Discovery and Development of Novel Betulinic acid Derivatives as Potential Anti-cancer Agents

THESIS
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by

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CERTIFICATE

This is to certify that the thesis entitled **Discovery and Development of Novel Betulinic acid Derivatives as Potential Anti-cancer Agents** which is submitted for award of Ph.D. Degree of the Institute embodies original work done by him under my supervision.

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PARENTS

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(Praveen. R)

ABSTRACT

Betulinic acid is a pentacyclic lupane-type triterpene. One of the most widely reported sources of betulinic acid is the birch tree where both betulinic acid and betulin can be obtained in substantial quantities. Betulinic acid was reported to be selective to melanoma and neuroectodermal tumors. It was shown to act through induction of apoptosis, mainly through the induction of changes in mitochondrial membrane potential. It was found to have remarkable activity in animal tumor models and surprisingly did not have any toxicity [Pisha et al., 1995; Schmidt et al., 1997; Fulda et al., 1999; Zuco et al., 2002].

In Chapter-1 of this thesis, betulinic acid was shown to possess broad-spectrum anti-cancer activity. Betulinic acid was screened using a panel of human cancer cell lines and was found to be cytotoxic and more sensitive to leukemia, lymphoma, melanoma, prostate, ovary, lung and colon cancers. About 500 novel betulinic acid derivatives with chemical modifications in C_2, C_3, C_{20} and C_{28} positions were screened for anti-cancer activity based on cytotoxicity to human leukemia (MOLT-4) cell line. Among the selected derivatives with IC_{50} less than 4 µg/ml, 25 (twenty five) derivatives had better broad-spectrum cytotoxicity than betulinic acid when tested in the panel of sensitive cancers. Further, the above derivatives were tested for selective cytotoxicity to cancer cells as compared to non-cancerous cells and 5 (five) derivatives were short-listed for further evaluation based on better cytotoxicity and specificity to cancer. The pro-apoptotic activity and in vivo anti-tumor activity in colon and melanoma human tumor xenografts has been demonstrated for betulinic acid and the most potent derivative - 1098.

In Chapter-2 of this thesis, the in vitro anti-angiogenic activity of betulinic acid has been demonstrated by its effect on human umbilical vein endothelial cells (ECV304). Betulinic acid was found to have weak inhibitory effects on the secretion by tumor cells of key angiogenic factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and Endostatin. In addition, the five short-listed derivatives from Chapter-1 were tested and found to inhibit vital processes of angiogenesis i.e. growth, migration and tube formation of endothelial cells. Further, one of the derivatives (1098) was tested in an in vivo

anti-metastatic model and was shown to significantly inhibit the formation of melanoma lung nodules.

In Chapter-3 of this thesis, betulinic acid and the five short-listed derivatives from Chapter-1 were tested using in vitro ADME assays. These compounds were found to have poor aqueous solubility at pH 7.4, had low to moderate permeability across lipid membranes, and had high plasma protein binding, possessed good in vitro metabolic stability and acceptable toxicity in animals. They did not inhibit key cytochrome P450 enzyme isoforms in vitro. Further, the pharmacokinetics of one derivative (1098) has been evaluated in animals.

Based on the selective anti-cancer activity and good anti-angiogenic potential, these molecules are promising anticancer agents for the clinical treatment of various forms of cancer. The most potent betulinic acid derivative - 1098 [3, (4-nitrobenzyl-oximino)-betulinic acid] was selected for advanced pre-clinical development studies based on favorable metabolism, pharmacokinetics and toxicity in animals.

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LIST OF ABBREVIATIONS/SYMBOLS

30G	- 30 gauge
ACN	- Acetonitrile
ADME	- Absorption, Distribution, Metabolism, and Elimination
ADMET	- Absorption, Distribution, Metabolism, Elimination and Toxicity
AKT	- also known as protein kinase B
AMMC	- [2-(*N*, *N*-diethyl-*N*-methylamino) ethyl]-7-methoxy-4-methylcoumarin
APN	- Amino peptidase N
ATCC	- American Type Culture Collection
AUC	- Area under the curve
b.wt.	- Body weight
BA	- Betulinic Acid
BAECs	- Bovine aortic endothelial cells
Bax	- Pro-apoptotic member of the Bcl-2 protein
BBB	- Blood Brain Barrier
BBMV	- Brush Border Membrane Vesicles
Bcl-2	- Human proto-oncogene located on chromosome 18
BCL-XL	- Anti-apoptotic protein of bcl-2 family
BFC	- 7-benzyloxy-4-trifluoromethylcoumarin
bFGF	- basic Fibroblast Growth Factor
BrdU	- Bromo deoxyuridine
BSA	- Bovine Serum Albumin
C_0	- Concentration at 0 min
cdk4	- Cyclin-dependant kinase 4
cDNA	- complementary DNA
CHO	- Chinese hamster ovary
CL	- Clearance
CMC	- Carboxy methyl cellulose
CNS	- Central nervous system
CYP	- Cytochrome P450
Deriv.	- Derivative
DMEM	- Dulbecco's Modified Eagle's Medium

DMF - Dimethyl Formamide

DMPK - Drug metabolism and pharmacokinetics

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

ECS - Endothelial Cell Specificity

EDTA - Ethylene di-amine tetra acetic acid

EGFR - Epidermal Growth Factor Receptor

EIA - Enzyme immuno assay

ELISA - Enzyme linked immuno sorbent assay

ERK - Extra cellular signal-regulated kinase

EtOH - Ethanol

FDA - Federal Drug Administration

FITC - Fluorescein isothiocyanate

GEM - Genetically engineered mouse

GLM - General Linear Model procedure

HEPES - N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)

HGF - Human Growth Factor

HIA - Human Intestinal Absorption

HIV - Human immunodeficiency virus

HPLC - High Performance Liquid Chromatography

hrs - hours

HUVECs - Human umbilical vein endothelial cells

i.p. - intra-peritoneal

i.v. - intravenous

IAM - Immobilized Artificial Membrane

IC_{50} - 50% Inhibitory Concentration

IND - Investigational New Drug

kD - Kilo Dalton

LC-ESMS - Liquid Chromatography Electron Spray Mass Spectrophotometer

LC-MS - Liquid chromatography Mass spectrophotometer

log D - logarithm of diffusion coefficient

log P - logarithm of partition coefficient

log Pe - logarithm of experimental permeability

LOQ	- Limit of Quantitation
LSMEANS	- Least square means
MeOH	- Methanol
MFC	- 7-methoxy-4-trifluoromethylcoumarin
min	- minutes
MLD	- Mean Lethal Dose
MMP	- Matrix metalloproteinase
MPT	- Membrane permeability transition
MTT	- [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
N/T	- Normal/Tumor
NADH	- Reduced Nicotinamide Adenine Dinucleotide
NADPH	- Reduced Nicotinamide Adenine Dinucleotide Phosphate
NCE	- New Chemical Entity
NCI	- National cancer institute
ND	- Not done
NDDS	- New drug delivery system
NFkappaB	- Nuclear factor kappa B
NIN	- National institute of nutrition
NMR	- Nuclear Magnetic Resonance
NSCLC	- Non small cell lung cancer
O.D.	- Optical density
p.i	- post inoculation
PAMPA	- Parallel artificial membrane permeability assay
PARP	- Poly (ADP-ribose) polymerase
PBS	- Phosphate buffered saline
PDGF	- Platelet Derived Growth Factor
PEG 400	- Poly ethylene glycol 400
PIGF	- Placental Growth Factor
PPB	- Plasma protein binding
PS	- Phosphatidyl serine
PT	- Permeability Transition
PTC	- Primary Tumor Cells
PTPC	- Permeability transition pore complex

PVDF	- Poly Vinylidene di-fluoride
RAID	- Rapid access to intervention development
rpm	- revolutions per minute
RT	- Retention time
RTK	- Receptor Tyrosine Kinase
s.c.	- sub-cutaneous
S.No.	- Serial Number
SAR	- Structure activity relationship
SAS	- Statistical analysis system
SD	- Standard deviation
SDS	- Sodium dodecyl sulfate
SEM	- Standard error mean
Spp.	- Species
SRB	- Sulforhodamine B
T/C	- Tumor/Control
T/E	- Tumor/Endothelial
TLC	- Thin Layer Chromatography
Tmax	- Time to reach maximum concentration in plasma
TPA	- Tissue plasminogen activator
TSG	- Tumor suppressor gene
uPA	- Urokinase plasminogen activator
UV/Vis	- Ultraviolet/Visible
UV-C	- Ultraviolet-C
V_d	- Volume of distribution
VEGF	- Vascular Endothelial Growth Factor
WHO	- World Health Organization
XTT	- Sodium 3, 3'-{1-[(Phenyl amino) Carbonyl]-3, 4-Tetrazolium}-Bis (4-Methoxy-6-Nitro) Benzene Sulfonic Acid Hydrate

