP90 contains nine helixes and eight anti-parallel beta pleated sheets that are folding into various alpha/beta sandwiches (Didenko, et al., 2012). Three areas, the ATP binding, protein binding and dimerizing regions, all in particular are highly important to its function.

Function

HSP90's role of chaperonin and transporter can be described well by its interaction with transforming cellular signal molecules and the proteasomes that may or may not degrade them. HSP90 is a major helper in assembling and causing the ATP-dependent folding of S26, the importance of this is found in the fact that the S26 proteasome targets virtually all eukaryotic proteins for degradation and are usually marked for destruction through the polyubiquitation pathway (Pearl and Prodromou, 2006). As previously stated, the S26 proteasome performs proteolysis on virtually all ubiquinated proteins which includes some tyrosine kinases, such as Her-2 (p185erbB2) which is commonly overproduced in cancerous tumors and p60v-src which is the transforming agent coded for by the Rous sarcoma virus (Mahalingam, et al., 2009). In the cases of both Her-2 (p185erbB2) and p60v-src, studies using benzoquinone ansamycin antibiotics (BA) have indicated that HSP90's ATPase active site is being blocked in a way similar to geldanamycin would and therefore the chaperonin is unable to adequately complex the aforementioned tyrosine kinases (Hadden, et al., 2006)

HSP70

Members of the HSP70 family are strongly upregulated by heat stress and toxic chemicals, particularly heavy metals such as arsenic, cadmium, copper, mercury, etc. HSP70 was originally discovered by FM Ritossa in the 1960s when a lab worker accidentally boosted the incubation temperature of Drosophila (fruit flies). When examining the chromosomes, Ritossa found a "puffing pattern" that indicated the elevated gene transcription of an unknown protein. This was later described as the "Heat Shock Response" and the proteins were termed the "HSP" (Ritossa and Vonbostel, 1964).

Structure

All of the HSP70 proteins have three major functional domains.

1) An N-terminal ATPase domain-binds ATP (Adenosine triphosphate) and hydrolyzes it to ADP (Adenosine diphosphate). The exchange of ATP drives conformational changes in the other two domains.

2) A substrate binding domain contains a groove with an affinity for neutral, hydrophobic amino acid residues. The groove is long enough to interact with peptides up to seven residues in length.

3) A C-terminal domain rich in alpha helical structure acts as a 'lid' for the substrate binding domain. When an HSP70 protein is ATP bound, the lid is open and peptides bind and release relatively rapidly. When HSP70 proteins are ADP bound, the lid is closed, and peptides are tightly bound to the substrate binding domain.

Function

HSP70 is essential for the restoration of normal ribosome assembly, promotion of new ribosomal synthesis, and acceleration of the recovery of nucleolar morphology after heat shock by ATP dependent mechanisms. Stress response is vital for cell recovery and survival after various harmful stresses. These stresses normally act to damage proteins, causing partial unfolding and possible aggregation. By temporarily binding to hydrophobic residues exposed by stress, HSP70 prevents these partially-denatured proteins from aggregating, and allows them to refold. Therefore, if HSP70 is produced in sufficient amounts after ischemia, this protein may protect against neuronal death. HSP70 also aids in transmembrane transport of proteins, by stabilizing them in a partially-folded state. Finally, HSP70 seems to be able to participate in disposal of damaged or defective proteins. Interaction with CHIP (*C*arboxyl-terminus of *HSP70 I*nteracting *P*rotein)–an E3 ubiquitin ligase–allows HSP70 to pass proteins to the cell's ubiquitination and proteolysis pathways.

HSP60 (GroEL)

GroEL is a 60D (actually 59D) HSP. To function properly, it requires the cochaperone protein GroES. (GroEL and GroES are also sometimes referred to as chaperonin and cochaperonin, or chaperonin 60 and chaperonin 10 for their molecular weights respectively) (Sigler, et al., 1998). In eukaryotes the proteins HSP60 and HSP10 are structurally and functionally nearly identical to GroEL and GroES, respectively. This is the reason that GroEL and its homologues are often referred to as "HSP60" proteins.

Structurally, GroEL is a dual-ringed tetra decamer, with both the cis and trans rings consisting of seven subunits each. The inside of GroEL is hydrophobic, and is likely where protein folding takes place (Sigler, et al., 1998).

GroEL is a very complicated molecule and, doubtless, there are many number of ways in which a polypeptide can interact with it. Merely by way of example, it is known that certain larger polypeptides, which can't fit inside the cis chamber, extend into the trans portion and interact in some (unknown) way with the trans heptamer (Kirchhoff, et al., 2002). Since GroEL has changed hardly at all over the last several billion years, it seems far more likely that the rest of the genome has adapted to GroEL, and uses it in various specialized ways.

HSP27 (Small HSP)

HSP27 is a chaperone of the sHSP – small HSP- group among ubiquitin, α-crystallin, HSP20 and others. The common functions of sHSP are chaperone activity, thermotolerance, inhibition of apoptosis, regulation of cell development and cell differentiation and they take part in signal transduction. HSP27 appears in many cell types, especially all types of muscle cells. It is located mainly in the cytosol, but also in the perinuclear region, endoplasmatic reticulum, and nucleus. It is overexpressed during different stages of cell differentiation and development. Probably it is essential for development in general: knock-out mice do not survive.

Structure of sHSP

sHSP have some structural features in common: Very characteristic is a homologue and highly conserved amino acid sequence, the so-called α-crystallin-domain at the C-terminus. These sequences consist of 80 to 100 residues with a homology between 20% to 60% and form β-sheets, which are important for the formation of stable dimers(Van Monfort et al. 2001; Kim et al. 1998).

The N-terminus consists of a less conserved region, the so-called WD/EPF domain, followed by a short variable sequence with a rather conservative site near the C-terminus of this domain. The C-terminal part of the sHSP consists of the above mentioned α-crystallin domain, followed by a variable sequence with high motility and flexibility (Gusev, et al., 2002).

This C-terminal tail appears in many mammalian sHSP (e.g. mouse HSP25, αA-crystallin) and has no homology. It is highly flexible and polar because of its negative charges (Liao, et al., 2002). Probably it functions as a mediator of solubility for hydrophobic sHSP and it stabilizes the protein and protein/substrate complexes (Lelj-Garolla, et al., 2005, Lindner, et al., 2000).

Functions

General functions are the thermotolerance in-vivo, the cytoprotection, and the support of cell survival under stress conditions. Special functions are manifold and complex. In-vitro it acts as an ATP-independent chaperone by inhibiting protein aggregation and by stabilizing partially denatured proteins, which ensures refolding by the HSP70-complex. HSP27 is also involved in the apoptotic signaling pathway. HSP27 interacts with the outer mitochondrial membranes and interferes with the activation of cytochrome c/Apaf-1/dATP complex and therefore inhibits the activation of procaspase-9 (Sarto, et al., 2004). The phosphorylated form of HSP27 inhibits Daxx apoptotic protein and prevents the association of Daxx with Fas and Ask1 (Charette, et al., 2000).

A well documented function of HSP27 is the interaction with actin and intermediate filaments. It prevents the formation of non-covalent filament/filament interactions of the intermediate filaments and protects actin filaments from fragmentation. It also preserves the focal contacts fixed at the cell membrane (Sarto, et al., 2004).

Another function of HSP27 is the activation of the proteasome. It speeds up the degradation of irreversibly denatured proteins and junk proteins by binding to ubiquitinated proteins and to the 26S proteasome. HSP27 enhances the activation of the NF-κB pathway, that controls a lot of processes, such as cell growth and inflammatory and stress responses (Parcellier, et al., 2003). The cytoprotective properties of HSP27 result from its ability to modulate reactive oxygen species and to raise glutathione levels. Probably HSP27 is involved in the process of cell differentiation and It is possible that HSP27 plays a crucial role in the termination of growth. (Arrigo, et al., 2005).

2.4.2. HSPs relevant for neuroprotection

HSP are induced by stressful stimuli and are thought to assist in the maintenance of cellular integrity and viability (Sharp, et al., 1999; Kiang, et al., 1998). HSP overexpression has been observed in-vivo and in-vitro under a range of conditions, including hypoxia, nutrient deprivation, ischemia, and thermal stress. Briefly, upregulation of HSP27, HSP70, and HSP90 mRNA and protein after several insults has been detected in numerous neuronal cultures and brain tissue extracted from animals exposed to stress (Feder and Gretchen, 1999; Wagstaff, et al., 1996; Higashi, et al., 1994; Kawagoe, et al., 1993a, b). HSP70 is the major inducible HSP and its one of the most extensively studied in the central nervous system (Latchman, 2004; Yenari, 2002). Following a variety of central nervous system insults, HSP70 is expressed at especially

high levels and is present in the cytosol, nucleus and endoplasmic reticulum. The stress proteins of the HSP70 family function as chaperones interacting transiently with many proteins in an ATP dependent manner. HSP70's increased expression led investigators to study its potential role in the protective effect of induced tolerance, a phenomenon whereby a prior sublethal insult leads to protection against a subsequent severe insult. For instance thermal or chemical stress protects against excitotoxic insults such as glutamate exposure in cultured neuronal cells and in whole animal models (Yang, et al., 1999). Prior heat shock, sublethal ischemia or chemical toxins protect against subsequent ischemia (Gass, et al., 1995). Induction of HSP70 and protection from subsequent injury has been demonstrated in neuron cultures and in whole animal models of thermal stress, global and focal cerebral ischemia (Massa, et al., 1996; Parsell, et al., 1994). The expression of HSP70 also correlated with the period within which tolerance was observed, leading some to believe that HSP70 may explain the observed phenomenon (Yang, et al., 1998)

Experiments in in-vivo models of epilepsy and stroke indicate that transgenically overexpressed or virally delivered HSP are able to reduce lesion size and enhance cell survival (Akbar, et al., 2003; Kalwy, et al., 2003; Tsuchiya, et al., 2003; Kelly, et al., 2002, 2001a, b,; Hoehn, et al., 2001; Lee, et al., 2001a, b; Rajdev, et al., 2000; Yenari, et al., 1998; Plumier, et al., 1997). The great majority of these studies have involved HSP70, while the protective effect of HSP27 in-vivo has only been investigated in a model of chemical-induced cell death in the hippocampus (Wagstaff, et al, 1999). Thus HSP70 plays a dominant role in the stress induced adaptive responses of the brain cells.

2.4.3. HSP70 expression and stroke protection

Endogenous expression and temporal profile in stroke

Several groups using various models of experimental nervous system stress and injury have studied the anatomic and temporal expression of HSP70. In a model of global cerebral ischemia, HSP70 mRNA was detected in the hippocampus within hours of ischemia onset and decreased when neurons were lost. Protein expression followed a few hours later (Vass, et al., 1988). Brief periods of ischemia (3-8 minutes) resulted in HSP70 protein expression within neurons and some glia after 24 hours, but longer durations of ischemia (10-20 minutes) (Massa, et al., 1996; Freeman, et al., 1995; Foster and Brown, 1997;Vass, et al., 1988) showed decreased HSP70 protein expression. Following 60 minutes of transient focal cerebral ischemia, HSP70

protein expression of similar patterns were observed with persistent expression as far out as 7 days.(Massa, et al., 1996; Kinouchi et al., 1993). HSP70 was observed within neurons and astrocytes at the infarct periphery, but only endothelial cells expressed it within brain regions where ischemia was the most severe (striatum) (Kinouchi et al., 1993). With increasing duration of MCAo, graded levels of HSP70 expression are observed within neurons, microglia and endothelial cells that decreased after most severe ischemic insults. It has previously been suggested that glia can transfer HSP70 to neurons; therefore, neuronal HSP70 expression may be linked to a potential protective mechanism by glial cells (Massa, et al., 1996; Soriano, et al., 1994; N'owBk, et al., 1994).

Evidence of HSP70 mediated neuroprotection in stroke

Nervous system ischemia can be modeled in-vitro by exposing cultures to ischemia like conditions such as substrate and/or oxygen deprivation (Lee, et al., 2001; Papadopoulos, et al., 1996) excitotoxin exposure (Flink, et al., 1997) or incubation in simulated ischemic buffers. Recent work from different investigators showed that cultured hippocampal, but not cortical neurons from transgenic mice overexpressing HSP70 were protected from excitotoxin exposure and oxygen and glucose deprivation (Lee, et al., 2001). Glial cultures isolated from this same mouse strain were also resistant to substrate deprivation. Xu et al (Xu and Giffard, 1997) and Papadopoulos et al (Papadopoulos, et al., 1996) used such retroviral vectors to overexpress HSP70 in astrocyte cultures. Exposure to isolated glucose or combined oxygen and glucose deprivation led to robust glial survival following either insult. Conversely, suppression of HSP70 in hippocampal neuron cultures with an antisense oligonucleotide worsened injury following heat shock (Sato, et al., 1996). Using this approach, Sato et al (Sato, et al., 1996) found that the protection from induced tolerance was reversed with HSP70 blockade using antisense oligonucleotides.

Observations from these results suggest that HSP70 protects against many kinds of central nervous system injury, and that the protective effects may be related to the nature and severity of the insults. It has previously been shown that the response to heat shock and HSP70 expression can vary depending on the type and age of the cell (Nishimura, et al., 1999) therefore, it is likely that the protective effects may similarly be dependent on such factors.

At the in-vivo level, recently one group has shown that recombinant Fv-HSP70 protein mediates neuroprotection after focal cerebral ischemia in rats (Zang, et al., 2010). Another group with constitutive overexpression of HSP70 in one strain using a cytomegalovirus (CMV) enhancer combined with a β-actin promoter resulted in near complete protection determined by overall reduction in infarct size (Plumier, et al., 1997). In an MRI based study lesion volume measured using T2 weighted MRI was shown to be significantly lesser in HSP70 overexpressed mice as compared to wild type mice in a MCA occlusion model of permanent cerebral ischemia (van der weed et al 2005). Another group using a similar model but a different mouse strain found only improved hippocampal neuron survival by 24 h; however, overall infarct size was not affected (Plumier, et al., 1997). In this latter group, HSP70 expression was under the control of a β-actin (constitutive) promoter. A few groups have studied permanent MCAo in various HSP70 transgenic mice strains with conflicting results. These differing results could be due to background strain differences and the limitations of transgenic animals, such as developmental alterations in other biochemical systems caused by transgene overexpression. The extent of HSP70 expression might also be different between the transgenic strains, since different promoters were used. The transgenic model on containing the CMV enhancer increased transgene expression by 5-10 folds (Rajdev, et al., 2000). In contrast, the model using the f3-actin promoter alone resulted in only 2 fold increases (Lee, et al., 2001). Therefore, higher transgene levels could account for the marked protection in the first study (Rajdev, et al., 2000). and the less robust or lack of protection in the latter two studies (Lee, et al., 2001; Plumier, et al., 1997) .These data indicate that HSP70 is protective at the in-vivo level in adult wild type animals. Further these studies suggest that augmenting the endogenous protective response to stress by inducing HSP70, at an appropriate time window limits the progression of the ischemic cascade in a model, which incorporates focal ischemia.

2.4.4. Postulated mechanisms of HSP70 mediated neuroprotection

2.4.4.1. HSP70 and chaperone action

The HSP70 family of stress proteins constitutes the most conserved and best studied class of HSP. Human cells contain several HSP70 family members including stress inducible HSP70, constitutively expressed HSC70, mitochondrial HSP75, and GRP78 localized in the endoplasmic reticulum (Jaattela, 1999). Under normal conditions, HSP70 function as ATP-dependent molecular chaperones by

assisting the folding of newly synthesized polypeptides, the assembly of multiprotein complexes, and the transport of proteins across cellular membranes.

2.4.4.2. Antiapoptotic effect

Another protective effect of HSP is related to their ability to interfere with apoptotic pathways. Two main pathways of apoptosis have been described. One involves the Bcl-2 family (including Bid, Bim, Bad, and others), which may function as context-specific sensors for cell damage and converge on the mitochondria to trigger their permeabilization (intrinsic pathway), whereas the other implicates plasma membrane proteins of the TNF receptor family known as death receptors (extrinsic pathway). At least in some cell types, signals originating from death receptors require the mitochondria, thus depend on the Bcl-2 family of proteins (Jacobson, et al., 1997; Solary, et al., 2000). The mitochondrial pathway is initiated by the release into the cytosol of soluble apoptogenic molecules that include cytochrome c, AIF (apoptosis inducing factor), Smac/Diablo, HtrA2/Omi, and the endonuclease G (Daugas, et al., 2000; Joza, et al., 2001; Du, et al., 2000). HSP function at key regulatory points in the control of apoptosis by directly interacting with different apoptotic proteins. HSP70 prevents both caspase-dependent and caspase-independent apoptosis. HSP70 was initially proposed to rescue cells from a later phase of apoptosis than any other known survival enhancing drug or protein. In TNFα-treated human cervix carcinoma ME-180 cells, HSP70 overexpression did not prevent caspase-3 activation but inhibited downstream morphological changes that are characteristic of dying cells (Jaattela, et al., 1998). It was subsequently reported that elevated levels of HSP70, as a consequence of transient or stable transfection, could prevent both caspase activation and apoptosis-associated nuclear changes (Buzzard, et al., 1998). In the intrinsic pathway to cell death, HSP70 overexpression did not inhibit cytochrome c release from the mitochondria while preventing caspase-3 activation (Li, et al., 2000). These observations were subsequently related to HSP70 ability to directly bind Apaf-1 through its ATPase domain, thereby preventing the recruitment of procaspase-9 to the apoptosome (Saleh, et al., 2000; Mosser, et al., 2000; Beere, et al., 2000). HSP70 can also prevent caspase-independent cell death, e.g., in the presence of exogenous caspase inhibitors (Saleh, et al., 2000) or in cells in which the Apaf-1 gene has been deleted (Ravagnan, et al., 2001). This anti-apoptotic effect of HSP70 was related to the protein ability to neutralize AIF apoptogenic effects by direct binding to the protein. The ATPase domain of HSP70 is not required for

this interaction, indicating that distinct parts of the protein modulate the caspase-dependent and caspase-independent pathways, respectively. HSP70 can also protect the cells from energy deprivation and/or ATP depletion associated with cell death (Wong, et al., 1998). HSP70 has also been shown to associate with the pro-apoptotic proteins p53 and c-Myc, but the functional impact of these interaction remain unknown (Jolly and Morimoto, 2000).

2.4.4.3. Anti-inflammatory action

In addition to the cytoprotective effect of HSP70, an anti- inflammatory effect of HSP70 has also been reported (Kim, et al., 2012). For example, up-regulation of HSP70 expression by heat shock inhibits the inflammatory stimuli-dependent activation of NF-kB, which is responsible for inducing the production of various pro-inflammatory cytokines (Krappmann et al., 2004). Previously it was reported that the LPS-induced production of proinflammatory cytokines, including interleukin 1 (IL-1) and interleukin 6 (IL-6), was inhibited in peritoneal macrophages prepared from transgenic mice expressing HSP70 compared with their wild-type controls (Tanaka et al., 2007). Furthermore, indomethacin-induced expression of mRNA for some cytokines and chemokines (IL-10, IL-6, and MIP-2) in the small intestine was suppressed in transgenic mice expressing HSP70. It was considered that expression of HSP70 suppresses the expression of these genes in the small intestine through its inhibitory effect on nuclear factor B and that this effect is involved in the protective role of HSP70 against NSAID-induced lesions of the small intestine. As an anti-inflammatory molecule, HSP70 decreases the release of inflammatory mediators in models of endotoxemia and cardiopulmonary bypass- induced inflammation (Hayashi, et al., 2002; Van Molle, et al., 2002; Klosterhalfen, et al., 1996). Earlier work has documented HSP70 expression in microglia after experimental stroke (Soriano, et al., 1994), and recently it is shown that MMP-9, one of several genes regulated by NF-kB, was reduced in cultured HSP70 overexpressing astrocytes exposed to in-vitro ischemia (Lee, et al., 2004). Anti-inflammatory mechanism leading to in-vivo stroke protection in transgenic models overexpressing HSP70 has been recently reported (Zang Z, 2008).

2.4.5. Evidence of HSP70 mediated neuroprotection in chronic neurological diseases

HSP have well-characterized roles in facilitating protein folding in de novo protein synthesis and during refolding of partially denatured proteins that arise after cellular stress (Hartl and Hayer-Hartl, 2002; Morimoto, et al., 1997). Their ability to recognize and bind to denatured or partially unfolded proteins allows HSP to counter denaturation, misfolding, and irreversible aggregation of proteins. Family members of HSP have been implicated in the solubilization of aggregated proteins (Stege, et al., 1995). Because alteration of aggregation kinetics can affect the progression of the neurodegenerative disease (Wolozin and Behl, 2000), upregulation of HSP could alleviate neurodegeneration by modulating protein misfolding in affected neurons. This concept has led to a quest for pharmacological agents that can induce HSP in neuronal cells as a therapeutic approach for combating neurodegeneration. Animal models of neurodegenerative diseases have demonstrated the beneficial of effects of HSP70 overexpression (Muchowski and Wacker, 2005). Upregulation of a set of HSP, rather than a single HSP, will likely yield added benefits (Patel, et al., 2005; Jana, et al., 2000).

2.5. Clinical, experimental assessment of stroke outcome and relevant animal models.

2.5.1. STAIR recommendations for the evaluation of neuroprotective agents

The plethora of failed clinical trials with neuroprotective drugs for acute ischemic stroke have raised justifiable concerns about how best to proceed for the future development of such interventions. Preclinical and clinical testing of neuroprotective drugs is an important aspect of assessing their therapeutic potential, but guidelines concerning how to perform preclinical and clinical development of putative neuroprotective drugs for acute ischemic stroke are lacking. The Stroke Therapy Academic Industry Roundtable (STAIR) was established in order to address the challenges encountered in finding an effective neuroprotective therapy for acute stroke (Fisher, et al., 2007; Stroke Therapy Academic Industry Roundtable, 1999). To date, the STAIR group has met six times discussing and revising their recommendations for preclinical and clinical stroke trials (Fisher, et al., 2009; Saver, et al., 2009; Fisher, et al., 2007; Fisher, 2005; Fisher, 2003; STAIR Group, 2001; STAIR Group, 1999). Recommendations provided by the STAIR consortia emphasize the design quality of both experimental and clinical stroke trials.

Recommendations for the conduct of preclinical studies

This conference of academicians and industry representatives was convened to suggest such guidelines for the preclinical evaluation of neuroprotective drugs and to recommend to potential clinical investigators the data they should review to reassure themselves that a particular neuroprotective drug has a reasonable chance to succeed in an appropriately designed clinical trial. With respect to experimental animal stroke trials, STAIR revised recommendations have highlighted the need for investigators to consider factors such as species and gender differences, clinical relevance of animal models, dose-response determinations, therapeutic time windows, BBB permeability and tissue drug levels, treatment randomization, physiological monitoring, and at least 2 outcome measures covering both acute and long-term endpoints. Following are the key points which are required to be considered while carrying out preclinical evaluation of neuroprotective agents. Adequate dose–response curve defined. 2) Document that the drug accesses the target organ, the brain. 3) Define the therapeutic time window in well-characterized animal stroke models. 4) All animal

treatment experiments should be done in a blinded, randomized manner with control of physiological variables with predefined inclusion/exclusion criteria using an adequate sample size based on an appropriate sample size estimate. 5) Both histological and functional outcomes should be assessed acutely and long term. 6) Efficacy studies should be performed initially in young healthy male animals using permanent occlusion modeling in most cases. 7) Initial studies should be performed in rodents and then studies in gyrencephalic species should be considered. 8) Additional studies with promising agents should be performed in female animals, aged animals, and animals with co-morbid conditions such as hypertension, diabetes, and hypercholesterolemia. 9) Relevant biomarker endpoints such as diffusion/perfusion MRI and serum tissue injury markers should be considered. 10) Interaction studies with commonly used medications should be performed.

Recommendations for the conduct of clinical trials

As for the recommendations regarding carrying out of clinical trials, various aspects of the design of clinical trials for promising new acute stroke drugs were considered. Emphasis was made on the design of both initial and pivotal clinical trials of single and combination therapies for acute ischemic stroke. Importance of carefully designed and implemented phase I and II trials to derive relevant, valuable information needed to proceed to phase III trials with promising interventions was felt. The phase III trial should evaluate drug efficacy in an appropriately targeted stroke population evaluated by a meaningful and reliable outcome measure. Following points highlight the key considerations to be addressed in clinical trials pertaining to evaluation of new neuroprotective drugs.

Considerations for designing phase IIb stroke trials

1) Route of administration 2) Dose range 3) Duration of treatment 4) Time from stroke onset to initiation of treatment (a key variable) 5) Pharmacokinetic profile 6) Side effects and their frequency (with attention to side effect management) 7) Interactions with other commonly used medications 8) Drug distribution to the proposed site of action 9) Refinement and identification of the target population (e.g., drugs without preclinical evidence of activity in white matter ischemia should not be studied in patients with subcortical stroke or even large cortical events with attendant subcortical injury) 10)

Obtain evidence measurement of therapeutic activity by evaluation of clinical and/or surrogate markers (hints of potential effectiveness)

Considerations for designing phase III acute stroke trials

1). Dose selection based on preclinical and phases 1 and 2 data 2) Time window for initiation of drug 3) Patient selection based on mechanisms of action 4) Outcome measures: one type of primary outcome or a global assessment 5) Severity of stroke population to be studied 6) Length of follow-up period 7) Use of surrogate markers to provide support of drug efficacy 8) Prespecification of covariate analyses 9) Fostering of appropriate and effective relationships between sponsors, academicians, and investigators. Although the recommendations cannot be validated until effective therapies based on them emerge from clinical trials, it is hoped that adherence to them might enhance the chances for success.

2.5.2. Clinical stroke assessment methods

Stroke is a complex condition which can affect many aspects of body function. Stroke scales are assessment tools designed to quantify different aspects of the effect of stroke, recovery and impairment following stroke. There is no gold standard scale which incorporates all criteria of an ideal stroke scale (Lyden and Hanston, 1998). Therefore, several separate scales are generally recorded for each patient, all of which fall into one of four broad categories: pathology; impairment; disability (activity); or handicap (participation). Impairments are usually described as the symptoms and signs of stroke so impairment scales numerically record specific findings of detailed neurological examination. This type of scale is particularly important in assessing patients almost immediately after stroke onset. Disability or activity refers to the personally meaningful functions or activities the patient can achieve, such as bathing and dressing. These scales measure functional outcome and are used to assess stroke- related disability and performance in occupational functions (Wolfe, et al., 2000). All stroke scales have their advantages and disadvantages (Kasner, 2006; Lyden and Hanston, 1998) never the less below mentioned are different stroke scales used frequently.

Table 2: List of frequently used clinical stroke assessment scales

Sr.No	Assessment Parameter	Stroke Scale
1	Level-of-consciousness scale	Glasgow Coma Scale
2	Stroke deficit scales	NIH Stroke Scale, Canadian Neurological Scale
3	Global disability scale	Rankin Scale
4	Measures of disability/activities of daily living (ADL)	Barthel Index, Functional Independence Measure (FIM)
5	Mental status screening	Folstein Mini-Mental State Examination, Neurobehavioral Cognition Status Exam (NCSE)
6	Assessment of motor function	Fugl-Meyer Motor Assessment Scale Motricity Index
7	Balance assessment	Berg Balance Assessment
8	Mobility assessment	Rivermead Mobility Index
9	Assessment of speech and language functions	Boston Diagnostic Aphasia Examination Porch Index of Communicative Ability (PICA) Western aphasia Battery
10	Depression scales	Beck Depression Inventory (BDI) Center for Epidemiologic Studies Depression (CES-D) Geriatric Depression Scale (GDS) Hamilton Depression Scale
11	Measures of instrumental ADL	PGC Instrumental Activities of Daily Living Frenchay Activities Index
12	Family assessment	Family Assessment Device (FAD)
13	Health status/ quality of life measures	Medical Outcomes Study (MOS) 36-Item Short-Form Health Survey Sickness Impact Profile (SIP)

2.5.3. Non-invasive brain imaging strategies for stroke

Need for imaging strategies in stroke

Imaging modalities are required to differentiate between ischemic and hemorrhagic stroke types. Several clinical scales have been developed for this purpose, chiefly relying on the fact that hemorrhagic strokes are more often associated with symptoms of severe stroke. But none had sufficiently good sensitivity and specificity to be used to initiate antithrombotic treatment, identify those few patients with underlying tumors or infections mimicking a stroke. Imaging added to clinical data allows a more precise prediction of outcome than clinical parameters alone. Imaging can diagnose the cause of deteriorating neurological features after stroke (Edlow, et al., 2008; Wardlaw, et al., 2004). For example, it might show hemorrhagic transformation of the infarct, or

infarct swelling with midline shift, or a new infarct elsewhere in the brain, or hydrocephalus requiring drainage.

Different imaging modalities

MRI, CT and PET are the most commonly used imaging modalities in stroke patients and many research protocols (Wardlaw, et al., 2004). There are many advantages and limitations of one imaging technique over the other. But MRI is the most appropriate imaging modality which offers wide spread advantages compared to other modalities.

Magnetic resonance imaging (MRI)

MRI uses the magnetic properties of the protons present in tissue and produces images of greater soft tissue contrast. This is currently the most widely used imaging modality in stroke research due to the wide spread benefit it offers. Following are the list of advantages an MR imaging technique offers (Beckmann, et al., 2001).

Advantages: Very early detection (in minutes) of the type of stroke (Ischemic/Hemorrhagic) is possible with grater sensitivity. Unlike CT the signal intensity in case of hemorrhage remains for a longer time offering advantage of accurate detection. The perfusion/diffusion mismatch studies would precisely detect penumbral region which can be quantitatively detected. As MRI offers greater sensitivity even a minute effect of the drug treatment can be accurately detected which is not a possibility in many of the other imaging studies. Techniques like functional imaging are possible only with MRI and are highly valued in stroke research. With the availability of DWI or ADC imaging future damage can be detected as early as possible i.e. within minutes and treatment benefits can be known very early. Unlike CT and PET, Multiple MRI scans are very safe.

Limitations: Patients using paramagnetic devices like pacemakers, metallic devices like intra ocular bodies, intracranial aneurysm clips are contraindicated. Some Patients may feel unpleasant because of claustrophobia.

2.5.4. Animal models of stroke

With a view toward developing an ideal neuroprotective drug for stroke, not only the basic concept of the disease but also the basic clinical signs such as hemiplagia, memory and learning disabilities should be determined. A key factor in this process was

the development of animal model that mimics the neurological consequences of stroke (Mergenthaler and Meisel, 2012; Longa, et al., 1989; Tamura et al., 1981;). While there are so many numbers of animal model of stroke, but suitable animal model to fit stroke clinically should be investigated (Krafft, et al., 2012; Hong, et al., 2000). The following are the some of the animal models for the different type of ischemia. They are generally divided into two categories, global and focal ischemia.

Animal models of global ischemia

In global cerebral ischemia, the reduction of blood flow occurs in the whole brain or a major portion of forebrain. It is thought to model the selective neuronal loss observed after a heart attack or coronary artery bypass surgery (Hunter, et al., 1995 & 1995). Several global ischemia animal models have been designed, (Hunter, et al., 1995). The three most widely used global ischemia models are four-vessel occlusion (4-VO) and two-vessel occlusion (2-VO) combined with hypotension in the rat, and two-vessel occlusion in the gerbil. Mice two vessel occlusion (2-VO) is reported but, variability is high and survival rates are low (Yonekura, et al., 2004; Panahian, et al., 1996). Overall, gerbil vessel occlusion is most widely studied model because the operation is simpler than rat.

Animal models of focal ischemia

Focal ischemic stroke models, whether in larger mammals such as cats, dogs or non-human primates, or in rodents, usually involve occlusion of one MCA (Lipton, 1999; Kawamura, et al., 1991; Koizumi, et al., 1986). Focal ischemia is differentiated from global ischemia in two ways. First, even at the core of the lesion, the blood flow is almost always higher than during global ischemia, so that longer insults are required to cause damage than for global ischemia. Secondly, there is a significant gradation of ischemia from the core of the lesion to its outermost boundary, and hence there are different metabolic conditions within the affected site. Presence of long lasting neurological deficit in the model, which is in accordance with human disease condition, makes this model suitable for evaluating putative neuroprotective agents targeting neuroprotection along with functional recovery.

Almost all focal ischemic models, involve occlusion of one MCA (Longa, et al., 1989; Tamura, et al., 1981), which supplies the lateral surface of cerebral cortex

and various subcortical structures, notably the caudate putamen (Guyton and Hall, 2001b), and involves in processing of sensorimotor information.

Several experimental models are available for focal cerebral ischemia studies, (Hunter, et al., 1998 & 1995), in which MCA is occluded either transiently or permanently (Longa, et al., 1989; Tamura, et al., 1981). Depending upon the occlusion site of MCA, focal ischemia can be induced by two procedures, proximal and distal MCAo (Roof, et al., 2001). Proximal MCAo typically causes infarction in the cerebral cortex and caudate putamen (Longa, et al., 1989), while distal occlusion results in infarctions generally limited to neocortex (Stroemer, et al., 1998).

The transient MCA occlusion (MCAo) model

This is most widely used model in experimental stroke research which involves the removal of the obstruction and return of flow (reperfusion) through the native vessel in a way that may not lead only to postischemic injury, but also the added insult of reperfusion injury, with stretching of BBB, reduction of edema and extrusion of immunologically active cells (Small, et al., 1996). The intraluminal filament model is extensively used method where MCA is occluded using either coated or uncoated nylon suture. Koizumi et al 1986, first introduced this method by occluding MCA using a silicon coated 4-0 nylon suture, it was further improved by Longa et al., 1989, by using 4-0 uncoated nylon suture whose tip was blunted by heating near flame. Various modifications have been done to improve its variation in infarct area (Ludmila, et al., 1996).

Endothelin-1(ET-1) induced occlusion is another model of reperfusion injury (Hunter, et al., 1999), in which endothelin1, is injected intracranially in order to block MCA (Sharkey, et al., 1996). ET-1 is a potent vasoconstrictor. It reduces regional cerebral blood flow and produces ischemic injury when being injected directly into brain tissue (Windle, et al., 2006) or adjacent to the MCA (Nikolova, et al., 2009). The magnitude and duration of reduction of cerebral blood flow is variable, dose dependent (Nikolova, et al., 2009), and strain dependent (Horie, et al., 2008), persistent up to 7-16 hours (Biernaskie, et al., 2001). ET-1 has a much less potent effect for producing an infarct in mice than in rats (Horie, et al., 2008).

From the point of resemblance to clinical stroke condition, temporary occlusion model better recapitulates the clinical situation, where reperfusion, spontaneous or with thrombolytic treatment would be expected. This model is

characterized by presence of ischemic core and penumbra in which neuronal death due to both necrotic and apoptotic pathways are evident.

Permanent focal ischemia

In this model arterial blockage is maintained throughout the experiment, usually for 1 to several days. It is done either by intraluminal filament method (Longa, et al., 1989) or by occluding MCA by craniotomy (Tamura, et al., 1981).

Photochemical induced Infarction

Photochemically induced focal cerebral thrombosis provides a precise location and size of infracted cortical area. This model involves intravenous administration of photosensitive dye like rose Bengal, and irradiation of specific area of brain with focused light beam of specific wavelength (De Ryck, et al., 1989). This treatment induces the generation of free radical and thus resulting platelets aggregation and thrombosis. Electron microscopy and light microscopy studies showed intravascular thrombotic material, red blood cell stasis, and platelet aggregates adhering to luminal surfaces inside blood vessels, with intravascular thrombosis that is responsible for the occurrence of ischemia leading to infarction. Increased permeability through disruption of the BBB by the photochemical reaction is also involved in this type of model. The main advantage of this method is that invasive procedures are minimal (Hunter, et al., 1998 &1995,). This method is suitable for studying antiplatelets and thrombolytic agents.

2.5.5. Measurement of ischemic stroke damage in animal models

The measurement of dynamic changes in the ischemic brain has attracted growing attention (Ejaz, et al., 2012). Ischemic brain injury in both focal and global ischemia models evolves as a progressive sequence of cellular and molecular events (Dirnagl, et al., 1999). In these section methods for analyzing brain injury and dynamic changes in the brain during ischemia and reperfusion, cerebral blood flow analysis and physiological variables are described. Regional microvascular tissue perfusion (cerebral blood flow) is normally monitored before, during, and after focal ischemia, using laser Doppler flowmetry (Okun, et al., 2007). Blood pressure, rectal temperature, and blood gases are measured during the operation in rats and mice. Normally, under controlled conditions using heating pads, there are no significant differences in temperature or blood pressure monitored at pre-, intra-, and post-ischemic time points in most studies.

However, measuring and maintaining these parameters is important for proper interpretation of the technical procedure causing stroke, outcomes among animals.

Quantification of cerebral infarction

The size of the brain infarct in focal cerebral ischemia increases during the period of reperfusion. This has been shown in animal models of stroke and in human stroke patients (Lathia, et al., 2010]. The infarct volume is normally analyzed after 12-24 hours in transient and permanent focal ischemia models. The brain is removed and coronal sections are cut (2 mm-thick slices in rats or 1-2 mm thick slices in mice) through the entire rostro-caudal extent of the cerebral cortex. The slices are immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC). An edema index is calculated by dividing the total volume of the hemisphere ipsilateral to the MCAo by the total volume of the contralateral hemisphere. An infarction index, the actual infarcted lesion size adjusted for edema, is then calculated for each animal (Arumugam, et al., 2010).

Blood-brain barrier function

Albumin leakage, a quantitative index of endothelial barrier dysfunction, can be applied to the brain for determination of BBB integrity. Albumin extravasation in the brain can be quantified either by fluorescence imaging of the leakage of fluorescein isothiocyanate-labeled albumin, or sulforhodamine (Texas Red)-labelled albumin from cerebral venules, or from the clearance of the same fluorochromes from blood to the artificial CSF perfusing the brain surface (Thompson, et al., 2006; Deryck M, 1990).

Brain edema measurement

The brains are immediately removed and divided into contralateral and ipsilateral hemispheres. The tissue samples are weighed on an electronic analytical balance to the nearest 0.1 mg to obtain the wet weight. The tissue is then dried at 90-100°C for 24 hours to determine the dry weight. Brain water content (%) is calculated as {(wet weight-dry weight)/wet weight} × 100.

Magnetic resonance imaging (MRI)

The introduction of in-vivo MRI has enabled non-invasive examinations of morphological and functional changes in the normal and diseased biological systems. Recently similar approaches have became available for experimental studies in research

animals as well. The application of imaging modalities has brought the advantage of non invasive in-vivo monitoring of pathological changes for longer durations, and therefore offers in-depth information in real time. However, most of the studies are performed on small bore dedicated preclinical MR scanners to obtain high spatial resolution and sensitivity. Limited availability of such machines and high cost makes it difficult for research studies involving animal applications.

2.5.6. Experimental assessment of neurological impairment

MCAo in rat typically results in extensive neuronal death in dorso lateral cortex and caudate putamen, as well as secondary neuronal damage in remote brain regions, such as the thalamus (Tamura, et al., 1981),. Depending upon the severity and location of infarction rat can show different neurological deficits such as motor deficit, hemipalgia, cognitive deficits, neglect and tactile extinction. These functional consequences of focal ischemic stroke injury can be assessed by either semiquantitative methods using a neurological deficit score or by the use of several behavioral tests that are sensitive to a particular location (Corbett and Nurse, 1998; Aronowski, et al., 1996; Markgraf, et al.,1992).

Remarkable work in the design of neurological deficit scales to be used for animals has been done in several labs (Zousinger, et al., 2000; Bederson, et al., 1986; Longa, et al., 1989). Following are different scales used in experimental models to assess neurological deficit.

2.5.7. Behavioral tests to assess neurological deficit following MCAo

Several behavioral tests have been employed to assess neurological outcome following stroke in rats, with modest success in linking ischemic injury volume and location to behavior (Schallert, et al., 2000; Bland, et al., 2000; Aronowski, et al., 1996). Standard methods have yet to be established regarding measurements of animal behavioral deficits and their recovery after focal ischemia in rodents. The summary of various behavioral tests that are used for rat model of stroke is given here in the table (Table 7).

Table 3:. A four point grading system of neurological examination post MCAo in lab animals

Grade	Neurological deficit and severity
0	No observable deficit, normal
1	Forelimb flexion, moderate deficit
2	Decreased resistance to lateral push (and forelimb flexion) without circling, severe deficit
3	Decreased resistance to lateral push (and forelimb flexion) with circling, very severe deficit

Table 4:. A five point grading system of neurological examination post MCAo in lab animals

Grade	Neurological deficit and severity
0	No neurological deficit
1	Failure to extend left fore paw fully) a mild focal neurological deficit,/ Moderate deficit
2	Circling to the left) a moderate focal neurological deficit
3	Falling on the left) a severe focal deficit
4	Animal did not walk spontaneously and had a depressed level of consciousness

Table 5: Five point neurological deficit score to assess post ischemic motor and behavioral deficits

Grade	Neurological deficit and severity
0	No detectable deficit
1	Forelimb flexion (mild/moderate or severe) that includes wrist/elbow/shoulder flexion with thorax twisting
2	Stimulated (if lifted by tail as to align the animal at around 45º with the plane) circling towards paretic side
3	Spontaneous circling towards paretic side
4	No spontaneous motion/absence of exploratory behavior/ falling on the paretic side following lateral push or without push, unconsciousness or death

Table 6:. Six point neurological deficit score to assess post ischemic motor and behavioral deficits

Grade	Neurological deficit and severity
5	Rats were held gently by the tail suspended one-meter above the floor, and observed for forelimb flexion. Normal rats extend both forelimbs toward the floor. Rats that extended both forelimbs toward the floor and had no other neurological deficit
4	Rats with consistent flexion of the forelimb contralateral to the injured hemisphere, 'varying from mild wrist flexion and shoulder adduction to severe posturing with full flexion of wrist, elbow and adduc-tion with internal rotation of the shoulder
3	Rats were placed on a large sheet of soft, plastic coated paper that could he gripped firmly by their claws. With the tail held by hand, gentle lateral pressure was applied behind the rat's shoulder until the forelimbs slid several inches. Normal or mildly dysfunctional rats resisted sliding equally in both directions. Severely dysfunctional rats with a con-sistently reduced resistance to lateral push toward the paretic side
2	Rats were then allowed to move on the floor and were observed for circling behavior if pulled by tail. Rats circling toward the paretic side
1	Rats were allowed to move about freely and were observed for circling behavior. Rats that circled spontaneously toward the paretic side
0	Rats without any spontaneous motion

Table 7:.- Various behavioral tests, behavioral task and nature of assessment following MCAo in rat

Behavioral test	Behavioral task	Behavioral assessment
Montoya's staircase test	Reaching and grasping of food pellets	Fine movements of forepaws
Grid walking test	Walking on a grid	Limb misplacement asymmetry while moving around a grid
Rota-rod test	Walking on a rotating rod	Time of staying on a rod
Running wheel test	Running in a wheel	Forelimb slips during running
Beam-walking test	Walking on a beam	Ability to maintain balance and hind limb slips during walking
Limb-placing test	Forelimb and hind limb placement	Response to proprioceptive and Forelimb slips during running
Tape test	Removing of sticky tapes from forepaws	Tactile stimuli Extinction, preference For removing of adhesive stimuli
Sensory inattention test	Orientation to sensory (visual, olfactory or tactile) stimuli	Tendency to orientate and investigate impinging stimuli
Cylinder test	Forelimb usage in vertical movements	Asymmetry in forelimb usage
Radial arm maze	Memorizing the location of food	Spatial memory
Passive avoidance test	Avoidance of aversive stimulus	Associative memory
Water-maze test	Memorizing the location of a hidden platform	Spatial memory

2.6. Unmet need

Stroke is the third leading cause of death globally, with more than 85% of deaths from stroke occurring in developing countries. Annually, 15 million people worldwide suffer a stroke. Of these, 5 million die and another 5 million are left permanently disabled, placing a burden on family and community (Mukherjee and Patil, 2011). Stroke burden is projected to rise from around 38 million disability-adjusted life years (DALYs) globally in year 1990 to 61 million DALYs in year 2020 (Feigin, et al., 2009). The fact that the loss of quality-adjusted life-years caused by stroke is greater than that of any other disease implies that the economic burden of stroke to humankind is also great. In the United States, the total direct and indirect cost of stroke for year 2008 is estimated at $65.5 billion (Feigin, et al., 2009). Although most strokes occur in older patients, there has been an alarming increase in stroke incidence in patients between 45 and 65 years of age (World Health Organization report, 2010).

Current management of ischemic stroke suffers from large dependence on preventive strategies. Preventive strategies include strict control of blood pressure, diabetes management, use of antiplatelets, anticoagulants, cholesterol lowering agents and life style management in those high risk patients. The incidence of stroke is declining in many developed countries, largely as a result of better control of high blood pressure, and reduced levels of smoking (European Stroke Organisation, 2008; Lawes, et al., 2003; Lewington, et al., 2002). However, the absolute number of strokes continues to increase because of the ageing population.

Currently available strategies to treat stroke are limited to thrombolytic therapy and use of surgery devices/techniques to reperfuse occluded arteries. Thrombolysis using tPA, a thrombolytic agent that can dissolve clots is the only approved therapy for the treatment of stroke available till date. Several factors confound the use of tPA, however, resulting in fewer than 5% of stroke patients being treated (Marler, 1995). For example, tPA must be given within 3 hours after symptom onset, but it can be fatal in hemorrhagic stroke and therefore requires that a computed tomography scan be performed prior to administration (Fisher and Bogousslavsky, 1998). Further, research suggests that less than a quarter of stroke patients arrive at the hospital within 3 hours of symptom onset, another factor reducing the eligible patient population for tPA (Fagan, et al., 1998). However though tPA is an effective clot burster, it does not address other vicious pathological events associated with cerebral ischemic injury to neurons.