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GENERAL INTRODUCTION

The Cancer problem

Cancer is a devastating disease - but largely preventable. Its impact can be reduced through basic research and improvements in treatment and care. Cancer rates could further increase by 50% to 15 million new cases in the year 2020 according to the World health organization (WHO) world cancer report [Stewart et al., 2003]. According to the report, in the year 2000, malignant tumors were responsible for 12 per cent of the nearly 56 million deaths worldwide from all causes. In many countries, more than a quarter of deaths are attributable to cancer. In 2000, 10 million people developed a malignant tumor and altogether 6.2 million died from the disease. Cancer has emerged as a major public health problem in developing countries, matching its effect in industrialized nations. The most common cancers worldwide, excluding non-melanoma skin cancers, are cancers of the lung, breast and colorectal tissue. As per figures for the year 2000, lung cancer is the most common cancer worldwide, accounting for 1.2 million new cases annually; followed by cancer of the breast, just over 1 million cases; colorectal, 940,000; stomach, 870,000; liver, 560,000; cervical, 470,000; esophageal, 410,000; head and neck, 390,000; bladder, 330,000; malignant non-Hodgkin lymphomas, 290,000; leukemia, 250,000; prostate and testicular, 250,000; pancreatic, 216,000; ovarian, 190,000; kidney, 190,000; endometrial, 188,000; nervous system, 175,000; melanoma, 133,000; thyroid, 123,000; pharynx, 65,000; and Hodgkin disease, 62,000 cases. The cancers which caused the greatest proportion of deaths were those of the lung, stomach and liver, because of the relative success of early intervention in breast and colorectal cancers.

Investigations into cancer causation had revealed that the most important human carcinogens include tobacco, asbestos, aflatoxins and ultraviolet light. In addition, nearly 20 percent of cancers were associated with chronic infections, the most significant ones being hepatitis B and C viruses (liver cancer), human papilloma viruses (cervival and ano-genital cancers) and *Helicobacter pylori* (stomach cancer). In developed countries chronic infection causation amounted to only 8 percent of all malignancies, whereas in developing countries up to 25 percent of tumors were associated with chronic infections. “Once considered a “Western” disease, more than 50 per cent of the world’s cancer burden, in terms of both numbers of cases and deaths, already occurs in developing countries. In addition to substantial opportunities for primary prevention, the emphasis is on the potential of early detection, treatment and palliative care [Stewart et al., 2003].

Need for the development of anti-cancer drugs

Great strides have been made in the effective treatment of some forms of cancer by means of chemotherapy used alone or in combination with other modalities. Unfortunately, however, the number of available clinically active antitumor agents remains quite small and the spectrum of clinical antitumor activity is generally rather limited. The ultimate potential of chemotherapy in cancer treatment still remains unrealized.

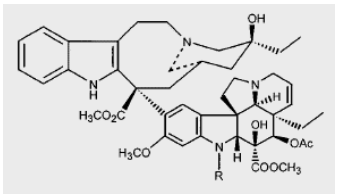
A quantum leap in effective cancer chemotherapy requires the discovery and development of new anticancer drugs with unprecedented antitumor activities, specificities, and mechanism of action. Anticancer drugs have well known therapeutic limitations, which have continued to stimulate the search for new agents with enhanced therapeutic efficacy. Earlier studies recognized that the growth of both normal and neoplastic cells is affected by intracellular levels of chemotherapeutic agents. Modification of drug activity, however, involved the use of modulating agents that may offer selective protection against toxicity of normal tissue without compromising anti-tumor activity. Increasing understanding of cellular and molecular biology of normal cell growth and proliferation appears to offer potentially important new targets for drug design and synthesis.

Why Natural compounds?

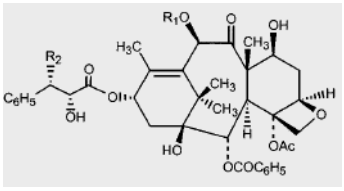
There are three main reasons why natural compounds are worth studying. First, natural compounds that show anticancer potential inhibit cancer by interfering with one or more of the mechanisms that researches now feel are central to cancer progression and fit into the mechanism-based approach as perfectly as a hand fits into a glove. Second, although the future does look bright for eventual success in the fight against cancer, we are not there yet. Much more work remains to be done. As a science, the field of natural compound research can contribute to a greater understanding of cancer and a faster development of successful therapies. Third, we must study natural compounds because they are already being used in cancer treatment. For better or worse, hundreds of thousands if not millions of patients around the world are experimenting with natural compounds in their efforts to heal themselves of cancer. Because the popularity of using natural compounds in cancer treatment appears to be growing rather than declining, we are compelled to study natural compounds so that we can properly guide the public [Boik, 2001(a)].

Plant derived anti-cancer drugs

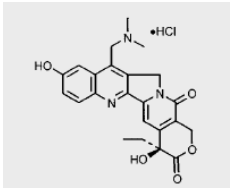
Historically, plants were a folkloric source of medicinal agents, and as modern medicine developed, numerous useful drugs were developed from lead compounds discovered from medicinal plants. Today, this strategy remains an essential route to new pharmaceuticals. Since 1961, nine plant-derived compounds have been approved for use as anticancer drugs in the US: vinblastine, vincristine, navelbine, etoposide, teniposide, taxol, taxotere, topotecan, and irinotecan [Cragg et al., 2005; Lee, 1999] (Figure - 1).



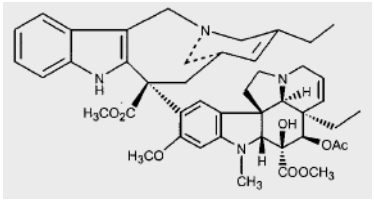
Vincristine, R=CH_3
Vinblastine, R=CHO



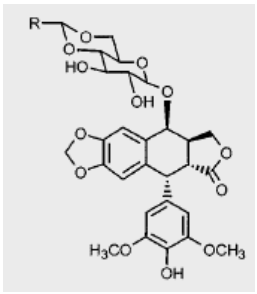
Taxol, R_1=$COCH_3$, R_2=$HNC0C_6H_5$
Taxotere, R_1=H, R_2=$NHCOOC(CH_3)_3$



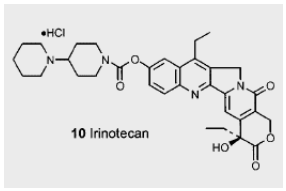
Topotecan



Navelbine



Etoposide, R=CH_3
Teniposide, R=



Irinotecan

Figure – 1

Plant-derived anti-cancer drugs

Accordingly, the preclinical development of bioactive natural products and their analogs as chemotherapeutic agents is a major objective of anti-cancer research programs. Three main research approaches in the drug discovery and development process are: (1) bioactivity or mechanism-of-action-directed isolation and characterization of active compounds, (2) rational drug design-based modification and analog synthesis, and (3) mechanism of action studies. Structural derivatization of natural compounds is aimed at increasing activity, decreasing toxicity, or improving other pharmacological profiles. Preclinical screening using

in vitro human cell line panels and selected *in vivo* xenograft testing is a major tool in identifying the most promising anticancer drug development targets. Structure refinement is also aided by four types of studies: (1) structure-activity relationship (SAR) studies including qualitative and quantitative methods, (2) mechanism of action studies including drug receptor interactions and specific enzyme inhibitions, (3) drug metabolism studies including identification of bioactive metabolites and blocking of metabolic inactivation, and (4) molecular modeling studies including determination of three-dimensional pharmacophores. Toxicological, production, and formulation concerns are addressed before clinical trials can begin [Lee, 1999].

Pharmacological effects of Triterpenes

Triterpenes represent a varied class of natural products. Thousands of structures have been reported with hundreds of new derivatives described each year [Connolly et al., 2000]. Triterpenoid-rich plants have been used in herbal medicine traditions more for their anti-inflammatory effects and their protective effects on the vascular system. They have been known to inhibit angiogenesis as well as invasion and metastasis. Some triterpenoids including those from the *Centella* and *Boswellia* species produce cytotoxicity against cancer cells [Boik, 2001(b)].