Numerous clinical trials indicate that thrombolytic therapy is effective in reducing the brain injury and disability from ischemic stroke as long as the treatment is given within the first 3 hours after symptom onset and ideally, within the first 90 to 120 minutes after symptom onset (Hacke, et al., 1998; Hacke, et al., 1995).

Although imaging techniques are available in diagnosing the type of stroke whether ischemic or hemorrhagic, a device that could accurately indicate the time of onset of an ischemic stroke is presently unavailable. Assuming the clinical diagnosis is clear as an ischemic stroke; thrombolytic therapy cannot be given unless the time of symptom onset is known. In patients who were asleep when symptoms began or who cannot communicate and were alone when the symptoms began, thrombolytic therapy is currently withheld. Current standard of care is that the therapy can only be given when there is positive information regarding symptom onset. High prevalence rates, mortality and limited therapeutics for stroke highlights large unmet need in the field of stroke therapy and necessitates investigation of new therapies with clinically relevant time window which can effectively mitigate the outcome of stroke.

Thus, there is a significant unmet need in stroke for a therapy that can salvage brain cells by delaying the onset of cell death in nutrient deprived tissues until blood flow can be resumed. While the cells in the core of the affected portion, the infarct, cannot be recovered, there is a large surrounding region that is mildly affected and represents tissue that may be salvaged. A neuroprotective strategy applicable to both ischemic and hemorrhagic stroke is advantageous because the type of stroke need not be determined prior to treatment.

Progressive elucidation of the complex pathophysiology involved in the ischemic cascade has led to the development of numerous candidate interventions. There has been a great voluminous experimental work in the past three decades on finding a treatment strategies aimed at achieving neuroprotection for stroke. Some promising strategies, for example are the use of receptor antagonists targeted to reduce the neuronal excitotoxicity, therapies to reduce the oxidative stress, antiapoptotic agents and others which reduce the inflammatory mediators have shown good efficacy in animal models (Takasago et al., 1997, free radical scavengers Lees, et al., 2006) but ultimately failed in the clinical trials owing to no therapeutic benefit or serious toxicities (Fisher, 2005; Fisher, 2003; Gladstone, et al., 2002; STAIR Group, 2001; STAIR Group, 1999). Failure in the translation of results from animal models to humans implicates potential limitations of the current drug development process. Careful evaluation in

metaanalysis suggests possible flaws at several stages. Some of these pitfalls to summarize include, 1) Preclinical studies have used very short time windows for drug administration, whereas clinical trials allow longer time windows. 2) Preclinical studies target the ischemic penumbra, whereas clinical trials do not. 3) Preclinical studies have demonstrated protection of gray matter, whereas clinical trials frequently enroll patients without specifying location of damage. 4) Optimal duration of neuroprotectant administration is unknown. 5) Preclinical studies have relied on infarct size to judge therapeutic efficacy, whereas clinical trials rely on behavioral outcomes. 6) Preclinical studies have relied on early outcomes, whereas clinical trials rely on late assessments. 7) Experimental stroke models are homogeneous, whereas human stroke is heterogeneous. 8) Choice of outcome measures can determine the success of a clinical trial more than actual drug efficacy. 9) Small trials are trying to answer questions that only large trials can answer. Incorporation of standardized guidelines for preclinical testing of putative neuroprotective therapies and modification of clinical trial design, methodology and reporting is the urgent need of the hour and this may improve chances for success (Fisher, et al., 2009; Saver, et al., 2009; Fisher, et al., 2007).

Because cerebral ischemia involves a cascade of injury pathways, it may be preferable to focus on drugs with multiple mechanisms of action and that target multiple pathways (Fernandez-Gomez, et al., 2008; Uchino, et al., 2007; Zador, et al., 2004; Naritomi, 2001). Most of the putative neuroprotective agents evaluated earlier had the potential to address either single component or had limited capacity (interms of time window) to modulate the complex injury pathways (Gladstone, et al., 2002). Historically, the simplest approach is to use a drug with multiple targets. The need for more novel approaches is presently felt and these should be evaluated, for example, combinations of single target agents within or across cell death/recovery pathways. An approach that takes into account stroke biocomplexity is to target processes within the dynamic network that activate ensembles to promote maintenance of homeostasis under stress. In this regard the present promising agents that have shown preliminary clinical success such as high dose albumin therapy (Palesch, et al., 2006), hyperacute magnesium therapy (Saver, et al., 2006) which act on multiple pathways provide proof for the hypothesis stated above. Similarly strategies under experimental evaluation like upregulation of cell survival/adaptive pathways also hold promise for similar reasons.

Promising new strategies presently which are undergoing intense preclinical research include, use of monoclonal antibodies, growth factors, gene therapy, strategies

to stabilize hypoxia inducible factor and upregulation of stress proteins. All these approaches have shown significant success in preclinical models of stroke. Strategies like upregulation of stress proteins such as HSP70 have shown remarkable ability to cause neuroprotection both in-vitro and in-vivo (Mehta et al., 2007). Studies employing animal models either overexpressing HSP70 or HSP70 delivered using viral vectors have demonstrated improvement from multiple neuronal injury mechanisms including cerebral ischemia (Zhan et al., 2010; Rajdev et al., 2000). Although these new strategies are promising from preclinical research point of view, translation to clinical success appears to be a limitation. Since there is no effective delivery system available yet to deliver such therapies clinically, need for pharmacological agent is expressed.

With these collective evidences we identified that presently there exist significant research gap in the field of preclinical interventional stroke. Some of them to highlight are, preclinical studies have used very short time windows for drug administration whereas clinical trials allow longer time windows, most of the studies have relied on early outcomes evaluating efficacy over 24 hours period whereas clinical trials rely on late assessments. Further, preclinical studies have employed healthy animals to study stroke outcome but, clinically stroke occurs in patients with co-morbid disease conditions. Preclinical studies have relied on infarct size to judge therapeutic efficacy, whereas clinical trials rely on behavioral outcomes.

We hypothesized that an augmentation of the endogenous protective responses employing a pharmacological agent at an appropriate time and at desirable magnitude could provide benefit by reducing the neuronal loss and associated deleterious cascade of events in the intra and extracellular environment. Further, in order to fill the research gap we aimed at late intervention (4 hour and 8 hour post stroke onset) with a pharmacologic agent with the potential to induce HSP70 to demonstrate stroke protection possibly mediated through chaperone action and mitigation of late events such as inflammation and apoptosis. MR imaging was incorporated in our study as it offers advantage of non invasiveness in order to examine the progression of neuronal damage along with the possibility to capture treatment related benefit which can be directly applied and compared to clinical setup. We aimed for late assessment of efficacy over 7 day period evaluating effect on infarct size, behavioral outcome and survivability. Further we also have evaluated efficacy in co-morbid model of stroke.

Aims and Objectives

3. Aims and objectives

The aim of these studies was to evaluate pharmacological induction of HSP70 as a neuroprotective strategy for the treatment of acute ischemic stroke.

The objectives of these studies were:

- To evaluate the potential of a small molecule TRC051384 for its ability to transcriptionally express (induce) HSP70.
- To evaluate if treatment with TRC051384 results into potentiation of endogenous stress response, chaperonic and anti-inflammatory action.
- To evaluate neuroprotective efficacy of TRC051384, a small molecule HSP70 inducer, in a clinically relevant animal model of ischemic stroke.
- To evaluate whether delayed intervention treatment with TRC051384 is still efficacious, thus offering wide window of opportunity to treat ischemic stroke.
- To evaluate the neuroprotective ability of TRC051384 in an in-vivo model of stroke with clinically important co-morbidities.

Materials and Methods

4 Material 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. Software
- 4.1.5. Chemicals, drugs and disposables
- 4.1.6. Solutions and buffers
- 4.1.7. Formulation and vehicle

4.2. In-vitro studies

- 4.2.1. HSP70 mRNA induction
- 4.2.2. Heat Shock Factor 1(HSF-1) activation assay and in-vitro chaperone assay
- 4.2.3. In-vitro potentiation of endogenous stress response
- 4.2.4. Inhibition of TNF-α expression in human acute monocytic leukemia (THP-1) cell line

4.3. In-vivo studies

- 4.3.1. Standardization of middle cerebral artery occlusion (MCAo) model of stroke in rats
  - 4.3.1.1. Surgical procedure for the occlusion of middle cerebral artery occlusion (MCAo)
  - 4.3.1.2. Procedure for recording neurological deficit (Neurological score)
  - 4.3.1.3. Procedure for the quantification of infarct and edema.
  - 4.3.1.4. Identification/selection of optimum intraluminal suture for MCAo
    - 4.3.1.4.1. Identification of optimum suture diameter
    - 4.3.1.4.2. Standardization of poly-l-lysine concentration to be used for coating intraluminal suture
  - 4.3.1.5. Effect of varying duration of ischemia (MCAo) on model characteristics
  - 4.3.1.6. Study of temporal profile of brain injury and neurological deficit in rats subjected to transient MCAo.
  - 4.3.1.7. Effect of permanent MCAo on model characteristics

4.3.2. A Feasibility study on non-invasive imaging and quantification of infarct in rat brain using clinical magnetic resonance scanner

4.3.2.1. MRI feasibility studies for imaging rat brain

4.3.2.2. Temporal profile of brain injury (Infarct) in transient ischemia model of stroke by MRI

4.3.3. Validation of MCAo model of stroke for evaluating the efficacy of neuroprotective agent.

4.3.3.1. Validation of transient ischemia model

4.3.3.2. Validation of permanent ischemia model

4.3.4. Pharmacokinetic profiling of TRC051384 in rat

4.3.5. Acute safety studies with TRC051384 in rat

4.3.6. In-vivo efficacy studies of TRC051384 in MCAo model of stroke

4.3.6.1. Efficacy studies in 3 hr transient MCAo model

4.3.6.1.1. Efficacy of TRC051384 by single dose administration in MCAo model of stroke

4.3.6.1.2. Efficacy of TRC051384 by multiple dose short duration administration in MCAo model of stroke

4.3.6.1.3. Efficacy of TRC051384 by multiple dose long duration administration in MCAo model of stroke

4.3.6.2. Efficacy studies in 2 hr transient MCAo model – Therapeutic time window and dose response

4.3.6.2.1. Efficacy of TRC051384 administered at 4 hrs after MCAo

4.3.6.2.2. Efficacy of TRC051384 administered at 8 hrs after MCAo

4.3.6.3. Efficacy studies of TRC051384 in permanent MCAo model

4.3.6.4. Efficacy studies in stroke with co-morbid conditions

4.3.7. Mechanistic studies

4.3.7.1. BBB Permeability studies

4.3.7.2. Gene macro array studies

4.3.7.3. HSP70 Immunohistochemistry and TUNEL staining

4.1. Materials

4.1.1. New chemical entities and reference compounds

TRC051384 was synthesized in-house by medicinal chemistry discovery laboratory, Torrent Research Centre. MK 801 (Cat. No: M-107, Sigma-adlrich, Germany) used as reference compound in this study.

4.1.2. Experimental animals

Sprague Dawley rat:

Sprague Dawley (SD) 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, 23-26 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.

Animal housing conditions:

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.)
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.)
Liquid chromatography tandem mass spectrometry (LC-MS/MS, Thermo Finnigan, U.S.A.)
MacLab 8/s (AD Instruments, Australia)
Micro vessel clip (Biomedical Research Instruments, U.S.A.)
Micropipette (Eppendorf, Germany)
pH meter (Eutech, Singapore)
Pressure transducer (SensoNor 840, SensoNor AS, Norway)
QUAD Bridge (AD Instruments, Australia)
Refrigerated bench top centrifuge (Eppendorf, Germany)
Small animal ventilator (Columbus, U.S.A.)
Surgical microscope (Sethi Surgicals, India)
Surgical instruments (Locally sourced)
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. Software

Adobe photoshop (version 6.0, U.S.A.)

Chart (version 5) (AD Instruments, Australia)

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

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

Scion Image (version beta 4.0.2, U.S.A.)

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

4.1.5. Chemicals, drugs and disposables

2,4,5 Triphenyl Tetrazolium Chloride (Sigma Aldrich, Germany)

Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4, Qualigen, India)

Halothane I.P. 85 (Raman & Weil Pvt. Ltd., India)

Hypodermic needle (BD, Singapore)

Non Absorbable surgical suture (Ethilon) U.S.P, 3-0 (Ethilon Johnson & Johnson, India)

Non Absorbable surgical suture (Ethilon) U.S.P, 4-0 (Ethilon Johnson & Johnson, India)

Medical oxygen (Locally sourced)

MK-801 (Sigma Aldrich, Germany)

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

Normal saline (NS, Claris Lifesciences, India)

Poly-l-lysine solution (Sigma Aldrich, Germany)

Potassium dihydrogen pohosphate (Merck, Germany)

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

Sodium Hydroxide (NaOH, S.D.Fine chem., India)

Sterile surgical gloves (Kemwell, India)

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

4.1.6. Solutions and buffers

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

2% TTC solution

TTC	2 gm
Phosphate buffer saline pH 7.4 q.s.	100 ml

4.1.7. Formulation and vehicle

All formulations were freshly prepared at the time of administration. Sterile normal saline was used as vehicle for both TRC051384 and MK 801 in this study.

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4.2. In-vitro studies

Following paragraphs enumerate the methods used in various in-vitro studies carried out with TRC051384. All of these studies were carried out in the cell culture facility of department of cellular and molecular biology of Torrent research centre.

4.2.1. HSP70 mRNA induction

HeLa cell line (CCL-2, ATCC) or rat primary mixed neurons were employed. Primary mixed cerebellar granule neuron culture was established from cerebellum of seven days old SD rat pups. Proliferation of non-neuronal cells was prevented by addition of Cytosine Arabinoside (10 µM) to culture. On day 5 in culture, proper neuronal morphology was ascertained microscopically. Induction of HSP70B mRNA (reference sequence NM_002155.3) was carried out by treatment of cells with TRC051384 (6.25 and 12.5 µM) for 4 hours duration for both HeLa cell line or rat primary mixed neurons and total RNA was isolated. For all RNA samples, cDNAs were synthesized and expression of HSP70B mRNA along with expression of 18S rRNA was monitored by real-time PCR employing ABI 7000 system. For human HSP70B, ABI Taqman gene expression assay (Hs00275682-s1) while for rat HSP70B, Taqman gene expression assay (Rn00583013-s1) were employed. HSP70B mRNA expression was normalized relative to the expression of 18S rRNA. The results were expressed as fold induction of HSP70B mRNA relative to vehicle treated control. Details of these procedures are described in appendix (*Appendix I*)

4.2.2. Heat Shock Factor 1(HSF-1) activation assay and in-vitro chaperone assay

HeLa cell transiently transfected with heat shock elements (HSE)-luciferase reporter (pHSE-Luc, BD Biosciences) and normalization vector, β-galactosidase (pβgal, BD Biosciences) were treated with vehicle or TRC051384 (12.5 & 25 µM) for 4 hours. Cell lysates were then prepared and analyzed for luciferase and β-galactosidase activity. Results were expressed as fold induction of HSF1 activation over that for vehicle control.

In-vitro chaperone assay was carried out as follows, HeLa cells co-transfected with plasmids encoding cytoplasmic firefly luciferase and b-galactosidase (BD Bioscience) were employed. Transfected cells were treated with either vehicle or TRC051384 (25 µM) for 4 hours. Cell culture medium was then replaced with cycloheximide (20 mg/ml) to inhibit further new protein synthesis during inactivation and recovery phases. Inactivation of luciferase was achieved by subjecting cells to sublethal heat shock (44 °C for 30 min). Cells were then allowed to recover at 37°C for 3 hours. Subsequently, cells were lysed and monitored for luciferase and β galactosidase activity

(Lu et al., 2002). The luciferase activity for each condition gives an idea about the extent of functional luciferase protein. Functional luciferase for each condition is normalized for transfection efficiency. Results are expressed as normalized luciferase activity.

4.2.3. In-vitro potentiation of endogenous stress response

Potentiation of endogenous stress response by TRC051384 treatment was monitored employing HeLa cells. The cells were subjected to heat shock by incubating cells at 42ºC for 1 hour. Potentiation of stress response was studied in three set of conditions as follows. a) Heat shock + TRC051384 (12.5 µM) treatment was conducted for various duration starting from 1hr till maximum of 6 hrs immediately after the heat shock application. b) Heat shock + vehicle treatment was conducted for various duration starting from 1hr till maximum of 6 hrs immediately after the heat shock c) Only TRC051384 (12.5 µM) treatment (without heat shock) was conducted for various duration starting from 1hr till maximum of 6 hrs At the end of each treatment, total RNA was isolated and cDNAs were synthesized. Expression of HSP70 mRNA along-with expression of 18S rRNA was monitored by real-time PCR employing ABI Taqman gene expression assay (Hs00275682-s1) and ABI 7000 system. HSP70 mRNA expression was normalized relative to the expression of 18S rRNA. The results were expressed as fold induction of HSP70 mRNA relative to vehicle treated control.

4.2.4. Inhibition of TNF-α expression in human acute monocytic leukemia (THP-1) cell line

Human monocytic leukemia cell line, THP-1, (ATCC, TIB-202), which grows in suspension form was treated with Phorbol Merystyl ester (PMA), (25 ng/ml) for 48 hours. At the end of PMA treatment, THP-1 cells were differentiated into macrophage-like adherent cells. Differentiated cells were then treated with either LPS (1 mg/ml) alone or with LPS (1 mg/ml) and TRC051384 at 6.25 and 12.5 µM for 4 hours. Total RNA was isolated and cDNAs were synthesized for all conditions. Expression of TNF-α mRNA along with expression of 18S rRNA was monitored by real-time PCR employing ABI 7000 system. For detection of human TNF-α, ABI Taqman gene expression assay (Hs00174128-m1) was employed. TNF-α mRNA expression was normalized relative to the expression of 18S rRNA. Results were expressed as % TNF-α expression. TNF-α expression for cells treated with LPS alone was considered as 100%. Details of these procedures are described in appendix (*Appendix-II*)

4.3. In-vivo studies

In-vivo studies carried out in the present work were comprised of three major activities. First part comprised of transient and permanent MCA occlusion model standardization, standardization of magnetic resonance imaging technique for brain infracts and edema characterization and validation of MCAo model in rats. In the second part, acute safety and pharmacokinetic parameters for TRC051384 were studied. Third part consisted of efficacy studies in which TRC051384 was evaluated for its effect in an animal model of stroke by various treatment protocols.

4.3.1. Standardization of MCA occlusion (MCAo) model of stroke in rats

Standardization of MCA occlusion model in rats was carried out in male SD rats of body weight range 240-300 grams. In the present study for MCAo, intraluminal suture technique was employed. The study was carried out with aim to produce a consistent and reproducible animal model of stroke that resembles in most aspects of human stroke including the associated neurological deficits so that it could be used for the evaluation of potential neuroprotective agents.

These standardization activities were carried out in different parts with specific aim in mind. The first part consisted of characterizing/selecting the optimum intraluminal suture, i.e. diameter of the suture and coating solution concentration to be used for successful MCAo and consistent results. In these preliminary studies the duration of ischemia produced was for 3 hours. Later in the second part we carried out the effect of varying duration of ischemia (produced using standardized suture diameter and coating solution) on infarct and edema in this model. The detailed methods for these studies are enumerated below. Grossly, The methods for surgical procedure to produce cerebral injury employing intraluminal suture technique, measurement of neurological deficit and terminal quantification of infarct and edema remains same throughout the activity. So the detailed procedures for them are described in common below here.

4.3.1.1. Surgical procedure for the MCAo

Pre-surgical preparation and aseptic care

The whole surgical procedure for MCAo was conducted in the surgery room pre-fumigated with room disinfectant. Further the surgery table was wiped with 70% IPA and sterile surgical instruments and gloves were used. Male SD rats were

anesthetized with 3% halothane and maintained using 1.5% halothane mixed with oxygen throughout the surgery and while reperfusion. Before making incision the clean shaved (Oster A5 with #40 blade) neck region was wiped with Povidone Iodine (Betadine) and further cleaned with 70% IPA. Rectal temperature was monitored using a rectal probe and body temperature was maintained around 37°C using a homeothermic blanket (Harward apparatus Inc, USA) through out the surgical procedure of vessel occlusion and while carrying out reperfusion procedures.

Procedure for MCAo and reperfusion

The basic surgical procedure consisted of blocking blood flow to the MCA with an intraluminal suture introduced through the extracranial internal cerebral artery (ICA). Under the operating microscope, the left common cerebral artery (CCA) was exposed through the midline incision; a self-retaining retractor was positioned between the digastric and sternomastoid muscles, and the omohybid muscle was divided. The occipital artery branches of the external carotid artery ECA were then isolated and tied using a cotton thread. The ECA was dissected further distally and tied using cotton thread along with the terminal lingual and maxillary artery branches, which were then divided. The ICA was isolated and carefully separated from the adjacent vagus nerve. Further dissection identified the ansa of the glossopharyngeal nerve at the origin of the pterygopalatine artery; this posteriorly directed extracranial branch of the ICA, ligated with the help of a cotton thread close to its origin. At this point, the ICA is the only remaining extracranial branch of the CCA.

Next, a cotton thread was tied loosely around the mobilized ECA stump, and a curved microvascular clip was placed across both the CCA and the ICA adjacent to the ECA origin. A 3-cm length of the monofilament polyamide suture was introduced into the ECA lumen through a puncture or through one of the terminal branches of the ECA. The cotton thread around the ECA stump was tightened around the intraluminal polyamide suture to prevent bleeding, and the microvascular clip was removed. The suture was then gently advanced from ECA to the ICA lumen; the position of the suture within the ICA lumen could be seen as it reached the base of the skull. After a variable length of suture (2.0 to 2.2cm) had been inserted into the ECA stump, resistance was felt and a slight curving of the suture or stretching of the ICA was observed, indicating that the tip of the suture had passed the MCA origin and reached the proximal segment of the anterior cerebral artery (ACA), which has a smaller diameter. At this point, the

intraluminal suture has blocked the origin of the MCA, occluding all sources of blood flow from the ICA, ACA and posterior cerebral artery (PCA). The time of onset of ischemia was recorded. The incision was closed (dermis, panniculus carnosus, subcutaneous tissue layers) quickly with 3-0 braded silk suture (a simple continuous pattern will facilitate reopening for reperfusion) and the rat carefully placed in a recovery cage to monitor recovery from anesthesia.

Reperfusion: In order to produce transient ischemia, reperfusion of occluded artery was carried out as per the procedure stated here. Shortly before the predetermined occlusion period should end, re-anesthetize the rat, disinfect the incision site with Betadine and 70% ethanol and reopen the incision by removing the closing sutures. Place a microclip on the CCA, as before. Withdraw the occluding suture partway from the ICA until the suture end is visible through the ICA. Do not fully remove the suture from the ICA/ECA. Place a microclip on the ICA above the end of the intraluminal suture. Completely remove the occluding suture and tightly tie off the ECA stump. At this time point the MCA had been completely reperfused and blood flow was reestablished. The time of onset of reperfusion was recorded. Microclip from the ICA and CCA were removed. Moistened the region with several drops of sterile saline and close the incision layers (dermis, panniculus carnosus, subcutaneous tissue) with 3-0 silk suture using a simple interrupted pattern.

In case of permanent occlusion the suture was not withdrawn and animals were allowed to recover till the end of stipulated time of the study.

Post operative care

Immediately after the completion of surgical procedure, rats were transferred to autoclaved cage lined with two layers of blotting paper kept in an incubator maintained at 37°C. After recovery from anesthesia and acclimatization to new home cage for 30 minutes, food was presented in glass petridish within the cage and water was provided in bottle with long nozzle to facilitate easy intake. Rats in the recovery cage were frequently monitored for their normal well being.

4.3.1.2. Procedure for recording neurological deficit (Neurological score)

Neurological scoring is done according the modified method described by (Longa, et al., 1989; Zousinger, et al., 2000). A five point neurological deficit scoring system is used to assess post-ischemic motor and behavioral deficits. Neurological

score for each animal is recorded during ischemia period, after reperfusion, at various time points. Following are various grades of deficit recorded and also mentioned the severity of deficit for each grade.

Grade 0 : No detectable deficit.

Grade 1 : Forelimb flexion that includes wrist/elbow/shoulder flexion with thorax twisting. Indicating mild neurological deficit.

Grade 2 : Stimulated (if lifted by tail as to align the animal at around 45º with the plane) circling towards paretic side. Indicating moderate neurological deficit.

Grade 3 : Spontaneous circling towards paretic side. Indicates severe neurological deficit.

Grade 4 : No spontaneous motion/absence of exploratory behavior/ falling on the paretic side following lateral push or without push, unconsciousness or death. Indicating very severe neurological deficit.

4.3.1.3. Procedure for the quantification of infarct and edema.

Infarct and edema quantification was carried out employing Triphenyl tetrazolium chloride (TTC) staining method terminally at the end of study period. Following paragraph enumerates the procedure used for staining brain slices using TTC.

Triphenyl tetrazolium chloride (TTC) staining

TTC staining was performed according to the procedure of Bederson, et al (Bederson, et al., 1998). Under deep ether anesthesia, perfuse the rats with cold phosphate buffered saline (PBS) via the left ventricle (transcardiac route). Isolate the brain tissue and place it at -70 ºC for about 15 minutes. Keep the hardened brain in Rodent Brain Matrix and cut coronally into seven 2-mm sections after leaving the initial 3mm with the help of a microtome blade. Stain the sections by immersing in 1% 2,3,5-triphenyltetrazolium chloride solution by keeping it at 37ºC in a water bath for 10 minutes. Put cover slip on each of the sections during staining to obtain uniform staining on both sides. Fix the stained slices in buffered 4% formaldehyde solution overnight before taking the scan. Each slice was scanned by a scanner for analysis of total infarct area, edema using scion image software (Scion, Frederick, MD).

Image analysis for quantification of infarct and edema

Following steps were employed in order to capture the images of TTC stained brain slices, quantification of area of damage using computer assisted software.

1) Stained slices were kept on an OHP sheet in a uniform pattern and scanned at a resolution of 400 dpi by keeping the cover open.
2) The images were saved in TIFF format
3) Saved images were opened through Adobe Photoshop (version-6.0).
4) From the tool bar that appears on the left select the “magic wand” tool and click on the infracted area. The area will be automatically selected based on the color differences.
5) In case of any kind of discrepancies in the automatically selected areas, manual selection can also be done by clicking over the appropriate areas.
6) Measurements that were not based on color differences can be made by selecting the magnetic lasso tool from the tool bar and marking out the areas of interest.
7) Areas selected with either of the tools were copied and saved in TIFF format.
8) Open the saved images of infarcts using the scion image analysis software.
9) Select “Edit” from the menu bar and click invert. Slices of infarct take up black color. Select “Options” from the menu bar and click “Threshold”.
10) Contrary to infarct slices, whole slices take up white color. To change its color to black, choose “EDIT” from the menu bar and select “Invert”. The color of the slice will be inverted to black.
11) By using the progress bar on the left the intensity of the black color was adjusted so that, it’s uniform across the slice.
12) Select the “analyze” option from the menu bar and click on “set scale”. Enter the resolution in the text box labeled “scale”.
13) Select the “analyze” option from the menu bar and select “show results” followed by “measure”.
14) The area of the slice will be displayed in the unit (usually mm2) entered in the “set scale” option.
15) The values can be copied to an excel worksheet for further analysis.

4.3.1.4. Identification/selection of optimum intraluminal suture for MCAo

4.3.1.4.1. Identification of optimum suture diameter

In order to study the impact of different sizes (Diameter) and tip properties of these vessel occluding sutures, we studied four different types of sutures (Ethilon, Johnson & Johnson) on various outcome parameters post MCAo. The details of suture type used and other experimental details were mentioned below (Table 8).

Table 8: Group details for the identification/selection of intraluminal suture diameter.

Groups	Suture size	Suture tip properties	Group size (n)	Model (Transient ischemia)	Parameters studied
4-0	4-0	Rounded	5	3 hour ischemia + 21 hour reperfusion	Infarct, Edema, Neurological deficit & survival
4-0-Bulb	4-0	Heat made bulb	5		
3-0	3-0	Rounded	5		
3-0-Bulb	3-0	Heat made bulb	5		

Three hour transient ischemia protocol was used according to the method mentioned in section above (Section; 3.2.1.1). Neurological deficit was recorded for each animal at two time points; first recording was made at 45 min post MCAo after ensuring that the animals have fully recovered from anesthesia. Second recording of neurological deficit was made just before termination at 24 hours post MCAo. Animals were then terminated brains were isolated to further quantify infarct and edema.

***Preparation of suture with rounded tip and heat flame made bulb*:**

Commercially available non absorbable polyamide monofilament [(3-0/4-0 polyamide suture (Ethilon, Johnson & Johnson)] was used. Suture was cut into 30 mm individual pieces using Microtome blade (high profile) from the straight part by excluding the curvatures. For the **preparation of sutures with rounded tip**, these 30mm pieces were gently rubbed by rotation on a sand paper from one end for 5-6 times. To exclude any suture with sharp edges the front rubbed end was checked under optical microscope (Magnification: 10X). The back end of the prepared suture could be grated by using an artery forceps to distinguish it from the front end. This procedure did not affect diameter or texture of the suture and produced sutures with blunt rounded tips which can be used for the purpose of MCAo.

For preparing sutures with tip rounded by heat made bulb, same 30 mm cut sutures were used. One end of the tip was taken close to the bottom end flame (Blue flame) by hand holding them. This procedure causes the tip held near flame to melt and form a uniform bulb around the tip. Due care should be taken not to take the suture too close to the flame as it may burn the tip. Check the front end (one with bulb) under optical microscope (Magnification: 10X) to exclude any suture with non uniform edges. This procedure produced sutures with slight increase in diameter at the tip, but is reported to cause more complete occlusion of MCAo hence increased success rate.

4.3.1.4.2. Standardization of poly-l-lysine concentration to be used for coating intraluminal suture

Two different concentrations of poly-l-lysine solution were used for coating the sutures (3-0 Ethilon, Johnson & Johnson). The detailed procedure for coating is mentioned below. These sutures were used for the occlusion of MCAo in rats in order to study the impact of coating on consistency of results. MCAo procedure remained same as mentioned in the earlier sections. Three hour transient ischemia protocol was used and 3-0 sutures with rounded tip were employed. Since these sutures were found to better in comparison to other sutures such as size 4-0 with rounded tips, size 4-0, 3-0 sutures with heat made bulb. The details of groups used and other experimental details are mentioned below (Table 9).

Table 9: Group details for the identification optimum poly-l-lysine concentration to be used for coating intraluminal suture

Groups	Suture size	Suture tip properties	Group size (n)	Model (Transient ischemia)	Parameters studied
Uncoated	3-0	Rounded	5	3 hour ischemia + 21 hour reperfusion	Infarct, Edema, Neurological deficit
Poly-L-lysine (0.01%)	3-0	Rounded	5		
Poly-L-lysine (0.01%)	3-0	Rounded	5		

Neurological deficit was recorded for each animal at two time points; first recording was made at 45 min post MCAo after ensuring that the animals have fully recovered from anesthesia. Second recording of neurological deficit was made just before termination at 24 hours post MCAo. Animals were then terminated brains were isolated to further quantify Infarct and edema.

Preparation of poly-l-lysine coated sutures

Poly-l-lysine coated sutures (with rounded tip) were prepared according to a modified method of Belayev et al. (1996). This coating increased the tissue adherence but does not affect diameter or texture of the suture. Procedure for suture preparation was as follows. Cut the suture into 30 mm individual pieces using sharp Microtome blade from the straight part by excluding the curved portion in the suture. Fill the coating solution (0.1% or 0.01% w/v poly-l-lysine) in a 2.0 ml centrifuge tube. Dip all 30 mm pieces in the solution completely for 10 minutes with intermittent shaking. Pull out the threads and put them individually in a vertical manner on a thermocol bed. Put them into an incubator maintained at 60ºC for 30 minutes for drying. Collect all sutures and keep them in a dry container. Care must be taken to use the same side (i.e. the side that touches the thermocol bed) for all handling purposes. The back end of the prepared suture can be crimped by using an artery forceps to distinguish it from the front end. Check the front end under optical microscope (Magnification: 10X) to exclude any suture with sharp edges.

4.3.1.5. Effect of varying duration of ischemia (MCAo) on model characteristics

Male SD rats were subjected to increased duration of MCAo such as 2 hour, 3 hour and 4 hour ischemia according to the procedure mentioned in the earlier sections. In this study 0.1% poly-l-lysine coated 3-0 size (3-0 Ethilon, Johnson & Johnson) polyamide monofilaments with rounded tip were used for the MCAo. Neurological scores were recorded at two time point i.e. 4 hr and 24 hr after the initiation of MCAo. Animals were then terminated at 24 hours post initiation of MCAo; brains were isolated to further quantify Infarct and edema. Group details employed and parameters measured are mentioned in table (Table 10) below

Table 10: Group details for the study of varying duration of ischemia on different parameters in rats

Groups	Suture size	Suture tip properties	Group size (n)	Duration of ischemia + reperfusion	Parameters studied
2 hr	3-0	Rounded	5	2 hr + 22 hr	Infarct, Edema, Neurological deficit
3 hr	3-0	Rounded	5	3 hr + 21 hr	
4 hr	3-0	Rounded	5	4 hr + 20 hr	

4.3.1.6. Study of temporal profile of brain injury and neurological deficit in rats subjected to transient MCAo.

Male SD rats (n=50) were subjected to transient MCAo for 3 hours and reperfused to various time points according to the procedure mentioned in the earlier sections. In this study 0.1% poly-l-lysine coated 3-0 size (3-0 Ethilon, Johnson & Johnson) polyamide monofilaments with rounded tip were used for the MCAo. Group of atleast 5 animals were allowed to reperfuse for 4 hours, 8 ours, 12 hours, 24 hours, 36 hours, 48 hours and 7 days. Animals were terminated at the end of their respective reperfusion times and brains were isolated to further quantify Infarct and edema. Neurological scores were recorded for each surviving animal at 1hr, 3hr, 24hr after initiation of MCAo, and there after daily for 7 days. Survivability was studied over 7 day's period.

4.3.1.7. Effect of permanent MCAo on model characteristics

Male SD rats were subjected to permanent MCAo for 24 hours according to the vessel occlusion procedure mentioned in the earlier sections. Effect of permanent MCAo was studied by dividing animal in to three groups (Table 11). Two groups consisted of permanent MCAo carried out using 0.1% poly-l-lysine coated 3-0 and 4-0 size (Ethilon, Johnson & Johnson) polyamide monofilaments with rounded tip and third group studied included permanent MCAo done using uncoated 3-0 sutures. Neurological scores were recorded at two time point i.e. 1 hr and 24 hr after the initiation of MCAo. All animals were terminated at 24 hours post initiation of MCAo; brains were isolated to further quantify Infarct and edema.

Table 11: Group details for the study of effect of suture with different diameter on different parameters in permanent MCAo in rats

Groups	Suture size	Suture tip properties	Group size (n)	Duration of ischemia	Parameters studied
4-0 Coated	4-0	Rounded	5	24 hr	Infarct, Edema, Neurological deficit & Mortality
3-0 Uncoated	3-0	Rounded	6		
3-0 Coated	3-0	Rounded	6		

4.3.2. A Feasibility study on non-invasive imaging and quantification of infarct in rat brain using clinical magnetic resonance scanner

Background and rationale

MRI is a well established diagnostic tool in clinical radiology. The introduction of in-vivo MRI has enabled non-invasive examinations of morphological and functional changes in the normal and diseased biological systems. In recent times its use has increased manifold in clinical diagnosis and management of number of diseases including central nervous system disorders. Recently similar approaches have became available for experimental studies in research animals as well. The application of imaging modalities has brought the advantage of non invasive in-vivo monitoring of pathological changes for longer durations, and therefore offers in-depth information in real time. However, most of the studies are performed on small bore dedicated preclinical MR scanners to obtain high spatial resolution and sensitivity. Limited availability of such machines and high cost makes it difficult for research studies involving animal applications. The present study explored the feasibility of using a clinical whole body MR-scanner to perform imaging in rat brain and specifically in models of stroke.

4.3.2.1. MRI feasibility studies for imaging rat brain

These studies were performed to optimize position of object inside the MR scanner, sequence parameters for imaging small structures with high resolution and for the application of standardized sequence parameters, from first part, for anatomical imaging, scanning MCAo rat brain to study feasibility of infarct quantification and its validation using TTC staining.

MRI scanner and RF coils

MRI was performed on a 1.5 T clinical MRI scanner (Fig 1a. Magnetom Symphony, Siemens Medical solutions, Erlangen, Germany) with a field gradient strength of 33 mT/m. Amongst other commercially available RF coils we used for our studies two different types of coils with the aim to identify the one which best suits the purpose of getting good resolution images with lower signal to noise ratio. For that in our initial experiments we used small loop flex coils (Fig.1b) (Loop Flex Coil, small; Siemens Medical Solutions, Erlangen, Germany) and CP small loop coil. These above mentioned surface coils have been designed for high-resolution imaging of structures near the surface (e.g. fingers, toes, ankle and wrist).

Standardization of object positioning and MR sequence parameters

Phantom studies: Phantom studies were performed using a water filled 50 ml plastic tube (Make: Tarson) as test object for initial selection of RF coils and optimization of scan time and sequence parameters. The 50ml water filled tube was chosen because it offers a good signal due to its water content and its contour and size resembles the dimensions of the head of a rat.

Positioning of the animal/object within the scanner

To achieve good images, it was required to stabilize and properly position the anesthetized rat, nose cone which delivers gaseous anesthesia and RF coil in the scanner. It was equally important to place the object be it the phantom or animal into the centre of the magnet. To achieve this, anesthetized animal was placed in a custom made plastic (PVC) holder (Figure.24c), with its snout in the anesthesia nose cone and the head of the animal positioned inside the loop of flex loop small RF coil (Figure 24b). In case of CP small loop coil the coil was placed above the phantom or on the top of the head in case of rat brain imaging. In addition the animal was also immobilized with strips of adhesive, to reduce movement artifacts induced by breathing.

MRI sequence parameters

Factory built sequences that are used for imaging of human body parts such as fingers or wrist are employed here and suitable modification were made in order to get images of rat brain with good resolution. To improve the signal to noise ratio (SNR) and to obtain images with high resolution and tissue contrast the following

parameters were systematically varied - field of view (FOV), TR (Repetition time) and TE (Echo time) ratio (TR:TE), number of acquisitions, matrix, and slice thickness. Coronal and sagittal T1- and T2-weighted (T2W) images were obtained using spin echo sequences. Diffusion weighted imaging (DWI) were acquired using echo planar spin echo sequences. Apparent diffusion coefficient (ADC) maps were constructed from diffusion weighted images.

Procedure for scanning object

Test object was placed inside flex loop small radiofrequency coil (radio frequency receiver coil) and integrated body coil acts as radio frequency transmit coil. Finalize the alignment and position of the tube inside the coil with the help of laser pointer in relation to the magnetic field. Initially acquire a globaliser sequence (T1 weighted image with a TR: TE 42:6 ms) to know the global position of the object. Then obtain two (Coronal and Sagital) T1-weighted images through the entire object. Obtain coronal T2-weighted images of 13 slices covering entire object using a fast spin-echo sequence [field of view(FOV) 50*50 mm, TR:TE 2500:100 ms, slice-thickness :1.5 mm, distance factor of 30%between slices and scan time 3.47 min].

Acquire DWI with a echo planar spin-echo sequence of contiguous 5 coronal slices covering the same area as scanned earlier (thickness: 2.2 mm, FOV) 103*103 mm, matrix 256*100, TR:TE 3200:97 ms, acquisition time 50 sec). Three sets of coronal images were used to quantify apparent diffusion coefficient of water ($ADCH_2O$), with equidistant diffusion gradient resulting in b values of 0, 500, and 1000 s/mm^2.

MRI protocols for anatomic structures and quantification total brain area and lesion area

Male SD rats that underwent transient intraluminal MCA occlusion for 2h and reperfused (MCAo) according to the procedure mentioned in earlier sections were employed. In addition age matched normal (n=3) rats were subjected to MRI scan for the study of identification of anatomical structures and quantification of total brain area. Body temperature was maintained around 37°C using a thermostatically controlled MRI compatible heating blanket through out the procedure of MR imaging.

Procedure for MRI Imaging

Anaesthetized rat in the supine position was placed in the animal holder with the head inside flex loop small radiofrequency coil. For anatomical imaging purpose serial T2 weighted images were acquired using multi slice MR sequences that were standardized earlier. The whole brain was scanned into 13 slices. For the quantification of infarct lesion MCAo animals were used. Serial DWI and T2W scans were acquired for one animal at 12hr, other animal at 24 hr, and third animal at 48 hrs post MCA occlusion. Coronal T2-weighted images through the entire brain were obtained using a turbo spin-echo sequence. To map the apparent diffusion coefficient of water, diffusion-weighted images were acquired with a spin-echo sequence. Three sets of coronal images were recorded to quantify ADC, with equidistant diffusion gradient resulting in b values of 0, 500, and 1000 s/mm2.

Quantification of brain area and lesion area on MR images

The total brain area for normal animals and MCAo animals, ipsilateral area and lesion area for MCAo animals only on each coronal section of the multi-slice anatomical T2-weighted images and ADC images was determined manually by an observer blinded to the experiment using SYNGO MR Image analysis software. ADC images (hypointense area on MR image) were analyzed to quantify the lesion area while T2 images (hyperintense area on MR image) were analyzed for brain edema according to a method described elsewhere (Gladstone, et al., 2002; Guzman, et al., 2000; Li, et al., 2000b; Beckmann, et al., 2001) The method involved marking of a region of interest with a selection tool available from the software which gave the values of area (in cm2) marked. A total of seven coronal T2 weighted images (excluding olfactory lobes and cerebellum) were used to calculate total brain area and five coronal ADC images (covering the entire area infarct area) were used to calculate infarct area.

Validation of MR measurements

To further confirm the lesion seen on MR images a terminal measurement of infarct area using TTC stained brain slices were performed. The animals were sacrificed after 12, 24 and 48hr post MCAo and immediately after MR scan and perfused with ice cold heparinized phosphate buffer saline. 2mm thick coronal sections of the frozen brain (10 mins) were stained using 2% TTC. Formalin fixed sections (24 hrs) were scanned using a document scanner (HP Desk jet) and images thus obtained were

analyzed for total area and lesion area using scion image software (Scion, Frederick, MD).

4.3.2.2. Temporal profile of brain injury (Infarct) in transient ischemia model of stroke by MRI

Male SD rats were subjected to transient MCAo for 2 hour and 3 hour according to the procedure mentioned in the earlier sections. In this study 0.1% poly-l-lysine coated 3-0 size (3-0 Ethilon, Johnson & Johnson) polyamide monofilaments with rounded tip were used for the occlusion of MCAo. Group details are as below (Table 12),

Table 12: Group details for the study of temporal profile of infarct progression measured using magnetic resonance imaging

Groups	Suture size	Suture tip properties	Group size (n)	Duration of ischemia + reperfusion	Parameters studied
2 hr	3-0	Rounded	5	2 hr + 46 hr	T2W and ADC imaging for infarct core and penumbra
3 hr	3-0	Rounded	5	3 hr + 45 hr	

T2W, T2 weighted; ADC, apparent diffusion coefficient

MRI protocol

MR imaging of rat brain was carried out according to the procedure mentioned earlier (section. 3.2.2.3). Briefly, each animal was imaged serially at 1hr, 4hr, 8hr, 12hr, 24hr, 36hr and 48 hr post initiation of MCAo in order to monitor the progression of infarct. At each time of imaging coronal T2-weighted images through the entire brain were obtained using a turbo spin-echo sequence. To map the apparent diffusion coefficient of water, diffusion-weighted images were acquired with a spin-echo sequence. The brain images thus obtained were used for analysis of infarct volume using MR image analysis software.

4.3.3. Validation of MCAo model of stroke for evaluating the efficacy of neuroprotective agent.

Animal model validation was performed to ensure sensitivity of model to capture the ability of neuroprotective agent to reduce infarct and edema. MK-801, a NMDA receptor blocker was employed for this purpose. Validations of transient ischemia as well as permanent ischemia model were carried out.

4.3.3.1. Validation of transient ischemia model

In this study MR imaging was used for the evaluation of efficacy of MK-801 in a 3hr transient ischemia model of stroke. Male SD rats were subjected to 3 hr transient ischemia according to procedure as mentioned in the above sections. 0.1% poly-l-lysine coated 3-0 size sutures with rounded tip were used for occlusion of MCA. Animal were further divided into MK801 treated and vehicle control groups. Details of the experimental groups and conditions are enumerated in table (Table 13) below.

Table 13: Group details for validation of transient ischemia MCAo model using MK-801

Groups	Dose	Dosing schedule	Group size (n)	Duration of ischemia + reperfusion	Parameters studied
Vehicle	Normal Saline, 2ml/kg	i.v bolus immediately after MCAo	5	3 hr + 45hr	T2W and ADC imaging for infarct core and penumbra, Neurological scores and survivability
MK-801	0.1mg/kg	i.v. bolus immediately after MCAo	5	3 hr + 45hr	

i.v., intra venous ; MCAo, middle cerebral artery occlusion; T2W, T2 weighted; ADC, apparent diffusion coefficient

MR imaging of ischemic rat brain was carried out according. Briefly, each animal was imaged serially at 1hr, 4hr, 8hr, 12hr, 24hr and 48 hr post initiation of MCAo in order to monitor the progression of infarct. At each time of imaging coronal T2-weighted images diffusion-weighted images were acquired. The brain images thus obtained were used for analysis of infarct volume using MR image analysis software.

4.3.3.2. Validation of permanent ischemia model

Male SD rats were subjected to 24 hr permanent ischemia according to procedure as mentioned in the above sections. 0.1% poly-l-lysine coated 3-0 size sutures with rounded tip were used for occlusion of MCA. Animal were further divided into MK801 treated and vehicle control groups. Details of the experimental groups and conditions are enumerated in the table (Table 14) below.

Table 14: Group details for validation using MK-801 of permanent MCAo model

Groups	Dose	Dosing schedule	Group size (n)	Duration of permanent ischemia	Parameters studied
Vehicle	Normal Saline, 2ml/kg	i.v bolus immediately after MCAo	5	24 hr	Infarct area by TTC staining, and survivability
MK-801	0.1mg/kg	i.v. bolus immediately after MCAo	5	24 hr	

MCAo, middle cerebral artery occlusion; TTC-Triphenyl tetrazolium chloride

Neurological scores were recorded at two time point i.e. 1 hr and 24 hr after the initiation of MCAo. 24 hours after the initiation of MCAo all animals were humanely sacrificed and brains were carefully isolated. Evaluation of efficacy of MK-801 was carried out by measuring infarct on TTC stained brain slices.

4.3.4. Pharmacokinetic profiling of TRC051384 in rat

Male SD rats of age ranging from 10-12 weeks were used in the study. Rats were divided into 2 groups, each had four rats. Pharmacokinetic profile of TRC051384 was studied by i.v and i.p route in two separate groups of rat. For i.v PK study, right external jugular vein of rats was cannulated and the cannula was exteriorized subcutaneously from the back of the neck, which was used for i.v. drug administration. Rats were administered TRC051384 at dose of 7.57mg/kg. For i.p. PK, group of rats were administered with 7.57mg/kg of TRC051384 by intraperitoneal route using 26G needle. Blood sampling was done from puncturing the sublingual vein. Detailed procedure for sublingual blood collection is described in the appendix (*Appendix III*).

Timed blood sampling was done and plasma separated from blood samples collected before dosing (0 hr) and 0.25, 0.5, 1, 2, 4, 6 and 8 hr post drug administration by either i.v. or i.p. route. An additional sample at 0.125 hr was also collected from rats administered TRC051384 by i.v. route.

The plasma samples were analyzed using LC-MS/MS in bioanalytical department, Torrent Research Centre. A method fit for purpose was developed; partial validation of analytical method was carried out. Described below are the key parameters that were validated. Repeat, sensitivity, intra and inter variability. The PK parameters were calculated using non compartmental model analysis module of winNonlin (version 5.1).

4.3.5. Acute safety studies with TRC051384 in rat

Male SD rats weighing 270-300g were used to study the cardiovascular and respiratory effects of TRC051384. The experiment was carried out on four groups, of which three were TRC051384 treatment groups and the fourth was vehicle control group. Each group comprised of 3 rats each.

Animal were anesthetized with intraperitoneal injection of urethane (1.25gm/kg), right external jugular vein was cannulated for drug administration and left common carotid artery was cannulated for blood pressure (BP) recording. The catheter inserted into the carotid artery is connected to a pressure transducer attached to a MacLab 8/s data acquisition system. Standard limb lead II ECG was recorded continuously in these animals for 4 hr using BIO Amp connected to MacLab 8/s. The heart rate was calculated as a derived parameter from the lead II ECG. Respiratory rate was recorded by positioning pulse transducer beneath the rats back. The pulse transducer attached to MacLab 8/s acquired the rhythmic thoracic movement resulting due to inspiration-expiration during respiration cycle. A stabilization period of 30 min was allowed until BP stabilizes before checking the drug effect. TRC051384 was administered by i.v route at dose of 7.54mg /kg, 3.77mg/kg and 1.88mg/kg. BP, ECG and respiratory rate were subsequently recorded for four hours from TRC051384 treated and vehicle treated rats. The BP, ECG and respiration data was analyzed using chart Pro (version 5.4.2) software to obtain effect on BP, heart rate and respiration rate. Peak effect on BP, ECG (change in amplitude of ECG pattern), heart rate and respiration rate were calculated for TRC051384 and vehicle group of animals.

4.3.6. In-vivo efficacy studies of TRC051384 in MCAo model of stroke

After the standardization and validation of animal model, we used these models to evaluate efficacy of TRC051384 by employing various treatment protocols in order to arrive at most efficacious dose or dosing regimen to be followed. Once the most effective dose regimen was identified we then aimed at studies to identify the therapeutic time window, efficacy in permanent ischemia models and efficacy studies in model of stroke with co-morbidities.

4.3.6.1. Efficacy studies in 3 hr transient MCAo model

4.3.6.1.1. Efficacy of TRC051384 by single dose administration in MCAo model of stroke

Male SD rats of body weight 250-300g were used; MCAo was occluded for 3 hours duration using intraluminal suture (0.1% poly-l-lysine coated 3-0 suture (Ethilon, Johnson & Johnson)) technique according to the procedure explained in the earlier sections. Jugular vein was cannulated, exteriorized from animal back for drug administration. Dose, time of drug administration and parameters measured are enumerated below (Table 15). Neurological scores were recorded at 1 hour and just before termination at 24 hours post MCAo. Infarct and edema were quantified terminally on TTC stained brain slices.

Table 15: Dose, treatment schedule and group details for single dose efficacy of TRC051384 in 3 hr transient ischemia MCAo model of stroke

Groups	Dose	Dosing schedule	Group size (n)	Duration of transient ischemia + reperfusion	Parameters studied
Vehicle	Normal Saline, 1ml/kg	Single i.v. bolus at 4 hr after MCAo	8	3 hr+ 21 hr	Infarct, and Edema by TTC staining, Neurological scores and survivability
TRC051384	3.78 mg/kg	Single i.v. bolus at 4 hr after MCAo	9	3 hr+ 21 hr	

i.v., intra venous ; MCAo, middle cerebral artery occlusion; TTC-Triphenyl tetrazolium chloride

4.3.6.1.2. Efficacy of TRC051384 by multiple dose short duration administration in MCAo model of stroke

Male SD rats of body weight 250-300g were used; MCA was occluded using 0.1% poly-l-lysine coated 3-0 suture (Ethilon, Johnson & Johnson) for 3 hours duration according to the procedure explained in the earlier sections. Jugular vein was cannulated, exteriorized from animal back for drug administration. Dose, time of drug administration and parameters measured are enumerated below (Table 16). MR imaging was used in this study for quantitative estimation of infarct and edema.

Table 16: Dose, treatment schedule and group details for efficacy of TRC051384 by multiple doses, short duration (4hr drug exposure) treatment in transient ischemia 3hr MCAo model of stroke

Groups	Dose	Dosing schedule	Group size (n)	Duration of transient ischemia + reperfusion	Parameters studied
Vehicle	Normal Saline, 1ml/kg	First dose beginning from 6 hrs, subsequent doses every 2 hrs to maintain drug exposure upto 10 hrs post MCAo, vehicle group received saline in similar fashion	8	3 hr+ 7 day	Penumbra recruitment into infarct core, Edema by MRI, Neurological scores and survivability
TRC051384	0.9 mg/kg as first dose, 0.45 mg/kg as subsequent dose by i.v. route		8	3 hr+ 7 day	

i.v., intra venous ; MCAo, middle cerebral artery occlusion; MRI, Magnetic resonance imaging

Magnetic resonance imaging (MRI) protocols for lesion area and edema quantification

Only animals which showed neurological deficit score (recorded ~45 min post MCAo) of at least 2 were included in the study and subjected to MRI scan. Diffusion-weighted (DW) images and T2-weighted (TWI) scans were acquired for each animal at 1, 3, 8, 12, 24, 36 and 48 hour post-initiation of MCAo (3 hours occlusion followed by 7 days reperfusion). Coronal T2-weighted images through the entire brain were obtained using a turbo spin-echo sequence. To map the apparent diffusion

coefficient (ADC) of water, diffusion-weighted images were acquired with a spin-echo sequence. ADC images were constructed from the diffusion-weighted images and were used to calculate the infarct area in each rat. Coronal sections covering the entire MCA supplied area were obtained and these sectioned images were used for the calculation of area of damage by drawing region of interest (ROI) using pixel intensity based measurements by means of image analysis software. The total brain area, ipsilateral area and lesion area on each coronal section of the multi-slice anatomical T2-weighted images and ADC images were determined manually by an investigator blinded to the experiment using SYNGO MR Image analysis software. ADC images were analyzed to quantify the lesion area while T2 images were analyzed for brain edema.

Neurological deficit assessment

Neurological deficit was recorded according to a 5 point score by investigator blinded to the treatment using following a previously described method (Longa et al.1989). Neurological behavior was assessed in the same animals that were used for measurement of infarction volume on MRI. All rats were neurologically examined after MCAo (intra ischemic period i.e. ~45 min post-initiation of ischemia once the animals have completely recovered from anesthesia) and post reperfusion at 3, 24, 48 hours and on day 7. Animals which had score of ~1 at first scoring time point (~45 min post MCAo) were not included in the study. Reduction in neurological deficit was assessed at 24 hours, 48 hours and on day 7 after MCAo.

4.3.6.1.3. Efficacy of TRC051384 by multiple dose long duration administration in MCAo model of stroke

Male SD rats of body weight 250-300g were used; MCA was occluded using 0.1% poly-l-lysine coated 3-0 suture (Ethilon, Johnson & Johnson) for 3 hours duration and reperfused for 7 days. Drug administration was done by i.p. route. Dose, time of drug administration and parameters measured are enumerated below (Table 17). MR imaging was used in this study for quantitative estimation of infarct and edema, recording of neurological scores were done in similar fashion as explained in above sections.

Table 17: Dose, treatment schedule and group details for efficacy of TRC051384 by multiple doses, long duration (2 day drug exposure) treatment in transient ischemia 3hr MCAo model of stroke

Groups	Dose (i.p. route)	Dosing schedule	Group size (n)	Duration of transient ischemia + reperfusion	Parameters studied
Vehicle	Normal Saline, 1ml/kg	First dose beginning from 4 hrs, subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo, vehicle group received saline in similar fashion	7	3 hr+ 7 day	Penumbra recruitment into infarct core, Edema by MRI, Neurological scores and survivability
TRC051384	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose by i.p. route		10	3 hr+ 7 day	

i.p., intra peritoneal; MCAo, middle cerebral artery occlusion; MRI, Magnetic resonance imaging

4.3.6.2. Efficacy studies in 2 hr transient MCAo model – Therapeutic time window and dose response

4.3.6.2.1. Efficacy of TRC051384 administered at 4 hrs after MCAo

Male SD rats of body weight 250-300g were used; MCA was occluded using 0.1% poly-l-lysine coated 3-0 suture (Ethilon, Johnson & Johnson) for 2 hours duration and reperfused for 7 days. Drug administration was done by i.p. route. Dose, time of drug administration and parameters measured are enumerated below (Table 18). MR imaging was used in this study for quantitative estimation of infarct and edema, recording of neurological scores were done in similar fashion as explained in above sections.

Table 18: Dose, treatment schedule and group details for efficacy of TRC051384 in multiple doses by i.p. route beginning from 4 hrs after 2hr MCAo

Groups	Dose (i.p. route)	Dosing schedule	Group size (n)	Duration of transient ischemia + reperfusion	Parameters studied
Vehicle	Normal Saline, 1ml/kg	First dose beginning from 4 hrs, subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo, vehicle group received saline in similar fashion	6	2 hr+ 7 day	Penumbra recruitment into infarct core, Edema by MRI, Neurological scores and survivability
TRC051384	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose		9	2 hr+ 7 day	

i.p., intra peritoneal; MCAo, middle cerebral artery occlusion; MRI, Magnetic resonance imaging

4.3.6.2.2. Efficacy of TRC051384 administered at 8 hrs after MCAo

Male SD rats of body weight 250-300g were used; MCA was occluded using 0.1% poly-l-lysine coated 3-0 suture (Ethilon, Johnson & Johnson) for 2 hours duration and reperfused for 7 days. Drug administration was done by i.p. route. Dose, time of drug administration and parameters measured are enumerated below (Table 19). MR imaging was used in this study for quantitative estimation of infarct and edema, recording of neurological scores were done in similar fashion as explained in above sections.

Table 19: Dose, treatment schedule and group details for efficacy of TRC051384 in multiple doses by i.p. route beginning from 8 hrs after 2hr MCAo

Groups	Dose (i.p. route)	Dosing schedule	Group size (n)	Duration of transient ischemia + reperfusion	Parameters studied
Vehicle	Normal Saline, 1ml/kg	First dose beginning from 8 hrs, subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo, vehicle group received saline in similar fashion	10	2 hr+ 7 day	Penumbra recruitment into infarct core, Edema by MRI & on day 7 by TTC, Neurological scores and survivability
TRC051384 (9.0/4.5mg/ kg)	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose		10	2 hr+ 7 day	
TRC051384 (4.5/2.25mg /kg)	4.5 mg/kg as first dose, 2.25 mg/kg as subsequent dose		10	2 hr+ 7 day	

i.p., intra peritoneal; MCAo, middle cerebral artery occlusion; MRI, Magnetic resonance imaging

TTC Staining

After 7 days of MCAo, all the surviving rats from vehicle and TRC051384 treatment groups (only 9.0/4.5mg/kg group) were killed and transcardially perfused with normal saline. The brains were removed immediately, sectioned coronally into seven slices of 2 mm thickness each covering the entire MCA supplied area, incubated in a 2% solution of TTC at 37^0C for 10 min, and then fixed by overnight immersion in a 4% buffered formalin solution. Each slice was scanned by a scanner for analysis of total infarct area, edema using scion image software (Scion, Frederick, MD) as mentioned in previous section.

4.3.6.3. Efficacy studies in permanent MCAo model

Male SD rats of body weight 250-300g were used; permanent MCAo was produced using 0.1% poly-l-lysine coated 3-0 suture (Ethilon, Johnson & Johnson). Drug administration was done by i.p. route. Dose, time of drug administration and parameters measured are enumerated below (Table 20). MR imaging was used in this study for

quantitative estimation of infarct and edema, recording of neurological scores were done in similar fashion as explained in above sections.

Table 20: Dose, treatment schedule and group details for efficacy of TRC051384 in multiple doses by i.p. route beginning from 4 hrs after permanent MCAo

Groups	Dose (i.p. route)	Dosing schedule	Group size (n)	Duration of permanent ischemia	Parameters studied
Vehicle	Normal Saline, 1ml/kg	First dose beginning from 4 hrs, subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo, vehicle group received saline in similar fashion	6	7 day	Penumbra recruitment into infarct core, Edema by MRI, Neurological scores and survivability
TRC051384	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose		9	7 day	

i.p., intra peritoneal; MCAo, middle cerebral artery occlusion; MRI, Magnetic resonance imaging

4.3.6.4. Efficacy studies in stroke with co-morbid conditions

Age matched old (24 to 26 week) Male SHR and SHR rats made diabetic with streptozotocin (STZ) injection were employed in this study. All animals allowed to get acclimatized with the laboratory condition and presented standard pelleted food and water *ad libitum* during acclimatization period.

Procedure for measurement of Blood pressure

Blood pressure was measured non-invasively (NIBP) using tail cuff method. Animals were kept restrained and kept acclimatized for 30 minutes before the reading was taken on each day. Before measurement on each day the instrument was switched on for 15 to 30 min to gain set temperature of 37^{o}C. The instrument had 6 channels so at a time 6 animals could be placed and BP recording could be obtained simultaneously for all the 6 rats. For each animal the BP was measured on 3 consecutive days before the day of surgery. On each day of recordings, 5 readings were obtained for each animal and data from day 2 and day 3 are averaged and was expressed as the mean BP for the respective animal.

Procedure for induction of diabetes

Animals were kept for 12 hrs fasting one day prior to STZ injection. Body weight was recorded for each animal. Streptozotocin (in 4.5 pH Citrate buffer), STZ injection was made to a group of SHR rats at dose 40 mg/kg by i.p route. Fasting (12 hrs) plasma glucose levels were measured for each animal at basal, on day 3, day 7, day 21 and one day prior to MCAo. Blood samples for estimation of fasting glucose levels were collected by sublingual root. MCAo was performed on 8 week diabetic rats.

Surgical induction of stroke and assessment of efficacy

MCAo was produced using intra luminal suture (0.1% poly-l-lysine coated 3-0 suture (Ethilon, Johnson & Johnson)) according to procedure as mentioned earlier. The duration of ischemia produced and reperfusion time, dose and duration of treatment with TRC051384 were enumerated below (Table 21). Neurological scores were recorded at 30 min, 24hr, and 48 hr and just before termination post MCAo. Efficacy assessment was carried out using TTC stained brain slices.

Table 21: Dose, treatment schedule and group details for efficacy of TRC051384 in transient MCAo model with co-morbid conditions.

Groups	Dose (i.p. route)	Dosing schedule	(n)	Model transient ischemia + reperfusion	End points
SHR-Veh	Normal Saline, 1ml/kg	First dose beginning from 8 hrs, subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo, vehicle group received saline in similar fashion	5	2 hr+7 day	Infarct by TTC method Neurological scores and survivability
SHR-TRC051384	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose		6	2hr+7 day	
SHR-D-Veh	Normal Saline, 1ml/kg		6	0.5hr+4 day	
SHR-D-TRC051384	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose		5	0.5hr+4 day	

i.p., intra peritoneal; MCAo, middle cerebral artery occlusion; TTC, Triphenyl tetrazolium chloride

4.3.7. Mechanistic studies

Male SD rat age (10-12 week) and body weight (250-300gm) matched animals were employed for these studies. A group of 22 rats were subjected to MCAo and 3 rats were sham operated and were used in BBB permeability studies. MCAo was produced using intra luminal suture (0.1% poly-l-lysine coated 3-0 suture (Ethilon, Johnson & Johnson)) according to procedure as mentioned earlier. The duration of ischemia produced and reperfusion time, dose and duration of treatment with TRC051384 were enumerated below (Table 22). Neurological scores were recorded for each animal at 45 min post MCAo in order to confirm successful occlusion.

Table 22: Dose, treatment schedule and group details for mechanistic studies of TRC051384

Study	Groups (group size)	Dose	Dosing schedule	Model (transient ischemia + reperfusion)
BBB permeation	Sham + TRC051384 (3)	6mg/kg, infusion into ipsilateral internal carotid artery, using infusion pump	Slow infusion over 1 minute at 6 hours post MCAo or sham operation	**NA**
	TRC051384 (3)			**2hr + 4 hr**
IHC for HSP70	Vehicle (3)	Normal Saline, 1ml/kg, i.p	First dose beginning from 4 hrs, subsequent doses every 2 hrs to maintain drug exposure upto time as per the study protocol post MCAo, vehicle group received saline in similar fashion	**2hr + 14hr**
	TRC051384 (3)	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose by i.p.		
Gene macro array	Vehicle (3)	Normal Saline, 1ml/kg, i.p		**2hr + 8hr**
	TRC051384 (3)	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose by i.p.		
TUNEL staining	Vehicle (3)	Normal Saline, 1ml/kg, i.p		**2hr + 46hr**
	TRC051384 (3)	9.0 mg/kg as first dose, 4.5 mg/kg as subsequent dose by i.p.		

i.p., intra peritoneal; MCAo, middle cerebral artery occlusion

Procedure for sham operation

Sham control groups were used to nullify the impact of ECA occlusion and surgical stress on stroke out come. The surgery procedure was same as for the MCAo animals except that the suture was not inserted deep in to the ICA. Instead it was kept at the ECA-ICA stump for one minute and further withdrawn.

4.3.7.1. BBB Permeability studies

Fifteen minutes after slow infusion of TRC051384 into the internal carotid artery ipsilateral to the occluded vessel, blood was collected from each animal from both sham operated and MCAo animals. Brain were perfused with 100 ml of sterile heparinized PBS (pH 7.4) using an infusion pump set at 800ml/hr flow rate. Both hemispheres were harvested and immediately stored in -70^{0}C deep freezer until analyzed for TRC051384 content. Plasma was separated from collected blood, plasma, ipsilateral and contralateral brain hemispheres were analyzed for TRC051384 content using LC-MS/MS in bioanalytical department.

4.3.7.2. Gene macro array studies

Injured hemispheres from vehicle treated animals and TRC051384 treated animals were collected at 10-hour post-initiation of tMCAo. Total RNA from each brain sample was extracted followed by cDNA preparation. Each sample of cDNA was analyzed on customized Taqman inflammation array employing ABI 7900 HT Fast- Real-Time PCR system. Taqman inflammation array comprised of a panel of 48 genes functionally associated with inflammation, stress response and apoptosis.

4.3.7.3. HSP70 Immunohistochemistry and TUNEL staining

At 16 hours after onset of stroke all rats were transcardially perfused with 4% paraformaldehyde in phosphate buffer saline (PBS) under halothane anesthesia and brains were isolated. 6 µm thick cryo-sectioned coronal slices were immunostained using primary mouse anti-HSP70 monoclonal antibody (SPA-810, Stressgen) and secondary rat absorbed biotinylated anti-mouse immunoglobulin (BA-2001, Vector). Control sections were incubated with normal serum instead of primary antibody and showed no immunoreactive product. Detailed method for HSP70 immunohistochemistry is described in appendix (*Appendix-IV*) Rat brains after deparaffinization were used for TUNEL staining. Apoptotic DNA fragment were visualized by ApopTag ® Peroxidase *In*

situ apoptosis detection kit (Millipore, S7100).Detailed method for the same is described in appendix (*Appendix V*)

Statistical analysis

Results of all in-vitro studies are expressed as mean±SD and the statistical significance of the results were assessed by two-tailed Student's t-test. The results of all animal studies unless stated are graphically represented as mean±SEM. Improvement in neurological score was assessed using RMANOVA with first score values as a covariate. Difference between two groups from in-vivo studies was assessed using non-parametric Wilcoxon test. Proportions of improved responses on survival data on a particular day was analyzed using chi square tests. In all studies p value <0.05 was considered as statistically significant. Statistical analysis has been performed using statistical analysis system (SAS Version-9.1).

Results

5 Results

5.1 In-vitro studies

5.1.1. HSP70B mRNA induction in HeLa and primary mixed neurons

5.1.2. HSF-1 activation and in-vitro chaperone activity

5.1.3. In-vitro potentiation of endogenous stress response

5.1.4. Inhibition of TNF- α expression in human THP-1 cell line

5.2 In-vivo studies

5.2.1 Standardization of middle cerebral artery occlusion (MCAo) model of stroke in rats

5.2.1.1 Surgical procedure for the occlusion of middle cerebral artery occlusion

5.2.1.2 Procedure for recording neurological deficit (Neurological score)

5.2.1.3 Procedure for the quantification of infarct and edema.

5.2.1.4 Identification/selection of intraluminal suture diameter

5.2.1.4.1 Identification of optimum suture diameter

5.2.1.4.2 Standardization of poly-l-lysine concentration to be used for coating intraluminal suture

5.2.1.5 Effect of varying duration of ischemia (MCAo) on model characteristics

5.2.1.6 Study of temporal profile of brain injury and neurological deficit in rats subjected to transient MCAo.

5.2.1.7 Effect of permanent MCAo on model characteristics

5.2.2 A Feasibility study on non-invasive imaging and quantification of infarct in rat brain using clinical magnetic resonance scanner

5.2.2.1 MRI feasibility studies for imaging rat brain

5.2.2.2 Temporal profile of brain injury (Infarct) in transient ischemia model of stroke by MRI

5.2.3 Validation of MCAo model of stroke for evaluating the efficacy of neuroprotective agent.

5.2.3.1 Validation of transient ischemia model

5.2.3.2 Validation of permanent ischemia model

5.2.4 Pharmacokinetic profiling of TRC051384 in rat

5.2.5 Acute safety studies with TRC051384 in rat

5.2.6 In-vivo efficacy studies of TRC051384 in MCAo model of stroke

5.2.6.1 Efficacy studies in 3 hr transient MCAo model

5.2.6.1.1 Efficacy of TRC051384 by single dose administration in MCAo model of stroke

5.2.6.1.2 Efficacy of TRC051384 by multiple dose short duration administration in MCAo model of stroke

5.2.6.1.3 Efficacy of TRC051384 by multiple dose long duration administration in MCAo model of stroke

5.2.6.2 Efficacy studies in 2 hr transient MCAo model – Therapeutic time window and dose response

5.2.6.2.1 Efficacy of TRC051384 administered at 4 hrs after MCAo

5.2.6.2.2 Efficacy of TRC051384 administered at 8 hrs after MCAo

5.2.6.3 Efficacy studies of TRC051384 in permanent MCAo model

5.2.6.4 Efficacy studies in stroke with co-morbid conditions

5.2.7 Mechanistic studies

5.2.7.1 BBB Permeability studies

5.2.7.2 Gene macro array studies

5.2.7.3 HSP70 Immunohistochemistry and TUNEL staining

5.1. In-vitro studies

5.1.1. HSP70B mRNA induction in HeLa and primary mixed neurons

Induction of HSP70B mRNA was carried out separately with the indicated dose(s) of TRC051384 for 4 hours duration in HeLa cell line and rat primary mixed neurons. TRC051384, dose dependently induced HSP70B mRNA by several hundred folds compared to vehicle control in both HeLa (Figure. 1) and rat primary mixed neurons (Figure. 2).

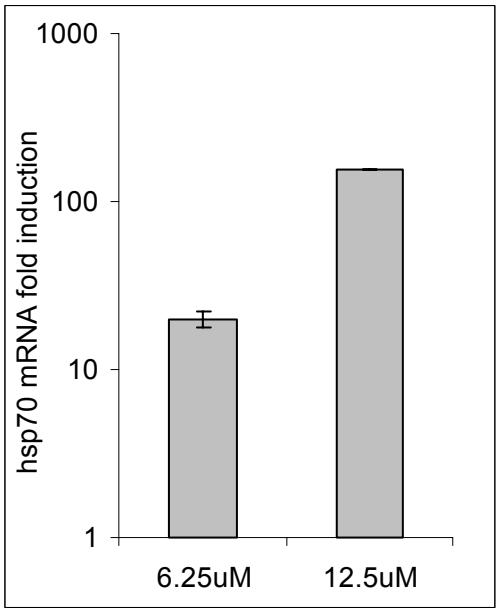

Figure 1: Induction of HSP70B mRNA in HeLa cells with 4 hours incubation with different doses of TRC051384 as compared to vehicle control. Values are expressed as mean±SD.

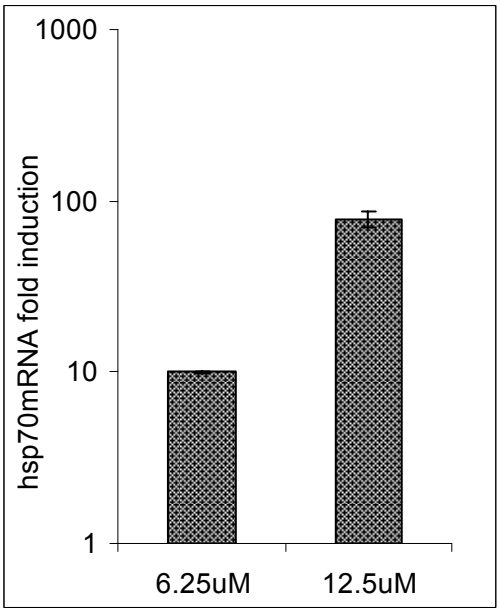

Figure 2: Induction of HSP70B mRNA expression in primary mixed neurons (cultured from neonatal rat cerebellum) with 4 hours incubation with different doses of TRC051384 as compared to vehicle control. Values are expressed as mean±SD.

5.1.2. HSF-1 activation and in vitro chaperone activity

Treatment with TRC051384 resulted in significant dose-dependent increase in HSF1 transcriptional activity (Figure 3a). In HeLa cells expressing firefly luciferase, inactivation of luciferase by heat shock and its subsequent recovery in absence and presence of TRC051384 was monitored. The luciferase activity for each condition gives an idea about the extent of functional luciferase protein. Treatment with TRC051384 resulted in significant recovery of luciferase activity as compared to vehicle treated cells (Figure 3b).

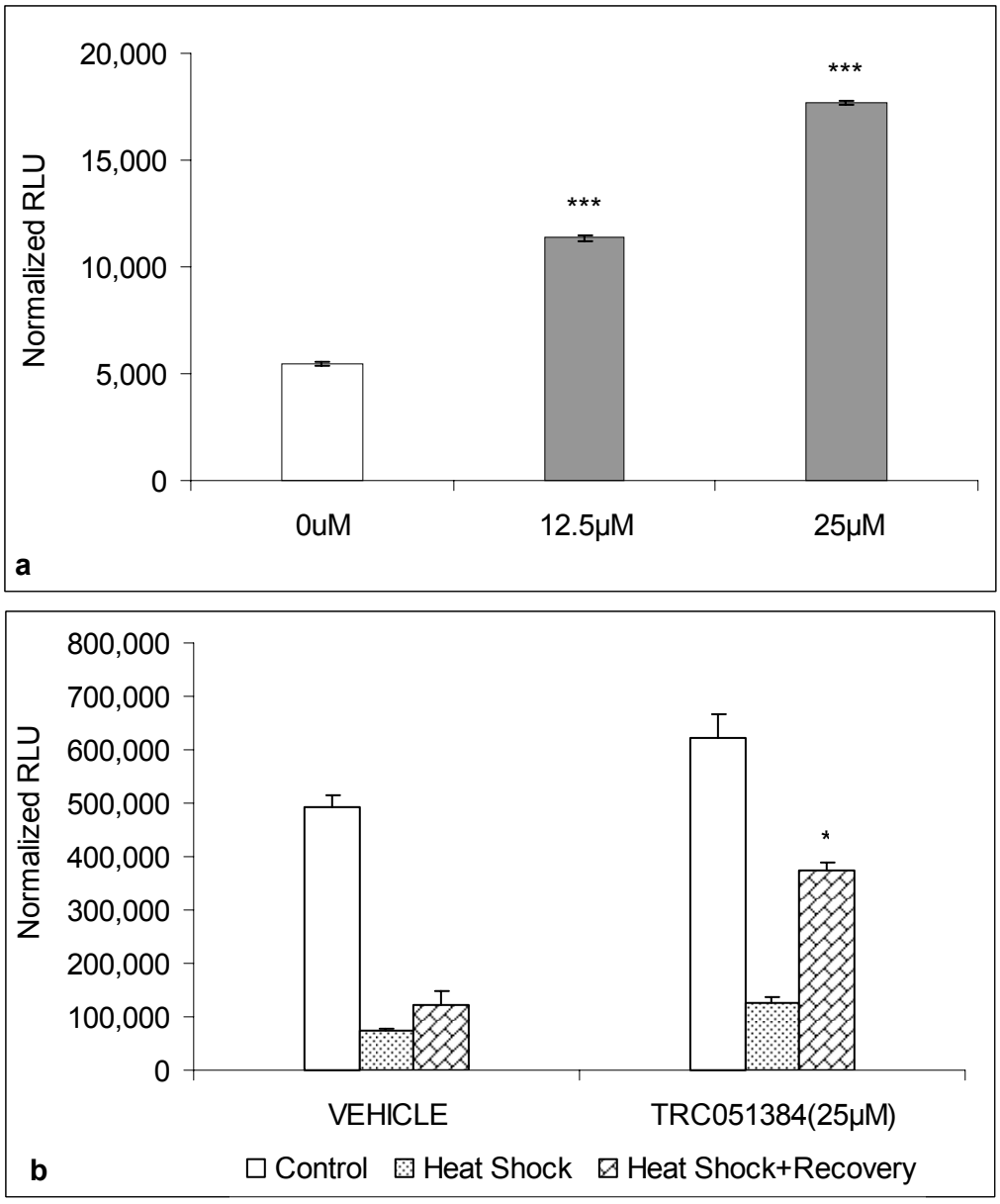

Figure 3: (a) Transcriptional activation of HSF1 by treatment with TRC051384. (b) Enhancement of in-vitro chaperone activity on treatment with TRC051384 compared with vehicle treated cells when subjected to heat shock followed by recovery. Values are expressed as mean±SD, ***p < 0.001 versus * p<0.05 vs vehicle/no treatment group.

5.1.3. In-vitro potentiation of endogenous stress response

Potentiation of endogenous stress response by TRC051384 treatment was monitored in HeLa cells. The cells were treated with TRC051384 immediately after the heat shock for indicated durations. Induction of HSP70 mRNA was monitored as an endogenous stress response. Treatment with TRC051384 along-with heat shockresulted in synergistic effect on HSP70 mRNA induction at all time-points studied (Figure 4).

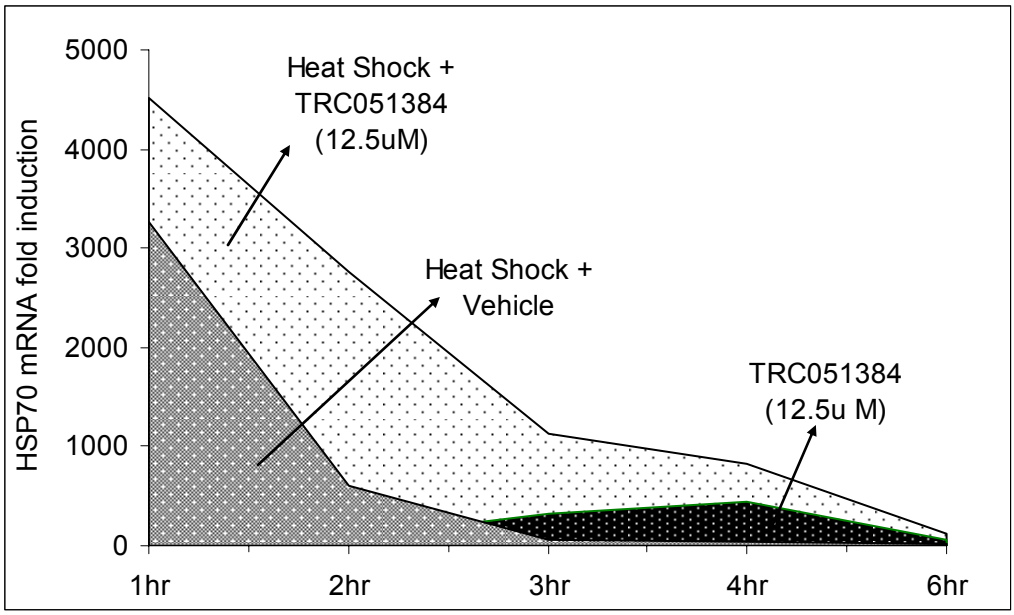

Figure 4: In-vitro potentiation of endogenous stress response by treatment with TRC051384

5.1.4. Inhibition of TNF- α expression in human THP-1 cell line

Differentiated THP-1 cells were stimulated with lipopolysaccharide (LPS) to induce TNF-α expression. Treatment with TRC051384 resulted in significant dose-dependent inhibition i.e. 60% inhibition ($p < 0.05$) at 6.25 µM and 90% inhibition ($p < 0.01$) at 12.5 µM of LPS-induced TNF- α expression in differentiated THP-1 cell line (Figure 5).

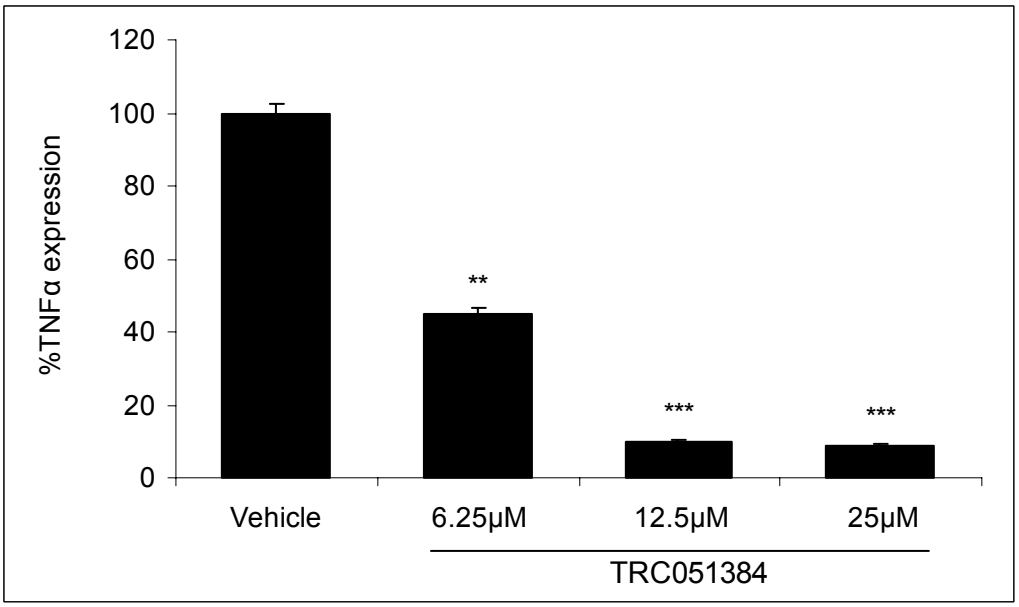

Figure 5: Inhibition of expression of LPS-induced TNF-α mRNA by treatment with TRC051384 in differentiated THP-1 cells. Values are expressed as mean ±SD. **p < 0.01; ***p < 0.001 versus vehicle/no treatment group.

5.2. In-vivo studies

5.2.1 Standardization of MCA occlusion (MCAo) model of stroke in rats

MCAo in rats is the most widely used model for studies of transient and permanent ischemic stroke. Several variations pertaining to the use of intraluminal suture for occluding MCA are reported. They include use of different diameter size, with or without coating and with or without heat made bulb in order to increase success rate, consistency and reduce mortality. We initially identified the optimum suture characteristics that were required to produce consistent cerebral injury which was sufficient enough to produce long term neurological deficit so effect of therapeutic agent can be evaluated.

5.2.1.1. Surgical procedure for the occlusion of middle cerebral artery occlusion

The technique of MCAo was successfully standardized in our laboratory. The surgical method for was practiced to result into successful occlusion in 75-80% of the animals. We had very few failure rates in these studies.

5.2.1.2. Procedure for recording neurological deficit (Neurological score)

The five point scoring method for the evaluation of neurological deficit was successfully standardized. In all our studies neurological assessment was carried out by a person who was blinded to the treatment groups.

5.2.1.3. Procedure for the quantification of infarct and edema.

The procedure for the quantification of infarct and edema using brain slices stained with TTC was successfully standardized. Staining of brain slices with 2% TTC solution yielded us the best contrast for easy visualization of infarct and edema. Quantification of infarct and edema was made using computer based image analysis software (Scion Image).

5.2.1.4. Identification/selection of intraluminal suture

5.2.1.4.1. Identification of optimum suture diameter

For the purpose of identification of suture diameter we employed 4 different sutures. Initially we kept the duration of ischemia constant i.e. we produced 3

hrs of transient ischemia and than reperfused for 21 hrs. The results of the effect of different sutures on infarct (Figure 6), edema (Figure 7), neurological deficit (Figure 8) and mortality (Figure 9) are depicted below.

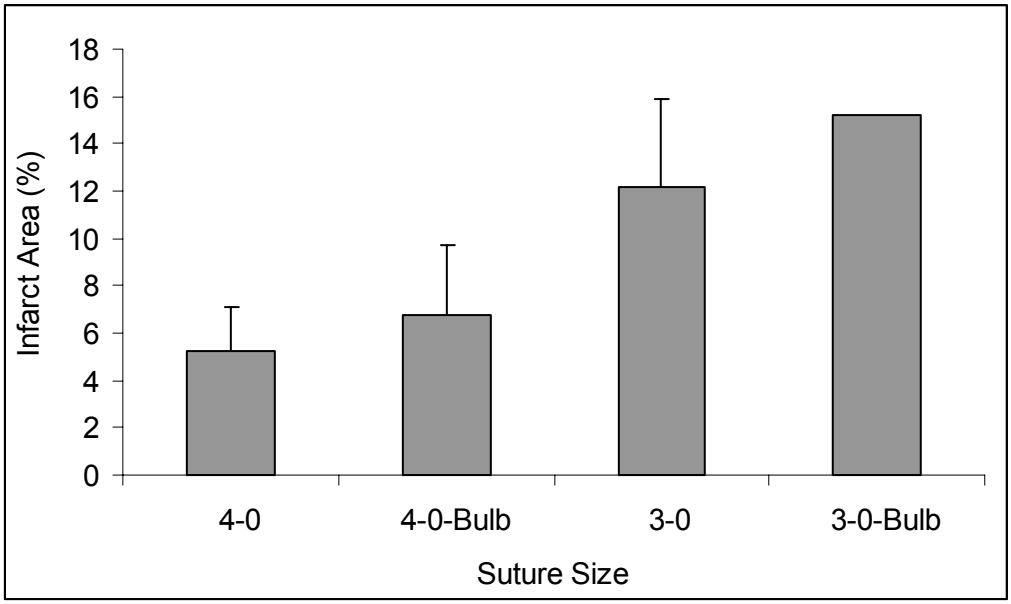

Figure 6: Effect of intraluminal suture of different diameter on infarct outcome measured at 24 hrs in transient 3hr MCAo model in rats. A bulb is made at the tip of the suture using flame heat to increase the diameter. Values represent Mean± SEM.

Suture sized 4-0 having smaller diameter has produced small infarct area (expressed as % of total brain area) 5.2% (5.2±1.9) & 6.8% (6.8±2.9) without bulb and with bulb respectively. Similarly 3-0 suture has produced relatively large infarct area i.e. 12.15% (12.15±3.8) and 15.2% (15.2±0) without bulb and with bulb respectively.

Similarly brain edema produced with 4-0 suture was 3.4% (3.4±1.6) and 4.1% (4.1±2.9) without bulb and with bulb respectively. Edema produced with 3-0 suture was relatively more as compared to 4-0 sutures. Brain edema was quantified to be 7.2% (7.2±3.9) and 15% without bulb and with bulb respectively.

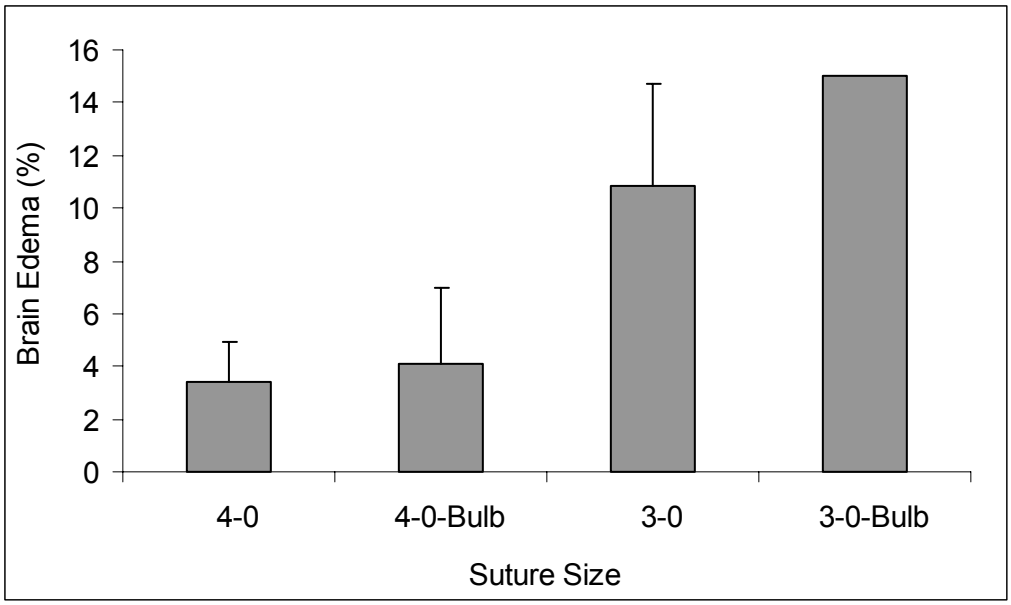

Figure 7: Effect of intraluminal suture of different diameter on brain edema measured at 24 hrs in transient 3hr MCAo model in rats. A bulb is made at the tip of the suture using flame heat to increase the diameter. Values represent Mean± SEM..

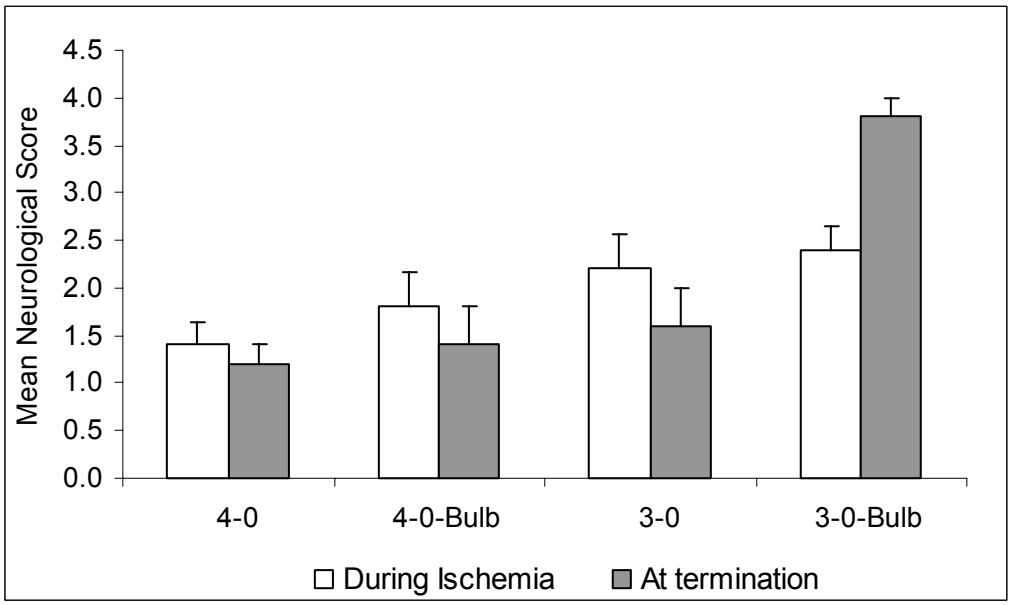

Figure 8: Effect of intraluminal suture of different diameter on neurological deficit measured at 24 hrs in transient 3hr MCAo model in rats. A bulb is made at the tip of the suture using flame heat to increase the diameter. Error bars represent ± SEM.