There still is a great need for the identification of novel biologically active triterpene compounds. Achieving the difficult goal of identifying novel triterpenes with beneficial biological activities and good drug-like properties could provide entirely new avenues of treatment for a diverse set of human ailments in which therapeutic options currently are limited. Therefore, there is a great deal of interest in probing the structural features responsible for the pharmacological effects, and to further optimize the activity profile. A class of pentacyclic triterpene was reported to be a selective inhibitor of human melanoma tumor growth by inducing apoptosis [Pisha et al., 1995]. A triterpene saponin from a Chinese medicinal plant in the Cucurbitaceae family has demonstrated anti-tumor activity [Kong et al., 1993]. Certain triterpene glycosides of the Iridaceae family inhibited the growth of tumors and increased the life span of mice implanted with Ehrlich ascites carcinoma [Nagamoto et al., 1988]. Soya saponin, also from the Leguminosae family, has been shown to be effective against a number of tumors [Tomas-Barberan etal, 1988]. Among these are included the

pentacyclic lupane-type triterpenes which are represented by a diverse assemblage of bioactive natural products. 3β-Hydroxy-lup-20(29)-en-28-oic acid (betulinic acid), a C-28 carboxylic acid derivative of the ubiquitous triterpene betulin, is a member of the class of lupane type triterpenes (Figure - 2). However, unlike betulin, the oxidized derivative betulinic acid possesses a number of intriguing pharmacological effects including anti-inflammatory, anticancer, and anti-HIV activities.

The lupane-type triterpene betulinic acid is found widely throughout the plant kingdom [Hayek et al., 1989]. One of the most widely reported sources of betulinic acid is the birch tree (*Betula* spp., Betulaceae, Figure 2) where both betulinic acid and betulin can be obtained in substantial quantities [O'Connell et al., 1988; Cole et al., 1991, Galgon et al., 1999]. Other known sources of betulinic acid include *Ziziphus* spp. (Rhamnaceae) [Pisha et al., 1995; Schuhly et al., 1999; Jagadeesh et al., 1998], *Syzygium* spp. (Myrtaceae) [Frighetto et al., 2005; Kashiwada et al., 1998; Chang et al., 1999], *Diospyros* spp. (Ebenaceae) [Recio et al., 1995, Higa et al., 1998, Singh et al., 1997] and *Paeonia* spp. (Paeoniaceae) [Ikuta et al., 1995, Lin et al., 1998, Kamiya et al., 1997].

In light of the tremendous interest generated with respect to the chemistry and pharmacological properties of these types of compounds, this research was undertaken in an effort to explore the potential of the pentacyclic triterpene betulinic acid found in abundance in the plant kingdom. Potent derivatives were selected and further studies were carried out to determine the potential of these derivatives for development as anticancer drugs.

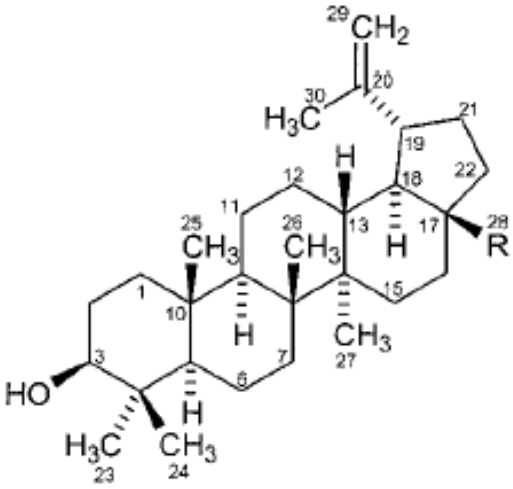


Figure - 2

Structure of betulin and betulinic acid (Top). A picture showing the shredding bark, fruits and leaves of Birch tree (*Zizipus jujuba*) (bottom)

Broad objectives of the research study

The first objective of this thesis work was to study the potential of betulinic acid and several hundred derivatives of betulinic acid with structural modifications at C_2, C_3, C_{20} and C_{28} positions for anti-cancer activity using a panel of human tumor cell lines. The derivatives would be screened using rapid cytotoxicity screening assays that will enable the selection of derivatives which are more potent than betulinic acid and act specifically against cancer cells. Efforts would be directed towards identifying the structure activity relationships. Further, the short-listed derivatives would be subjected to mechanism of action studies and *in vivo* efficacy studies.

The second objective of this thesis work was to study the anti-angiogenic potential of derivatives short-listed based on their anti-cancer potential. The anti-angiogenic studies would be carried out to determine the effect of these derivatives on human endothelial cells. The derivatives will be assessed on their ability to modify three key processes involved in angiogenesis i.e. growth, migration and tube formation by endothelial cells. The anti-angiogenic effect of selected derivative(s) would then be assessed in an *in vivo* efficacy model.

The third objective of this thesis work was to carry out early pre-clinical development studies with the short-listed derivatives so as to identify the potential for further clinical development. The derivatives would be screened using rapid preliminary ADME assays to study the solubility, permeability, metabolic stability, protein binding and inhibition of cytochrome P450 enzymes. Further, the toxicity and pharmacokinetics studies will be carried out to enable the selection of a lead compound for advanced pre-clinical development.

The broad objectives described above and the scheme of the research work to be carried out is summarized in the flow chart given in Figure – 3.