As for as neurological score was concerned, occlusion with 3-0 suture resulted into more severe neurological deficit as compared to 4-0 suture at all recording time points. We observed a very severe deficit with 3-0 suture with bulb (Figure 9). Mortality was observed when occlusion was made with 3-0 suture, 20% and 80% mortality was observed when MCA was occluded without bulb and with bulb respectively.

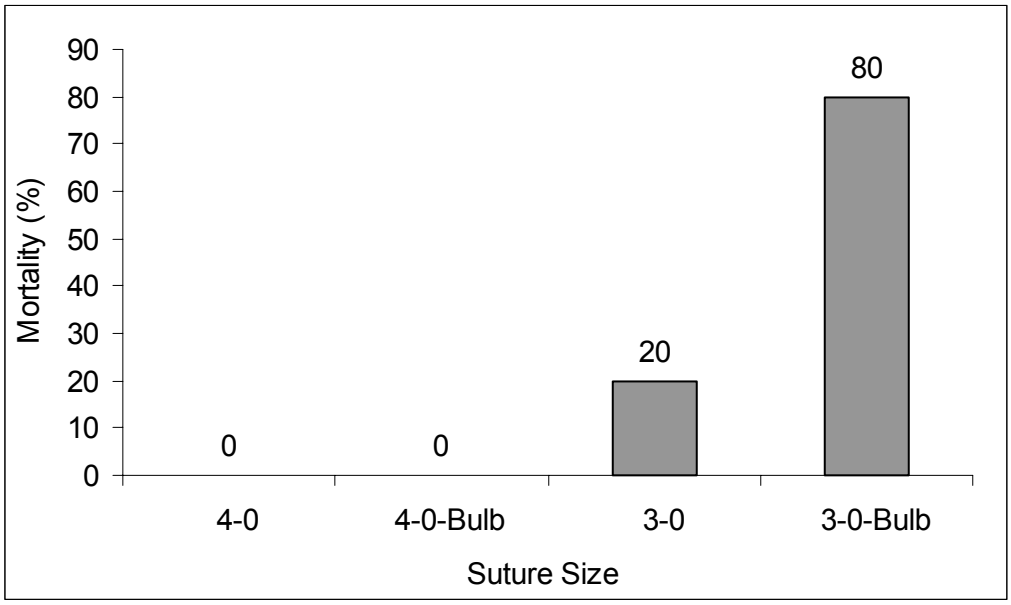

Figure 9: Effect of intraluminal suture of different diameter on mortality measured at 24 hrs in transient 3hr MCAo model in rats. A bulb is made at the tip of the suture using flame heat to increase the diameter.

5.2.1.4.2. Standardization of poly-l-lysine concentration to be used for coating intraluminal suture

From the initial studies we identified 3-0 suture produced larger infarct and edema, we employed 3-0 suture without bulb for further optimization of vessel occluder. Two different concentrations 0.01% and 0.1% of poly-l-lysine solutions were used for coating 3-0 sutures. We kept the duration of ischemia constant i.e. we produced 3 hrs of transient ischemia and than reperfused for 21 hrs. We compared effect of ploy-l-lysine coating on different parameters such as infarct (Figure 10), edema (Figure 11) and neurological deficit (Figure 12) with results produced using uncoated 3-0 sutures from previous study.

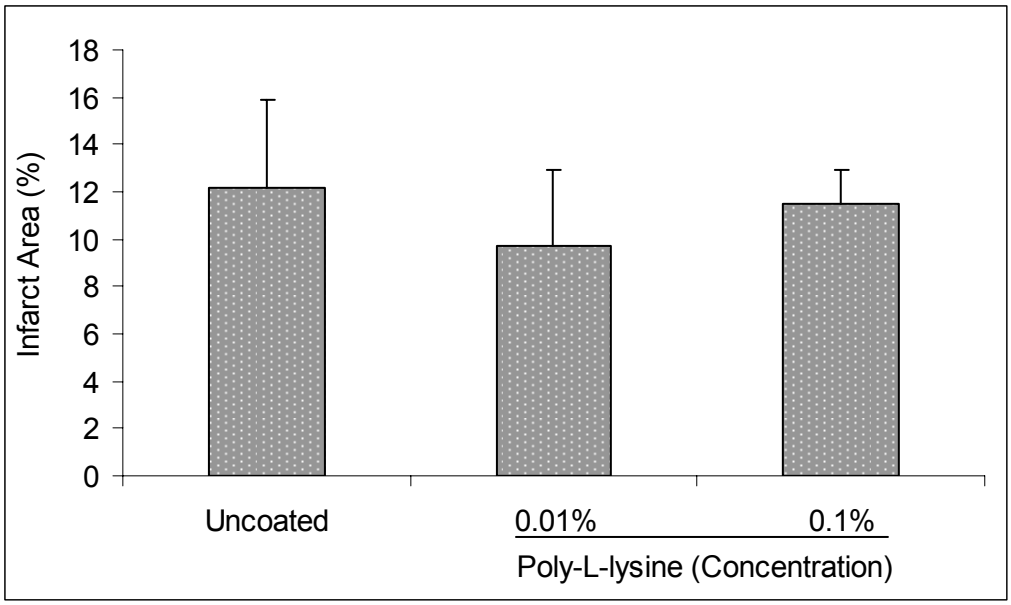

Figure10: Effect of intraluminal suture of different coating properties on infarct outcome measured at 24 hrs in transient 3hr MCAo model in rats. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated without and with different concentrations of poly-l-lysine solution were used. Values represent Mean± SEM.

Out of these experiments we found that MCAo with 3-0 suture coated with 0.1% poly-l-lysine resulted into more consistent infarct, edema and neurological deficit. We obtained % infarct area of about 13.07±1.47 with 3-0 suture coated with 0.1% poly-l-lysine as compared to 9.76±3.14 and 12.15±3.8 with 3-0 suture coated with 0.01% poly-l-lysine and uncoated sutures respectively.

Similarly brain edema produced about 6.11±1.25 with 3-0 suture coated with 0.1% poly-l-lysine as compared to 7.22±3.27 and 7.2±3.9 with 3-0 suture coated with 0.01% poly-l-lysine and uncoated sutures respectively.

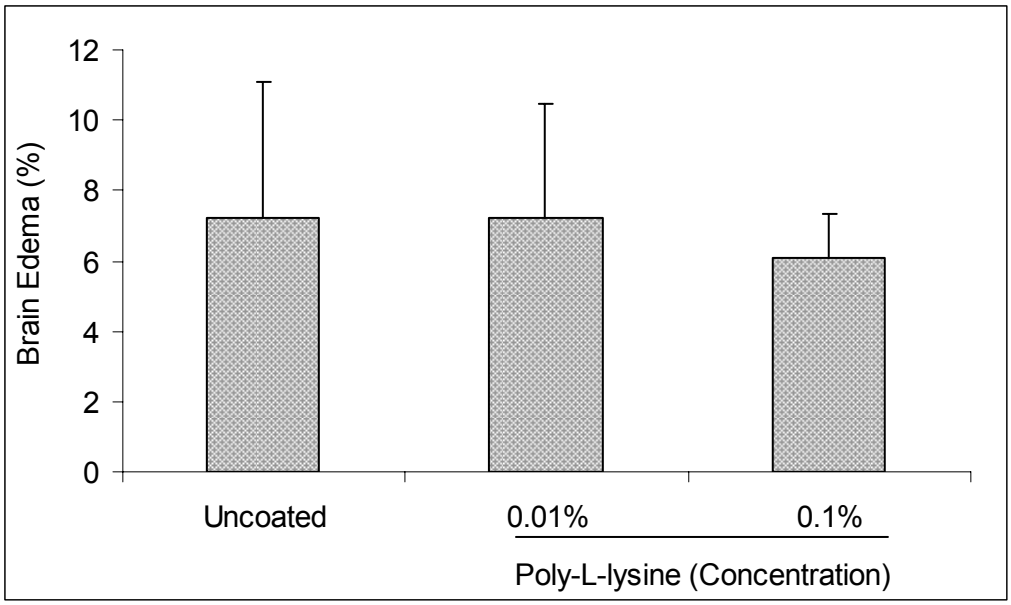

Figure11: Effect of intraluminal suture of different coating properties on brain edema measured at 24 hrs in transient 3hr MCAo model in rats. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated without and with different concentrations of poly-l-lysine solution were used. Values represent Mean± SEM.

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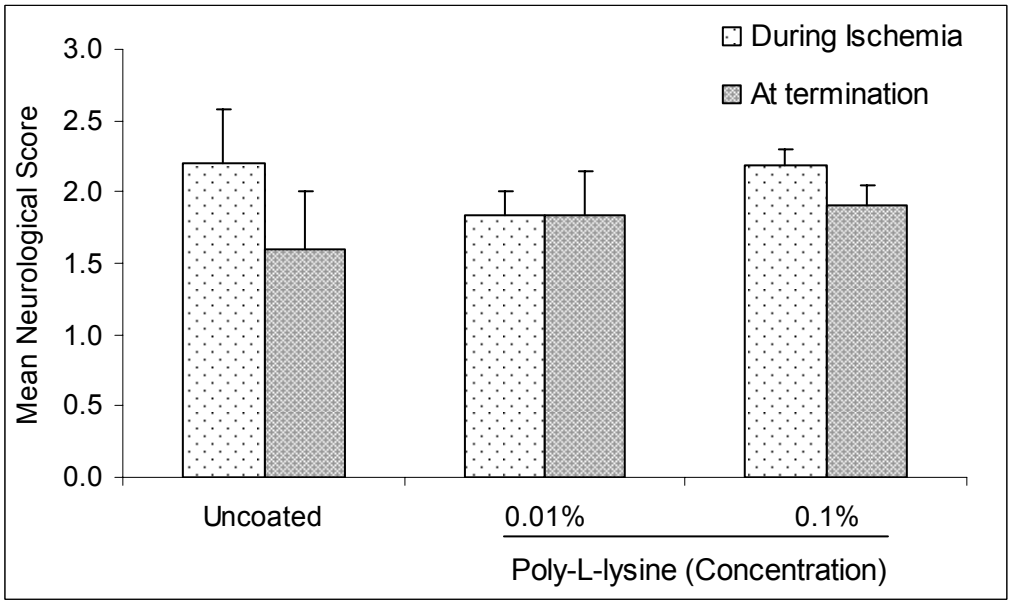

Figure12: Effect of intraluminal suture of different coating properties on neurological deficit measured at 24 hrs in transient 3hr MCAo model in rats. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated without and with different concentrations of poly-l-lysine solution were used. Error bars represent ± SEM.

As for as neurological score was concerned, we obtained more consistent deficit with score of 2.18±0.12 and 1.91±0.14 at 45 min and 24 hrs after MCAo with 3-0 suture coated with 0.1% poly-l-lysine. 3-0 suture coated with 0.01% poly-l-lysine and uncoated sutures produced more variable neurological deficits.

5.2.1.5. Effect of varying duration of ischemia (MCAo) on model characteristics

Once we identified the optimum suture diameter and concentration of poly-l-lysine coating solution we then aimed at effect of varying duration of ischemia on transient ischemia model outcome in rats. For this we subjected group of rats to 2hrs, 3 hrs and 4 hrs ischemia using 0.1% poly-l-lysine coated 3-0 sutures without bulb and these animals rats were reperfused for 22hrs, 21hrs and 20hrs respectively. Effect of different durations of ischemia on various parameters is depicted below.

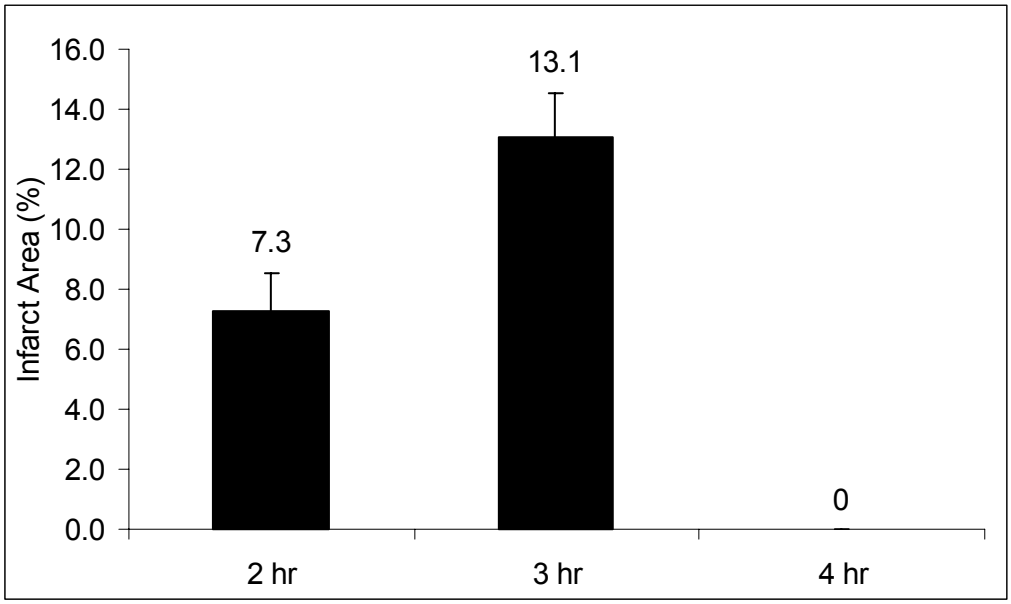

Figure13: Effect of different durations of transient MCAo on infarct outcome measured at 24 hrs using 2mm thick TTC stained brain slices in rats. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated with 0.1% concentration of poly-l-lysine solution was used. Values represent Mean± SEM.

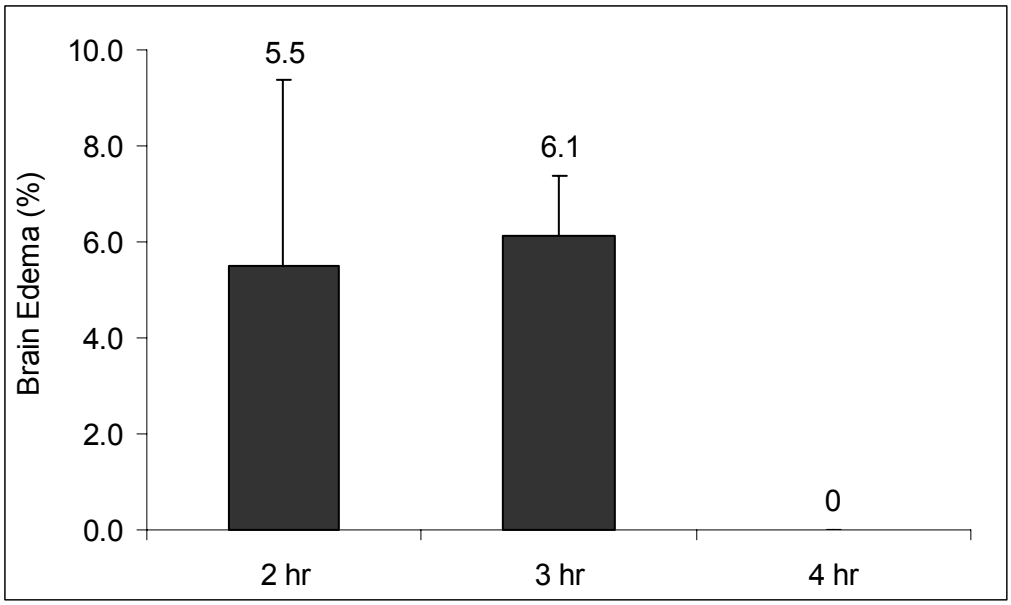

Figure14: Effect of different durations of transient MCAo on brain edema measured at 24 hrs using 2mm thick TTC stained brain slices in rats. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated with 0.1% concentration of poly-l-lysine solution was used. Values represent Mean± SEM.

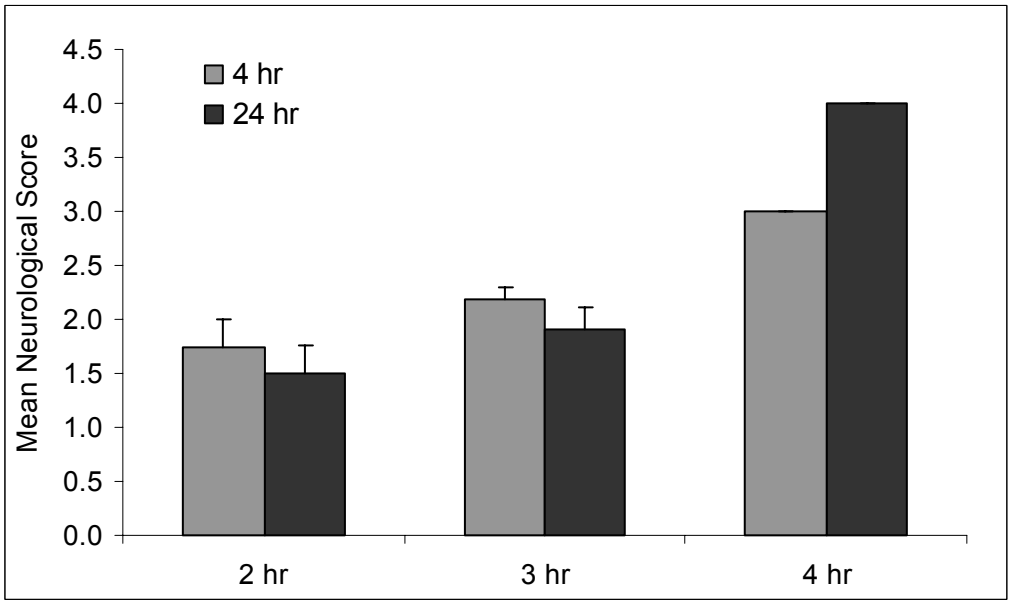

Figure15: Effect of different durations of transient MCAo on neurological score measured at 4 hrs & 24 hrs after MCAo. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated with 0.1% concentration of poly-l-lysine solution was used. Error bars represent ± SEM.

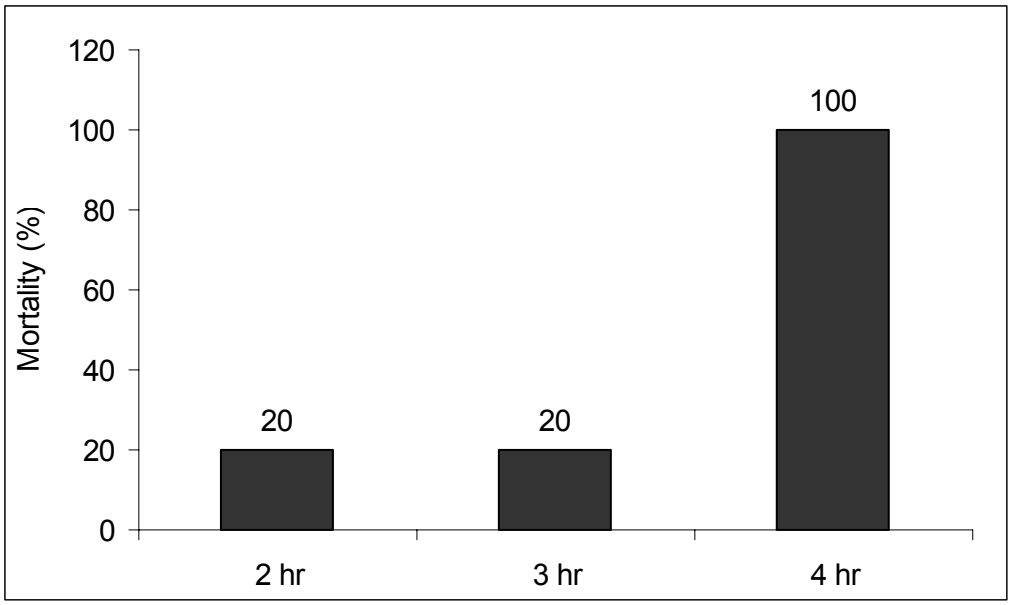

Figure16: Effect of different durations of transient MCAo on mortality measured at 24 hrs after MCAo. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated with 0.1% concentration of poly-l-lysine solution was used.

Out of these experiments we found that 4hrs ischemia produced very severe brain injury with none of the animals surviving 24hrs study period (Figure 16). MCAo for 3 hours period resulted into more consistent infarct (Figure. 13), edema (Figure 14) and neurological deficit (Figure 15) as compared to 2 hrs transient ischemia.

5.2.1.6. Study of temporal profile of brain injury and neurological deficit in rats subjected to transient MCAo.

Having established the optimum suture requirements and duration of ischemia to produce significant brain injury, we further have evaluated the temporal profile of brain injury (infarct and edema) to characterize the time at which peak infarct and edema occurs when MCAo was made for 3hrs duration using 0.1% poly-l-lysine coated 3-0 suture without bulb.

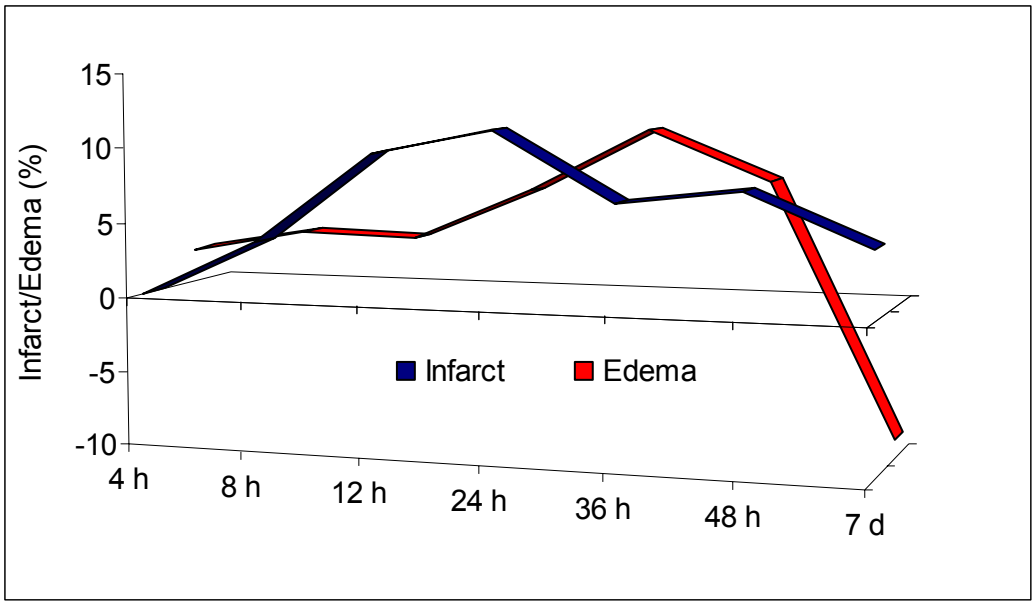

Figure17: Temporal profile of infarct and edema development measured from terminally sacrificed animals using TTC stained brain slices after 3 hr transient ischemia and reperfused to various time points till 7 days in rats. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated with 0.1% concentration of poly-l-lysine solution was use to occlude MCA.

Infarct and edema progression (Figure 17) was monitored by intermittent sacrifice of animals and we observed significant infarct has been developed by 12hrs post MCAo and by 24 hr peak infarct develops. Similarly peak edema was observed by 36hrs post MCAo. Both cerebral infarct and brain edema tend to resolve by 7 day observation period. Neurological deficit (Figure 18) was also monitored and we found that initially during ischemia period i.e. 1 hr post MCAo the deficit scores were higher which tend to reduce once the reperfusion of the occluded artery was initiated. Never the less there was still a significant deficit which persisted till 7 days study period.

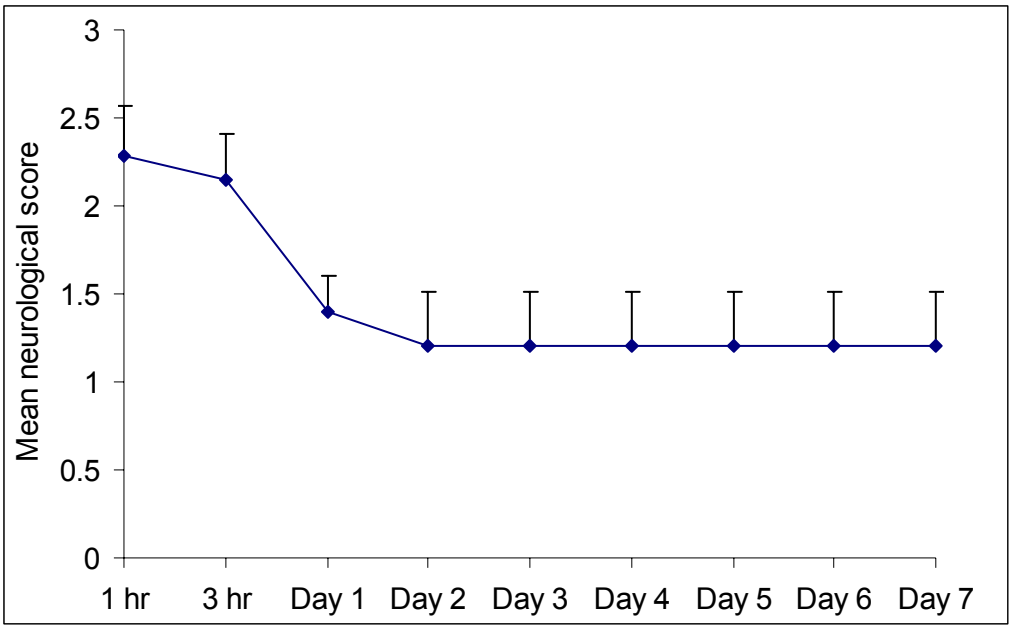

Figure 18: Temporal profile of neurological deficit development measured at indicated time points after 3 hr transient ischemia and reperfused to various time points till 7 days in rats. Error bars represent ± SEM.

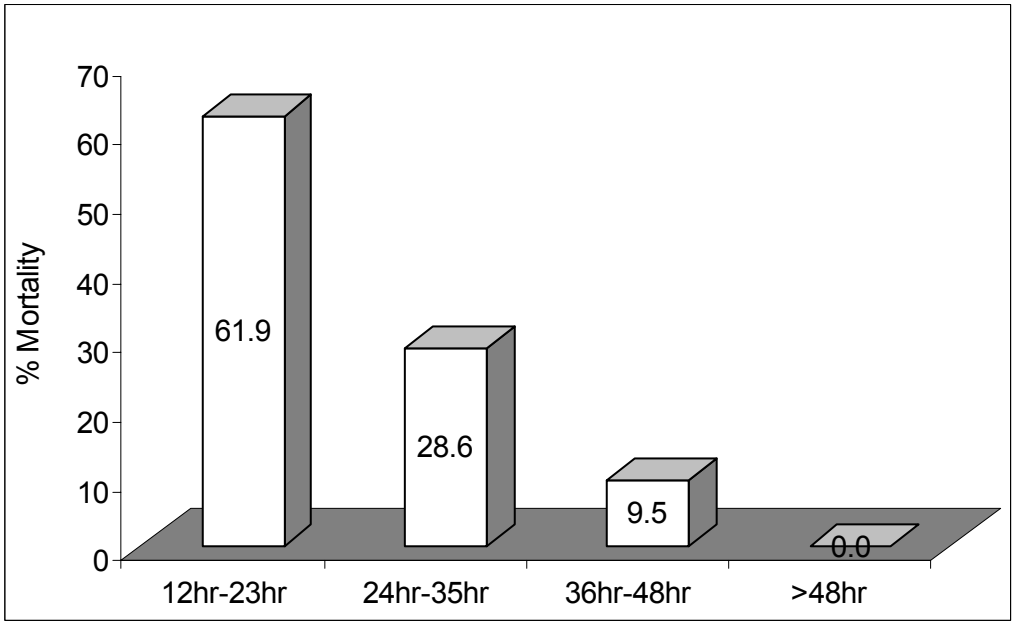

Figure 19: Effect on mortality at different intervals over 7 days period after 3 hr MCAo and reperfusion.

Mortality was also observed (Figure 19) in this study and we found an overall morality of 20% by day 7. Sub analysis of mortality data revealed that out of this 20%, ~62% mortality has occurred within the first 24hrs of initiation of MCAo, ~29% mortality by 36hrs and ~10% mortality by 48hrs post MCAo. There after till 7 days there was no mortality observed.

5.2.1.7. Effect of permanent MCAo on model characteristics

When rats were subjected to permanent MCAo using different sutures i.e. 4-0 and 3-0 with and without coating, we found significantly severe brain damage i.e. large infarct area (Figure 20), more severe brain edema (Figure 21) and neurological deficit (Figure 22) in these studies. Occlusion using 0.1% poly-l-lysine coated 4-0 sutures resulted into 9% (9.0±3.0) area of infarction, 18.4% (18.4±3.9) infarct area with 3-0 uncoated sutures and 20.9% (20.9±1.3) with 0.1% poly-l-lysine coated 3-0 sutures.

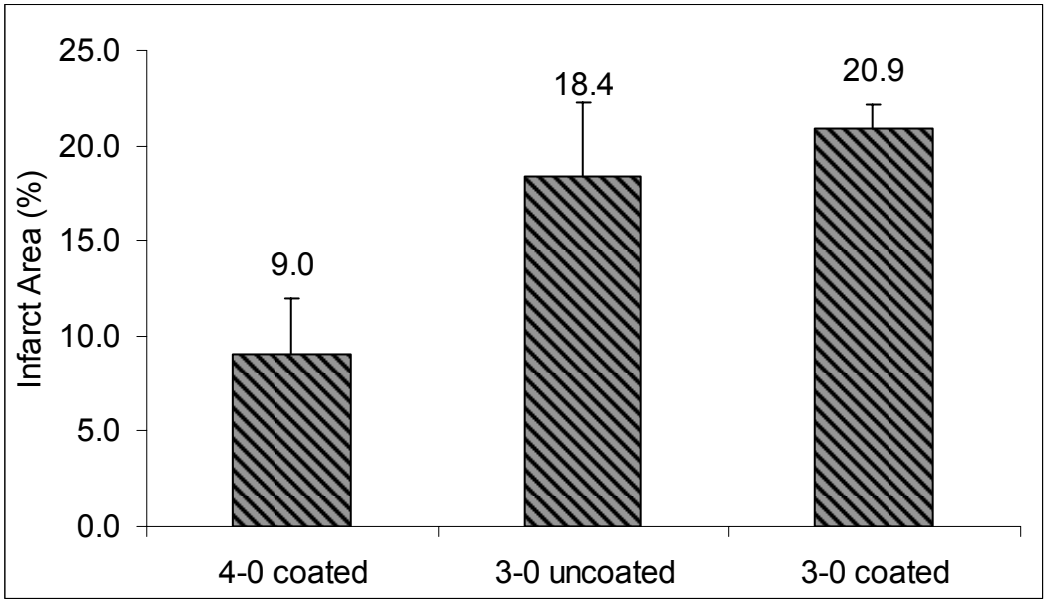

Figure 20: Effect of intraluminal suture of different diameter on infarct outcome measured at 24 hrs after permanent MCAo in rats. 4-0 and 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated without and with 0.1% poly-l-lysine solution were used. Values represent Mean± SEM.

Similarly brain edema using 0.1% poly-l-lysine coated 4-0 sutures resulted into 5.3% (5.3±1.5), 6.9% (6.9±1.5) with 3-0 uncoated sutures and 9.1% (9.1±1.0) with 0.1% poly-l-lysine coated 3-0 sutures.

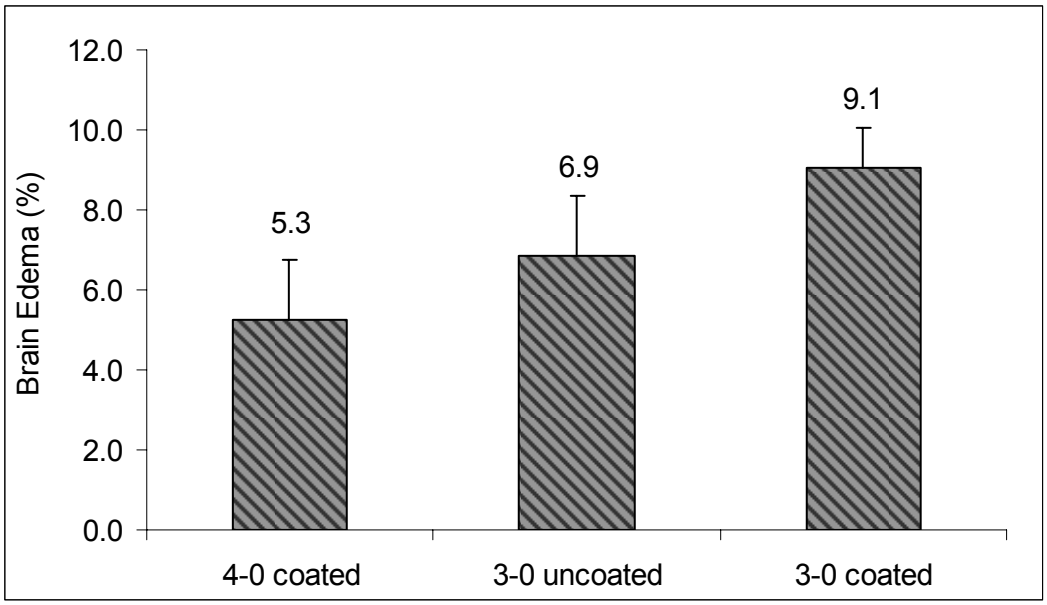

Figure 21: Effect of intraluminal suture of different diameter on brain edema measured at 24 hrs after permanent MCAo in rats. 4-0 and 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated without and with 0.1% poly-l-lysine solution were used. Values represent Mean± SEM.

As for as neurological score was concerned, occlusion with 0.1% poly-l-lysine coated 3-0 sutures resulted into more severe neurological deficit as compared to 0.1% poly-l-lysine coated 4-0 and uncoated 3-0 suture at all recording time points. Mortality of 20%, 14% and 37% (Figure 23) was observed when MCA was occluded 0.1% poly-l-lysine coated 4-0, uncoated 3-0 suture and 0.1% poly-l-lysine coated 3-0 sutures respectively.

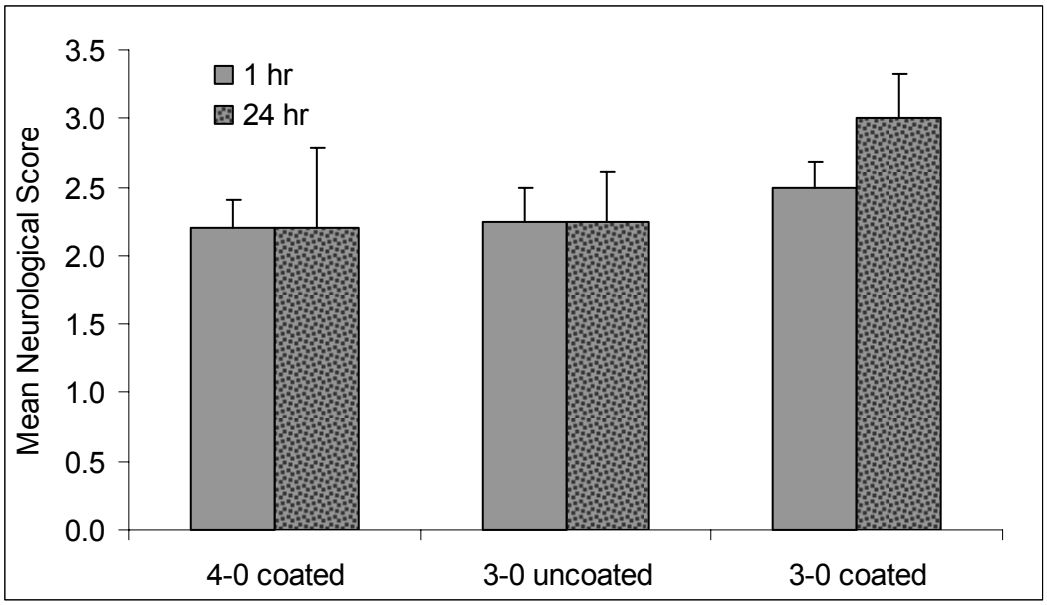

Figure 22: Effect of intraluminal suture of different diameter on neurological score measured at 1 hr & 24 hrs after permanent MCAo in rats. 4-0 and 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated without and with 0.1% poly-l-lysine solution were used. Error bars represent ± SEM.

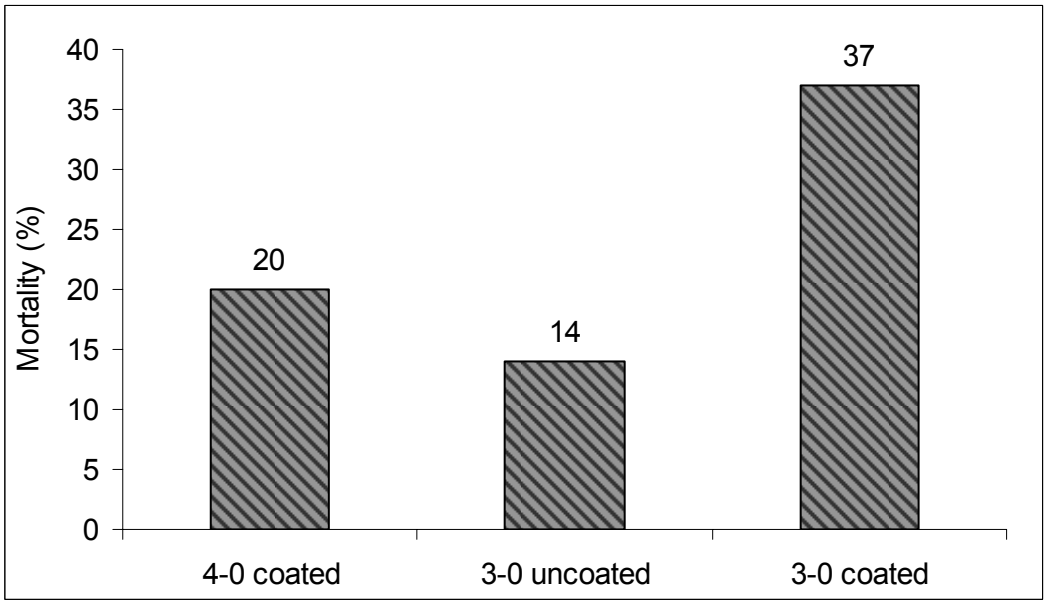

Figure 23: Effect of intraluminal suture of different diameter on mortality at 24 hrs after permanent MCAo in rats. 4-0 and 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated without and with 0.1% poly-l-lysine solution were used.

5.2.2. Feasibility study on non-invasive imaging and quantification of infarct in rat brain using clinical magnetic resonance scanner

5.2.2.1. MRI feasibility studies for imaging rat brain

MRI sequence parameters and Phantom studies

Phantom studies enabled us to identify the RF coil which best suits for acquiring images with good resolution. On the other hand, phantom studies allowed us to standardize various combinations of sequence parameters to be optimized for high resolution images with improved signal to noise ratio. With the use of flex loop small type of RF coil (Fig 24) we were able to get high resolution images of phantom (Fig 25). The sequence parameters used for T1, T2 and diffusion weighted measurements and the corresponding relative SNR are shown in Table 23.

Figure 24: MR scanner and coils Positioning of animal for imaging (a) 1.5 T clinical MRI scanner (Magnetom Symphony, Siemens Medical solutions) (b) small loop coil (Loop Flex Coil, small; Siemens Medical Solutions, Erlangen, Germany) (c) custom made animal holder with a facility to hold nose cone for anesthesia delivery (open arrow), and a notch for holding the RF coil (closed

arrow) and animal placed inside the opaque box (d) positioning the animal inside the RF coil covering the area of interest for imaging of rat brain.

Here in our study T1 weighted images were acquired with the purpose of finding the relative location of rat brain in the RF coil and MRI bore. This had enabled us to find out the axial, coronal and sagittal planes for the rat brain and further design the multi slice imaging parameters. For T1-weighted imaging the highest spatial resolution at a good signal-to-noise ratio and at an acceptable scanning time, was achieved using the following imaging parameters: TR/TE = 42/6 ms, a slice-thickness of 10 mm, a distance factor of 20%, a 250-mm FOV, and a 256*256-pixel matrix. 1 RF excitations were employed and summed for signal averaging to increase the SNR. Using these sequence parameters a spatial resolution of 1.0*1.0*10mm was achieved. The acquisition time was 34 seconds.

Figure 25: Results of the phantom study. Water filled plastic tube is used for initial standardization of various MR sequences like turbo spin echo (T2) and echo planar imaging (DWI). High resolution images of phantom were obtained with sequence parameters as mentioned in table 23 (a) T2 weighted image (b) diffusion image and (c) apparent diffusion maps of phantom in coronal plane.

For multi slice T2-weighted images the highest spatial resolution at a good signal-to-noise ratio and at an acceptable scanning time, was achieved using the following imaging parameters: TR/TE = 2500/100 ms, a slice-thickness of 1.5 mm, a distance factor of 30%, a 50-mm FOV, and a 192*192 pixel matrix. Four RF-excitations were employed to increase the SNR. A spatial resolution of 0.3*0.3*1.5 mm was achieved at a scan time of 3.47 min/slice.

For multi slice DW images the highest spatial resolution at a good signal-to-noise ratio and at an acceptable scanning time, was achieved using the following imaging parameters TR/TE = 3200/97 ms, a slice-thickness of 2.2 mm, a distance factor of 50%, a 103-mm FOV, and a 128*128 pixel matrix. A spatial resolution of 1.0*0.8*2.2mm was achieved at a scan time of 50 sec/slice.

Assessment of different anatomic structures of the rat brain

T2-weighted images typically offer a higher contrast between grey and white matter structures, compared to T1-weighted imaging and allow identification of different anatomic structures and tissues in the rat brain. The following anatomical structures could be easily identified when viewed in different planes (Figure 26). Olfactory bulb, structures of cerebrum and cerebellum can be seen from mid sagittal section. Neocortex and structures of cerebellum could be seen from axial section at the eye level. Thalami, lateral ventricles, fourth ventricle, hippocampus, caudate putamen were clearly seen from coronal sections. Thus the T2 weighted images obtained from standardized MR sequences as used in our study could be useful in the study of anatomical structures from small rodents.

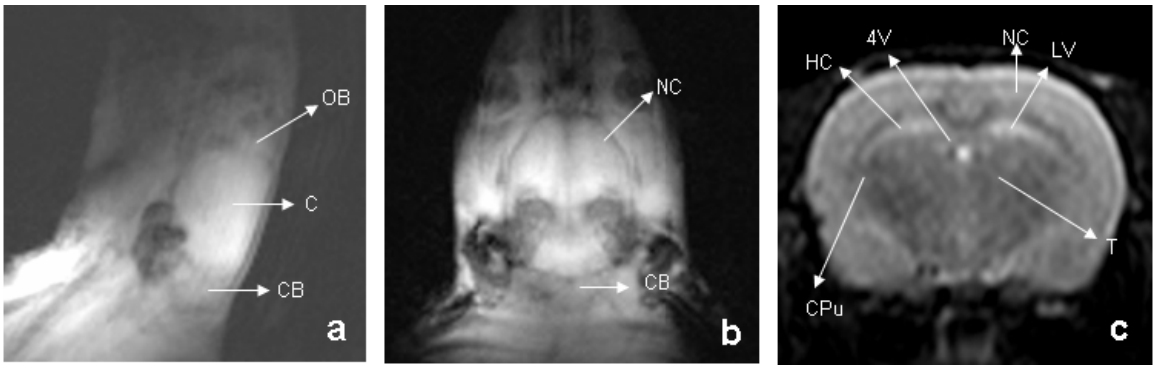

Figure 26: High-resolution MR images of rat brain in various planes (a) sagittal (b) axial and (c) coronal T2-weighted images at a good SNR. The following anatomical structures can be easily identified: olfactory bulb (OB), neocortex (NC), thalami (T), and cerebellum (CB) as well as lateral ventricles (LV), fourth ventricle (4V), hippocampus (HC), caudate putamen (CPu) and cerebrum (C).

Quantification of lesion area from MCAo animals

In figure 27 images of a rat brain subjected to MCAo and subsequent evolution of cerebral infarct were shown. The evolution was monitored by DW imaging and their corresponding ADC maps. Infarct lesion appears as a hyperintense area on DW images and hypointense area on ADC maps. Similarly in figure 28 panels of representative images were shown in which infarct on TTC stained slices can be seen as white area surrounded by pink area which is viable, on T2 weighted images corresponding area can be seen as a hyperintense area and on ADC maps hypo

intensity could be appreciated. These images were used for quantification of infarct (ADC images and TTC images) and edema (T2 images).

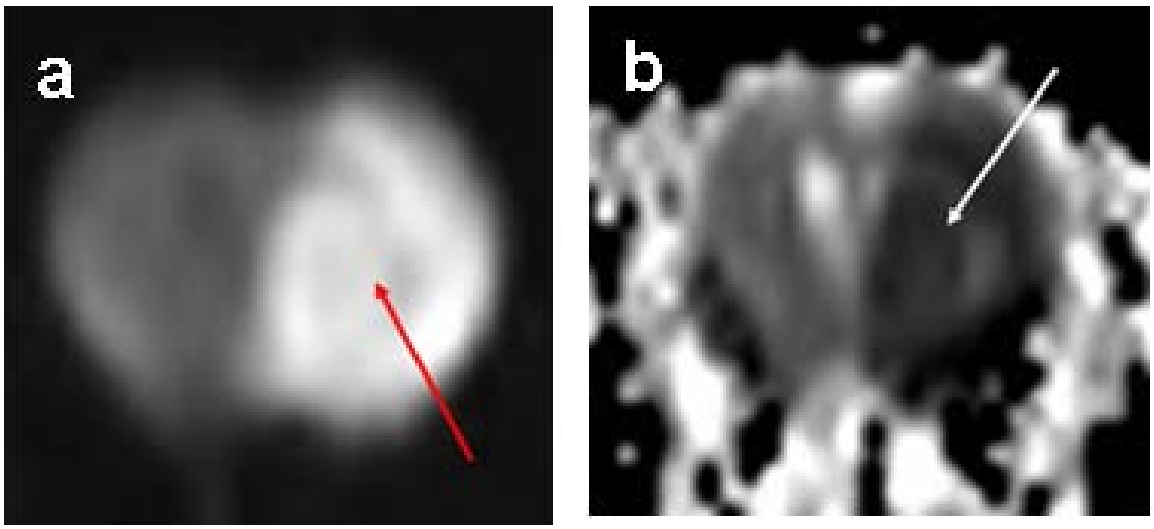

Figure 27: Demonstration of pathologic lesions in the rat brain of an MCA occluded rat. (a) Coronal diffusion weighted image showing hyperintense (arrow) region representing cytotoxic edema at 24 hr after MCAo (b) ADC images constructed using DWI shows hypointensity (arrow) in the corresponding lesion area representing reduced apparent diffusion coefficient of water protons.

5.2.2.2. Temporal profile of brain injury (Infarct) in transient ischemia model of stroke by MRI

Effect of transient ischemia i.e. 2hrs and 3hrs MCAo on infarct progression was monitored using MR imaging in rats. Having studied the feasibility of MR imaging of rat brain and non-invasive quantification of brain infarct we used this technique to further evaluate the temporal profile of infarct progression in the setting of transient ischemia. We were able to successfully image MCA occluded rat brains (Figure 29) for upto 48 hrs time point and were able to quantify brain infarction using ADC maps constructed employing diffusion weighted images. In both group of animal peak infarct occurred between 24 hrs-36 hrs post initiation of MCAo.

Sub group analysis into animals had low initial core damage (<1 cm^2 area of ADC deficit) and that had high initial core area (>1 cm^2 area of ADC deficit) reveled that the fall seen in initial ADC values in 2 hr and 3 hr model was from animals with high initial ADC values, otherwise there was no fall seen in animals with low initial ADC values after reperfusion. In animals with low initial infarct group both in 2 hr and 3

hr, there was more penumbra seen compared to animals with high initial infarct (Figure 30).

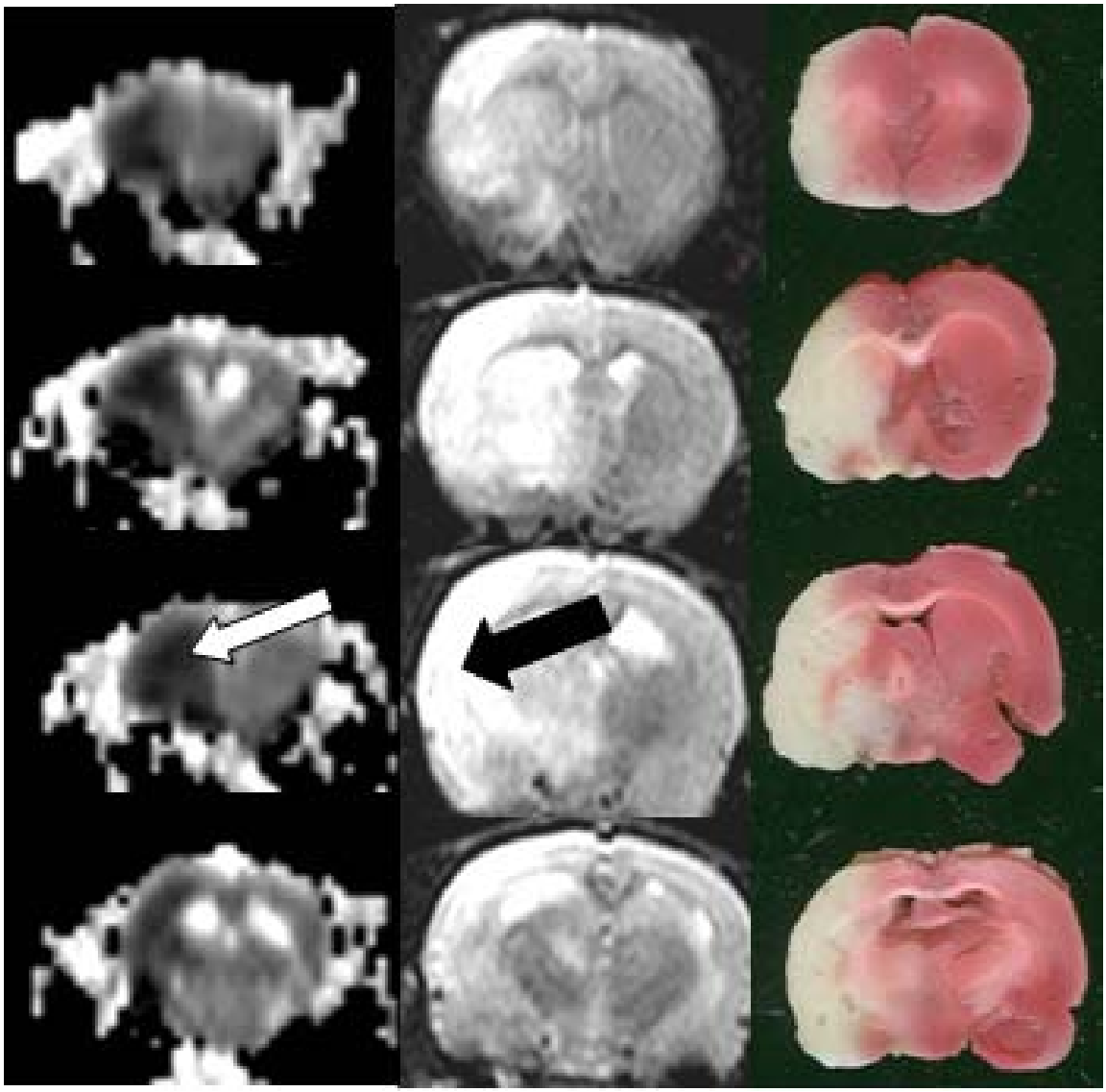

Figure 28: Representative images showing lesions in the rat brain at 24 hrs after the MCAo (a) ADC maps showing hypointensity (b) T2 images showing hyperintense region and (c) Tissue with unstained region (white) on TTC stained slices represents lesion

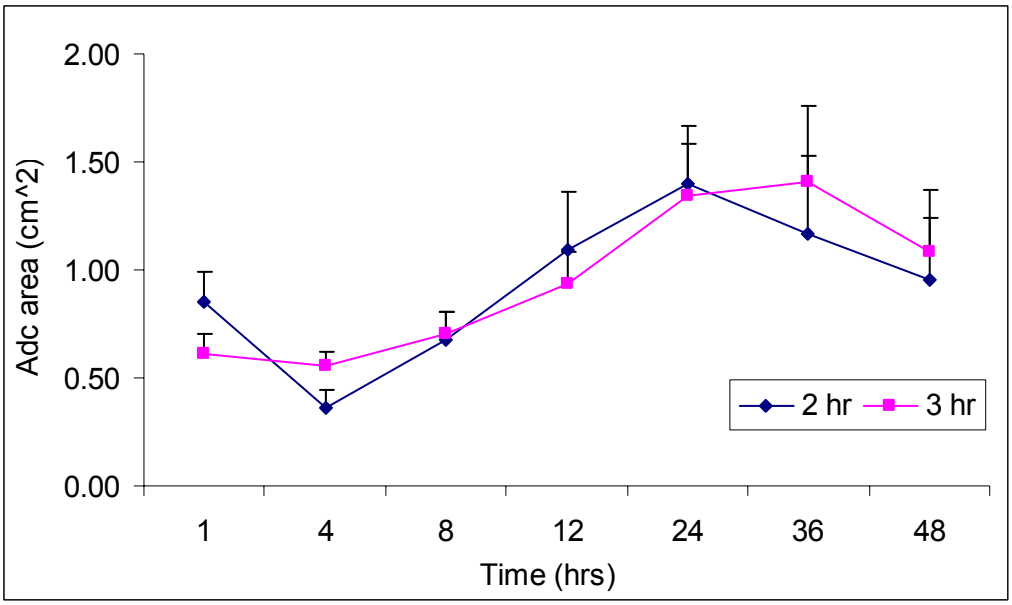

Figure 29: Temporal profile of infarct progression measured from ROI based analysis on ADC maps constructed using diffusion weighted MR serial images after 2hr and 3 hr transient ischemia and reperfused to various time points till 48hr in rats. 3-0 polyamide monofilament sutures (Ethilon, Johnson & Johnson) coated with 0.1% concentration of poly-l-lysine solution was use to occlude MCA. Values represent Mean± SEM. ROI, region of interest; ADC, apparent diffusion coefficient of water; MR, magnetic resonance.

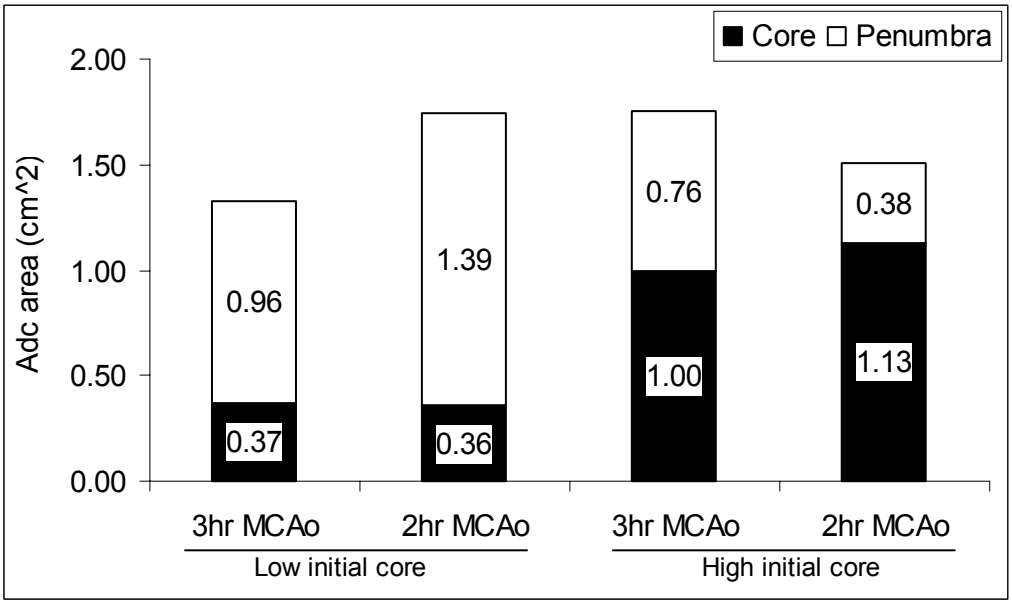

Figure 30: Effect of 2hr and 3hr MCAo on Infarct Core (measured at 1hr post MCAo) and Penumbra which further got infarcted over 48 hours post MCAo. Data represented shows that initial core segregated into low and high core are not different after 2hr or 3hr MCAo, but over next 48 hr significantly more penumbra becomes infarcted from low core group as compared to animal with high initial core. MCAo

5.2.3. Validation of MCAo model of stroke for evaluating the efficacy of neuroprotective agent.

5.2.3.1. Validation of transient ischemia model

Animal model validation experiments were carried out using a known NMDA receptor blocker MK-801 (Dizocilpine). MK-801 administered at a dose of 0.1mg/kg immediately following MCAo significantly reduced progression of infarct.

MK-801 treatment significantly reduced (98%, $p < 0.001$) the area of penumbra which got infarcted and recruited into initial core (Figure. 31, 32). Similarly, treatment with MK-801 significantly reduced (42%, $p < 0.05$) the brain edema (Figure. 33) as compared to control group of animals.

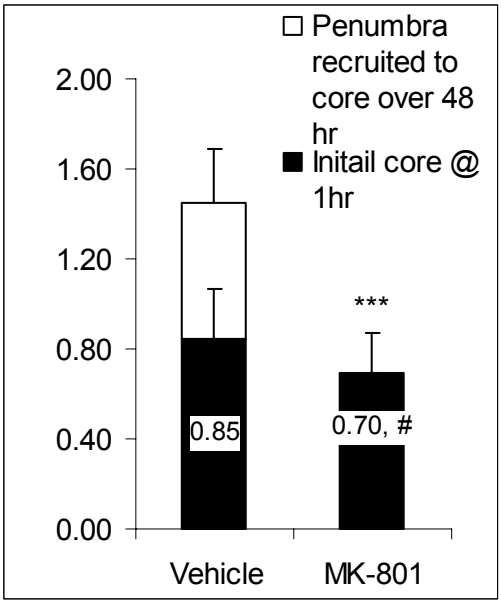

Figure 31: Core and Penumbra which further got infarcted over 48 hours post MCAo in MK-801 and vehicle control groups. Data represented shows that initial core was not significantly different (#p = 0.28) between TRC051384 and control group. Significantly lesser penumbra got infarcted in MK-801 treated group. Values represent Mean± SEM. ***p<0.001 vs. vehicle control

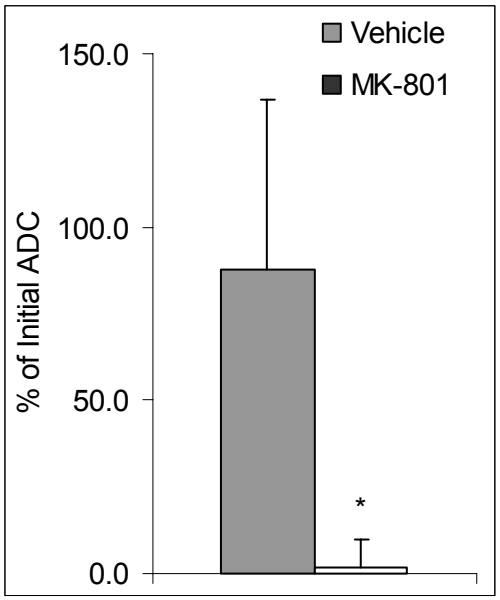

Figure 32: Data represents the reduction in recruitment of penumbra into the infarct core with MK-801 (0.1mg/kg, i.v.) treatment as compared to vehicle control measured up to 48 hours post MCAo on ADC maps using clinical MRI. Values represent Mean± SEM. *p<0.05 vs. vehicle control

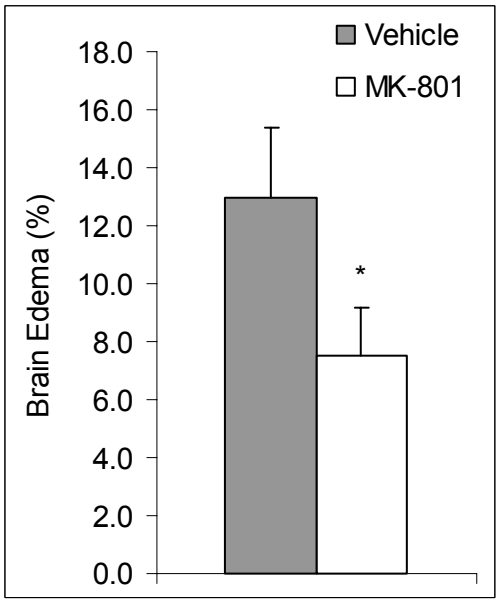

Figure33: Effect of MK-801 on brain edema, Values represent Mean± SEM. *p<0.05 vs. vehicle control

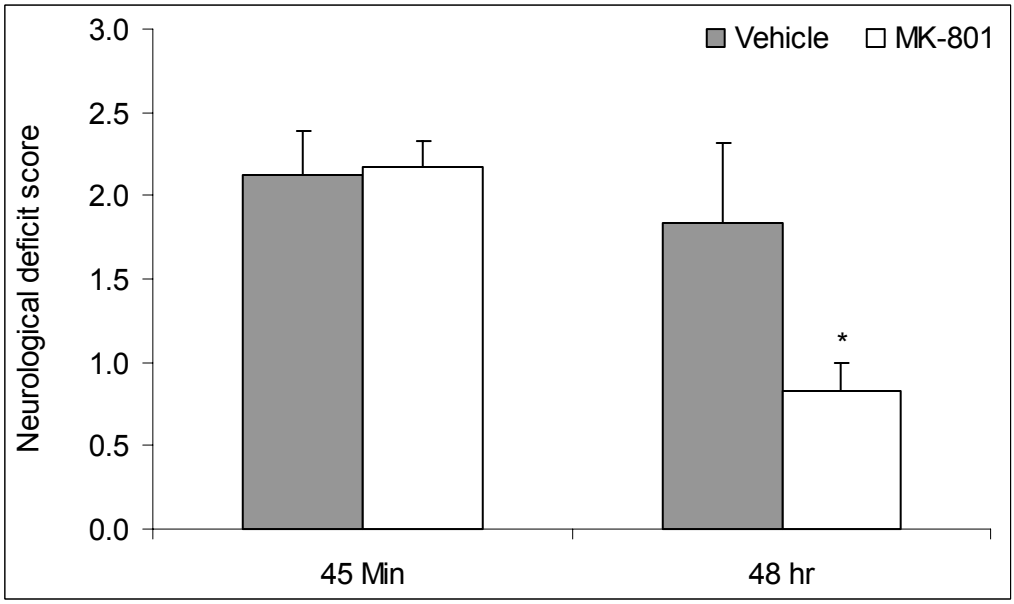

Figure34: Effect of MK-801 on mean neurological deficit, Values represent Mean± SEM. *p<0.05 vs. vehicle control

Similarly, MK-801 when intervened at 0.1mg/kg also showed significant reduction in neurological deficit on day 2 (54%, $p < 0.05$) as compared to control group of animals (Figure. 34).

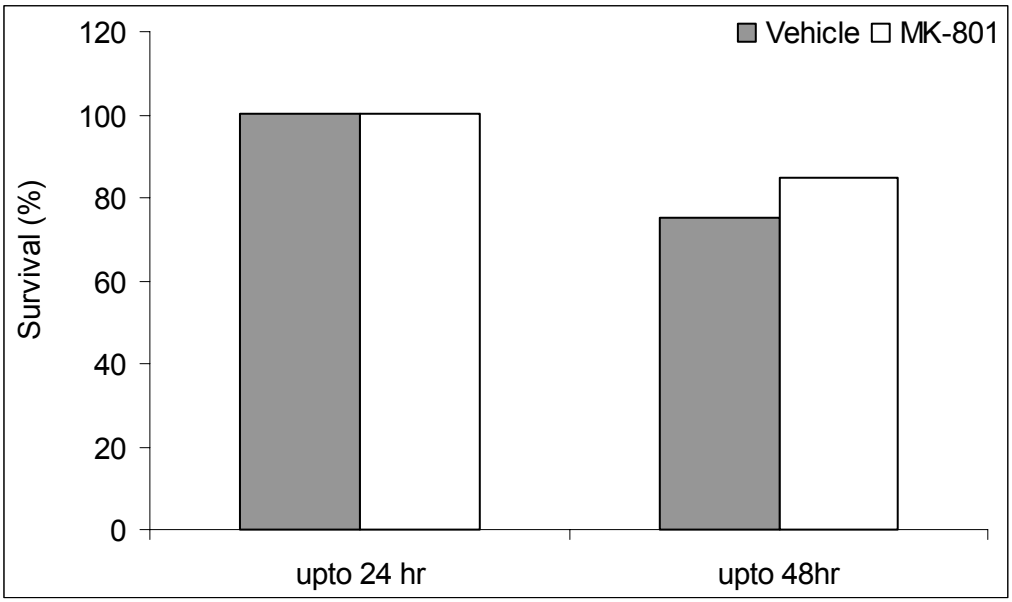

Figure35: Effect of MK-801 on survivability upto 48hrs in 3hr transient ischemia model in rats

Also treatment with MK-801 (although not significant) shows a trend towards greater survival of animals in comparison to control animals (Figure. 35).

5.2.3.2. Validation of permanent ischemia model

Treatment with MK-801 at 0.1mg/kg dose in a permanent ischemia model resulted into significant reduction (50%, $p<0.05$) in infarct area (Figure 36) as compared to vehicle control animals. Similarly treatment with MK-801 significantly improved the survival of animals (Figure 37). All animals treated with MK-801 survived the 24hrs study period where as only 62.5% animals from vehicle control group survived the 24 hr study period. Thus with the above mentioned two experiments it can be concluded that MK-801 demonstrates efficacy in both transient and permanent MCAo models and also here we have validated both these models further confirming that these models can be employed for evaluation of efficacy of potential neuroprotective agents.

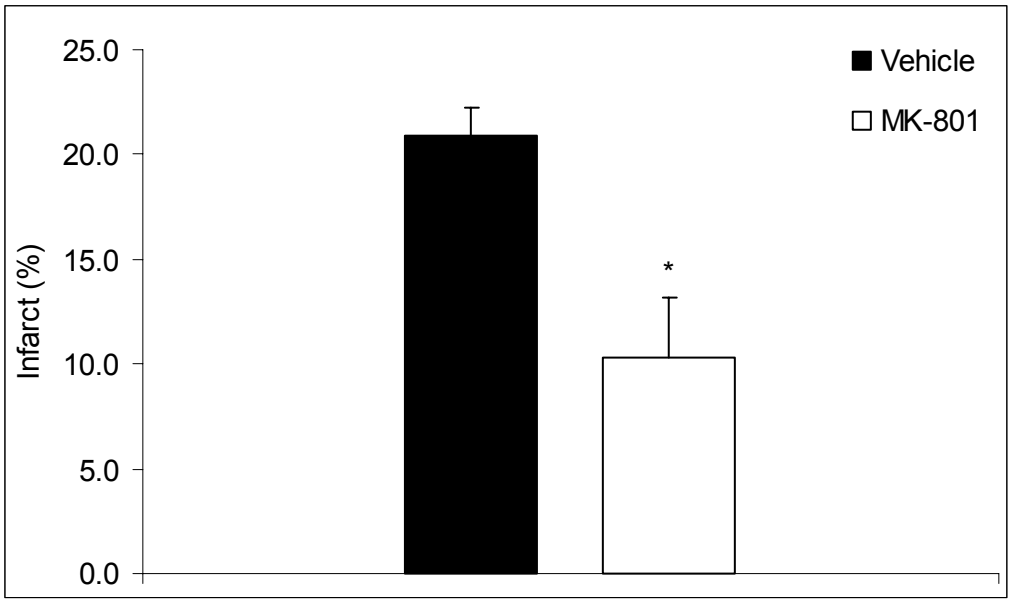

Figure 36: Effect of MK-801 on infarct area measured by TTC at 24 hrs post permanent MCAo in rats. Values represent Mean± SEM. *p<0.05 vs vehicle control

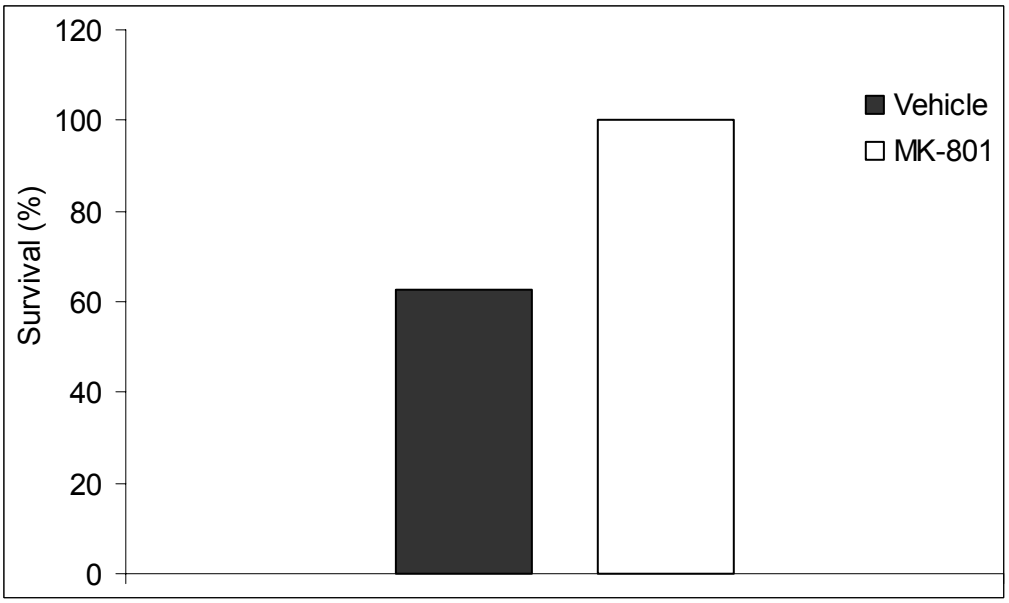

Figure37: Effect of MK-801 on survival upto 24 hrs post permanent MCAo in rats

5.2.4. Pharmacokinetic profiling of TRC051384 in rat

The plasma concentration-time profile of TRC051384 when administered by i.v. and i.p. route is shown in figure 38. The PK parameters computed from plasma concentration – time data are shown in table 24. TRC051384 showed rapid i.p.

absorption with C_{max} being reached within 0.17hrs of administration. Bioavailability by i.p. route was found to be 20%.

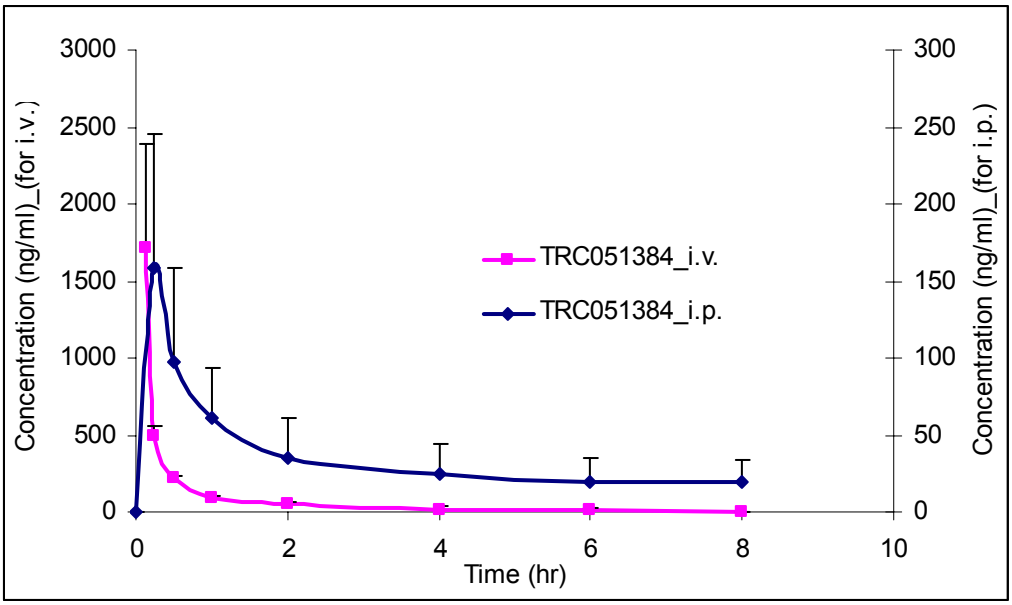

Figure 38: Plasma concentration vs time profile of TRC051384 administered as a single dose by i.v. route and i.p. route in rats. Values represent Mean± SEM.

Table 24: Pharmacokinetic parameters of TRC051384 administered by i.v. route and i.p. route in rats

Pharmacokinetic parameters	TRC051384 administered by i.v. route (Dose : 7.57mg/kg)	TRC051384 administered by i.p. route (Dose: 7.57mg/kg)
C_0 / C_{max}(ng/ml)	6267.5±1791.8	203.8±41.2
$t_{1/2}$ (hr)	2.23±0.52	2.01±0.25
AUC $_{(0-8)}$ (ng.hr/ml)	870.8±58.7	102.2±27.6
AUC $_{(0-\infty)}$ (ng.hr/ml)	971±65.7	142±38.7

Values represented are mean±SEM for 3-4 observations in each group

AUC, Area under the curve; C_{max}', maximum plasma concentration; $t_{1/2}$, half life

5.2.5. Acute safety studies with TRC051384 in rat

Acute safety study of TRC051384 at different doses was carried out in anesthetized rats and results are compiled in the following table 25. Results indicate that TRC051384 was better tolerated.

Table 25: Preliminary safety studies evaluating safety of TRC051384 on blood pressure, respiratory rate and ECG parameters administered by i.v. route.

Dose of TRC051384	Safety parameters			
	Effect on BP	Effect on Respiration Rate (RR)	Effect on Heart Rate (HR)	Effect on ECG
7.54mg/kg	3-5% increase in BP for 3 min	No Effect	No Effect	No Effect
3.77mg/kg	3-5% increase in BP for 1 min			
1.88mg/kg	1-2% increase in BP for 1 min			

5.2.6. In-vivo efficacy studies of TRC051384 in MCAo model of stroke

5.2.6.1. Efficacy studies in 3 hr transient MCAo model

5.2.6.1.1. Efficacy of TRC051384 by single dose administration in MCAo model of stroke

When TRC051384 was administered as a single i.v. dose at four hours after the initiation of MCAo, no reduction was observed in infarct area (Figure 39) as well as brain edema (Figure 40). At the same time no improvement was seen in survival also.

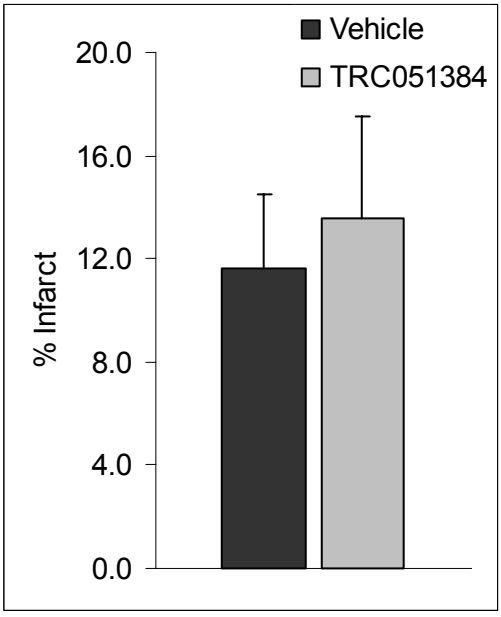

Figure 39: Effect of TRC051384 on % infarct area measured using TTC stained slices when administered as single dose (3.78mg/kg) by i.v. route in 3hr MCAo model of stroke. Values represent Mean± SEM.

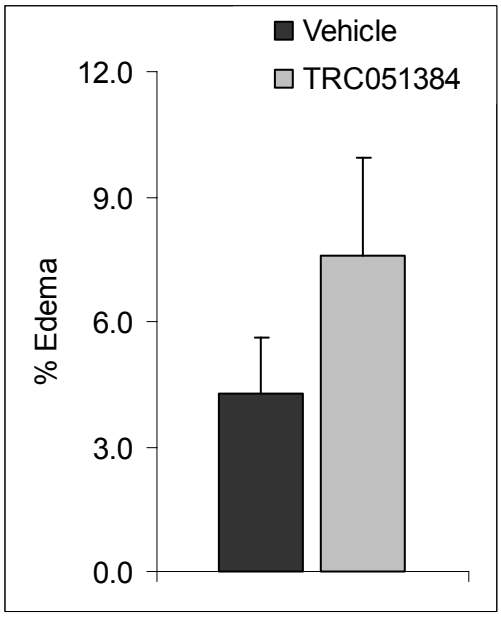

Figure 40: Effect of TRC051384 on brain edema when administered as single dose (3.78mg/kg) by i.v. route in 3hr MCAo model of stroke. Values represent Mean± SEM.

5.2.6.1.2. Efficacy of TRC051384 by multiple dose short duration administration in MCAo model of stroke

With learning from previous study that single dose exposure may not be sufficient to translate into efficacy, we here in this study had evaluated efficacy of TRC051384 administered as small multiple doses beginning from 6 hrs post MCAo. Additionally from here onwards we studied the rate of infarct and edema progression in these animals non-invasively by MRI. Diffusion weighted images were acquired to study effect on cytotoxic edema (cerebral infarct) and T2 weighted images were taken for the study of vasogenic brain edema.

We randomized animals to receive either the test compound or vehicle on the basis of initial infarct core recorded at 1 hour (First MR Scan) post MCAo. However, over the next 48 hours, no reduction was observed in the area of penumbra which got infarcted and recruited into initial core with TRC051384 treatment (Figure 41). Similarly, treatment with TRC051384 did not reduce the brain edema (Figure. 42) as compared to control group of animals.

We included animals with similar neurological deficit before randomizing them to receive either the test compound or vehicle. TRC051384 when intervened at 6 hours post ischemia showed no reduction in neurological deficit (Fig 43). Only trend improvement observed with TRC051384 treatment was on survival parameter. There

was a 27% improvement in survival observed with TRC051384 upto 7 days as compared to their respective control animals (Fig. 44).

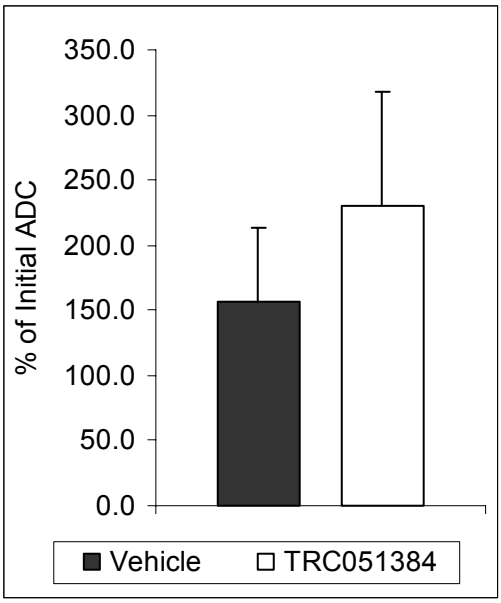

Figure 41: Effect of TRC051384 on area of penumbra got infarcted over 48hrs time measured using MRI. TRC051384 was administered at 0.9mg/kg as first dose beginning from 6 hrs, 0.45mg/kg as subsequent doses every 2 hrs to maintain drug exposure upto 10 hrs post MCAo in 3 hr transient ischemia model of stroke. Values represent Mean± SEM.

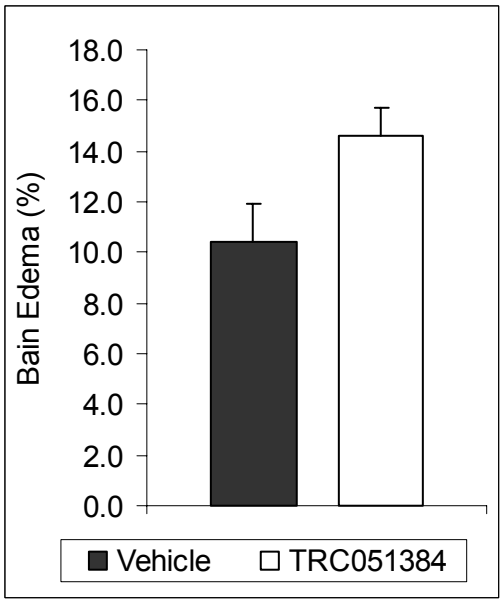

Figure 42: Effect of TRC051384 on brain edema. TRC051384 was administered at 0.9mg/kg as first dose beginning from 6 hrs, 0.45mg/kg as subsequent doses every 2 hrs to maintain drug exposure upto 10 hrs post MCAo in 3 hr transient ischemia model of stroke. Values represent Mean± SEM.

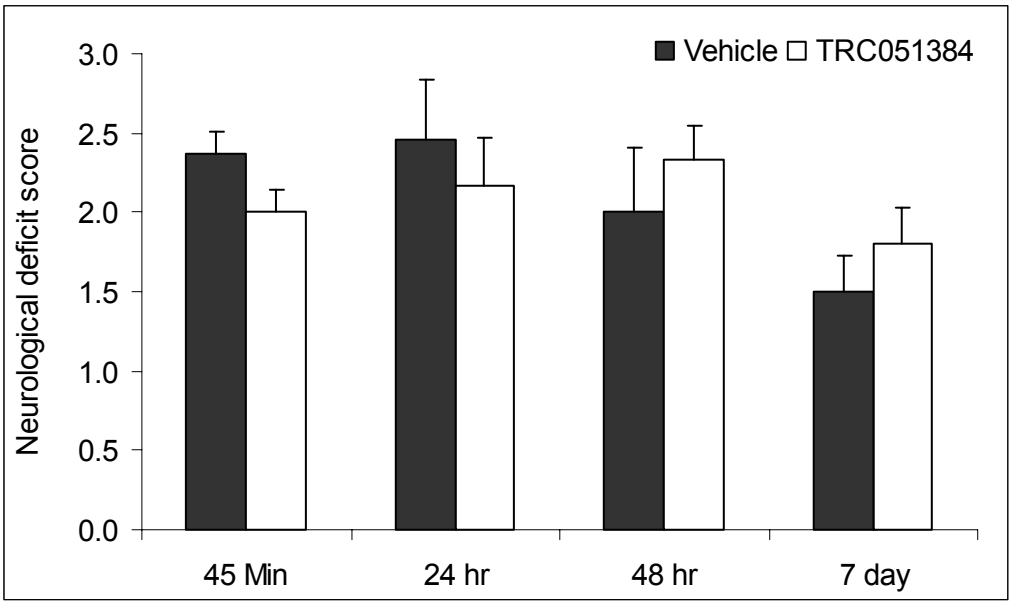

Figure 43: Effect of TRC051384 on neurological deficit in 3 hr MCAo model of stroke. TRC051384 was administered at 0.9mg/kg as first dose beginning from 6 hrs, 0.45mg/kg as subsequent doses every 2 hrs to maintain drug exposure upto 10 hrs post MCAo. Values represent Mean± SEM.

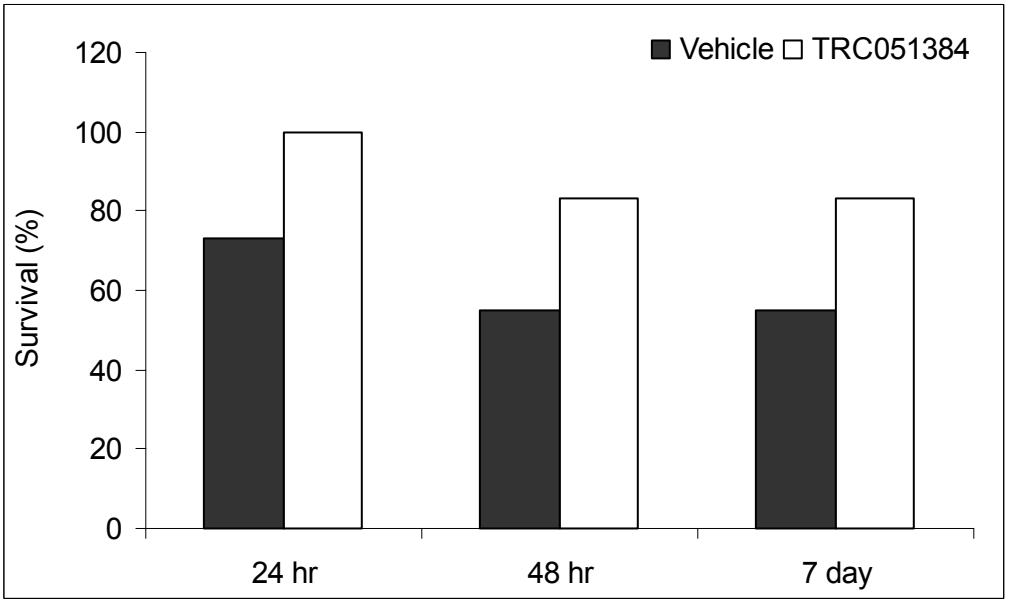

Figure 44: Effect of TRC051384 on % survival in 3 hr MCAo model of stroke. TRC051384 was administered at 0.9mg/kg as first dose beginning from 6 hrs, 0.45mg/kg as subsequent doses every 2 hrs to maintain drug exposure upto 10 hrs post MCAo.

5.2.6.1.3. Efficacy of TRC051384 by multiple dose long duration administration in MCAo model of stroke

With results hinting us towards that longer exposure of small doses of TRC051384 may be required for better treatment outcome we then evaluated efficacy of this test compound by long term administration i.e. 2 days of drug exposure. However TRC05138 treatment showed no reduction in the area of penumbra which got infarcted and recruited into initial core (Figure. 45). But, a sub group analysis of animals with low initial core and high initial core revealed that TRC051384 significantly reduced (87% as against vehicle control, $p < 0.05$) the area of penumbra which got infarcted and recruited into initial core (Figure. 45) in animals which had low initial infarct core. No reduction was seen in high initial insult group.

TRC051384 treatment did not result into any reduction of brain edema at the tested dose (Figure 46). A total of 70% animals were able to survive the 7 day study period in comparison to 30% survival seen in control animals (Figure 47).

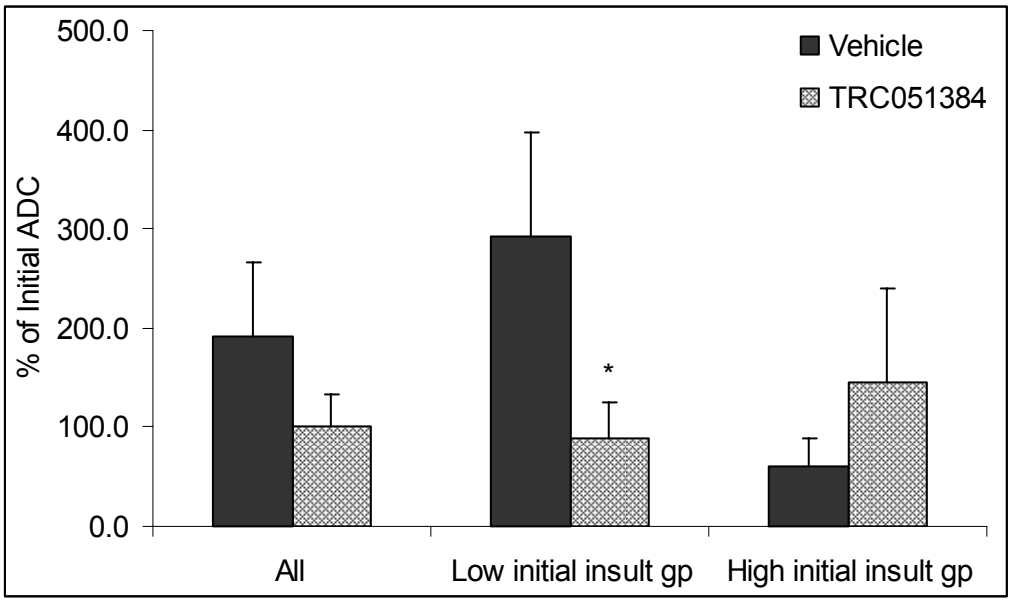

Figure 45: Effect of TRC051384 on area of penumbra got infarcted over 48 hrs time in low infarct and high initial infarct group of animals in 3 hr MCAo model of stroke measured using MRI. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg (i.p.) as subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *$p<0.05$ vs vehicle control

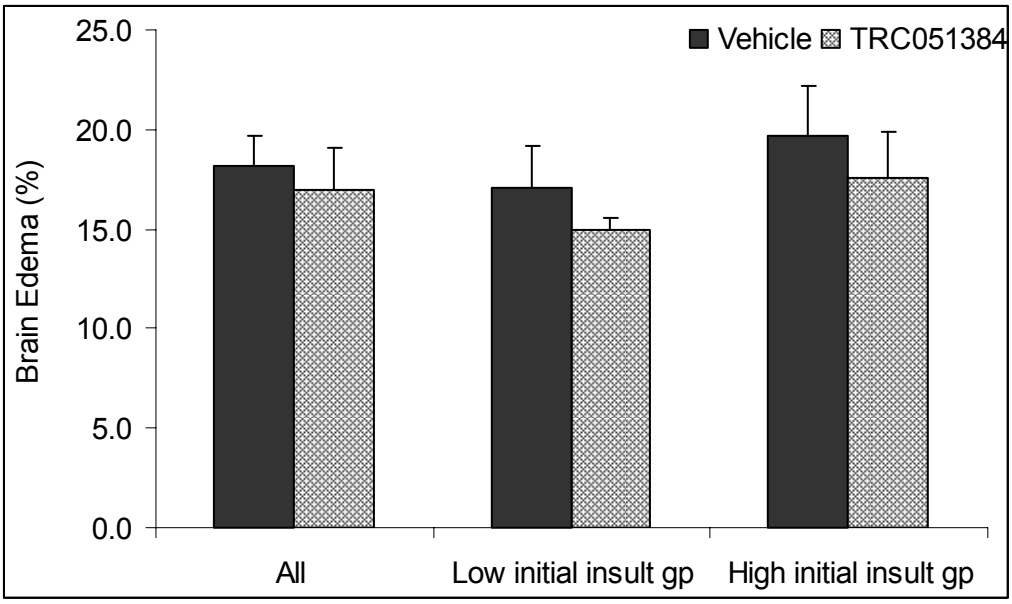

Figure 46: Effect of TRC051384 on brain edema in low infarct and high initial infarct group of animals in 3 hr MCAo model of stroke measured using MRI. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg (i.p.) as subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM.