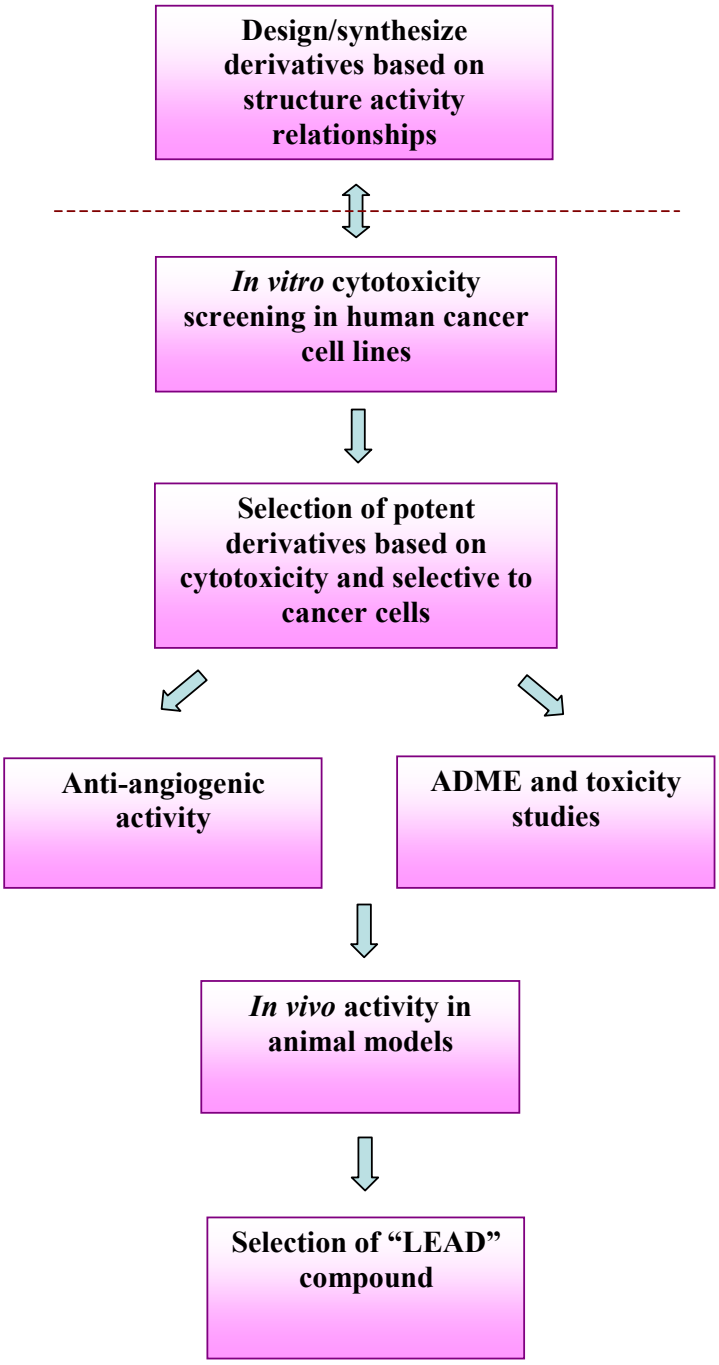


Figure - 3

Summary of objectives and scheme of research work

Scope of the research study

Since more than 60% of anti-cancer drugs are of natural origin, the present research work on betulinic acid and derivatives is an effort directed towards identifying potential anti-cancer drugs from the natural source for the treatment of certain forms of cancer where betulinic acid and derivatives are found to be effective. In general, treatment options have been found inadequate with several drugs providing either partial remissions of tumor or marginally extending the survival time. For example, in adult acute leukemia, which affects approximately five persons per 100,000 long term disease free survival is currently achieved in less than 50% of patients. Similarly, in melanoma, once the cancer advances and metastasizes (spreads) to other parts of the body, it is hard to treat and can be deadly. During the past 10 years the number of cases of melanoma has increased more rapidly than that of any other cancer. The latest figures from U.S. state that there were about 55,170 deaths from cancers of the colon and rectum [National Cancer Institute, 2006]. The studies that are proposed to be done in this thesis would help in identifying potent molecules of betulinic acid specific for such cancers, which have desirable drug-like properties that will enable better success rate in treating human cancers. The ultimate findings of the thesis, particularly the structure activity relationships, would provide a platform for further work to be carried out in this direction. Moreover, the findings of this research study would fulfill the requirements of the investigational new drug application (IND) to be filed to the drug approval authorities.

Limitations of the research study

The research work involves the use of *in vitro* systems and animal models for drug discovery. The correlation of these results to clinical situation would emerge only once clinical trials are performed. It is a well known fact that several drugs with good pre-clinical activity fail in clinical trials due to several factors, which include, but are not limited, to the differences between *in vitro* and *in vivo* systems and the large differences that exist between animal and human species due to differences in drug metabolism and other complex and yet unexplained factors.. These factors have been taken care of to some extent in this research study by incorporating human tumor cell lines, human tumor xenografts and human liver microsomes in the various research models. In general, it has been suggested that the *in vitro* human tumor cell line and the human xenograft models might have good clinical predictive value in

some solid tumors (such as ovary and NSCLC) under both the disease and compound oriented strategies, as long as an appropriate panel of tumors is used in preclinical testing [Nomikos et al., 2003].

Further, the use of selection filters, like solubility, permeability etc. would enable less compound drop-outs at the later part of drug development. The use of several *in vitro* assays carried out in the high-throughput mode would help to compress the development time, but has the disadvantage of producing many false positive or negative results. Efforts have been directed to carry out detailed investigation of few short-listed compounds in order to overcome these problems.

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CHAPTER - 1

IN VITRO AND IN VIVO ANTI-CANCER ACTIVITY OF BETULINIC ACID AND DERIVATIVES

1.1 INTRODUCTION

The discovery of novel anti-tumor agents from natural sources is largely based on testing for cytotoxic activity against cancer cell lines grown either *in vitro* or using *in vivo* models. Many of the naturally derived anti-cancer agents originally discovered using such assays, have been shown to exert their cytotoxic action through interaction with tubulin, and include agents, such as vinblastine, vincristine, colchicine, combretastatin and maytansine which promote the depolymerisation of tubulin, while, in the case of the taxanes, microtubules are "bundled" as a result of stabilization against depolymerization.

One of the greatest challenges faced by developers of new drugs and treatment strategies of cancer is the obvious need to test them in preclinical models that have a good probability of being predictive of similar activity in humans. The discovery that human tumor cell lines and primary biopsy human tumor specimens, can give rise to progressively growing and potentially lethal cancers in immunodeficient mice gradually resulted in a shift towards the use of human tumor xenografts for the study of virtually all other types of anticancer drugs and treatment strategies. Essentially every clinically approved anti-cancer drug was tested using these models, and showed positive anti-cancer effects before being evaluated in early, and then late phase clinical trials.

Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer, and while the actual compounds isolated from the plant frequently may not serve as the drugs, they provide leads for the development of potential novel agents. As new technologies are developed, some of the agents which failed earlier clinical studies are now stimulating renewed interest.

1.2 REVIEW OF LITERATURE

Several different *in vitro* and *in vivo* methods have been used for anti-cancer drug screening. Some of the most promising methodologies and models are described below.

1.2.1 Methodologies to study the anti-cancer potential of molecules

***In vitro* methods**

The clonogenic assay involves the growth on soft agar of colonies derived from freshly explanted human tissue. Compounds were tested against tumor colonies and activity defined by the growth inhibition of colonies. However, several limitations prevented the use of this assay for large-scale screening [Fiebig et al., 2004; Selby et al., 1983; Fiebig et al., 1987], the main criticism being that many tumor types have a low plating efficiency.

The *in vitro* human tumor cell line screen shifted the screening strategy from being ‘‘compound-orientated’’ to ‘‘disease-orientated.’’ Three assays have been extensively used to study cellular growth and viability in primary screens. Two are metabolic assays; the cellular reduction of a colorless tetrazolium salt (MTT or XTT) yields a colored formazan derivative in proportion to viable cell number. The assays MTT and XTT are dependent on cellular generation of cofactors: NADH and NADPH. XTT assay requires the addition of an electron transfer reagent, phenazine methosulfate. These assays may yield low levels of formazan due to depletion of glucose. The third assay is the SRB assay that binds to cellular proteins. This assay sometimes gives artifacts related to the presence of protein from dead cells. Studies show that all the above assays gave comparable results. [Rubinstein et al., 1990].

MTT assay is a simple colorimetric test of cell proliferation and survival [Mosmann, 1983] which has been adapted for measuring chemo sensitivity of both established tumor cell lines [Carmichael et al., 1987; Cole, 1986; Park et al., 1987] and fresh tumor samples [Campling et al., 1991, Suto et al., 1989, Twentyman et al., 1989]. It has been shown to be simple, rapid, inexpensive and reproducible in chemo-sensitivity testing for tumor cells [Boyd, 1989] and adaptable to high throughput screening [Hayon et al., 2003; Sarjent, 2003]. The dye MTT is taken into the cell through endocytosis and is reduced and accumulated in a population of acidic vesicles. Reduced MTT formazan is exocytosed to form needle-like formazan crystals at the cell surface. [Liu, 1999].

The present human tumor cell line *in vitro* screen is technically simple, relatively fast, cheap, reproducible, and provides valuable indicative data of mechanistic activity and target interaction. Yet it is not without its limitations. *In vitro* methods are susceptible to false-

positive and false-negative results. It is also clear that factors other than the inherent chemo-sensitivity of tumor cells significantly influence the outcome of chemotherapy *in vivo* (e.g., pharmacokinetics).

***In-vivo* methods**

Different animal models used for testing potential anti-cancer activity of molecules include hollow fiber model, human tumor xenografts, orthotopic transplantation models, autochthonous tumors and genetically engineered mouse models.

Based on previous microencapsulation and hollow fiber culture systems [Gorelik et al., 1987; Lanza et al., 1991; Lacy et al., 1991], Hollingshead and colleagues [Hollingshead et al., 1995] developed the hollow fiber model designed to identify *in vivo* activity of potential anticancer compounds. The assay assesses the pharmacologic capacity of compounds to reach two physiologic compartments within the nude mouse and shows a practical means of quantifying viable tumor cell mass.

Compounds defined as active in the Hollow fiber assay are evaluated in s.c. xenograft models using the most sensitive tumors identified by the hollow fiber assay [Plowman et al., 1999]. Xenograft tumors are generally established by the s.c. inoculation of tumor cells into nude mice ($1.0 * 10^7$ cells per mouse). Growth of solid tumors is monitored using *in situ* caliper measurements and models may be advanced stage or early stage [Plowman et al., 1997; Kelland, 2004]. Generally, activity is defined by tumor growth delay, optimal % T/C (T/C = median treated tumor mass/median control tumor mass) or net log cell kill. Drug-related deaths and body weight loss are used as parameters of toxicity.

It is becoming more and more appreciated that xenograft models should be characterized to ensure that the molecular drug target is expressed [Kelland, 2004; Decker et al., 2004; El Hilali et al., 2002; Bibby, 2004(a); Bibby, 1999(b)] and that xenograft studies should integrate both pharmacokinetic and pharmacodynamic investigation [Kelland, 2004; Decker et al., 2004; El Hilali et al., 2002; Suggitt et al., 2004; Kerbel, 2003; Peterson et al., 2004].