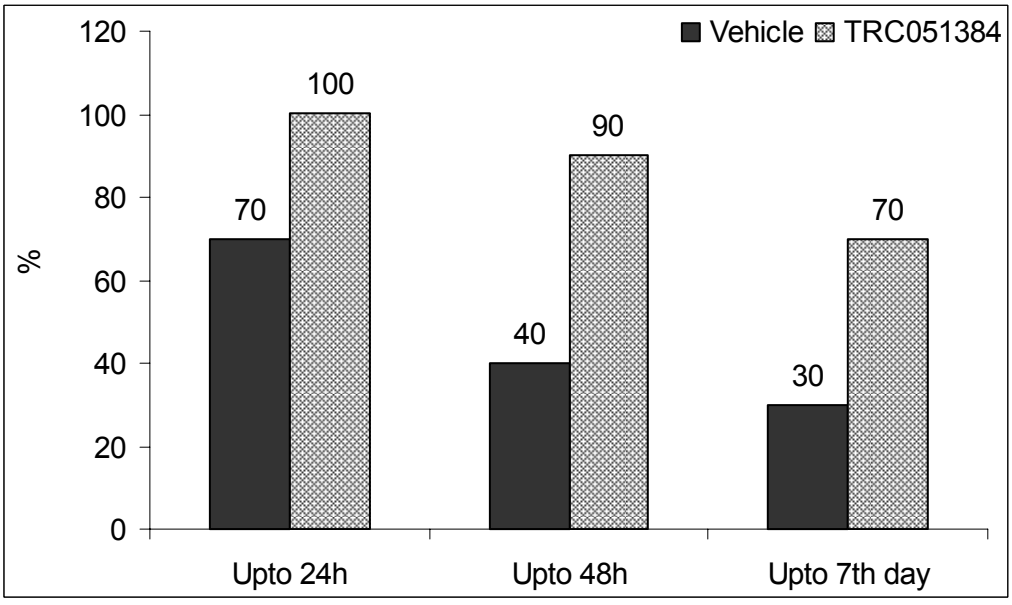

Figure 47: Effect of TRC051384 on % survival when administered as multiple doses upto 48 hrs in 3 hr MCAo model of stroke. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg (i.p.) as subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo.

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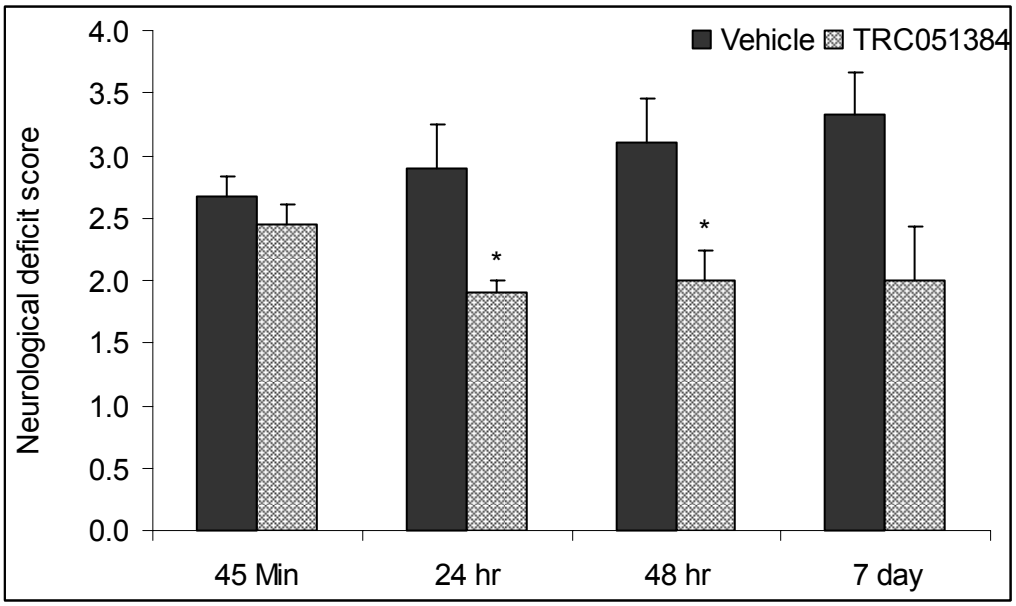

Figure48: Effect of TRC051384 on neurological deficit when administered as multiple doses upto 48 hrs in 3 hr MCAo model of stroke. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg (i.p.) as subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *p<0.05 vs vehicle control

On the other hand there was time dependant significant improvement in the neurological deficit (Figure 48) in TRC51384 treated animals. In conclusion the compound TRC51384 treatment initiated at 4 hrs post ischemia in 3 hr transient occlusion model showed significant reduction in the infarct progression rate in low initial ADC animals, greater improvement in neurological deficit as well as increased survival. Looking at the results in animals which had low initial infarct it was further decided that a 2 hr occlusion model (it is shown to have more salvageable penumbra) would be used to study the efficacy of compound TRC51384.

5.2.6.2. Efficacy studies in 2 hr transient MCAo model – Therapeutic time window and dose response

5.2.6.2.1. Efficacy of TRC051384 administered at 4 hrs after MCAo

On DWI (hence on ADC), there was no difference found in the area of brain damage (initial core, p=0.22) of rats at1 hour (First MR Scan) post MCAo in both TRC051384 treated and control groups of rats (Figure. 49).

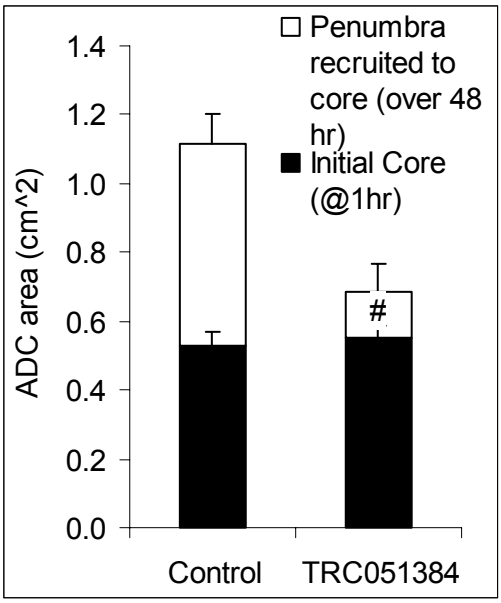

Figure 49: Effect of TRC051384 on core area and penumbra further got infarcted over 48 hours post MCAo measured using MRI in a 2hr MCAo model of stroke. Data represented shows that initial core was not significantly different (#p = 0.22) between TRC051384 and control group. Significantly lesser penumbra got infarcted in TRC051384 treated group. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg (i.p.) as subsequent doses every 2 hrs to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *p<0.05 vs vehicle control.

However, over the next 48 hours TRC051384 treatment significantly reduced (87% as against vehicle control, p <0.05) the area of penumbra which got infarcted and recruited into initial core (Figure. 50). We then evaluated if the reduction in infarct seen at all brain level (MCA supplied area) and TRC051384 treatment significantly reduced infarct at all brain levels (Figure 51). Similarly, treatment with TRC051384 significantly reduced (39%, p<0.05) the brain edema (Figure. 52) as compared to control group of animals. Also treatment with TRC051384 significantly increased the survival (50%, p< 0.05 on day 2 and 67.3%, p < 0.01 on day 7) of animals in comparison to control animals (Figure. 53).

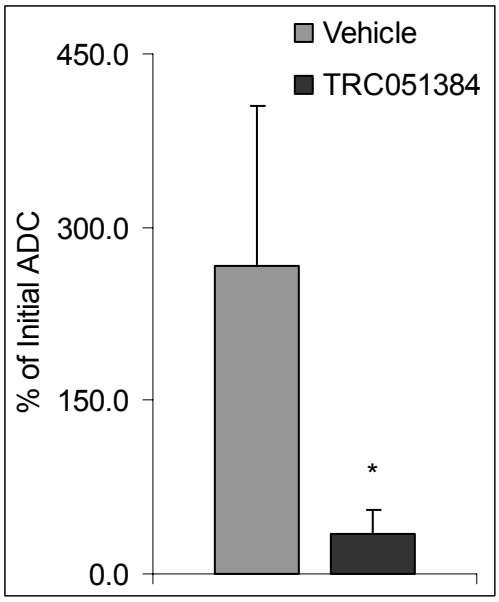

Figure 50: Data represents the reduction in recruitment of penumbra into the infarct core with TRC051384 treatment as compared to vehicle control measured up to 48 hours post MCAo on ADC maps using clinical MRI. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *p<0.05 vs vehicle control

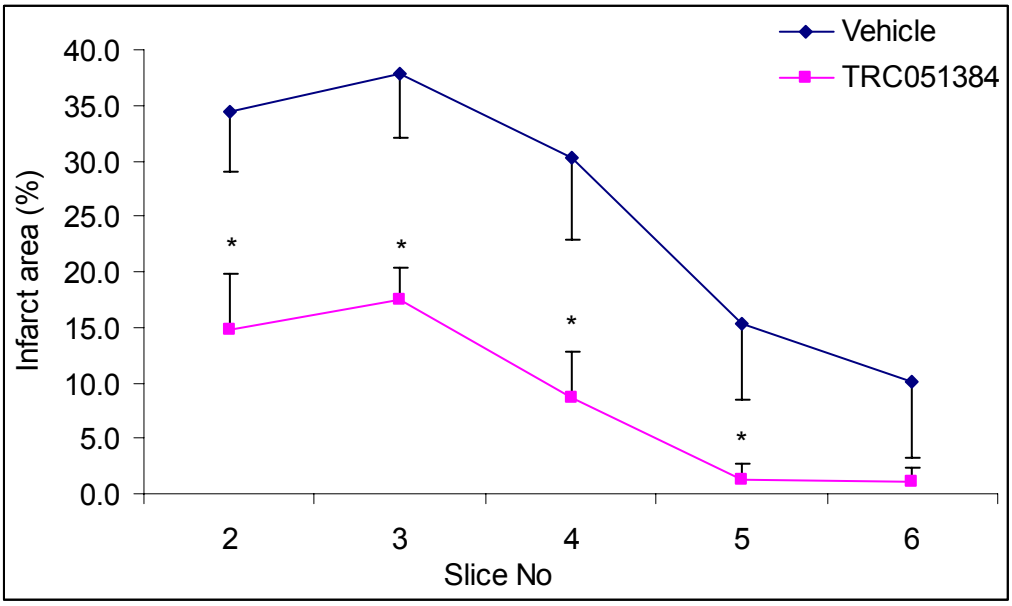

Figure 51: Effect of TRC051384 on infarct area at different slice levels in 2 hr MCAo model of stroke demonstrating protection of cortical and subcortical neurons. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo.Values represent Mean± SEM. *p<0.05 vs vehicle control.

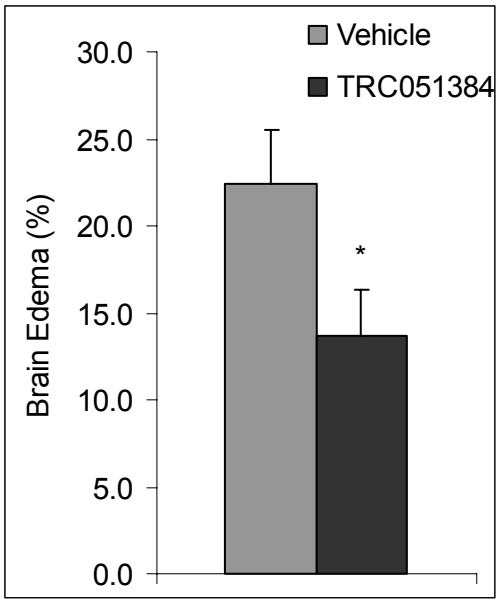

Figure 52: Brain edema reduction as seen with TRC051384 treatment, measured using T2-weighted images in a 2hr transient ischemia model of stroke. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *p<0.05 vs vehicle control.

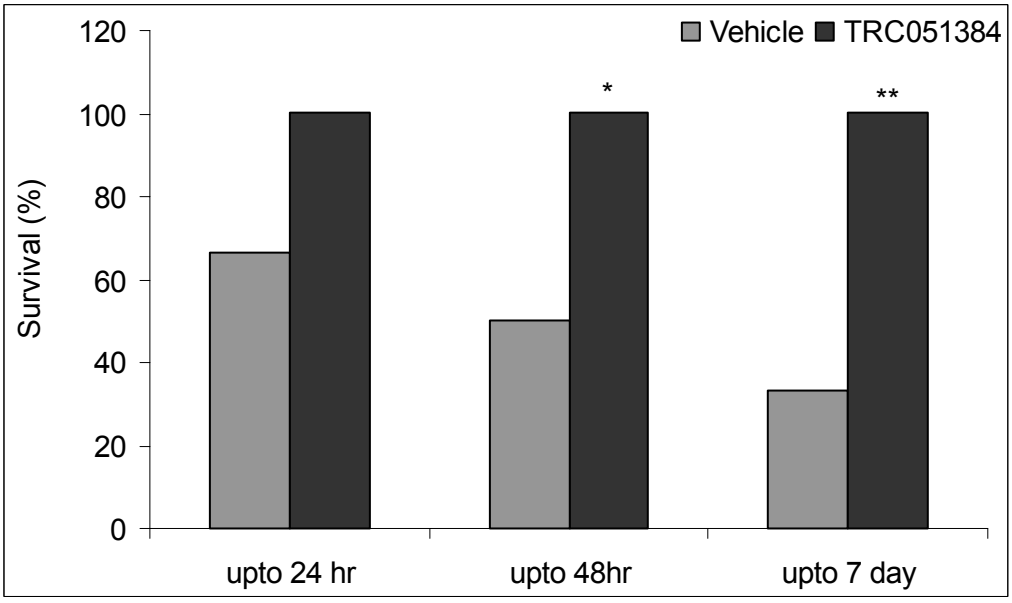

Figure 53: Effect of TRC051384 treatment on % survival in 2 hr MCAo model of stroke. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo. **p<0.01, *p<0.05 vs vehicle control.

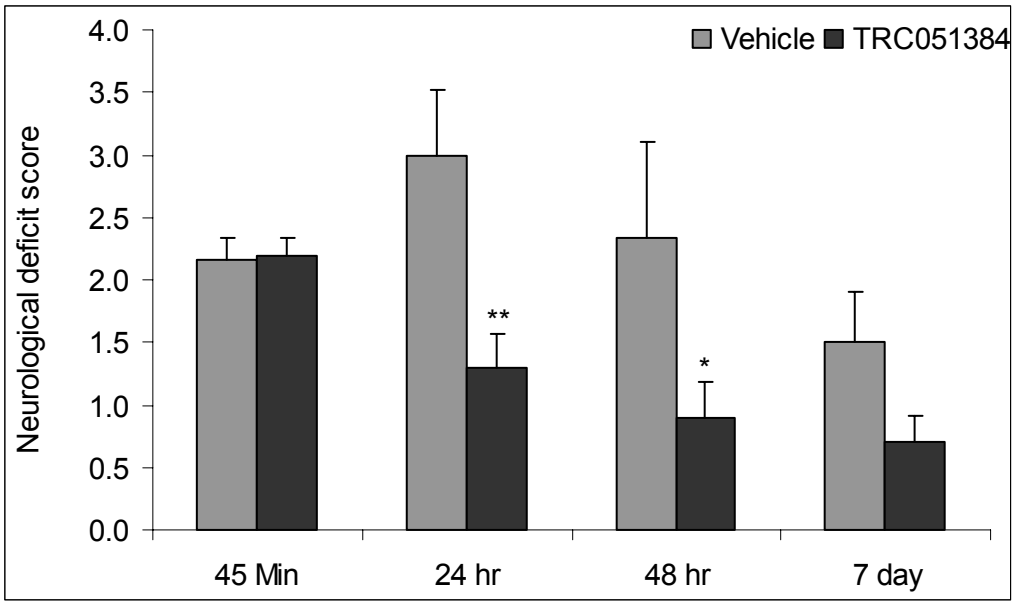

Figure 54: Effect of TRC051384 treatment on reduction in neurological deficit in 2 hr MCAo model of stroke. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *p<0.05, **p<0.01 vs vehicle control.

We included animals with similar neurological deficit before randomizing them to receive either the test compound or vehicle. TRC051384 when intervened at 4 hours post ischemia showed significant reduction in neurological deficit on day 1 (57%, p< 0.05), day 2 (61%, p < 0.05) and till the end of the study, i.e. day 7 (53%) post onset of ischemia, as compared to control group of animals (Figure. 54). Neurological deficit in the TRC051384 group was significantly improved at much earlier time points than vehicle control animals, although deficits gradually improved in both groups over 7 days period.

5.2.6.2.2. Efficacy of TRC051384 administered at 8 hrs after MCAo

Animals which had similar injury (Initial core, Figure. 55) were randomized into treatment and control groups. TRC051384 treatment at the dose of 9mg/kg by i.p. significantly reduced (84%, p < 0.001) the area of penumbra which got infarcted and recruited into initial core (Figure. 56). The rate of infarct progression was significantly reduced (Figure 57) and this reduction was evident at all brain levels (Figure 58) with

TRC051384 treatment. Where as, TRC051384 treatment at starting dose of 4.5mg/kg showed only a trend towards reduction of recruitment of penumbra.

Similarly, treatment with TRC051384 at both doses studied significantly reduced (18% and 29% respectively, $p < 0.05$) the brain edema (Figure. 59) as compared to control group of animals.

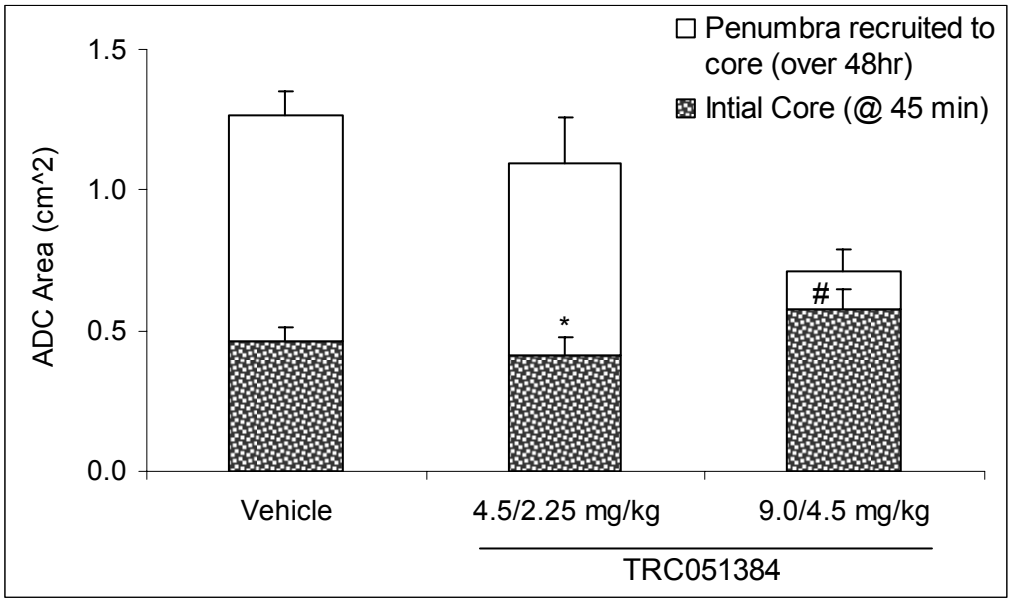

Figure 55: Effect of TRC051384 administered at 8 hrs post MCAo at indicated doses as compared to vehicle control group on core area and penumbra got infarcted over 48hr in 2 hr MCAo model of stroke measured on ADC maps using clinical MRI. Note that there is no difference in the initial core between vehicle control group and TRC051384 treated group. TRC051384 was administered at 9mg/kg or 4.5mg/kg (i.p.) as first dose beginning from 8 hrs, 4.5mg/kg or 2.25mg/kg (i.p.) as subsequent doses every 2 hrs in respective group of animals to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. **p<0.05 vs vehicle control.

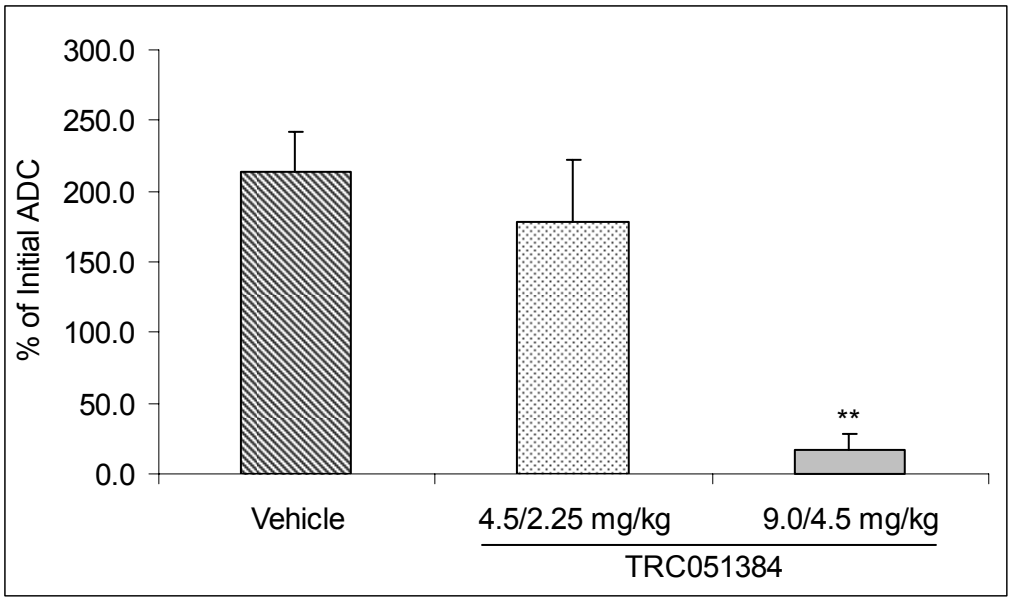

Figure 56: Effect of TRC051384 administered at 8 hrs on reduction in recruitment of penumbra into the infarct core with TRC051384 treatment at indicated doses as compared to vehicle control measured up to 48 hours post MCAo on ADC maps using clinical MRI. TRC051384 was administered at 9mg/kg or 4.5mg/kg (i.p.) as first dose beginning from 8 hrs, 4.5mg/kg or 2.25mg/kg (i.p.) as subsequent doses every 2 hrs in respective group of animals to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. **p<0.01 vs vehicle control

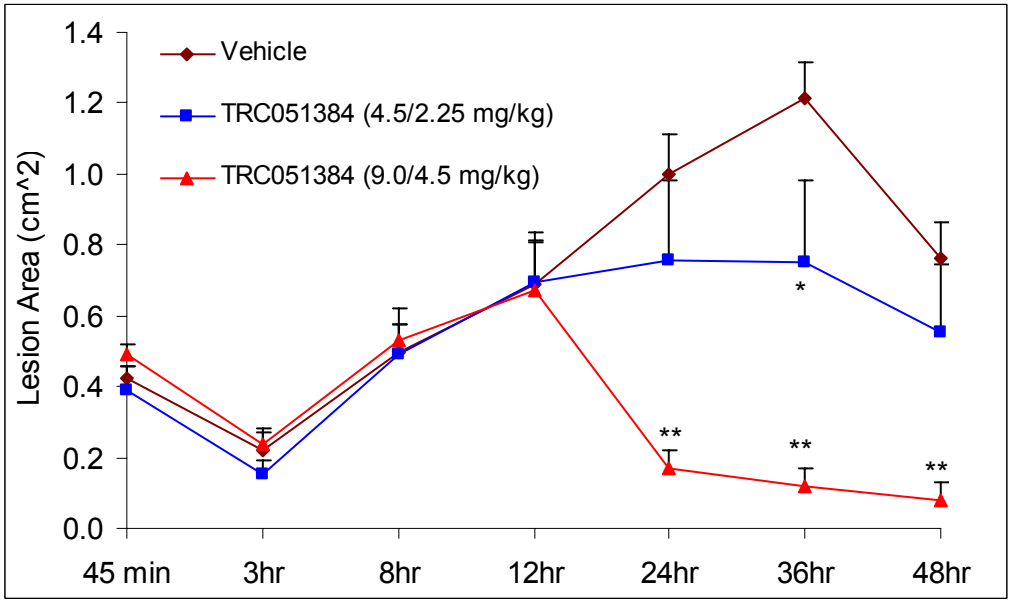

Figure 57: Effect of TRC051384 administered at 8 hrs post MCAo at indicated doses as compared to vehicle control group on reduction in rate of infarct progression over 48 hrs in 2 hr MCAo model of stroke measured using MRI. TRC051384 was administered at 9mg/kg or 4.5mg/kg (i.p.) as first dose beginning from 8 hrs, 4.5mg/kg or 2.25mg/kg (i.p.) as subsequent doses every 2 hrs in respective group of animals to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *p<0.05, **p<0.01 vs vehicle control

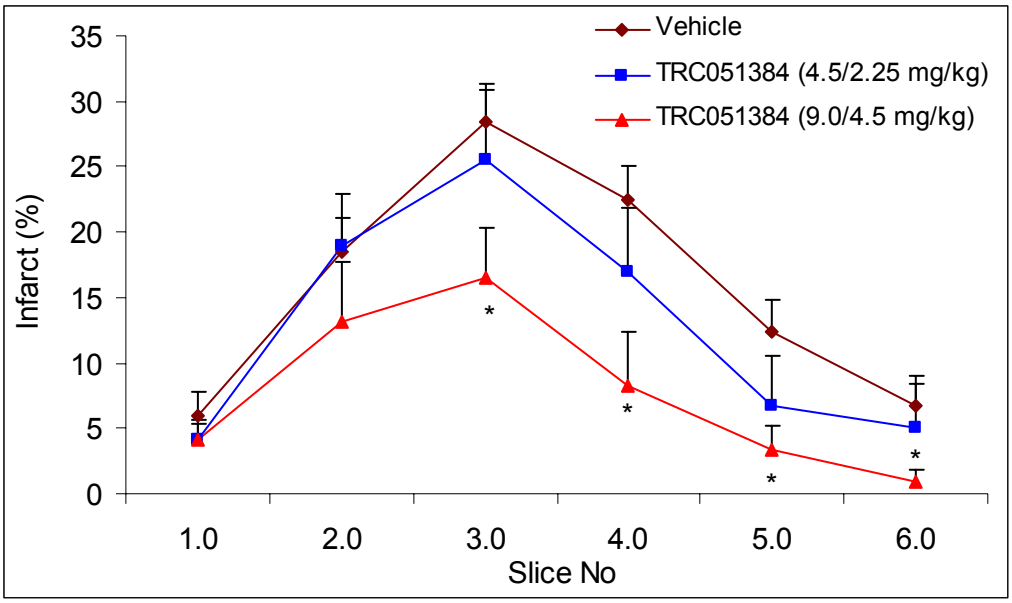

Figure 58: Effect of TRC051384 administered at 8 hrs post MCAo at indicated doses as compared to vehicle control on reduction in infarct area at different brain levels in 2 hr MCAo model of stroke measured using MRI demonstrating protection of cortical and subcortical neurons. TRC051384 was administered at 9mg/kg or 4.5mg/kg (i.p.) as first dose beginning from 8 hrs, 4.5mg/kg or 2.25mg/kg (i.p.) as subsequent doses every 2 hrs in respective group of animals to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *p<0.05 vs vehicle control

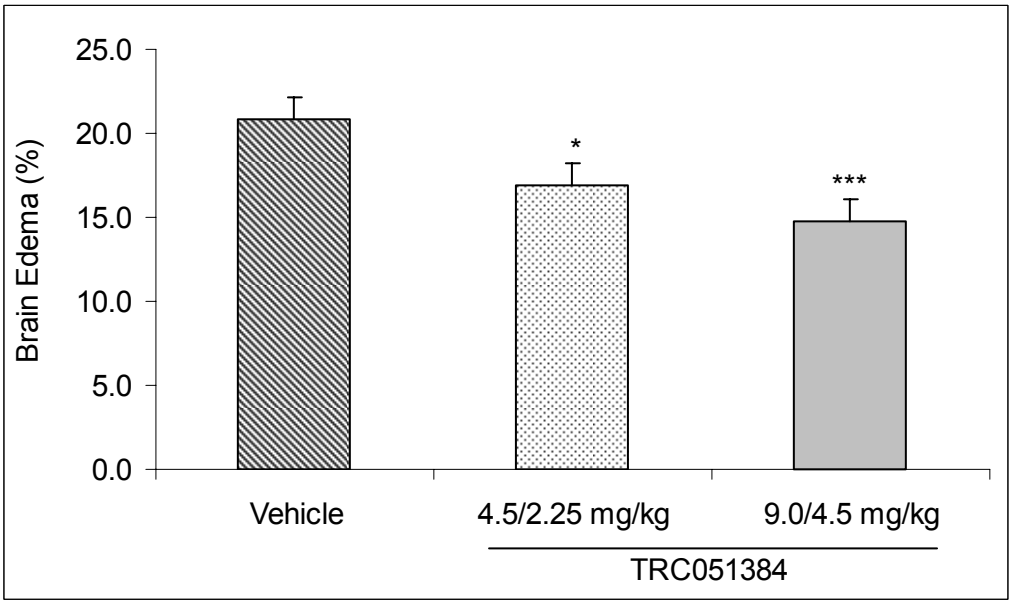

Figure 59: Brain edema reduction as seen with TRC051384 treatment at indicated doses as compared to vehicle control, measured using T2-weighted images in a 2hr transient ischemia model of stroke. TRC051384 was administered at 9mg/kg or 4.5mg/kg (i.p.) as first dose beginning from 8 hrs, 4.5mg/kg or 2.25mg/kg (i.p.) as subsequent doses every 2 hrs in respective group of animals to maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. ***p<0.001, *p<0.05 vs vehicle control.

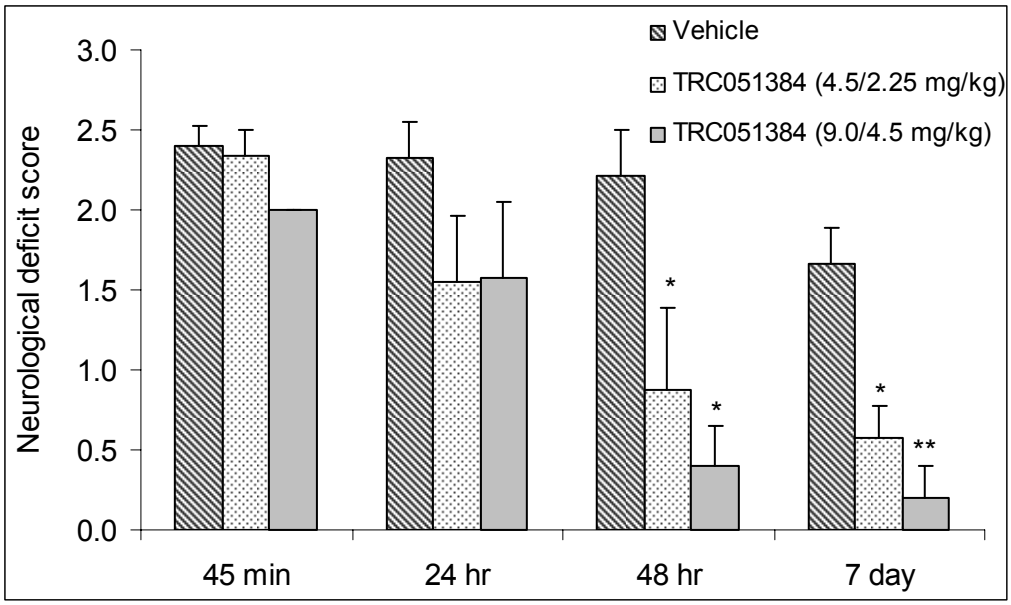

Figure 60: Effect of TRC051384 on reduction in neurological deficit in 2 hr MCAo model of stroke administered at 8 hrs post MCAo at indicated doses as compared to vehicle control. Values represent Mean± SEM .**p<0.01, *p<0.05 vs vehicle control.

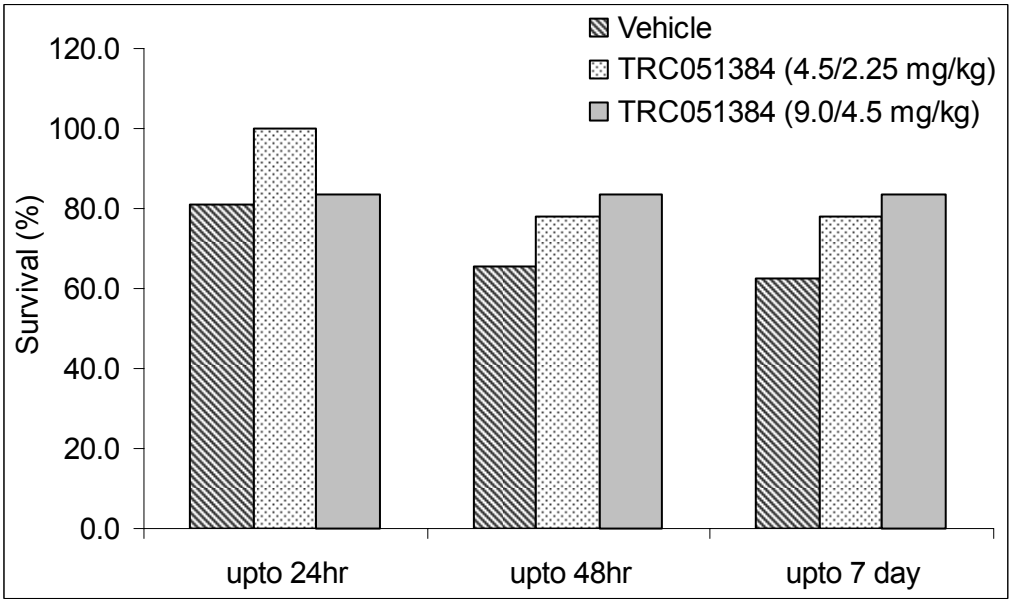

Figure 61: Effect of TRC051384 on % survival in 2 hr MCAo model of stroke administered at 8 hrs post MCAo at indicated doses as compared to vehicle control.

TRC051384 when intervened at 8 hours also showed significant reduction in neurological deficit on day 2 (60% and 82%, p< 0.05) and till the end of the study i.e. day 7 (65% and 88%, p< 0.05) at both tested doses respectively as compared to control

group of animals (Figure. 60). Moreover, we observed reduction in neurological deficit in significantly larger proportion of animals in test compound treated group. Neurological deficit in the TRC051384 group was significantly improved at much earlier time points than vehicle control animals, although deficits gradually improved in both groups over 7 days period. Also treatment with TRC051384 (although not significant) certainly shows a trend towards greater survival of animals in comparison to control animals (Figure. 61).

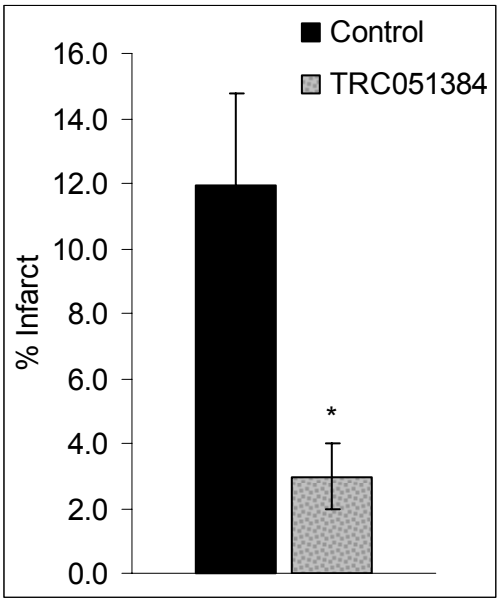

Figure 62: Effect of TRC051384 administered at 8 hrs post MCAo on reduction of infarct measured terminally at 7th day post MCAo after 2 hr MCAo model of stroke. Percentage infarct area was measured on 2mm thick TTC stained brain slices. Values represent Mean± SEM, *p<0.05 vs vehicle control

In order to assess whether the drug treatment related reduction in neuronal injury was further sustained we studied these animals up to 7 days (i.e. 5 days drug free duration). The histological outcome (TTC staining) 7 days after MCAo shows that TRC051384 treatment significantly reduced infarct (75%, p< 0.05) as compared to vehicle treated rats (Figure. 62).

5.2.6.3. Efficacy studies of TRC051384 in permanent MCAo model

We randomized animals to receive either the test compound (TRC051384 or vehicle) on the basis of initial infarct core recorded at 1 hour (First MR Scan) post MCAo. However, over the next 48 hours TRC051384 treatment significantly reduced (36% as against vehicle control, p <0.05) the area of penumbra which got infarcted and

recruited into initial core (Figure. 63). Similarly, treatment with TRC051384 at the tested dose showed a trend towards reduction in edema (Figure 64), improvement in neurological deficit (Figure 65) and better survival (Figure 66) in this permanent ischemia model.

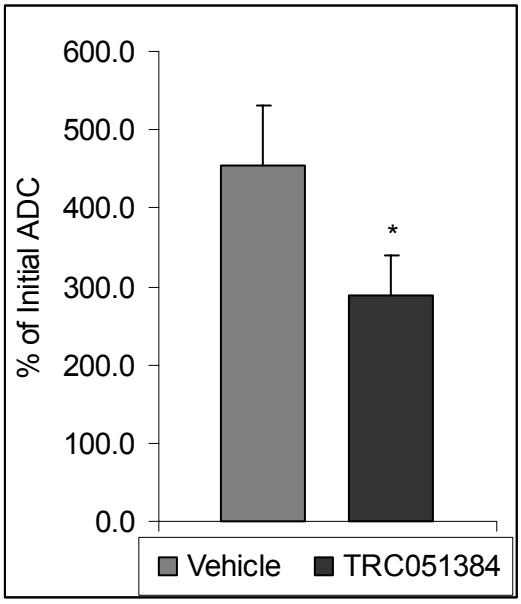

Figure 63: Effect of TRC051384 treatment on reduction in recruitment of penumbra into the infarct core with as compared to vehicle control measured up to 48 hours post permanent MCAo on ADC maps using clinical MRI. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM. *p<0.05 vs vehicle control

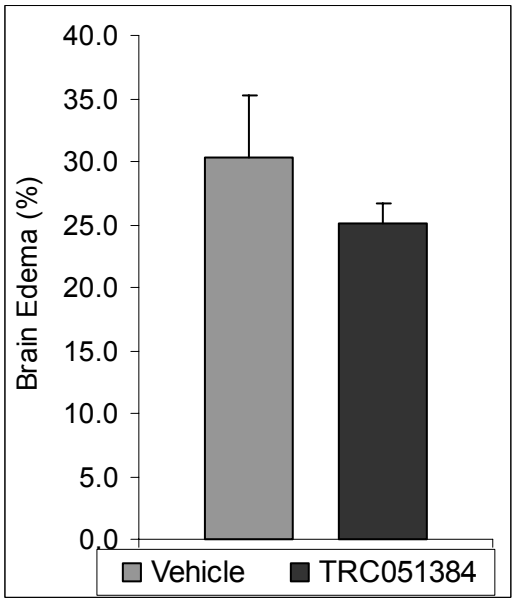

Figure 64: Effect of TRC051384 treatment on reduction in brain edema with as compared to vehicle control measured up to 48 hours post permanent MCAo on T2 weighted images acquired using clinical MRI. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM.

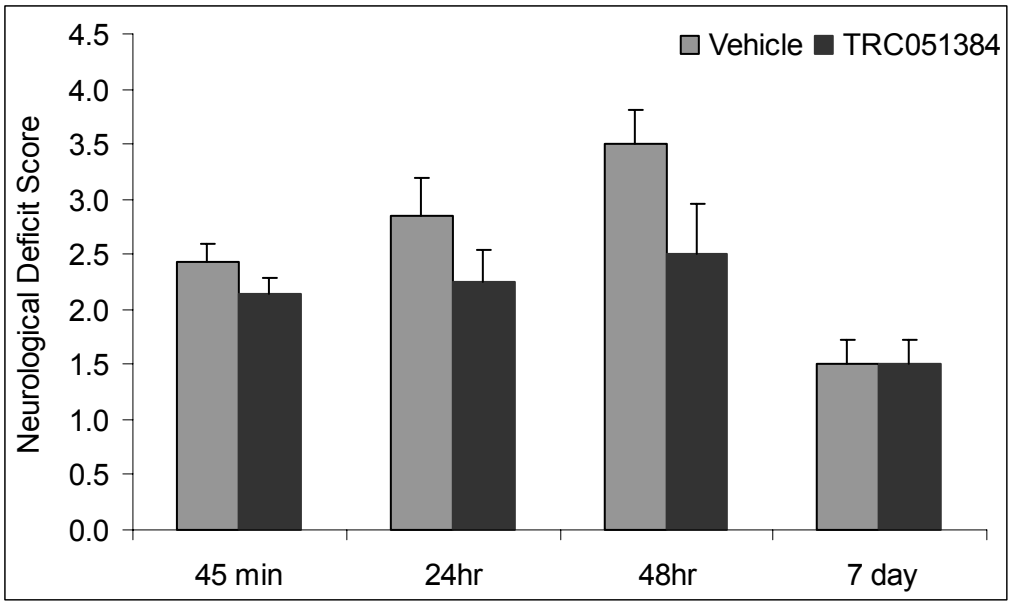

Figure 65: Effect of TRC051384 treatment administered at 8 hrs post permanent MCAo on mean neurological deficit. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo. Values represent Mean± SEM.

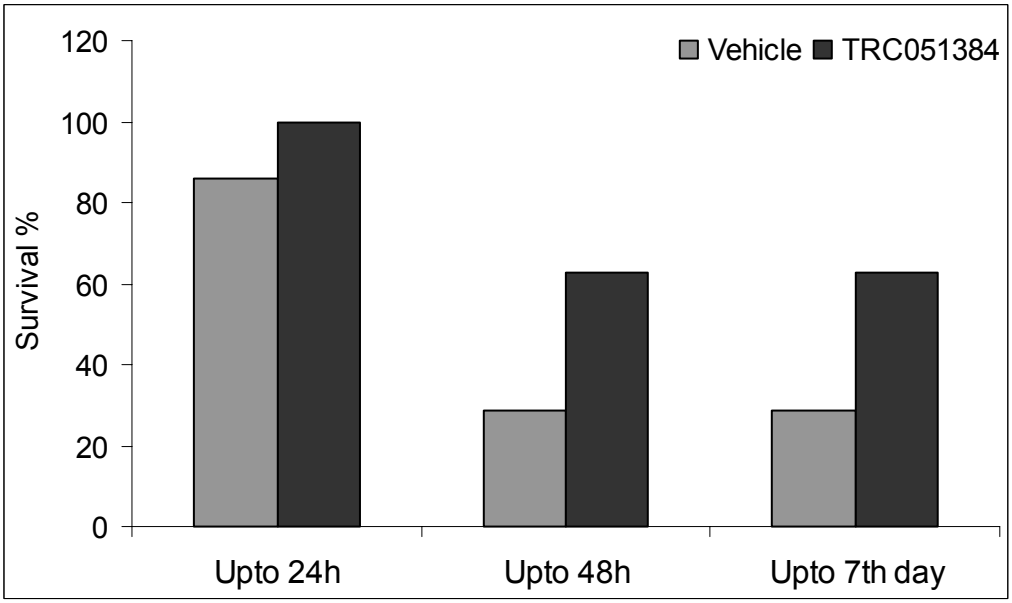

Figure 66: Effect of TRC051384 treatment administered at 8 hrs post permanent MCAo on % survival as compared to vehicle control. TRC051384 was administered at 9mg/kg (i.p.) as first dose beginning from 4 hrs, 4.5mg/kg as subsequent doses every 2 hrs maintain drug exposure upto 48 hrs post MCAo.

5.2.6.4. Efficacy studies in stroke with co-morbid conditions

Once having studied the neuroprotective effect of delayed treatment of TRC051384 in normal rats, we further aimed to test the efficacy of this compound in stroke model with co-morbidities. TRC051384 was evaluated by intervention beginning from 8 hrs after MCAo in SHR and SHR made diabetic by streptozotocin injection.