Orthotopic transplantation models attempt to mimic the morphology and growth characteristics of clinical disease [Paget, 1989; Fidler, 1986; Fidler, 1990; Hoffman, 1997;

Hoffman, 1999] and are thought to represent a more clinically relevant tumor model with respect to tumor site and metastasis [Killion et al., 1999]. One of the most obvious advantages of orthotopic systems is that attempts to target processes involved in local invasion (e.g., angiogenesis) can be carried out in a more clinically relevant site. In contrast to conventional s.c. tumor xenografts, limitations include technical skill, time, and cost. Therapeutic efficacy is also more difficult to assess in contrast to the relative ease of s.c. tumor measurements [Bibby, 2004].

Autochthonous tumors include spontaneously occurring tumors and induced tumor growth (e.g. by chemical, viral, or physical carcinogens). It is thought that autochthonous tumors may mimic human tumors more closely than transplanted tumors (i.e., s.c./orthotopic). Advantageous properties include orthotopical growth, tumor histology devoid of changes introduced by transplantation, and a route of metastasis through lymph and blood vessels that surrounded early tumor growth [Berger, 1999]. Despite such properties, the use of autochthonous tumor models has not been widespread due to several limitations. A large variability in take rate and growth exists, the large number of animals needed, time frames of several months to years exist for a single experiment due to long tumor latencies as opposed to weeks in transplanted xenograft models, and lack of spontaneous metastasis [Berger, 1999; Huss et al., 2001].

Genetically engineered mouse (GEM) models possess well-validated molecular/genetic characteristics (e.g., gene mutations), which ultimately facilitate the rational design of small molecule therapeutics. One of the first transgenic cancer models involved the constitutive expression of the c-myc oncogene [Macleod et al., 1999]. Later transgenic mouse models were developed which involved introducing a mutant TSG to the mouse germ line. One of the first TSG mutants was the Rb ‘‘knockout’’ mouse [Stewart et al., 1984]. Since the Rb knockout, many mutant TSG or knockout cancer-prone mouse models have been developed including p53 [Stewart et al., 1984; Jacks, 1992; Donehower et al., 1992], Apc [Purdie et al., 1994] and Nf-1 [Moser et al., 1993].

There are several existing mouse models of multistep tumorigenesis. The RIP-Tag mouse expresses the SV40 antigen under the control of the insulin promoter leading to the development of pancreatic islet cell carcinoma [Jacks et al., 1994]. Conditional

transgenic/knockout models involve spatial control over the initiation of oncogene expression and TSG inactivation, respectively, and have been used to create models of several types of cancer [Hanahan, 1985]. The Cre-Lox system is the most widely used for both transient conditional knockout [Tuveson et al., 2002] and oncogene expression [Le et al., 2001].

Unlike xenograft models, GEM models possess well validated drug targets and may potentially offer a more appropriate preclinical model in which to test modern small molecule therapeutics. Additionally, GEM tumors develop autochthonously/*in situ* and therefore may be more biologically representative of a particular tumor type in humans than transplanted xenografts. Despite such promise, GEM models are not without limitation. Compared to the traditional xenograft model, GEM models are expensive and time consuming. Their use is often restricted by intellectual property rights and patents [Lakso et al., 1992]. In addition to embryonic lethality, mice often do not develop the expected tumor type as they may die prematurely from a different tumor type caused by the constitutive expression of oncogene/TSG. Species-specific differences also exist in the role of different genes in different cell types, which can lead to different mutant phenotypes in both man and mouse [Weiss et al., 2003].

1.2.2 Anti-cancer activity and mechanism of action of betulinic acid

Cytotoxicity of betulinic acid

Previous reports indicated that betulinic acid was a melanoma-specific cytotoxic compound; [Pisha et al., 1995] however; more recent evidence indicates that betulinic acid possesses a broader spectrum of activity against other cancer cell types [Fulda et al., 1999; Schmidt et al., 1997; Zuco et al., 2002].

Pro-apoptotic activity of betulinic acid

Betulinic acid was shown to act through induction of apoptosis [Pisha et al., 1995] independent of the cell's p53 status; [Zuco et al., 2002; Fulda et al., 1997; Selzer et al., 2000] however; another study suggests that betulinic acid may induce p53 upregulation [Rieber et al., 1998] in metastatic melanoma cells. Incubation of betulinic acid with melanoma cells *in vitro* resulted in the appearance of characteristic surface blebbing and cytoplasmic shrinking that are indicative of the induction of apoptosis. Further evidence supporting the induction of

apoptosis came from investigations showing the formation of characteristic high-molecular-weight DNA fragments and flow cytometry studies [Pisha et al., 1995]. It was reported that betulinic acid induces apoptosis through the induction of changes in mitochondrial membrane potential, production of reactive oxygen species, and permeability transition pore openings [Schmidt et al., 1997]. These processes lead to the release of mitochondrial apoptogenic factors, activation of caspases, and DNA fragmentation [Fulda et al., 1997; 1998; 2000]. In addition, researchers had shown that betulinic acid exhibited increased efficacy against melanoma cells grown at a reduced pH (<6.8) [Noda et al., 1997; Wachsbeberger et al., 2002]. This finding was of interest since many tumors produce an acidified extracellular environment that may help in the absorption of betulinic acid (a weak organic acid) by the tumor. It was also demonstrated that heat sensitization resulted in the increased susceptibility of tumor cells to betulinic acid possibly because of altered (lowered) intracellular pH [Wachsbeberger et al., 2002].

Further, it has been shown recently that cdk4 protein is an early target of betulinic acid-induced apoptosis and unrestricted ERK signaling favors betulinic acid-induced apoptosis. [Rieber et al., 2005]

Betulinic acid activates NF-kappaB in a variety of tumor cell lines. But activation of NF-kappaB by betulinic acid promotes betulinic acid-induced apoptosis in a cell type-specific fashion indicate that NF-kappaB inhibitors in combination with betulinic acid would have no therapeutic benefit or could even be contra productive in certain tumors, which has important implications for the design of betulinic acid-based combination protocols [Kasperczyk et al., 2005].

Anti-angiogenic effect of betulinic acid

A previous investigation had shown that betulinic acid inhibited the *in vitro* activity of aminopeptidase N [Melzig et al., 1998], an endogenous angiogenic factor, but failed to inhibit the enzyme *in vivo*. Betulinic acid does, however, inhibit mitochondrial function in endothelial cells [Kwon et al., 2002(a)].

Other activities of betulinic acid

Betulinic acid has also been shown to protect congenital melanocyte naevi cells from UV-C-induced DNA strand breakage independent of p53 and p21. It was speculated that betulinic acid may be acting through an antioxidant mechanism [Salti et al., 2001]. However, betulinic acid was shown to potentiate the activity of bleomycin, a mediator of DNA strand breakage, through inhibition of DNA polymerase-b in P-388D1 cells [Ma et al., 1999]. Additionally, betulinic acid exhibits weak inhibitory effects against topoisomerase I and IIa, but does not stabilize the topoisomerase IIa–DNA complex [Syrovets et al., 2000]. Further, it has been reported that betulinic acid induces differentiation as well as cell death in normal human keratinocytes (NHK). [Galgon et al., 2005]. Betulinic acid transiently activated the EGFR/AKT cell survival pathway and induced survivin expression, contributing to less sensitivity in human melanoma cells. The data suggest that a combination of the EGFR inhibitor and betulinic acid may be a better clinical option to treat human melanoma [Qui et al., 2005].