Table 25: Profiling details of SHR and SHR diabetic animals before MCAo

Group	Age (Week) Mean±SD	Body weight (g) Mean±SD	Blood pressure (mmHg) Mean±SEM	Blood sugar (mMol) Mean±SEM
SHR-Veh	24±1.2	254±6.2	171±1.9	5.3±0.23
SHR-TRC051384	25±1.8	245±7.8	182±2.3	5.2±0.38
SHR-D-Veh	25±1.5	265±6.5	186±11.7	26.5±1.4
SHR-D-TRC051384	24±2.0	254±8.0	189±4.9	26.5±1.1

SHR, spontaneously hypertensive rat; SHR-D; SHR diabetic

We observed significant reduction in infarct (Figure 67) in both SHR animals (32% reduction, $p<0.05$ vs respective control) and SHR-diabetic (24% reduction, $p<0.05$ vs respective control) treated with TRC051384 measured using TTC stained slices

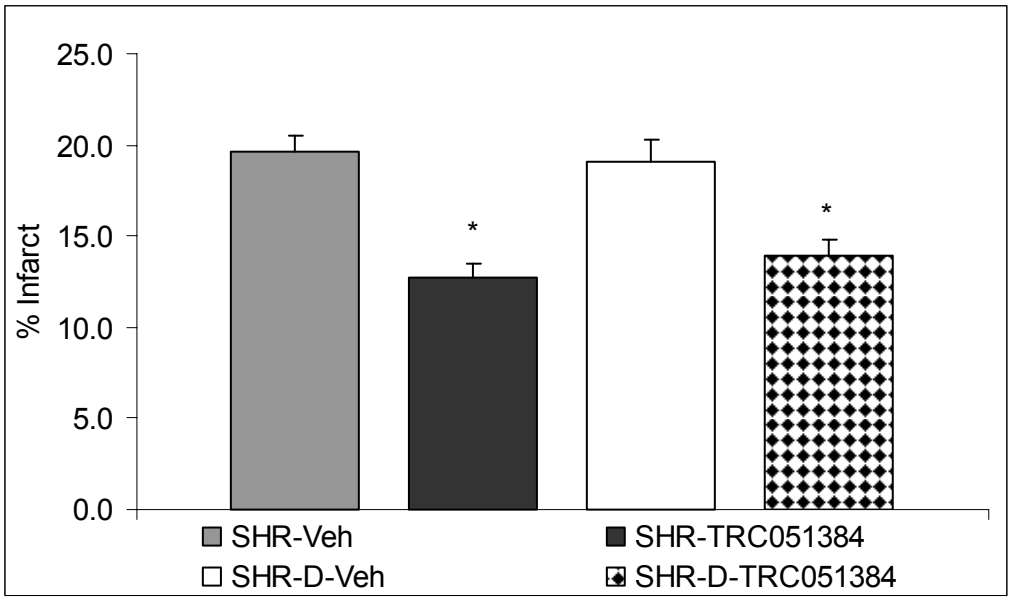

Figure 67: Effect of TRC051384 administered at 8 hrs post MCAo on reduction of infarct measured after MCAo in hypertensive (SHR) and hypertensive made diabetic (SHR-D) rat model of stroke. Values represent Mean± SEM. *p<0.05 vs respective vehicle control

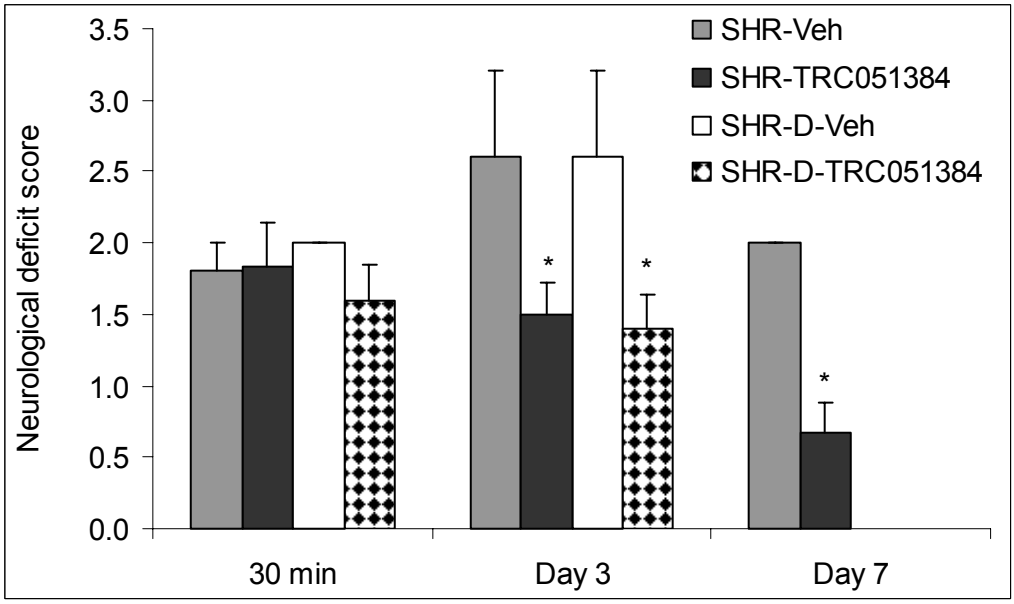

Figure 68: Effect of TRC051384 administered at 8 hrs post MCAo on reduction neurological deficit measured after MCAo in hypertensive (SHR) and hypertensive made diabetic (SHR-D) rat model of stroke. Values represent Mean± SEM. *p<0.05 vs respective vehicle control

Similarly TRC051384 treatment in this study resulted in significant reduction in neurological deficit (Figure 68) on day 3 day 7 in SHR and on day 3 in SHR-D group of animals in comparison to respective controls.

5.2.7. Mechanistic studies

5.2.7.1. BBB Permeability studies

Results indicated in figure 69 shows TRC051384 penetrates BBB and shows significantly higher levels in ipsilateral hemisphere (Damaged side) as compared to contralateral hemisphere (Normal side) of the brain.

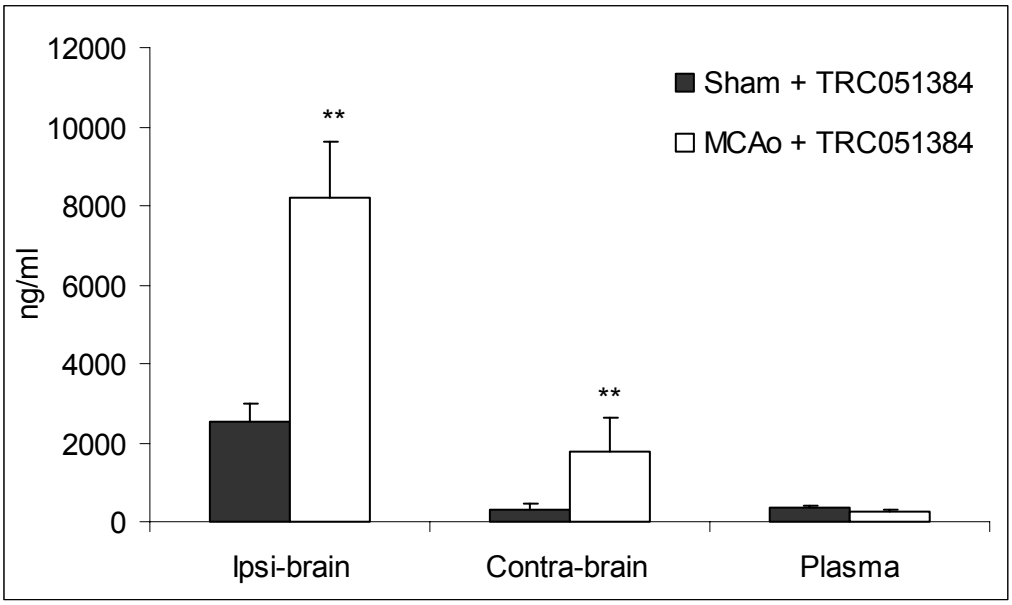

Figure 69: TRC051384 concentration levels in different brain hemispheres and plasma of MCAo rats. Values represent Mean± SEM. **p<0.01 vs respective sham control

5.2.7.2. Gene macro array studies

Modulation of gene expression profile by TRC051384 was studied in injured hemisphere of brain from stroke affected animals. Stroke-induced upregulation of pro-inflammatory cytokines such as TNF-α, interleukin 6 (IL-6), IL-1a and b are significantly ($p < 0.05$ versus control) abrogated by treatment with TRC051384. In addition, treatment also significantly ($p < 0.05$ versus control) lowered, stroke-induced expression of cell adhesion molecules such as E-Selectin, P-Selectin, intracellular cell adhesion molecule (ICAM) and key pro-inflammatory mediators such as MCP-1, MIP-α, CINC, NOS2 and COX2 (Figure. 70&71).

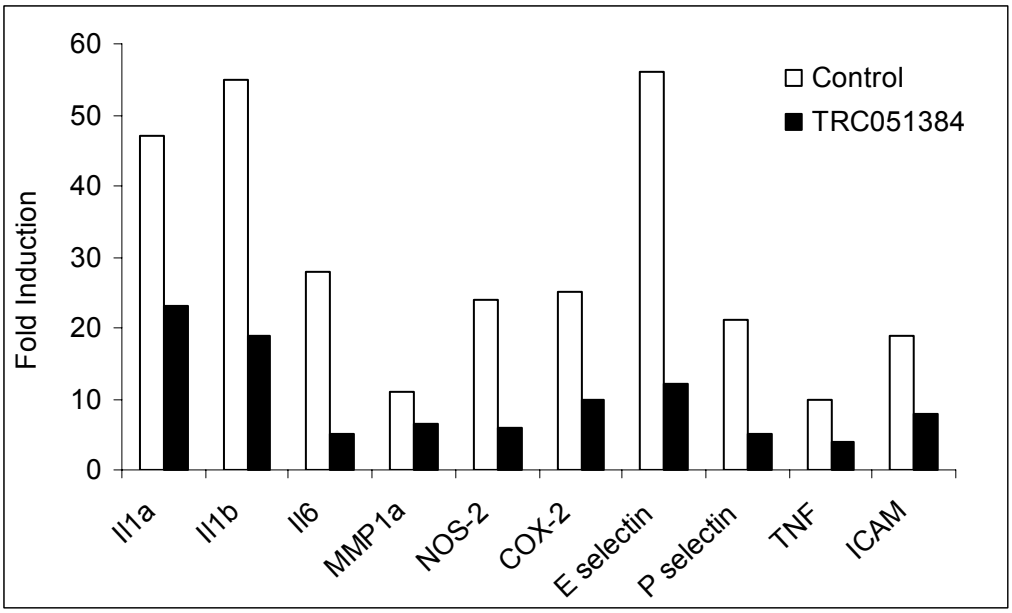

Figure70: Abrogation of inflammation associated gene expression by treatment with TRC051384 in the affected brain as compared to control rats, measured with real-time PCR based low density array system. All values are expressed as mean ±SEM; *p < 0.05; **p < 0.01; versus the vehicle control

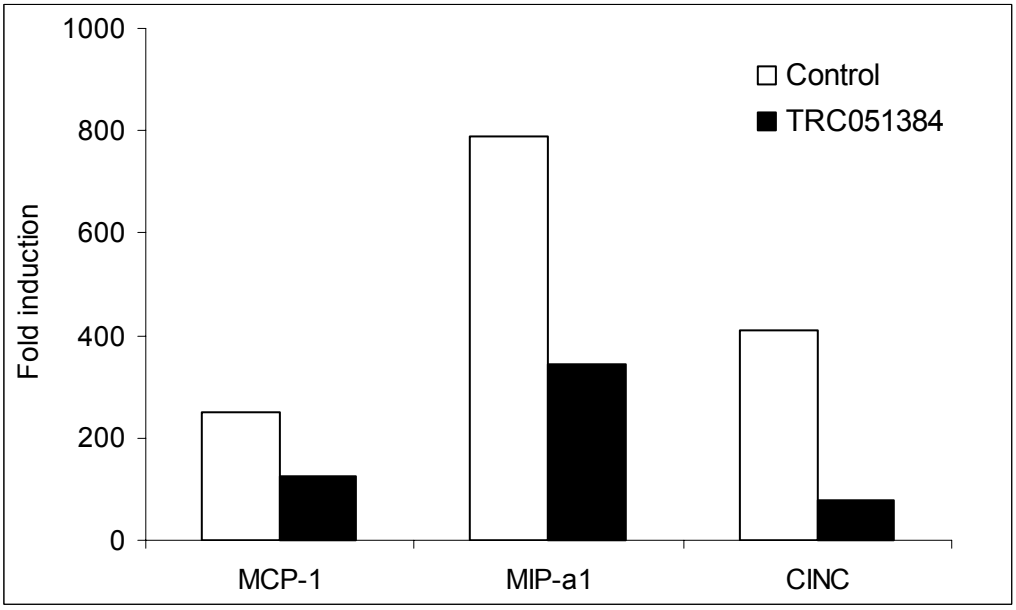

Figure71: Abrogation of inflammation associated gene expression by treatment with TRC051384 in the affected brain as compared to control rats, measured with real-time PCR based low density array system. All values are expressed as mean ±SEM; *p < 0.05; **p < 0.01; versus the vehicle control

5.2.7.3. HSP70 Immunohistochemistry and TUNEL staining

HSP72 immunohistochemistry has further confirmed that the compound TRC051384 has the potential to induce HSP72 in rat brain. The coronal brain sections from TRC051384 treated rats have shown greater HSP72 proteins as seen with higher count of HSP72 immunopositive stained cells in the penumbral region (Figure. 72) and a milder staining at the infarct core.

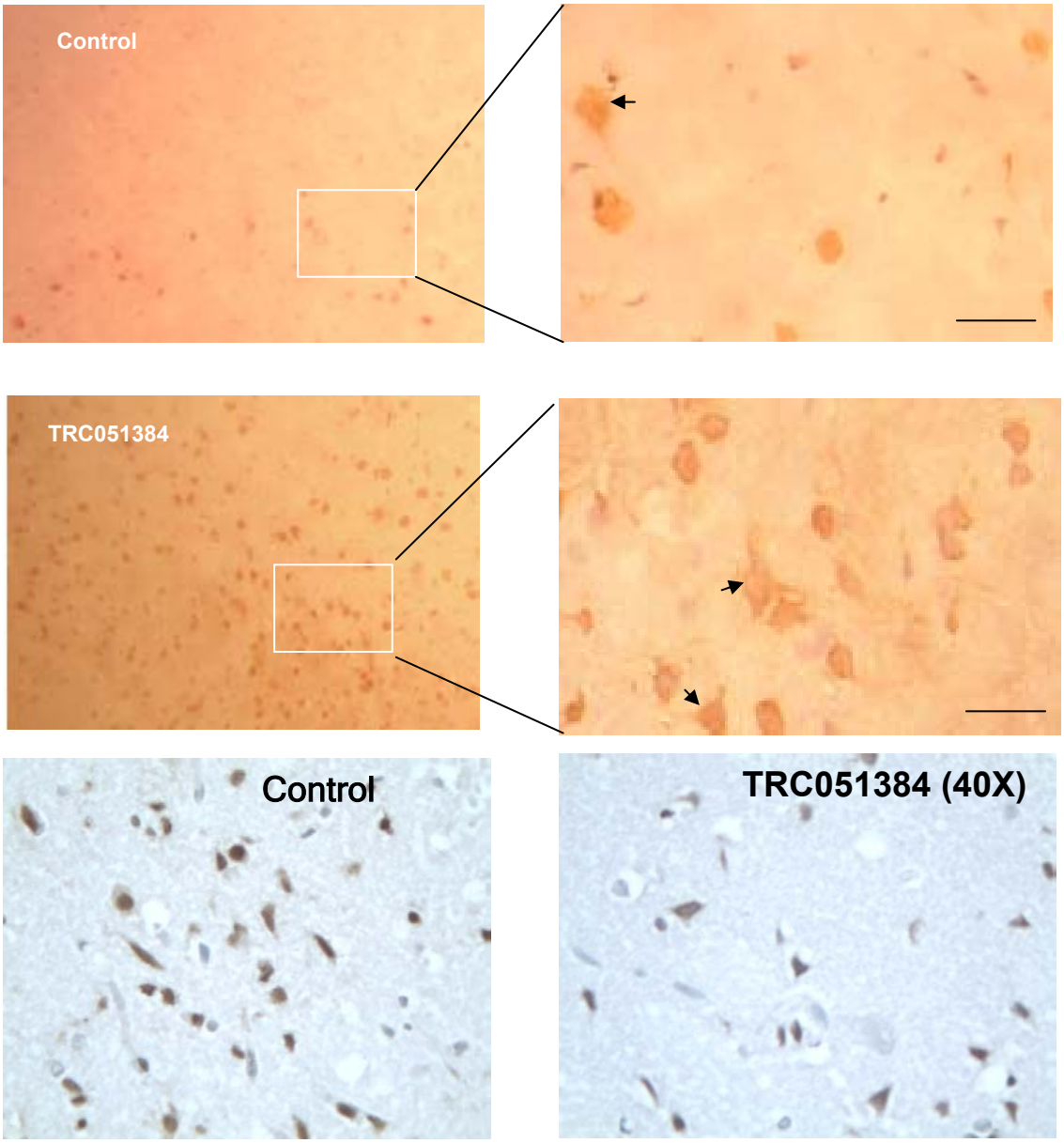

Figure 72: Representative photomicrographs of brain sections showing enhanced expression of HSP72 in the affected region in TRC051384 treated rats compared to control rats. Also shown on the lower panel, TRC051384 treatment significantly reduced apoptotic cells presence. Scale bars: 25 mm (arrow showing HSP72 positive neurons).

TRC051384 treatment induced HSP72 positive cells which were seen till 72 hours post ischemia. Both neuron and glia showed enhanced HSP72 expression with TRC051384 treatment. TUNEL staining (Figure 72) confirms in addition to immunohistochemistry findings that drug induces HSP70 in rat brain and this induction of HSP 70 reduces the apoptosis of neuronal and non-neuronal cells.

Discussion

6. Discussion

Stroke is clearly a devastating disease that affects millions of people worldwide, and contributes to high morbidity and mortality. It is a common clinical problem with increasing prevalence, serious consequences, unsatisfactory therapeutic options and an enormous financial burden to the society. Projections for the period 2006-2025 estimate new cases of ischemic stroke at 1.5 and 1.9 million cases in men and women respectively, highlighting the increasing profile of stroke in the future (Ankolekar, et al., 2012; Zille, et al., 2012; Mukherjee and Patil, 2011; Feigin, et al., 2009). Current management of ischemic stroke suffers from large dependence on preventive strategies. Thrombolysis using tPA is the only approved therapy available to date for the treatment of stroke. Compounding the problem further is the issue of limited time window for treatment with tPA, resulting in fewer than 5% of stroke patients being treated. An attempt to increase this time window beyond 3 hrs by conducting large scale clinical trials has been reported to be achieved with limited success (Fagan, et al., 1998). Hence there exist a huge unmet need and there is an urgent need for a therapeutic agent which can abrogate the deleterious cascade of events which leads to neuronal death in stroke.

Given the multifactorial nature of stroke, targeting more than one mechanism has been looked at as a plausible strategy. There are several parallel pathways in which ischemia can lead to tissue damage (Zille, et al., 2012; Woodruff, et al., 2011; Lipton, 1999; Pulsinelli, 1992). Subsequent extension of the damage in the penumbra is contributed by free radical injury, inflammation and apoptosis which are further potentiated by reperfusion occurring several hours to days post ischemia (Woodruff, et al., 2011). Hence interventional modalities which target free radical generation, inflammation and apoptosis are expected to reduce the delayed extension of brain damage post ischemic insult. Emerging experimental evidence support this hypothesis as several putative neuroprotective agents have been shown to protect extension of infarct to the penumbra in various animal models when administered soon after the initiation of ischemic insult. Such therapies which have shown significant protection of penumbra include antioxidants (Takasago et al., 1997), free radical scavengers (Lees, et al., 2006, and antiapoptotic agents (Amemiya, et al., 2005) amongst others. Inspite of demonstrating a clear preclinical efficacy these therapies were not reported to be translated into effective bedside therapies (Fisher, 2005; Fisher, 2003; Gladstone, et al., 2002; STAIR Group, 2001; STAIR Group, 1999). The reason for this, being, all of them

had very short window for efficacy, thus limiting their clinical utility. These pre-clinical results support mechanism based neuroprotective efficacy with very early administration but do not adequately substantiate whether robust neuroprotection is possible with longer, clinically relevant, treatment delay.

On the other hand several strategies are reported which address the multiple components of the disease pathology and have been shown to offer good efficacy. The need for more of such novel approaches is presently felt and these should be evaluated, for example, an approach that takes into account stroke biocomplexity is to target processes within the dynamic network that activate ensembles to promote maintenance of homeostasis under stress. In this regard the present promising agents that have shown preliminary clinical success such as high dose albumin therapy (Palesch, et al., 2006), hyperacute magnesium therapy (Saver, et al., 2006) which act on multiple pathways provide proof for the hypothesis stated above. Similarly strategies under experimental evaluation like upregulation of cell survival/adaptive pathways also hold promise for similar reasons.

Maintenance of homeostasis is a fundamental mechanism that the body has conserved evolutionarily. One such significantly studied homeostatic mechanism in experimental stroke is induction of HSP (Turturici, et al., 2011; Evans, et al., 2010; Sato, et al., 1996). Several in-vitro and in-vivo experimental evidence suggest that augmentation of this endogenous mechanism offers stroke protection, that too, with a clinically relevant time window for treatment. At the in-vivo level, recently one group has shown recombinant Fv-Hsp70 protein mediates neuroprotection after focal cerebral ischemia in rats (Zang, et al., 2010). Another group with constitutive overexpression of HSP70 in one strain using a cytomegalovirus enhancer combined with a β-actin promoter resulted in near complete protection determined by overall reduction in infarct size (Badin, et al., 2009). Most of the previous experiments have employed either HSP70 over expressed animal models or recombinant protein delivered using viral vectors, which as of today is difficult to administer in the clinic. Thus targeting HSP70 induction employing a pharmacological agent presents attractive strategy (Evans, et al., 2010). According to our knowledge, we for the first time have shown that a pharmacological induction of HSP70 using TRC051384 demonstrates neuroprotection upon delayed intervention in an experimental model of focal cerebral ischemia.

The first challenge was to identify an animal model of stroke which resembles the pathobilology associated with human ischemic stroke. Further such model should allow

for the therapeutic evaluation of a neuroprotective agent. There are mainly two factors that influence the selection of in vivo stroke models for preclinical trials. These are the potential protection mechanism of the neuroprotective candidate and the highest achievable model quality with a particular lab setting. We employed focal cerebral ischemia model produced by occlusion of MCA using intraluminal suture method in rats. This choice was made for the following reasons as stated here. There are several animal models of cerebral ischemia reported in the public literature. Rodent models of cerebral ischemia can be classified as global or focal and as reversible or irreversible. Global ischemia models mimic the clinical conditions of brain ischemia following cardiac arrest or profound systemic hypotension, focal models represent ischemic stroke, the most common clinical stroke subtype. Most human ischemic strokes are caused by occlusion of the MCA (del Zoppo GJ, et al., 1992) and so animal models were developed to induce ischemia in this arterial territory. The great advantage of these techniques is that the thread (suture used for occlusion of artery) can either be left in place for permanent occlusion or withdrawn any time to permit controlled reperfusion, and the presence of a significant ischemic penumbra early after occlusion makes them particularly suitable for studies of neuroprotection (Sicard and Fisher, 2009; Hsu, 1993). At the same time, most importantly, intraluminal suture method is less traumatic, offers more success rate and produces wide spread infarction involving cortical and subcortical regions (entire MCA supplied area) and allows prompt evaluation of neurological deficit which are severe and consistent (Longa, et al., 1989).

We used rats to induce focal cerebral ischemia. Rats are the most commonly used animals for several reasons, including: (1) their resemblance to humans in their cerebral anatomy and physiology, (2) their small size which enables easy analysis of physiology and brain tissue, (3) their low cost, (4) the remarkable genetic homogeneity within strains, and (5) greater public and institutional ethical acceptability of use relative to larger animals (Sicard and Fisher, 2009).

Here in our study we first attempted to standardize both transient and permanent ischemia models to be used for therapeutic evaluation of TRC051384. Transient focal ischemia has been shown to produce varying degrees of ischemic damage depending on the duration of ischemia (Durukan and Tatlisumak, 2009). Importantly, after transient ischemia, brain damage results from both the ischemia and the effects of reperfusion (reperfusion injury). Compared to permanent occlusion, which mimic only a minority of human strokes where there is no recanalization, transient models better correlate with

conditions such as therapy-induced thrombolysis, spontaneous thrombolysis, and transient ischemic attack. However, both permanent and transient ischemia models are needed prior to clinical drug development studies because of the heterogeneity of human stroke (Durukan and Tatlisumak, 2009; Fischer M, 2003).

The intraluminal MCAo models can be induced using different filaments. It is required that a proper choice of these filaments (sutures) to be used for occlusion of cerebral artery is necessary as it is reported that physical characteristics of these sutures influence outcome variation by causing insufficient occlusion, premature reperfusion, and/or filament dislodgement. We initially have identified the best suture properties required for producing consistent infarction, edema and neurological deficit. For this we made use of several suture types of varying diameter and coating properties to produce MCAo in order to achieve improved success rate, consistency and reduced variability. We found that when 3-0 sutures (tip rounded by rubbing on sand paper) coated with 0.1% poly-l-lysine produced most consistent results at our hand. These findings are in agreement with previously reported studies where Belayev et al reported increased infarct volume and experimental consistency with poly-l-lysine coated filaments as compared to uncoated sutures (Belayev, et al., 1996). Several others have made use of silicone-coated sutures to produce MCAo because these reduce the problems of subarachnoid hemorrhage (Schmid-Elsaesser, et al., 1998) and variability (Aspey, et al., 1998, Spratt, et al., 2006). At the same time it was observed by few that additional coating of the silicone with poly-L-lysine may further enhance the suture efficiency (Lourbopoulos, et al., 2008; Bouley, et al., 2007). At the same time when sutures with heat made bulb were used we observed variable results and higher mortality. In similar studies Tsuchiya et al. (Tsuchiya, et al., 2003) showed that using flame blunted monofilaments to induce MCAo caused a 40% rate of subarachnoid hemorrhage, and % coefficient of variation was greater than 100%. In another study (Schmid-Elsaesser, et al., 1998), models using heat-blunted 3-0 filaments had a success rate of 46%, with 44% occurrence of SAH. In our study with the use of 0.1% poly-L-lysine coated 3-0 sutures and animals of body weight range used here, we observed a success rate of 80-85% which is in close agreement with studies performed in other labs as well. When the occluder is matched to animal size, improved success rates and reduced sub arachnoid hemorrhage rates are reported. In rat models of 60-min transient MCAo to 24-h permanent MCAo using correct sized occluder, the success rate was

found to be 88-100%, and the SAH rate to be 4% (Candelario-Jalil, et al., 2008; Tsubokawa, et al., 2007; Khan, et al., 2006; Solaroglu, et al., 2006).

In studies of MCAo induced stroke strict control of some experimental conditions such as temperature and use of anesthetic agent is very important as these conditions are expected to influence outcome. Brain temperature during hypoxia affects brain metabolism significantly (Winn, et al., 1981). Hypothermia reduces (Florian, et al., 2008; Miyazawa, et al., 2003; Ohta, et al., 2007) and hyperthermia exacerbates (Kim et al 1996; Noor, et al., 2003; Noor, et al., 2005) ischemic brain injury, hence, fluctuation in animal body temperature will increase the variability of stroke outcome. For these above stated reasons we have maintained body temperatures of rats to 37 ± 1^{0}C both during induction of ischemia and while performing reperfusion using thermostatic controlled homeothermic blankets. Similarly choice of anesthesia is also very important. It is suggested that when designing a preclinical study for neuroprotection, the protection provided by anesthetics should be taken into account. Many commonly used anesthetics have been shown to have neuroprotective effects against cerebral ischemic injury. These anesthetics include isoflurane (Kawaguchi, et al., 2004; Xiong, et al., 2003), sevoflurane (Nakajima., et al 1997; Payne, et al., 2005), desflurane (Erdem, et al., 2005; Tsai, et al., 2004), halothane (Haelewyn, et al., 2003), xenon (David, et al., 2008), nitrous oxide (Abraini, et al., 2004; Haelewyn, et al., 2008), barbiturates (Warner, et al .,1996), propofol (Bayona, et al., 2004), ketamine (Proescholdt, et al., 2001), and the local anesthetic lidocaine (Siniscalchi, et al., 1998; Weber and Taylor, 1994). We used halothane as an anesthetic agent and animals were kept anesthetized for a very short period during the MCAo procedure (10-15 mins) and were reanesthetized again during reperfusion. Use of halothane allowed us faster recovery of animals from anesthesia and early measurement of neurological deficit. Although halothane is reported to cause protection against focal cerebral ischemia such an effect was seen only with longer duration of anesthesia of greater than 2 hr (Haelewyn, et al., 2003) and short duration of anesthesia as used in our study were found not to influence the outcome (Haelewyn, et al., 2003)

In order to evaluate the temporal profile of infarct development, edema formation and neurological deficit we conducted a study where animals were terminated intermittently at predetermined time point upto 48hrs after 3hr MCAo and reperfusion. These studies were conducted with an objective in mind to identify when does the peak injury occurs in this model so that a realistic time window can be identified for initiation of

neuroprotective therapy beyond which time it may be impossible to show the protective effect. At the same time we wanted to understand the extent of neurological deficit which occurs in our set up i.e. 3 hr MCAo and the natural process of functional recovery following cerebral ischemia. Peak infarction measured on TTC stained brain slices of intermittently sacrificed animals was observed between 12-24 hrs post MCAo. Similarly peak brain edema observed at 24-36 hrs post MCAo. Neurological deficit recorded using the method described by Longa et al (Longa, et al., 1989) was found to be persisted till the end of 7 days study period. These results are in accordance with guidance provided for validating neuroprotective efficacy, that functional tests with a slow or absent natural recovery process may be most appropriate, such as forelimb flexion, gait disturbance, and lateral resistance (Reglodi, et al., 2003). It is also suggested by several groups that to qualify for neuroprotection studies, the neurological scale must be able to detect the major ischemia induced behavioral changes, including motor, sensory, motion coordination, spontaneous activity, reflexes, consciousness, and alertness changes. In this regard the neurological scoring method used presently in our study detects the behavioral aspects mentioned above and has been widely used in preclinical neuroprotective stroke studies. (Becker, et al., 2001)

We chose to work with ischemia duration of greater than two hours in our study. Optimum duration of ischemia is very essential parameter in working with intraluminal models since this has direct effect on model severity and consistency. Transient Ischemia of durations less than 2 hr have been proposed to produce mild neuronal damage, higher variability (Yang, et al., 2001, Selman, et al., 1994) and on the other hand transient ischemia of greater than 4 hrs was found to be very severe with greater mortality. Occlusion duration of 2hr- 3hr time is preferable for transient ischemia because of increased consistency and similar results have been reported with different occlusion methods (Drummond, et al., 1995).

In all our studies quantification of infarct area and edema were carried out using TTC stained brain slices collected from terminally sacrificed animals. Among a number of histopathological methods, TTC and hematoxylin-eosin staining are the two most commonly employed. Infarct quantification by TTC is more rapid, easy and economic than hematoxylin-eosin (Bederson, et al., 1986; Lundy, et al., 1986). TTC serves as a proton acceptor for many pyridine nucleotide-linked dehydrogenases (such as succinate dehydrogenase); it is reduced by these enzymes in viable brain tissue into a red, lipid-

soluble formazan, while infarcted or non viable tissue remains unstained (Bederson, et al., 1986; Liszczak, et al., 1984).

We then explored the feasibility of using a clinical MRI for the study of infarct and edema progressions measured non-invasively and have quantitative estimation done on images thus obtained. We sought the major advantage of using MRI would be that this technique would allow us to monitor pathological changes or treatment related improvement in the same animal non-invasively over a long period of time greatly eliminating the animal to animal variation observed in such studies. We have successfully standardized the anatomical imaging of small animal brain and have made quantitative estimation of brain infarction and edema in a MCAo model on images acquired using different imaging modalities such as DWI and T2 weighted imaging. We used 1.5 tesla clinical magnetic resonance scanner and suitably modified various pulse sequences to be able to image small rat brain. Experimental stroke models in small rodents are frequently studied with MRI methods. The application of MRI has brought the advantage of non invasive in vivo monitoring of pathological changes for longer durations, and therefore offers in-depth information in real time. However, most of the studies are performed on high resolution (>4 tesla) small bore dedicated preclinical MR scanners to obtain high spatial resolution and sensitivity. But, limited availability of such machines and high cost makes it difficult for research studies involving animal applications. For this purpose there are several groups who have used clinical MR scanners with suitable modifications for imaging small animals (van der Weerd, et al., 2005; Neumann-Haefelin, et al., 2000) MR imaging generally require classic MR imaging modalities such as T1, T2 imaging and newer techniques include diffusion and perfusion imaging (Hoehn, et al., 2001; Hesselbarth, et al., 1998). DWI sequences are especially sensitive to early ischemia, and many studies have utilized this resource in animal imaging, which is capable of visualizing the penumbra - the potentially salvageable area at risk (Tatlisumak, et al., 2004; Hoehn, et al., 2001; Xue, et al., 1999). Similarly T2 - weighted MRI was used by many researchers which have been proposed to provide information on brain edema (Pedrono, et al., 2010).

We then performed serial MRI after 2hr and 3hr transient focal cerebral ischemia and monitored infarct and edema progression over 48hr time. These studies allowed us to monitor infarct progression over long time and we found in both 2hr and 3hr MCAo model peak infarct was observed at 24hr post initiation of MCAo. More over we found that in 2hr ischemia model, being relatively milder had small infarct core and larger area

of penumbra was recruited over 48hrs time. On the other hand 3hr ischemia model which is proposed to be a severe injury model had large core and less penumbra area to be infarcted over time. Infarct progression was quantified on ADC maps constructed by MR image analysis software using diffusion weighted pulse sequence and brain edema was quantified on T2 weighted images. Such imaging techniques i.e. DWI and T2 imaging to obtain Information related to cerebral infarct progression, penumbra and edema have been successfully used in similar studies in MCAo models to understand disease progression or to monitor efficacy of a neuroprotective agent (van der Weerd, et al., 2005; Neumann-Haefelin, et al., 2000; Guzman, et al., 2000; Li, et al., 2000b)

Once having standardized the animal models of transient and permanent MCAo we than have validated these models to check if such models can be used for the evaluation of therapeutic efficacy of newer neuroprotective agents. For this purpose we made use of a reference compound MK-801. The N-methyl-D-aspartate (NMDA) antagonist MK-801 was used for this study because this drug has been studied widely and has demonstrated efficacy in similar models (Schabitz, et al., 2000; Minematsu, et al., 1993). MK-801 has demonstrated significant efficacy in our models as shown by reduction in recruitment of penumbra to the infarct core in transient model and significant reduction in infarct area measured using TTC stained slices in permanent ischemia model.

We then moved further for the evaluation of our identified NCE i.e. TRC051384 for its neuroprotective effect in various in-vitro and in-vivo system. A series of new chemical entities were designed and synthesized at Torrent Research Centre (Kumar, et al., 2005) and they were screened sequentially in vitro, in HeLa and a primary neuronal cell based assay system for the upregulation of HSP70B gene expression, cytotoxicity and inhibition of production of inflammatory cytokine TNF-α. Active non-cytotoxic molecules from the in vitro screens along with having reasonable aqueous solubility were short-listed. TRC051384, a novel small molecule with ability to induce HSP70 proteins, was short-listed to study its potential benefits in stroke.

Under non-stress conditions, we show that TRC051384 increased HSP70B mRNA expression in both HeLa and primary mixed neuronal cells. HSP70 induction upon treatment with TRC051384 involves activation of transcription factor HSF1. Transcription factor HSF1, once activated, binds with HSE and induce transcription of target genes such as HSP70 (Brown, 2007). HSP70 induced by TRC051384 is functionally active and translates into increased chaperone activity. Treatment with

TRC051384 resulted in significant recovery of luciferase activity in HeLa cells expressing cytoplasmic firefly luciferase, which were exposed to sublethal heat shock (which inactivates and reduces the luciferase activity). This demonstrates significant enhancement of chaperone activity due to TRC051384 treatment in vitro. Additionally our compound possesses strong anti-inflammatory activity. We have observed such anti-inflammatory activity in vitro with TRC051384 treatment as demonstrated by the inhibition of LPS induced TNF-α expression. We further evaluated if these in vitro effects seen with TRC051384 (HSP70B induction, elevated chaperonic and anti-inflammatory activities) can be translated effectively in an in vivo model. Thus we have evaluated effect of TRC051384 treatment in a rat model of cerebral ischemia and reperfusion. We hypothesized that TRC051384 treatment is expected to modulate delayed events of tissue damage occurring in penumbra there by offer neuronal protection arising from ischemic stroke.

Using MRI and TTC in this study, we have shown that, TRC051384 belonging to substituted 2-propen-1-one class, which is a potent inducer of HSP70, significantly reduces stroke associated neuronal injury and reduces neurological deficit in a rat model even when administered 4 hours and/or 8 hours post onset of focal ischemia. In this study we have shown the neuroprotective effect of TRC051384 by delayed intervention treatment in transient MCAo, permanent MCAo and in transient MCAo produced in hypertensive and diabetic animals.

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. In disease conditions like stroke where immediate systemic availability of drug for its quick action is required and at the same time the patient is usually in unconscious state the preferred route of drug administration is by parenteral route. For this purpose we established the pharmacokinetic profile of TRC051384 by i.v and i.p. route which helped us to decide the dosing frequency required to maintain steady plasma concentration of TRC051384. Further in order to understand preliminary safety and tolerability of TRC051384 before extensive efficacy studies effect of different doses of TRC051384 on cardiovascular and respiratory system was evaluated. TRC051384 was found to be better tolerated at all doses tested with negligible effect on BP and no effect on other parameters such as ECG, heart rate and respiration rate. Similarly, TRC051384

administered as multiple doses (9.0mg/kg as first dose and 4.5mg/kg subsequently repeated every 2 hours by i.p. route) over 48 hours was also found to be better tolerated with no change in BP. Since the change of blood pressure affects regional cerebral blood flow and hence stroke outcome (Drummond, et al., 2000; Kawaguchi, et al., 2004) blood pressure monitoring during experiments are recommended.

We initially have identified by conducting various studies the optimum dose of TRC051384 and duration of treatment required for translating in-vivo efficacy. We found from these studies that drug exposure duration of approximately 40 hrs is required for most significant efficacy. Once we established this, our aim further was to identify the therapeutic window that TRC051384 offers for the treatment of ischemic stroke, which was one of the major objectives of these studies. For this we evaluated TRC051384 for the ability to reduce infarct and neurological deficit in rat model of transient and permanent ischemia when treatment was initiated as delayed as 4 hours and 8 hours post ischemia onset.

Many of the research efforts towards neuroprotection with putative neuroprotective agents have not translated into effective clinical therapies for ischemic stroke (del Zoppo, et al., 1997; Gladstone, et al., 2002; Brown, 2007). One major reason for this could be the experimental design, in which most of these neuroprotective agents have been evaluated either prophylactic or been administered soon after the initiation of ischemic insult, thus questioning their clinical utility. Some of the neuroprotective agents have failed in delayed intervention protocols as the molecular targets, the test agent addresses, is an early mediator in the sequence of events involved in the injury cascade post ischemic insult (Zaleska, et al., 2009). We therefore evaluated effect of TRC051384 treatment in a rat model of stroke upon delayed intervention i.e. treatment was initiated 4 hours and 8 hours after initiation of MCAo. Further in our study MRI was used as it offers advantage of non invasiveness in order to examine the progression of the core and penumbra over a period of time. The imaging modalities, which earlier have been used extensively, such as T2 weighted and ADC (Guzman, et al., 2000; Li, et al., 2000b; van der Weerd, et al., 2005) helped in monitoring the development of brain edema and extent of penumbra being further recruited into the ischemic core. Hence, disease monitoring in individual rats was done in this study by serial magnetic imaging with a 1.5 T clinical MR machine at various time points post-initiation of MCAo. Animals were monitored till the end of 7 days though MRI was restricted to 48 hours post stroke onset, due to MR fogging (O'Brien, et al., 2004). In this study, since ADC maps, a sensitive tool

to monitor extent of tissue injury was used; the changes in blood flow/perfusion, blood gases and blood pressure were not monitored before and during ischemia. The ADC map generated at 1 hour post-initiation of ischemic insult was used to randomize the animals with similar initial infarct core to the treatment groups. TRC051384 significantly reduced the area of penumbra being recruited to infarct, hence reduced the rate of infarct progression observed till 48 hours even with delayed intervention beginning either at 4 hours (in 2hr, 3hr transient ischemia models and permanent MCAo model) or 8 hours (in 2hr transient ischemia model) after the ischemia onset. On TTC stained brain slices of terminally (on seventh day) sacrificed rats we saw reduced infarct size with TRC051384 treatment as compared to control animals, demonstrating the sustained effect of TRC051384 on reducing neuronal injury associated with MCAo.

Our results show that the extent of penumbra salvaged upon delayed intervention of 4 and 8 hours (in 2hr MCAo model) is similar. These results hence infer that a similar salvageable penumbra is present in rats even at 8 hours as that was present at 4 hours after onset of ischemic insult. The neuroprotection shown by TRC051384 even with a delayed intervention at 8 hours could be attributed to its multiple mechanisms of action i.e. HSF1 activation and resultant elevated chaperone and anti-inflammatory activity, which addresses multiple targets in the sequence of events involved in the injury cascade post ischemic insult.

Vasogenic edema contributes significantly in the early mortality associated with ischemic stroke. We here evaluated the ability of TRC051384 to reduce brain edema in MCA occluded animals. TRC051384 treatment significantly reduced brain vasogenic edema calculated using coronal T2-weighted images. Lesion detected on T2-weighted images provides the earliest and best information about vasogenic edema (Mack, et al., 2003; Neumann-Haefelin, et al., 2000). These observations are in agreement with earlier published reports in transgenic overexpression of HSP70 (Zheng, et al., 2008) or pharmacological induction of HSP70 using pretreatment with geranylgeranylacetone (Nagai, et al., 2005) which have shown reduction in vasogenic edema in transient and permanent ischemia models. Brain edema in stroke is a major contributor for early mortality. Vasogenic edema which results from blood brain barrier disruption leads to increased intracranial pressure and brain tissue herniation which contribute to neurological impairment and mortality (Gerriets, et al., 2004). Early reduction of brain edema has been correlated well with increased survival from many preclinical and clinical studies. We observed similar results from the current study in which TRC051384

treatment has improved the survival over 7 days. We observed a significantly improved survival with TRC051384 treatment initiated at least at 4 hours. The observed difference in effect on survival with TRC051384 treatment initiated at 4 hours and 8 hours could be attributed to the early and greater reduction of brain edema with 4 hours intervention. But, we conclude that, although we observed a marginal improvement in survival with treatment at 8 hours it still holds a greater clinical significance.

The long term neurological deficit associated with stroke implies poor prognosis along with immense social and financial burden. Any therapy which can bring functional improvement would mean a significant clinical success. Further, reduction in the incorporation of penumbra to the infarct core along with reduction in cytotoxic and vasogenic edema has been shown to reduce the neurological deficit associated with stroke (Yasuda, et al., 2005; Yu,et al., 2003; Zhang,et al., 2002). TRC051384, along with reduction in infarct and brain edema also showed significant reduction in neurological deficits, which were evaluated by observers blinded to the treatment. Sufficient earlier evidence has demonstrated that overexpression of HSP70 or pre-treatment with agents that induce HSP70 have reduced development of infarction, reduced vasogenic edema and reduced neurological deficit (Masada, et al., 2001; Uchida, et al., 2006), but unlike our group here, none of them have showed neuroprotection in stroke with delayed intervention of 8 hours.

Stroke is a complex disorder precipitated by a variety of genetic and environmental factors. The clinical variability of stroke, mainly in terms of duration, localization and severity of ischemia, as well as the patients' age and coexisting systemic diseases, poses huge challenges for clinical research (Bacigaluppi, et al., 2010). One major problem that is nonetheless present and difficult to overcome is that stroke research is mostly conducted in young healthy laboratory animals and thus does not consider that the vast majority of strokes in humans that occur in subjects affected by a multiplicity of other co-morbidities and risk factors, such as arterial hypertension and diabetes (Bacigaluppi, et al., 2010). In consideration to above mentioned reasons in our study we have evaluated the efficacy of TRC051384 additionally in SHR and SHR made diabetic by streptozotocin injection subjected to MCAo. We found TRC051384 to significantly effective in reducing neuronal injury associated with MCAo in these models, thus our results hold more clinical significance.

There are many mechanisms postulated by which HSP70 offers such widespread neuroprotection. In our study we have shown that TRC051384 crosses BBB

and by studying HSP72 immunoreactivity and TUNEL, that treatment with TRC051384 has significantly induced HSP72 proteins predominantly in the penumbral region, in the brain of stroke-induced animals. These results are in agreement with earlier published reports (Kokubo, et al., 2003; Kinouchi, et al., 1993). TRC051384 treatment induced HSP72 positive cells which were seen till 72 hours post ischemia indicating a sustained heat shock response. Apart from their chaperone and anti-inflammatory activity, HSP70 has been shown to inhibit both apoptotic and necrotic cell death. It is well known that caspases are a family of intracellular proteins involved in the initiation and execution of cell apoptosis. The induction of apoptosis through extrinsic or intrinsic death mechanisms results in the activation of initiator caspases, where caspase-3 is a common and key executor caspase (Graham and Chen, 2001; Ashe and Berry, 2003). There is a large amount of evidence indicating that cerebral ischemia can induce the activation of caspases including caspase-3, the upregulation and activation of which have been found to precede neuronal death. Caspase-mediated neuronal death after transient focal cerebral ischemia is more extensive than after the permanent one and may contribute to the delayed loss of neurons from the penumbral region of infarcts (Graham and Chen, 2001; Love, 2003). Recent work in different cell types has identified specific effects of HSP70 on regulation of cell death both by direct interaction with proteins involved in apoptosis and indirectly by influencing expression of levels of apoptosis regulatory proteins (Mehta, et al., 2007; Li, et al., 2000a; Sreedhar and Csermely, 2004). Through the TUNEL staining method studied here we have also shown the anti-apoptotic activity of induced HSP70, which must have contributed to the neuroprotection seen with TRC051384 treatment.