Co-operation with other drugs

Betulinic acid cooperated with anticancer drugs to induce apoptosis and to inhibit clonogenic survival of tumor cells. Combined treatment with betulinic acid and anticancer drugs acted in concert to induce loss of mitochondrial membrane potential and the release of cytochrome c and Smac from mitochondria, resulting in activation of caspases and apoptosis. Overexpression of Bcl-2, which blocked mitochondrial perturbations, also inhibited the cooperative effect of betulinic acid and anticancer drugs, indicating that cooperative interaction involved the mitochondrial pathway. Notably, cooperation of betulinic acid and anticancer drugs was found for various cytotoxic compounds with different modes of action (e.g., doxorubicin, cisplatin, Taxol, VP16, or actinomycin D. Importantly, betulinic acid and anticancer drugs cooperated to induce apoptosis in different tumor cell lines, including p53 mutant cells, and also in primary tumor cells, but not in human fibroblasts indicating some tumor specificity. These findings indicate that using betulinic acid as sensitizer in chemotherapy-based combination regimens may be a novel strategy to enhance the efficacy of anticancer therapy, which warrants further investigation. [Fulda et al., 2005]

In vivo activity of betulinic acid

Betulinic acid is active *in vivo* against TPA-induced tumors [Yasukawa et al., 1991; Yasukawa et al., 1995] and ovarian [Zuco et al., 2002] and melanoma [Pisha et al., 1995] xenografts in mice. Remarkably, betulinic acid exhibited no toxic effects in mice even at a concentration of 500 mg/kg. However, doses of betulinic acid as low as 5 mg/kg were determined to significantly impede tumor development [Pisha et al., 1995]. Even more striking are the results of a study in which betulinic acid was withheld from mice infected with melanoma cells for 41 days and then administered as a six-dose regimen (50 mg/kg each) every third day. At the time of autopsy (71 days), the mice exhibited greater than 80% regression in tumor size [Pisha et al., 1995]. Combined, these encouraging findings have made betulinic acid a very attractive candidate for the clinical treatment of various forms of cancer.

1.2.3 Derivatives of betulinic acid

Studies have been performed to derive synthetic betulinic acid analogs in an effort to establish meaningful structure-activity relationships. Three positions in betulinic acid, the C-3 hydroxyl, C-20 alkene, and C-28 carboxylic acid moieties, have served as the target for most derivatization studies. The 3β-hydroxyl moiety found in betulinic acid represents a readily available position for chemical modification. However, only a limited number of synthetic C-3 betulinic acid derivatives have been reported and tested for cytotoxicity. Some of these modifications include oxidation to a ketone, acetylation, and formation of various nitrogen-containing analogs (amine; oxime). Despite these efforts, very little can be deduced regarding the role that derivatization of C-3 in betulinic acid may play in controlling its anticancer activity. It was determined that although the introduction of an oxime moiety at C-3 did not exert a considerable impact on the cytotoxicity of betulinic acid, it may result in the potential loss of selectivity against melanoma cells. Oxidation of the C-3 hydroxyl group in betulinic acid to a ketone yields the highly cytotoxic derivative betulonic acid; however, this also results in a loss of specificity against melanoma cells [Kim et al., 1998a]. An acetylated derivative, 3-O-acetyl betulinic acid, appears to retain the cytotoxicity associated with betulinic acid [Kinoshita et al., 1999; Hata et al., 2002; Lee et al., 1996]. An interesting C-3 benzyl ester derivative has also been synthesized [Kim et al., 1998a]. This compound was found inactive against both melanoma- and non-melanoma-derived cancer cell lines. It was

speculated that the bulky benzyl group was responsible for the loss of activity; however, the concurrent modification of the C-28 position in this molecule to a methyl and the lack of additional related derivatives for comparison hampered efforts to draw further conclusions in this regard. Additional studies are needed to probe the potential for further C-3 modifications that may alter the cytotoxicity and selectivity of betulinic acid.

A limited number of studies have examined changes in the cytotoxicity profile of betulinic acid derivatives in which the C-20 side chain has been modified. Minor changes, such as the introduction of a ketone or oxime moiety at C-29, resulted in a loss of activity [Kim et al., 2001a]. However, hydrogenation of the C-20 double bond does not adversely affect the cytotoxicity of betulinic acid [Kim et al., 1998a]. Based on these studies, the C-20 side chain of betulinic acid does not appear to be a useful site for structural modifications [Kim et al., 2001a].

A modest series of C-28 betulinic acid derivatives have been synthesized and tested for biological activity. In all cases, a C-28 carbonyl was found essential for preserving cytotoxicity [Kim et al., 1998a; Jeong et al., 1999; Hata et al., 2002]. A series of C-28 amino acid conjugates have also been examined for their cytotoxic properties [Jeong et al., 1999]. Several of these derivatives, such as the leucine, alanine, and valine methyl esters and the glycine free acid conjugate, were noted to exhibit melanoma-specific cytotoxic properties similar to betulinic acid. In addition, the amino acid conjugates displayed improved water solubility profiles when compared to betulinic acid. Interestingly, the 28-O-β-D-glucoside of betulinic acid did not exhibit any significant cytotoxicity [Chatterjee et al., 1999]. These results demonstrate the existence of a variety of structural constraints at C-28 that are necessary to preserve the biological activity of betulinic acid.

Several other betulinic acid derivatives, obtained from various sources, have been tested for anticancer activity. For example, 7β-hydroxy, 6α, 7β-dihydroxy, and 1β, 7β-dihydroxy derivatives were obtained as microbial transformation products of betulinic acid [Kouzi et al., 2000]. All of these compounds exhibited slightly reduced biological activity. A 23-hydroxyl derivative, however, was found to possess cancer growth inhibitory properties similar to betulinic acid [Ye et al., 2001]. Further comparative studies were conducted using oleanane- and ursane-type triterpenes of similar structures [Kinoshita et al. 1999; Hata et al., 2002]. 3-

β-O-phthalic esters were synthesized from betulinic acid. It was discovered that hemiphthalic esters had better cytotoxicity than starting compounds betulinic acid or betulin [Kvasnica et al., 2005].

A semi-empirical molecular-orbital method demonstrates that the cytotoxicity of betulinic acid derivatives can be predicted by several physical parameters (such as heat of formation, hydrophobicity (log P), water solubility, ionization potential, electron affinity, dipole moment), but not by molecular size (maximum length and width). [Ishihara, 2005] Based on the above studies, these compounds may offer additional opportunities to dissect the structure–activity relationship associated with the anticancer activity of betulinic acid. More studies are needed to probe the potential for enhancing the biological activity of betulinic acid through the modification of other positions in its core.

1.3 MATERIALS AND METHODS

1.3.2 Betulinic acid derivatives

Several derivatives of betulinic acid were synthesized by Process chemistry and Medicinal chemistry laboratories, Dabur Research Foundation. The pentacyclic triterpene of betulinic acid was retained and structural modifications were carried out at C-2, C-3, C-20 and C-28 positions based on structure activity relationships. The synthesized and fully characterized derivatives were given number codes (from 321 onwards) and submitted for activity testing. The synthetic strategy and structure of all the derivatives has been patented and published by us previously [Mukherjee R et al., 2004a; Mukherjee R et al., 2004b; Mukherjee R et al., 2004c; Ramadoss et al., 2003].

1.3.1 Cell culture

Human tumor cell lines and fibroblast cell line (CHO) were purchased either from American Type Culture Collection [ATCC, USA] or National Cell Science Centre [NCCS, Pune, India] and cultured in Dulbecco's Modified Eagles Medium [DMEM, Gibco BRL, USA], containing L-glutamine, 25mM HEPES and supplemented with 10% heat-inactivated fetal

bovine serum [Gibco BRL, USA], penicillin (100 units/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL) in the form of antibiotic solution [Hyclone, USA]. Cultures were maintained in a humidified incubator at 37 ^{0}C and 5% CO_2 [BB16, Heraeus, Germany]. Tissue culture grade, sterile plastic-ware (Nunc, Denmark) was used.

1.3.3 Cytotoxicity assay

Cells were collected from 70-80% confluent cultures by trypsinization (0.25% trypsin and 0.02% EDTA) and seeded in 96-well plates at 5000 to 30,000 cells/well in cell culture medium for 24 hours in a CO_2 incubator. The test substance was dissolved in DMSO (Merck, India) and further dilutions were made in cell culture medium such that the final DMSO concentration in the well even at the highest concentration is less than 1%. After 24 hours the cells were incubated with the above test substance to obtain drug concentrations in the range of 0.5 to 20 μg/mL. After 72 hours of incubation in a CO_2 incubator, cytotoxicity was measured by the tetrazolium-based MTT assay adapted from previously published methods [Mossman, 1983; Carmichael et al.., 1987; Cole, 1986; Park et al.., 1987]. Briefly, 25 μL of MTT (5 mg/ml, Sigma, USA) was added to each well of the 96-well plate and incubated at 37^{0}C for 3 hours. MTT was converted to greenish-brown colored formazan by mitochondrial dehydrogenase enzyme present in viable cells. For adherent cells, the medium in the wells was gently pipetted out and replaced with 150 μL of DMSO and kept with gentle shaking for 15 minutes to dissolve formazan crystals. For suspension cultures, formazan was dissolved by direct addition of 50 μL of sodium dodecyl sulfate (SDS) acidified with 1N HCl, was added to the wells followed by incubation for one hour and mixing the contents using a pipetman. The optical density (O.D.) in the wells was measured at 540 nm (for adherent cells) or 570 nm (for suspension cells) using a multi-well spectrophotometer (Anthos HTII, Austria). Percentage cytotoxicity was calculated using the formula given below:

$$\% \text{ cytotoxicity} = \left(1 - \frac{X}{R_1}\right) * 100$$

where X= O.D. of wells containing the test substance and R_1= O.D. of control wells. Each experiment was repeated thrice and IC_{50} values (half-maximal cytotoxicity) were calculated by employing non-linear regression analysis using Prism® software.

1.3.4 Selection of active compounds

Derivatives with IC_{50} < 4 μg/ml (≈10 μM) in the most sensitive cell line, acute lymphoblast leukemia (MOLT-4) in the MTT cytotoxicity assay were selected for further studies as previously described [Mukherjee R et al., 2004c]. The mean cytotoxicity of a selected derivative was calculated as the average IC_{50} in sensitive cell lines. If the mean cytotoxicity of a derivative was lesser than betulinic acid it was selected. These compounds were screened for cytotoxicity to normal mouse spleenocytes and Chinese hamster ovary (CHO) cells. Cancer cell specificity of test compound was calculated using the ratio IC_{50} in normal / IC_{50} in cancer cell line and termed N/T. If N/T was greater than 2, the compound was designated as more specific to cancer cells than normal cells. Such derivatives were selected if they had >95% chemical purity, have good yield of synthesis and are novel and patentable.

1.3.5 Active caspase-3 immunoassay

Acute lymphoblast leukemia cells (MOLT-4) were collected at 70% confluence and plated in a 6-well plate (Nunc, Denmark) at a density of 8 - 10 x 10^5/well. Test compounds were added to the treated wells at IC_{50} concentration in triplicates. The untreated wells were controls. The plate was incubated at 37^oC, 5% CO_2 incubator. Samples were drawn at 5 hrs, 17 hrs and 24 hrs and measured for levels of active caspase-3 using Quantikine Human active Caspase-3 Immunoassay [Catalog Number KM300, R&D Systems, Inc., USA] by following manufacturer's instructions.

Briefly, after induction of apoptosis, 2 μL of 5 mM biotin-ZVKD-fmk per 1 mL of culture medium was added to obtain a final concentration of 10 μM. The cells were incubated with the biotin-ZVKD-fmk inhibitor for 1 hour. The cell culture sample was centrifuged at 1000 x g for 5 minutes. The supernatant was discarded and cells were resuspended in PBS. Cells were again centrifuged at 1000 x g for 5 minutes and the supernatant was discarded. Extraction Buffer (1X) containing Protease Inhibitors was added at 1 mL per 1 x 10^7 cells and vortexed for 1 minute and allowed to sit for 2 hours at room temperature. Immediately prior to assay, samples were diluted 10 - 20 fold with a calibrator diluent.

Standard or sample was added to the above (100 µL /well), covered with the adhesive strip provided and incubate for 2 hours at room temperature. Each well was aspirated and washed, repeating the process four times for a total of five washes. The plate was inverted and blotted against clean paper towels. 100 µL of active Caspase-3 Conjugate was added to each well, covered with a new adhesive strip and incubate for 1 hour at room temperature. The aspiration/wash steps were done as above. 100 µL of substrate solution was added to each well and incubated for 30 minutes at room temperature protected from light. 100 µL of stop solution was added to each well and gently tapped to ensure thorough mixing.

The optical density of each well was determined within 30 minutes, using a microplate reader (Anthos HT II, Austria) set to 450 nm with wavelength correction set to 540 nm or 570 nm. The active caspase-3 large subunit concentration (ng/mL) of each sample was determined from a standard curve and multiplied by the dilution factor.

1.3.6 Annexin V-FITC apoptosis detection assay

Lung carcinoma cells (A549) were collected at 70% confluence and plated in a 6-well plate (Nunc, Denmark) at a density of 2.5 x 10^5/well. Test compounds (2 mg/ml in DMSO) were added at 5 µg/mL in triplicate wells. Camptothecin (positive control) was added to the respective wells 3-6 hrs prior to termination. The untreated wells were controls. The plate was incubated at 37^{o}C, 5% CO_2 incubator. The cells were measured for apoptosis using TACS Annexin V-FITC Apoptosis Detection Kit [Catalog Number TA4638, R&D Systems, Inc., USA] by following manufacturer's instructions. Briefly, after overnight incubation the medium was removed and cells were harvested by using trypsin (0.25% trypsin, 0.03% EDTA). Trypsinized cells were centrifuged at 1000 rpm for 10 min. The cells were washed with cell culture medium, centrifuged at 1000 rpm for 10 min, supernatant was discarded and cells were gently resuspended in the Annexin V Incubation Reagent at a concentration of 1 x 10^5 - 1 x 10^6 cells/100 µL and incubated in the dark for 15 minutes at room temperature. 400 µL 1X Binding Buffer was added to the samples. The samples were analyzed by flow cytometer [FACS Caliber, BD, USA] within one hour for maximal signal.

1.3.7 Tumor xenograft assay

Human tumor xenograft assay was adapted from previously published methods [Plowman et al., 1997; Kelland, 2004].

Animals

Athymic nude mice, age 6-8 weeks, weighing around 20 gms were obtained from National Centre for Laboratory Animals Sciences (NCLAS, NIN, Hyderabad, India) and maintained in sterile isolators at the Small animal facility, Dabur Research Foundation. Animal experiments were carried out as per guidelines of Institutional Animal Ethics Committee (IAEC), Dabur Research Foundation. These animals were quarantined for 1 week prior to beginning the study. The mice were divided in to groups of six each and numbered for identification. Each group was kept in sterile acrylic cages. Autoclaved standard food pellets (Gold feed, Delhi) and water (Aquaguard pure) through a sterile glass bottle fitted with a nozzle, was available for 24 h. The animals were kept in an air-conditioned laboratory (room temperature maintained between 22-26^{o}C) with 12h light and dark cycle.

Test compounds

Test compounds were provided by NDDS and Medicinal chemistry labs, Dabur Research Foundation. For intravenous administration, 1098 was dissolved in co-solvents Dimethylacetamide (20%), PEG400 (20%), Tween 80 (20%) and water for injection (q.s. to 100%) at a concentration of 2 mg/ml. For oral and intra-peritoneal administration, betulinic acid was administered as Zizyphus Jujuba extract, prepared as described previously [Ramadoss et al., 2001], containing 33% betulinic acid. The extract was freshly reconstituted as a suspension in saline at a concentration of 5 mg/ml.

Tumor propagation

A single cell suspension of tumorigenic cancer cells (1 - 3 x 10^7 cells/100 μL) harvested at exponential growth phase by trypsinization [0.25% trypsin and 0.02% EDTA] was subcutaneously injected in abdominal area using a 30G sterile needle. The cells were made to form a plug under the skin at a point distant from the site of injection so that the cell suspension does not ooze out from the injection site. The remaining cell suspension was checked to ensure sterility.

Study design

Betulinic acid and 1098 doses were selected based on preliminary toxicity studies conducted in mice using a range of doses. Tumor bearing mice were randomized by tumor volume and kept in groups of six animals per cage. Animals were dosed based on bodyweight. Betulinic acid was administered by two routes (oral and intra-peritoneal) at a dose of 100 mg/kg b.wt. (equivalent to 33 mg/kg of betulinic acid) every day for a period of 2 weeks. 1098 was dosed intravenously at a dose of 10 mg/kg b.wt. every day for 2 weeks. Oral dosing was done using an oral gavage tube, intra-peritoneal dosing by using a short-beveled 24G needle on the lower left quadrant of the abdomen, and intravenous dosing was done through a peripheral tail vein using a 30G needle. Tumor volume, bodyweight and signs of toxicity were recorded once every four days. Tumor growth was monitored by measuring perpendicular tumor dimensions using a vernier caliper and calculating tumor volumes using the formula given below:

Tumor volume = $0.4 * W^2 * L$, where W = smaller diameter, L = larger diameter.