To elucidate the probable mechanisms which must have contributed to the neuroprotection, a real-time PCR based low density array was used to study modulation of gene expression profile by TRC051384 in stroke model, involving panel of 48 genes functionally associated with inflammation process, stress response and apoptosis. Mapping of the gene response is an important approach to understand functional interactions of different molecular pathways in brain ischemia. In recent years, gene microarray technology has gained increasing popularity for gene expression studies. This technology allows detection and quantification of the differential expression of multiple genes simultaneously in a single experiment. Identification of novel modulators of ischemic neuronal death helps in developing new strategies to prevent the stroke-induced neurological dysfunction. Transient MCAo resulted in selective increased mRNA

levels of genes involved in stress, inflammation, transcription and plasticity, and decreased mRNA levels of genes which control neurotransmitter function and ionic balance (Raghavendra, et al., 2002). Stroke-induced upregulation of pro-inflammatory cytokines were abrogated by treatment with TRC051384. In addition, treatment also lowered stroke-induced expression of cell adhesion molecules. Abraham et al., (Abraham, et al., 2002) have reported earlier that, a peak increase in mRNA expression of inflammation mediators was observed during 24 hour post-injury, which is the critical time window of the maturation of ischemic injury. These genes included cytokines (e.g., IL-1 IL-6, and tumor necrosis factor [TNF]-α), chemokines (e.g., macrophage inflammatory protein 1 and macrophage inflammatory protein-2), and cell adhesion molecules (i.e., intercellular adhesion molecule-1 (ICAM-1) and E-selectin (ELAM-1), and they are upregulated through activation of nuclear factor kB (NF-kB). HSP are implicated in reduction of inflammation associated with stroke (Giffard, et al., 2008) mainly acting through the NF-kB and IKK pathway. Previously it is reported in other labs that inhibition of NF-kB activation with the proteasome inhibitor MLN519 has shown to effectively attenuate upregulation of several inflammatory genes including IL-1 IL-6, TNF-alpha ICAM-1, and ELAM-1, reduce neutrophil and macrophage infiltration, and consequently decrease infarction after transient MCAo in rats (Lu, et al., 2004). Both in vivo and in vitro studies in over the last few years have continued to produce corroborating evidence that HSP70 induction can protect against a variety of insults including stroke, trauma, and hemorrhage (Manaenko et al., 2010; Jones et al., 2011). Although the mechanism has been largely limited to its chaperone functions, recent work indicates that HSP70 also strongly modulates inflammatory pathways (Kim et al., 2012). There exists significant literature evidence to believe inflammation modulating properties of HSP70 in variety of CNS insults.

HSP70, perhaps the most studied of the HSPs with respect to its role in inflammation. While their chaperone activities appear to prevent apoptosis and stabilize cytoskeletal structures, HSP70 has also been shown to interact with proinflammatory factors such as NF-kB, matrix metalloproteinases and reactive oxygen species, leading to an anti-inflammatory state (Giffard et al., 2008). Intracellular overexpression of HSP70 or its intracellular induction by heat stress has been shown to reduce inflammatory cell production of nitric oxide and iNOS expression while decreasing NF-kB activation in astrocytes (Feinstein et al., 1996). HSP70 can also prevent responses to inflammatory cytokines such as TNF- α and IL-1 (Van Molle et al., 2002), while overexpression of

HSP70 in macrophages blocked LPS-induced increases in TNF- α, IL-1, IL-10 and IL-12 (Ding et al., 2001). Similarly in an experiment involving murine macrophages the lipopolysaccharide-induced cyclooxygenase-2 gene expression was reduced in heat shocked macrophage cells (Ialenti et al., 2005). In another study overexpression of HSP70 lead to reduced IL-6 release induced by UV-light in WN113 cells (Simon et al., 1995). Western blot analysis of astrocyte cultures transfected with retroviral vectors containing hsp70 exposed to oxygen-glucose deprivation followed by reperfusion showed that Hsp70 over-expression suppressed MMP-2 and MMP-9 (Lee et al., 2004). Human arterial endothelial cells (HAEC) were pretreated with hyperthermia for 60 min at 42 degrees, pretreatment of hyperthermia upregulated HSP70 and reduced TNF-alpha-induced up regulation of E-selectin and VCAM-1 in the endothelial cells (Nakabe et al., 2007).

In experimental stroke, overexpression of HSP70 was associated with reduced production of TNF- α, iNOS, ICAM-1 and IL-1beta (Zheng et al., 2008). In a model of intracerebral hemorrhage, upregulation of HSP70 decreased TNF-a expression and attenuated BBB disruption, edema formation, and neurological dysfunction (Manaenko et al., 2010). Lee et al showed that matrix metalloproteinase-9 (MMP-9), one of the several genes regulated by NF-kB, was reduced in cultured HSP70-overexpressing astrocytes exposed to ischemia-like insults. Consistent with the notion that HSP70 may regulate inflammatory protein expression at the transcriptional level, MMP-9 mRNA was also lower in HSP70-transfected cells (Lee et al., 2004). However, HSP70 expressed in astrocytes seems not only to decrease expression of MMP-9 at both the transcriptional and translational levels, but also to decrease MMP-2 (Lee et al., 2004). However, much of this research of HSP70 overexpression has been conducted in transgenic models or by gene transfer (Giffard et al., 2008; Yenari et al., 2005; Zheng and Yenari 2006). Recently in an another study application of a research tool geranyl acetone (GA) in cerebral ischemia and brain hemorrhage has determined that HSP70 induction by GA represses expression of pro-inflammatory markers (MMP-9, uPA, IL-6 and MIP-1), reduces infarct size, and downregulates apoptotic pathways in stroke and hemorrhage (Kwon et al., 2008; Lu et al., 2002; Sinn et al., 2007; Manaenko et al., 2010). In an animal model of sepsis induced by cecal ligation and double puncture (2CLP), adenoviral vector expressing HSP70 treatment of septic rats at the time of 2CLP reduced IL-6 and CINC-1 abundance (Weiss et al., 2007). In an experimental study in ischemic acute renal failure (ARF) in rats, heat pretreatment induced HSP70 in kidneys

of ARF rats as well as MCP-1 mRNA and protein level were significantly reduced (Jo et al., 2006). These above in-vitro and in-vivo findings are in agreement with present findings in our study where we show that TRC051384 treatment induces HSP70 in the brain and has significantly abrogated stroke associated proinflammatory markers in an experimental model of stroke. Therefore, HSP70 induction may be a viable approach since it does appear to have several mechanisms of action in addition to its ability to suppress inflammation (Giffard and Yenari 2004). Inflammation macro-array thus enabled to identify possible molecular responses influenced by TRC051384 in stroke model. The identified anti-inflammatory mechanisms may have contributed to the observed improvement in stroke-induced neuronal injury and edema reduction by TRC051384 treatment.

In conclusion we showed for the first time that treatment with TRC051384, an HSP70 inducer and potent anti-inflammatory agent significantly reduces infarct size, brain edema, and neurological deficit in an experimental model of stroke in rats even if administered 8 hours post onset of ischemia. Thus, there is high potential for TRC051384, which can be further developed as a novel pharmacological agent for ischemic stroke.

Summary and Conclusions

7. Summary and conclusions

Stroke is clearly a devastating disease that affects millions of people worldwide, and contributes to high morbidity and mortality. It is a common clinical problem with increasing prevalence, serious consequences, unsatisfactory therapeutic options and an enormous financial burden to the society (Mukherjee and Patil, 2011). Projections for the period 2006-2025 estimate new cases of ischemic stroke at 1.5 and 1.9 million cases in men and women respectively, highlighting the increasing profile of stroke in the future (Feigin, et al., 2009). Worldwide 6.15 million people die of stroke every year contributing to 10.8% of total number of deaths by different causes (World Health Organization report, 2010). The financial burden of stoke impacts both patients and society as a whole in terms of premature death, long term disability, restricted social functioning, cost of treatment and loss of productivity (Caro, et al., 2000). The huge economic and social burden of stroke is attributed to its high prevalence, hospitalization rates, morbidity and mortality, and its association with long term disability in survivors (O'Brien, et al., 1996).

Current management of ischemic stroke suffers from large dependence on preventive strategies. Preventive strategies include strict control of blood pressure, diabetes management, use of antiplatelets, anticoagulants, cholesterol lowering agents and life style management in those high risk patients (European Stroke Organisation, 2008). The incidence of stroke is declining in many developed countries, largely as a result of better control of high blood pressure, and reduced levels of smoking. However, the absolute number of strokes continues to increase because of the ageing population.
The pathophysiology of stroke is complex and involves numerous processes which grossly lead to loss of cellular homeostasis resulting into neuronal cell death. There are several parallel pathways in which ischemia can lead to tissue damage (Woodruff, et al., 2011; Lipton P, 1999; Pulsinelli, 1992). Ischemic neuronal damage is characterized by presence of central core and a potential salvageable tissue known as penumbra which surrounds the core. Subsequent extension of the damage in the penumbra is contributed by free radical injury, inflammation and apoptosis which are further potentiated by reperfusion occurring several hours to days post ischemia (Woodruff, et al., 2011). Hence interventional modalities which target free radical generation, inflammation and apoptosis are expected to reduce the delayed extension of brain damage post ischemic insult. Such therapies which have shown significant protection of penumbra include antioxidants (Takasago et al., 1997, free radical scavengers Lees, et

al., 2006, and antiapoptotic agents (Amemiya, et al., 2005) amongst others. Most of the putative neuroprotective agents evaluated earlier had the potential to address either single component or had limited capacity (interms of time window) to modulate the complex injury pathways (Gladstone, et al., 2002).

Maintenance of homeostasis is a fundamental mechanism that the body has conserved evolutionarily. One such significantly studied homeostatic mechanism in experimental stroke is induction of HSP70 (Turturici, et al., 2011; Evans, et al., 2010; Sato, et al., 1996). Transient ischemia induces HSP70 in the brain and the ability of neuronal population to survive an ischemic trauma is correlated with increased expression of HSP70. HSP70 mRNA was induced in neurons at the periphery of ischemia. It is proposed that the peripheral-zone of ischemia (penumbra) can be rescued by pharmacological agents. It was in this zone that HSP70 protein was found to be localized primarily in neurons (van der weed et al 2005; Rajdev, et al., 2000). There is accumulating evidence that HSP70 protects neuronal cells from a variety of stimuli, both in vitro and in vivo. Previous work from different investigators showed that cultured hippocampal, but not cortical neurons from transgenic mice overexpressing HSP70 were protected from excitotoxin exposure and oxygen and glucose deprivation (Lee, et al., 2001). Glial cultures isolated from this same mouse strain were also resistant to substrate deprivation. Xu and Giffard (Xu and Giffard, 1997) and Papadopoulos et al (Papadopoulos, et al., 1996) used such retroviral vectors to overexpress HSP70 in astrocyte cultures. Exposure to isolated glucose or combined oxygen and glucose deprivation led to robust glial survival following either insult. Conversely, suppression of HSP70 in hippocampal neuron cultures with an antisense oligonucleotide worsened injury following heat shock (Sato, et al., 1996). It is shown experimentally that HSP70 is induced in penumbral regions and amplification of such a response using HSP70 over expressing transgenic models or viral delivery of HSP70 protein has offered stroke protection (Zhan et al., 2010; Rajdev et al., 2000). Additionally HSP70 expressed in penumbra is reported to have an anti-inflammatory and anti-apoptotic activity in animal models of stroke (Kokubo et al., 2003; Pratt and Toft, 2003). Thus induction of HSP70 offers a potential target for the treatment of stroke (Mehta et al., 2007). These data indicate that HSP70 is protective at the in-vivo level. Further these studies suggest that augmenting the endogenous protective response to stress by inducing HSP70, at an appropriate time window limits the progression of the ischemic cascade in a model, which incorporates focal ischemia. Most of the previous experiments have employed

either HSP70 over expressed animal models or recombinant protein delivered using viral vectors, which as off today is difficult to administer in the clinic. Thus targeting HSP70 induction employing a pharmacological agent presents attractive strategy (Evans, et al., 2010).

With these collective evidences we hypothesized that an augmentation of the endogenous protective responses employing a pharmacological agent at an appropriate time and at desirable magnitude could provide benefit by reducing the neuronal loss and associated deleterious cascade of events in the intra and extracellular environment. Further, we aimed at late intervention with a pharmacologic agent with the potential to induce HSP70 to demonstrate stroke protection possibly mediated through chaperonic action and mitigation of late events such as inflammation and apoptosis. MR imaging was incorporated in our study as it offers advantage of non invasiveness in order to examine the progression of neuronal damage along with the possibility to capture treatment related benefit which can be directly applied and compared to clinical setup.

Male SD rats subjected to MCAo using intraluminal suture technique were used as animal model for studies designed to test our hypothesis. The great advantage of MCAo model is that the thread (suture used for occlusion of artery) can either be left in place for permanent occlusion or withdrawn any time to permit controlled reperfusion, and the presence of a significant ischemic penumbra early after occlusion makes them particularly suitable for studies of neuroprotection (Sicard and Fisher, 2009; Hsu, 1993). This model has allowed us to evaluate efficacy At the same time, most importantly, intraluminal suture method offers more success rate and produces wide spread infarction involving cortical and subcortical regions and allows prompt evaluation of neurological deficit which are severe and consistent (Longa, et al, 1989). We have standardized these models in our lab to produce consistent injury and subsequently have validated using reference compound MK-801.

Feasibility of using a clinical MRI to study disease progression and evaluation of treatment benefit was carried out. We sought the major advantage of using MRI would be that this technique would allow us to monitor pathological changes or treatment related improvement in the same animal non-invasively over a long period of time greatly eliminating the animal to animal variation observed in such studies. We have successfully standardized the anatomical imaging of small animal brain and have made quantitative estimation of brain infarction and edema in a MCAo model on MRI images.

We then moved further for the evaluation of our identified NCE i.e. TRC051384 for its neuroprotective effect in various in-vitro and in-vivo system. A series of new chemical entities were designed and synthesized at Torrent Research Centre and they were screened sequentially in vitro, in HeLa and a primary neuronal cell based assay system for the upregulation of HSP70B gene expression, and inhibition of production of inflammatory cytokine TNF-α. TRC051384, a novel small molecule with ability to induce HSP70 proteins, was short-listed to study its potential benefits in stroke. Under non-stress conditions, we show that TRC051384 increased HSP70B mRNA expression in both HeLa and primary mixed neuronal cells. HSP70 induced by TRC051384 is functionally active and translates into increased chaperone activity and potentiation of endogenous stress response. We further evaluated if these in vitro effects seen with TRC051384 (HSP70B induction, elevated chaperonic and anti-inflammatory activities) can be translated effectively in an in vivo model.

Using MRI and TTC in this study, we have shown that, TRC051384 belonging to substituted 2-propen-1-one class, which is a potent inducer of HSP70, significantly reduces stroke associated neuronal injury and reduces neurological deficit in both transient and permanent MCAo model in rat. In all these efficacy studies TRC051384 treatment was initiated after 4 hours and/or 8 hours post MCAo. Further In this study we have shown the neuroprotective effect of TRC051384 by delayed intervention treatment in transient MCAo, permanent MCAo and in transient MCAo produced in hypertensive and diabetic animals. In these models TRC051384 was shown to penetrate BBB, induce HSP72 in the ischemia penumbra, has shown antiapoptotic action and anti-inflammatory properties.

Thus with this study we showed for the first time that treatment with TRC051384, an HSP70 inducer significantly reduces infarct size, brain edema, and neurological deficit in an experimental model of stroke in rats even when administered 8 hours post onset of ischemia. Thus, there is high potential for TRC051384, which can be further developed as a novel pharmacological agent for the treatment of ischemic stroke.

To conclude:

- TRC051384, a small molecule shows the potential to transcriptionally induce HSP70 in different cell.
- TRC051384 treatment results into chaperonic activity, potentiation of endogenous stress response, and anti-inflammatory action.
- TRC051384 demonstrates significant neuroprotection in rat transient and permanent MCAo model which resemble human focal cerebral ischemia.
- TRC051384 is found to be efficacious even after delayed intervention thus offering wide window of 8 hours to treat ischemic stroke.
- TRC051384 demonstrates neuroprotection activity in MCAo model of stroke in hypertensive and diabetic rats which are important clinical co-morbidities.

Specific Contributions

8. Specific contributions

1) The hypothesis that pharmacological induction of HSP70 by means of a small molecule HSP70 inducer results into protection from ischemic stroke and hence offer as a potential neuroprotective strategy has been established by:
    a. Demonstrating the possibility of transcriptionally over expressed HSP70 leading to potentiation of endogenous stress response and anti-inflammatory activity in an in-vitro system by treatment with TRC051384.
    b. Showing that such treatment with TRC051384 translates into in-vivo efficacy in a clinically relevant animal model system.
    c. Further, demonstrating for the first time, by delaying treatment with TRC051384, that such a strategy offers wide window of opportunity for the pharmacologic treatment of ischemic stroke which meets the most significant clinical unmet need.
    d. Shown that this strategy is also useful in ameliorating ischemic stroke when present along with clinically important co-morbidities, hypertension and diabetes.
2) Standardized animal models of transient and permanent ischemic stroke that represent human stroke with and without spontaneous recanalization of occluded cerebral vessel. Evaluated temporal profile of infarct and edema progression, neurological deficit and survivability measured over longer period, enabling one in evaluating sustained neuroprotective effect caused by acute treatment.
3) Established feasibility of imaging small animal stroke using clinical MR scanner and successfully used MRI in the evaluation of neuroprotective agent. MRI is currently the gold standard in clinical radiology for the early diagnosis and measurement of treatment outcome in many stroke types. Thus offering direct comparison of results in the clinical setup.
4) Contributed to the preclinical development of a novel neuroprotective strategy in accordance with the STAIR guidelines which are intended for the successful translation of stroke therapy from bench to bedside.
5) Identified TRC051384 as a small molecule inducer of HSP70, which can be further developed as a putative neuroprotective agent.

Future Scope of Work

9. Future scope of work

The primary objective of evaluating a pharmacologically induced HSP70 as a neuroprotective strategy for the treatment of ischemic stroke was confirmed in the present study. Here we have shown that treatment with TRC051384 a small molecule induces expression of HSP70 in multiple cell types, such induction is transcriptionally mediated and results in to potentiation of endogenous stress response. Further it is demonstrated that application of TRC051384 treatment leads to HSP70 expression in-vivo and results into neuroprotection in a clinically relevant and well established animal model of ischemic stroke. Significant appreciation for HSP70 induction as a neuroprotective strategy for ischemic stroke can be made from the results of present study that this strategy offers wide window of opportunity for the treatment of stroke. In the present study we have shown for the first time that delayed treatment with TRC051384 results in to amelioration of many deleterious aspects of ischemic stroke such as reduction in infarct size, brain edema, improvement of neurological deficit and survival. It is noteworthy that this strategy is also protective in stroke with co-morbid conditions. With these collective observations here we have shown confirmatory evidence in support of the hypothesis that augmentation of endogenous HSP70 using a pharmacologic intervention TRC051384 applied within clinically relevant time window results in protection from deleterious outcome of ischemic stroke.

The plethora of failed clinical trials with neuroprotective drugs for acute ischemic stroke have raised justifiable concerns about how best to proceed for the future development of such interventions. In order to successfully translate preclinical efficacy findings to the clinical set up several recommendations have been made by group of experts from academics and industry jointly known as STAIR recommendations. Recommendations provided by the STAIR consortia (STAIR group, 1999; STAIR group, 2001; Fisher, et al., 2009) emphasize the design quality of both experimental and clinical stroke trials. With respect to experimental animal stroke studies, we adhered to most aspects of STAIR recommendations in the present study. Following aspects such as demonstration of therapeutic time window, dose response studies, and evidence for drug accesses target organ, efficacy studies in both permanent and transient ischemia models, use of imaging as a biomarker, study efficacy in animals with co-morbidities and assessment of drug effect on histological and functional measures that too in a blinded fashion were all met while TRC051384 was evaluated in-vivo.

There are few additional recommendations that STAIR emphasizes to consider include evaluation of efficacy of potential neuroprotective agents in gyrencephalic species. Gyrencephalic denotes brains, such as that of humans, in which the cerebral cortex has convolutions. So evaluation of efficacy in animals such as non human primate i.e. monkeys that have gyrencephalic brain are recommended. Such studies will greatly help in increased confidence for the molecule under evaluation to show a clinical success.

To further the scope of neuroprotective ability of TRC051384 and to address the large clinical unmet need for ischemic stroke, it is required to evaluate the efficacy in well designed clinical trails. Before clinical evaluation it is essential that safety issues if any with target and/or drug candidate be addressed as the cost of clinical development escalates substantially as compared to preclinical phase. Detailed studies with aim to establish the safety and toxicity potential and identification of no observed adverse effect level (NOAEL) are required to be conducted to proceed for clinical evaluation. Similarly from the point of view of feasibility of treatment in clinical trails TRC05134 might be given as continuous infusion since every 2h treatment for 2d as done in the present study is impractical clinically. It will be useful to study experimentally using an implantable continuous infusion pump for delivery of TRC051384 and detailed pharmacokinetic parameters are studied by continuous infusion route.

Carefully designed clinical trails beginning with early proof of concept studies in man are essential for translation of efficacy from bench to bed-side. Such studies are strongly recommended by STAIR committee that early proof of concept studies with the aim of refinement and identification of the target population most suited to get benefit are required before large scale pivotal clinical trails are conducted. The future clinical development plan for evaluation of efficacy TRC051384 as a neuroprotective agent would be aimed at delayed intervention with mean time for treatment initiation of 8 hours in ischemic MCA stroke patients with or without co-morbid condition. End points for evaluation would be assessment of neurological deficit employing standardized clinical stroke assessment scale, neuronal injury assessed employing MR imaging. These above mentioned studies would help in establishing HSP70 induction using pharmacological agent as a potential therapeutic strategy which is expected to address large clinical unmet need for stroke and other similar ischemic disorders.

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Appendices

Appendix-I: To determine the ability of test compounds to up-regulate the expression of HSP-70B gene using the Real-time protocol for mRNA quantitation

Principle of the procedure:

Real time PCR is a technique that is used for the quantitative measurement of gene expression levels in cells or tissues. The technique is based on the use of a fluorescent reporter dye at 5'end of the probe and a quencher dye at the 3' end of the probe to monitor the PCR reaction as it occurs. The fluorescence of the reporter molecule increases as products accumulate with each successive round of amplification. The point at which the fluorescence rises appreciably above the background is defined as the threshold cycle and is used for the determination of initial copy number

Following Steps are involved to complete screening of compound by Real Time PCR.

Induction with compound

RNA Isolation & Quantitation

DNase Treatment

cDNA synthesis

Real time PCR Reaction

Equipments:

20x 18s rRNA Taqman probe and primer mix – (ABI)
20x hsp70b Taqman probe and primer mix – (ABI)
25mM EDTA (MBI Fermentas)
2x Taqman universal master mix (Applied Biosystems (ABI))
96-well plates for real time PCR.
Centrifuge (5417 R, Eppendorf)
Centrifuge (Sigma, 3K15)
CO2 Incubator (Forma Scientific)
Cyclo-mixer (Thermolyne)
DEPC treated water (DEPC 0.01% v/v)
Dnase (MBI Fermetas)
Dnase buffer (10X, MBI Fermentas)
Eppendorf tubes (1.5 & 0.5ml DEPC treated, Axygen)

Filter Tips (Axygen)
High capacity cDNA archive kit (Applied Biosystems)
Inverted Microscope. (Carl-Zeiss)
Microbiological Safety Cabinet (Class-II, Heto-Holton)
Micropipettes (Eppendorf)
Optical seals
PCR machine (MJ Research)
Real Time PCR, SDS 7000 (ABI)
Rnase away (MBP)
Spectramax Plus (Molecular devices)
Thermomixer (Eppendorf)
Tips (DEPC treated, Axygen)
TRI Reagent /Trizol (Sigma/Invitrogen)

Reagents

75% Ethanol
Calcium and Magnesium free Phosphate Buffered Saline (Hi-Media)
DEPC
Fetal Bovine serum (Hyclone)
Minimum Essential Medium (MEM)
RNase away (Molecular Bioproducts)

Methodolody:

DAY 0: Split

Hela cells were seeded (ATCC no:-CCL-2) in a T-75 flask at a density of $2.5X10^6$ cells/flask. (Passage number should not exceed 583). Trypsinization and cell count was performed.

DAY 1: Cell seeding

Next day, cells were seeded in a 96 well flat bottom plate (Corning), at a density of 20,000 cells/well and allowed to recover for 24hrs. Trypsinization and cell count was performed

DAY 2: Induction with test compounds

A 100 ul of minimum essential medium was dispensed in each well of a 96 well round bottom plate. (This plate does not contain the cells.) A 200X stock of the test compounds was prepared in the appropriate solvent and 1ul was added to each well containing the culture medium.

Entire culture medium was removed from the plate containing the cell monolayer. A 100µl of new culture medium was added to each well. The medium containing the test compound (from the above plate) is transferred to the plate containing the cell monolayer. Each test compound was tested in triplicate. Plate was incubated at 37^0C in CO2 incubator for 4 hours. At the end of the incubation period, the culture medium was removed gently from all the wells without disturbing the cell monolayer.

RNA Isolation & Quantitation

1. A 120ul Trireagent (Sigma)/Trizol (Invitrogen) was added to each well & mixed 10times. Lysis was confirmed microscopically. (RNase away is used to decontaminate working surfaces and pipettes.)
2. The Trireagent/ Trizol colleted from three wells (Triplicates) in a 0.5ml DEPC treated micro centrifuge tube & incubated it for 10 min at room temperature followed by addition of 80ul chloroform/0.5ml tube and shake well for 15 second and incubated at RT for 2 to 3 min.
3. Tube was centrifuged at 12000g for 15 min at 4o C and upper phase ~ 200ul upper phase was colleted in separate 0.5 ml micro centrifuge tube.
4. 180ul of isopropyl alcohol was added in tube & incubated it for 10 min at RT. Followed by centrifugation at 12000g for 10 min at 4^0C.
5. Discard the supernatant & formed RNA pellet at bottom washed with 360ul, 75% Ethanol. Centrifuge the tube at 7500g for 5 min at 4^0C and supernatant was discarded and allowed pallet to air dry. Reconstituted the RNA pellet in 15ul DEPC treated H_2O and incubated the tube at 55^oC for 10 min in thermomix.

RNA Quantitation:

1. Diluted the RNA sample 1:50 (2ul sample+98ul of DEPC Treated H_20)
2. 100 µl of the diluted sample added in a quartz cuvette (100ul capacity) and absorbance was measured at 260 nm and 280 nm.
3. Quality of the RNA preparation determined from the OD (260nm)/OD (280nm) ratio. A ratio of 1.8 – 2.0 is indicative of a good quality RNA preparation.
4. Amount of RNA calculated using the following relationship:

5. OD value of 1 at 260nm corresponds to an RNA concentration of 40ug/ml. (OD = Optical density)

DAY 3: DNase Treatment: To remove the genomic DNA contamination

Mixture of the 1.5ul of 10x DNase Buffer with 1.0ul of DNase was incubated with RNA sample (1ug) at and final volume was adjusted up to 15ul with Milli Q H2O and incubated 37^0C for 30 minutes the final. Once the reaction is over, add 1.5ul of 25mM EDTA to each tube and incubate at 65^0C for 10 minutes.

cDNA Synthesis:

RNA converted into single stranded cDNA with the reverse transcriptase enzyme using random hexamers and dNTPs.

Following Components were added as per table for cDNA synthesis from RNA Samples

Components	Volume require for 1Rx
10Xreverse transcritase buffer	2.5ul
25X dNTPs	1µl
10X Random primer	2.5µl
Multiscribe reverse transcritase	1.25µl
DEPC treated H2O	13.625µl
DNase treated RNA sample (1µg)	4.125µl
Total	25µl

If No. of samples more than one than proceed the following way.

1. Aliquot the master mixes into the eppendorf tubes and 4.125µl of RNA sample added to individual tubes. Final volume for each reaction is 25µl.
2. A reaction was incubated at 25^0C for 10 minutes followed by incubation at 37^0C for 120 minutes.

Real time PCR Reaction was Setup

Following components were added as mentioned in table.

Each condition runs in duplicate. So PCR prepare the master mix for 2.3 reactions.

Components	Volume require for 1Rx	Volume require for 2.3 Rx
2X Universal master mix	6.25 µl	14.375µl
20X human hsp Probe & Primer master mix	0.625µl	1.437µl
20X 18S Probe & Primer master mix	0625µl	1.437µl
Milli Q H2O	2.5µl	5.75µl
cDNA	2.5 µl	5.75µl
Total	12.5µl	28.75µl

12.5 µl of the reaction mixure was pipetted per well in a 96-well plate. Plate was sealed with optical adhesive cover and samples spun at 3000g for10 minutes. ABI 7000 SDS was set under universal cycling conditions and run was performed

Appendix-II: To determine the ability of test compounds to inhibit the expression of TNF-α gene using the Real-time protocol for mRNA quantitation.

Principle of the procedure

Real time PCR is a technique that is used for the quantitative measurement of gene expression levels in cells or tissues. The technique is based on the use of a fluorescent reporter dye at 5'end of the probe and a quencher dye at the 3' end of the probe to monitor the PCR reaction as it occurs. The fluorescence of the reporter molecule increases as products accumulate with each successive round of amplification. The point at which the fluorescence rises appreciably above the background is defined as the threshold cycle and is used for the determination of initial copy number**.**

Following Steps are involved to complete screening of compound by Real Time PCR.

Induction with TPA

Compound addition

RNA Isolation & Quantitation

cDNA synthesis

Real time PCR Reaction

Instruments:

20x 18s rRNA Taqman probe and primer mix – (ABI)
20x TNF-α Taqman probe and primer mix – (ABI)
25mM EDTA (MBI Fermentas)
2x Taqman universal master mix (Applied Biosystems (ABI))
96-well plates for real time PCR.
Centrifuge (5417 R, Eppendorf)
Centrifuge (Sigma, 3K15)
CO2 Incubator (Forma Scientific)
Cyclo-mixer (Thermolyne)
DEPC treated water (DEPC 0.01% v/v)
Dnase (MBI Fermetas)
Dnase buffer (10X, MBI Fermentas)

Eppendorf tubes (1.5 & 0.5ml DEPC treated, Axygen)
Filter Tips (Axygen)
High capacity cDNA archive kit (Applied Biosystems)
Inverted Microscope. (Carl-Zeiss)
Microbiological Safety Cabinet (Class-II, Heto-Holton)
Micropipettes (Eppendorf)
Optical seals
PCR machine (MJ Research)
Real Time PCR, SDS 7000 (ABI)
Rnase away (MBP)
Spectramax Plus (Molecular devices)
Thermomixer (Eppendorf)
Tips (DEPC treated, Axygen)
TRI Reagent /Trizol (Sigma/Invitrogen)

Reagents

75% Ethanol
Calcium and Magnesium free Phosphate Buffered Saline (Hi-Media)
DEPC treated water
Dexamethasone
Ethanol
Fetal Bovine serum (Hyclone)
RNase away (Molecular Bioproducts)
RPMI Medium
Thiophorbol ester (TPA)

Methodology

DAY 0: Split

THP-1 cells were seeded (ATCC no TIB 202 in a T-75 flask at a density of $2.5X10^6$ cells/flask**.**

DAY 1: Cell seeding

Next day, cells were seeded in a 6 well flat bottom plate at a density of 30,000 cells/well in 2 ml culture medium and Induced with TPA (25ng/ml). Cells were incubated with TPA for 48hrs at 37^oC in CO2 incubator and cells were counted

DAY 3: Test compounds addition

1. A 1000µl of medium was removed from each well and transferred to1.5 ml microcentrifuge tube. A 200X stock of the test compounds was prepared in the appropriate solvent and 10µl was added to each well containing the culture medium.
2. The medium containing the test compound (from the tube) was transferred to the plate containing the cells monolayer.
3. Dexamethasone is a positive control for TNF-α Inhibition. So diluted the dexamethasone & add 100µl of 1µM stock (Final concentration was 50nM) in plate containing the cells monolayer.
4. Plate was incubated at 37^0C in CO_2 incubator for 6 hours. So total time was 42hrs+6hrs with compound.
5. At the end of the incubation period, the culture medium was removed gently from all the wells without disturbing the cell monolayer.
6. Ethanol control cells were not adherent to well so remove cells with medium in 1.5ml micro centrifuge tube & Centrifuge it at 3000rpm for 5 min.

RNA Isolation & Quantitation

1. 1ml of Trireagent (Sigma)/Trizol (Invitrogen) was added to each well & mix it 10 times. Lysis was confirmed microscopically. The Trireagent/ Trizol was collected form each well to 1.5ml DEPC treated microcentrifuge tube & incubated it for 10 min At room temperature.
2. 200µl of chloroform/0.5ml added to tube and Shake well for 15 second and incubated at RT for 2 to 3 min followed by Centrifugation at 12000g for 15 min at 4°C
3. ~ 300µl upper phase was collected and separated 0.5 ml micro centrifuge tube. 500µl isopropyl alcohol added in tube, which contain upper phase & incubated it for 10 min at RT followed by centrifugation the at 12000g for 10 min at 4^0C. Supernatant was discarded and RNA pellet formed at bottom washed with 1ml, 75% Ethanol.
4. Tube centrifuged at 7500g for 5 min at 4^0C & supernatant was discarded and air pellet were allowed to air dry.
5. RNA pellet was reconstituted in 25µl DEPC treated H_2O & incubated at 55^oC for 10 min in thermomix.

RNA Quantitation:

1. Diluted the RNA sample 1:50 (2µl sample+98µl of DEPC Treated H_20)
2. 100 µl of the diluted sample added in a quartz cuvette (100µl capacity) and the absorbance was measured at 260 nm and 280 nm.
3. Determined the quality of the RNA preparation from the OD (260nm)/OD (280nm) ratio. A ratio of 1.8 – 2.0 is indicative of a good quality RNA preparation.
4. Calculated the amount of RNA using the following relationship:OD value of 1 at 260nm corresponds to an RNA concentration of 40µg/ml. (OD = Optical density)

DAY 3:

cDNA Synthesis:

RNA was converted into single stranded cDNA with the reverse transcriptase enzyme using random hexamers and dNTPs**.**

Following Components added as per table for cDNA synthesis from RNA Samples.

Components	Volume require for 1Rx
10Xreverse transcritase buffer	2.5ul
25X dNTPs	1µl
10X Random primer	2.5µl
Multiscribe reverse transcritase	1.25µl
DEPC treated H2O	12.75µl
DNase treated RNA sample (50ng/µl)	5µl
Total	25µl

Each sample of RNA was diluted in to such way that final concentration should be 50ng/µl

If No. of sample more than one than proceed the following way.

1. Aliquot the master mixes into the eppendorf tubes (20µl per tube) and 5µl of RNA sample added to individual tubes. Final volume for each reaction was 25µl.
2. Incubate the reactions at 25^0C for 10 minutes followed by incubation at 37^0C for 120 minutes.

Real time PCR Reaction Setup

Following Components added as per table.

Each condition ran in duplicate. So PCR prepared the master mix for 2.3 reactions.

Note: If the No. of samples are higher than prepare the master mix 2.3Rx*No samples

Components	Volume require for 1Rx	Volume require for 2.3 Rx
2X Universal master mix	6.25 µl	14.375µl
20X human TNF-α Probe & Primer master mix	0.625µl	1.437µl
20X 18S Probe & Primer master mix	0625µl	1.437µl
Milli Q H2O	2.5µl	5.75µl
cDNA	2.5 µl	5.75µl
Total	12.5µl	28.75µl

12.5 µl of the reaction mixture pipetted per well in a 96-well plate. Plate was sealed with optical adhesive cover. Samples spun at 3000g for 10 minutes. ABI 7000 SDS was set up under universal cycling conditions, run was performed

Appendix -III : Sublingual Blood collection and Plasma separation

Sublingual blood collection:

Rats were anesthetized in induction chamber with 6% isfoflurane in medical oxygen, remove from induction chamber and handled in supine position with head toward 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 vein was punctured with the help of 26 gauge hypodermic needle and blood flowing out was collected in an eppendorff tube containing 20IU 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 eppendorff tube was gently mixed with heparin and centrifuged at 4000G for 15 min at 4°C. The straw coloured supernatant, plasma was aspirated using micropipette and was collected in another eppendrof tube.

Appendix –IV : Immuno Cytochemistry for inducible HSP 70 in Rat brain

Chemicals, drugs and disposals:

4% Para formaldehyde
DAB substrate kit (vector lab)
DPX mountant
Ethyl alcohol,
Haematoxyline
Hydrogen peroxide
IPA
Methanol
Mouse Anti-Hsp70 Monoclonal Antibody, Product#: SPA-810(Stressgen)
Proteinase –K (Merck)
Sodium phosphate monobasic and dibasic (Sigma)
Vectastain ABC kit (vector lab)
Xylene

Methodology

Animals with TMCAO/PMCAO were transdcaridcally perfused with ice cold heparinzed PBS, volume 50 ml @ of 600 ml/hr, 24hr post surgery. Brain was isolated and stored at –70.C for 10 min and section of 2 mm thickness were taken. Slice number 4^{th} and 5^{th} were put into the two different cassettes and were transferred in 4% Para formaldehyde(ice cold) in 0.1M PB X 8-12 hour. Fixed sections were embedded in paraffin by paraffin embedder. Sections of 6µm thickness were taken by microtome & 5 slides from each block were taken. Following preparation of slide further procedure has been carried in two days.

On day 1 following procedure followed,

1. Slides were place in oven at 60°C for 45 mins and cleared in two changes of xylene X 5 minutes each. Excess liquid was removed by shaking off and rehydrated slides in two changes of fresh absolute ethyl alcohol/ IPA X 5minutes each.
2. Shake off excess liquid and place slides in fresh 80% ethyl alcohol/IPA X 3 minutes. Slides were rinsed gently in running tap water X 30 seconds (avoid a

direct jet which may loosen the sections) and placed in PBS wash bath for further rehydration X30 min. at RT

3. Slides were placed on a flat level surface such that they didn't touch each other do not let the sections to dry out at any time. Incubated with 0.05% Proteinase K in PBS X 15 minutes at 37^0C followed by incubation with 0.05% Proteinase K in PBS X 15 minutes at RT and rinsed with PBS
4. Quench slides in 0.3% hydrogen peroxide in MeOH X 30 minutes followed by wash in PBS 2X10min
5. Blocked with blocking solution from Vectastain kit (1:75 dilution in PBS) X 60 min. using humidity chamber. Wiped off excess and incubated with 1°Ab (SPA810--1:1000) (in PBS containing blocking solution from Vectastain kit) in humidity chamber at 4°C X O/N.

On day 2,

1. Slide were wshed in PBS 10 min x 2 and incubated in biotinylated 2 °Ab (1:200 dilution in PBS) for 60 min. at RT. Dip in 0.1M PO_4 buffer 10 min x 1. Followed by vectastain for 60 mins. Again in 0.1M PO_4 buffer 10 min x 1.and then 0.1M Tris 10 min x 1.
2. Revelation has been carried out by incubating in DAB for 6 mins in dark under hood followed by wash with dH_2O as quickly as possible by taken care that section sit in dH_2O don't lead to altered morphology.
3. Transfer slides in to 0.1M PO_4 buffer followed by counter stain with haematoxylin for 30 seconds.
4. Washed it thoroughly with running tap water and decolorize with acid alcohol for 1 minute and kept in water for 30 seconds.
5. Allowed it to air dry and cleared in xylene for 2 minutes. Mounted in DPX and allowed it to day for overnight

Appendix V: Tunnel Staining

Chemicals

Anti-digoxignenin conjugate

dH_2O

Equilibrium Buffer (1X)

Ethanol (70, 95, 100%)

Hydrogen peroxide (3%)

MethygGreen

N-Butanol (100%)

Peroxidase Substrate

Phosphate buffer saline

Proteinase K

TdT enzyme

Xylene

Reagents Preparation

Proteinase K	
Proteinase K	1.5µl
PBS	150µl
3% H2O2 solution	
35% H2O2 solution	750 µl
PBS	25ml
Working TdT enzyme	
TdT enzyme	33 µl
Reaction Buffer	77 µl
Working Wash/Stop Buffer	
Stop/wash Buffer	1 ml
Milli Q water	34 ml
Peroxidase Substract	
DAB substract	3 µl
DAB dilution Buffer	147µl

Methodology

In brief, rat brain was deparaffinized in xylene and hydrated in series of graded alcohol to water. A protein digestion was carried out with proteinase K (20 µg/mL) to specimen for 15 minutes at room temperature. Quenched the slides with 3.5% hydrogen peroxide in PBS for 5 minutes at room temperature. Gently tap off excess liquid and carefully blot or aspirate the section. Immediately apply 75 µl/section of equilibration buffer directly on the specimen. Apply working strength TdT enzymes 55 µl incubate at humidified chamber at 37^0C for 1 hour followed by addition of working strength stop/wash buffer for 15 seconds and incubate for 10 minutes at room temperature. Apply room temperature AntiDigoxignin conjugate to the slide 65 µl, incubate in a humidified chamber for 30 minutes at room temperature. Wash in 4 changes of PBS. Gently tap off excess liquid and carefully blot or aspirate around section. Apply enough Peropxidase substrate to completely cover the specimen. Stain for 3-6 minutes at room temperature. Wash in dH_2O. Counter stain with 0.5% methyl green in coplin jar for 10 minutes. Wash the specimen in 3 changes of dH_2O in a coplin jar, wash the specimen in 3 changes of 100%N-Butanol in a coplin jar, dehydrate the specimen and mount with DPX**.**

List of Publications & Presentations

12. List of publications and presentations

Patents

Kumar P., Mohanan A., Argade N., Hadole C., Mandhare A., Gupta R., Deshpande S., **Jamadarkhana P**., Joshi P. Inventor; Torrent pharmaceuticals ltd., Novel Substituted Piperidones as HSP Inducers. United States Patent US 20100190824, 2010 Jul 29.

Srivastava S., Chhipa L., Gupta R.C., Deshpande S., Chaudhari A., Mohanan A., Dutt C., Chauthaiwale V., Badanthadka M., **Jamadarkhana P.G**. Inventor; Torrent Pharmaceuticals ltd., Novel fused thiazolo and oxazolo pyrimidinones. United States Patent US 20110130414, 2011 Jun 2.

Papers

Mohanan A, Deshpande S, **Jamadarkhana PG**, Kumar P, Gupta RC, Chauthaiwale V, Dutt C (2011) Delayed intervention in experimental stroke with TRC051384--a small molecule HSP70 inducer. *Neuropharmacology* **60**:991-9.

Jamadarkhana P, Chaudhary A, Chhipa L, Dubey A, Mohanan A, Gupta R, Deshpande S (2012) Treatment with a Novel Hypoxia-Inducible Factor Hydroxylase Inhibitor (TRC160334) Ameliorates Ischemic Acute Kidney Injury. *Am J Nephrol* **36**:208-18.

Jamadarkhana PG, Raval H, Badgujar N, Mohanan A, Non-invasive imaging and quantification of infarct in rat brain using clinical magnetic resonance scanner: a feasibility study. In communication phase with Indian Journal of Pharmacology.

Poster presentations

D. Joshi, A. Mohanan, **Prashant GJ**, A. Dubey, S. Deshpande, P. Kumar, RC Gupta, C Dutt. “Small molecule inducers of HSP70 (HSP70) prevent infarct progression and edema in a rodent model of focal ischemia with reperfusion” Abstract submitted for poster presentation at the 4th annual conference of Global College of Neuroprotection and Neuroregeneration. Garmisch-Partenkirchen, Germany. 14 - 16 March 2007.

Prashant Jamdarkhana and Anookh Mohanan. Use of clinical MR scanner for imaging rat brain. Abstract submitted for poster presentation at National Conference on Emerging Trends in Life Sciences Research. BITS-Pilani, India. 6-7 March 2009.

Biography

Brief biography of the candidate

Mr. Prashant G J, 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 Gulbarga, he opted pharmacy as his profession and graduated B. Pharm from H.K.E's Collage of pharmacy affiliated to Gulbarga University Gulbarga, Karnataka.Having decided to pursue higher education, he qualified all India Graduate Aptitude Test in Engineering (GATE) examination and selected Pharmacology as specialization subject for M. Pharm. He was guided through his M. Pharm by Dr. M.S Harish at Department of Pharmacology, Govt College of Pharmacy, affiliated to Rajiv Gandhi University of Health Sciences Bangalore. He was ranked second in Pharmacology discipline in M. Pharm.

Immediately after completing M. Pharm, Mr. Prashant G J 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 first, under supervision of Dr. Deepa Joshi and later under the supervision of Dr.Anookh M, Head, Department of Pharmacology, Torrent Research Centre.

Mr. Prashant G J has attended various conferences and workshops, have published paper in journals of international repute and participated in poster presentation in conferences of national repute.

Brief biography of the supervisor

Dr.Anookh M is presently heading the Department of Pharmacology, R&D centre of Torrent Pharmaceuticals Ltd, Gandhinagar.

Having a basic degree in pharmacy (B.Pharm) from Dr MGR Medical University, Madras, Tamil Nadu, his research aptitude has made him to qualify all India Graduate Aptitude Test in Engineering (GATE) examination and took him to Jadavpur Uniersity, Calcutta, West Bengal where he completed his M.Pharm in pharmacology discipline in the year 1996. .

Immediately after completing M. Pharm, Dr.Anookh M joined in Department of Pharmacology at Torrent Research Centre, Gandhinagar. While working as scientist in Torrent Research Centre, he got himself registered and completed Ph.D., with B.I.T.S., Pilani first, under supervision of Dr. A. Shankarnarayanan in the year 2004. Presently Dr.Anookh M is heading the department of pharmacology of Torrent Research Centre. His activities, in addition to administrative responsibilities, involved significant contribution to overall drug discovery process at Torrent research Centre. He served as a leader of various in-house and collaborative discovery projects. Activities involve identifying therapeutic areas which have clinical unmet needs, identify and evaluate new relevant (validated / semi-validated) targets, lead teams of scientists in setting up relevant in-vitro / in-vivo models to screen NCE's for activities and design experiments for mechanistic studies. As project leader – lead, inter departmental project teams which involve planning, co-ordination, and trouble shooting, and achieve project goals within the timelines.

In addition to vast research experience of 15 years, Dr.Anookh M has to his credit several awards and fellowships, has participated in many conferences, published patents, participated in poster presentations, published original research articles in journals of international repute.