Animal were sacrificed if tumor volume exceeded 5 gms. Final evaluation was made after the end of treatment. The different groups were compared on the basis of treated/control (T/C) values and statistical analysis.

1.3.8 Statistical Analysis

In vitro data

The results were expressed as mean ± SEM. The student's unpaired t-test was used to compare the means of two groups. Difference were considered significant when $p < 0.05$.

Tumor data

All results were expressed as mean ± SD.

Multiple comparison procedure was carried out using option in LSMEANS statement with the ADJUST=Dunnett in PROC GLM of SAS 9.1.3 for the data on *in vivo* activity of betulinic acid. The factor-analytic covariance approximation was used and adjusted as "Dunnett-Hsu". PROC GLM of SAS 9.1.3 with LSMEANS statement was carried out to compare LSMEANS of treated group with that of control group for the data on *in vivo* activity of the 'hit' compounds 1098 and 937.

The non-control levels were tested and declared significantly different than the control if the associated p-value was less than 0.05 level of significance.

1.4 RESULTS AND DISCUSSION

1.4.1 Anti-cancer activity of betulinic acid

Betulinic acid was screened for cytotoxicity in a panel of 23 human tumor cell lines. It shows an IC_{50} value less than 10 μg/ml (≈ 20 μM) in cell lines representing leukemia, lymphoma, colon, lung, prostate, ovary and melanoma (Table - 1). The cytotoxicity of betulinic acid against melanoma cell lines was reported previously [Pisha et al., 1995], whereas the broad-spectrum activity against human leukemia, lymphoma, colon, lung, ovary and prostate has been demonstrated here for the first time. Betulinic acid was most potent in leukemia and lymphoma (IC_{50} < 1 μg/mL) in Jurkat E6.1, CEM.CM3, U937 and BRISTOL-8 cell lines. The results show that betulinic acid possesses broad-spectrum anti-cancer activity in different types of cancers. Betulinic acid was however non-cytotoxic (IC_{50} > 10 μg/ml) to cell lines of pancreas, cervix, breast, oral, myeloma and glioblastoma.

1.4.2 Cytotoxicity and cancer specificity of betulinic acid derivatives

Betulinic acid derivatives were screened using three screens: primary cytotoxicity screen, secondary cytotoxicity screen and cancer specificity screen. Out of the 500 derivatives screened for cytotoxicity in the primary screen in a leukemia cell line (MOLT-4), 182 derivatives had $IC_{50} \leq 4$ μg/mL (Figure - 4). These derivatives were selected for secondary screening in the panel of sensitive cancer cell lines. 25 derivatives were better than betulinic acid in terms of broad-spectrum cytotoxicity based on mean cytotoxicity values (Table - 2). Among these derivatives 1098 and 937 were significantly more cytotoxic than betulinic acid ($p < 0.05$). These compounds were also found to possess remarkable specificity towards cancer cells as compared to spleenocytes as shown by values of normal/tumor (N/T) > 2 in six and four cell lines respectively (Table - 3), whereas betulinic acid had specificity to three

cell lines. Derivatives 998, 455, 1065, 829, 807, 940 and 789 had specificity to two cell lines each. Since broad-spectrum activity was a criterion for selection, derivatives 1098, 937, 1065, 829 and 807, which were selective to at least two cancers and have purity greater than 95% were "short-listed" for further studies. The other compounds screened had specificity to only one out of the six sensitive cell lines screened, and were not selected for further studies.

The cancer specificity of the "short-listed" derivatives was further confirmed by testing in a normal fibroblast cell line (CHO) using a larger panel of cancer cell lines (Table - 4). Results show that derivatives 1098, 937, 1065, 829 and 807 had cancer specificity to three or more cell lines compared to betulinic acid (specific to two cell lines). Hence the cancer specificity or selectivity of "short-listed" compounds 1098, 937, 1065, 829 and 807 was confirmed. The cytotoxicity and specificity of some of these short-listed derivatives was published by us [Mukherjee R et al., 2004c].

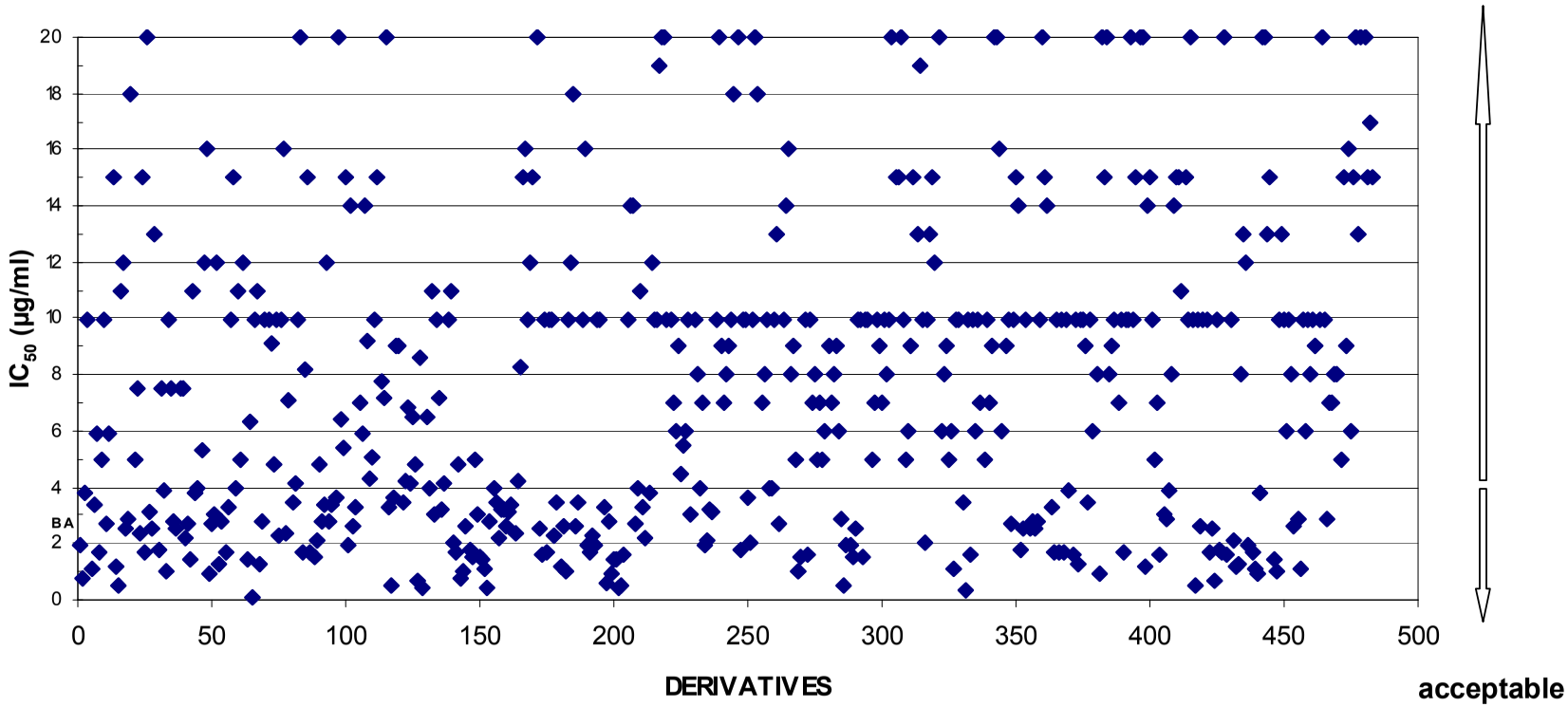

Figure-4

Primary screening of derivatives of betulinic acid in Molt-4 cell line

The graph shows mean IC_{50} of three independent experiments of about 500 derivatives that were screened using the primary cytotoxicity screen. Cytotoxicity was assessed by MTT method as described in Methods. The IC_{50} of betulinic acid is represented by the point BA. The points below BA have more potent cytotoxicity whereas points above BA have less potency, as represented by 'Acceptable' (i.e. $\leq$ 4 µg/ml) and 'Unacceptable' (> 4 µg/ml), respectively. Where IC_{50} was greater than the highest concentration tested the compounds have been plotted at that concentration.

Table - 1

Cytotoxicity of betulinic acid on a panel of human tumor cell lines

S.No	Cancer type	**Cell line**	**IC_{50} (µg/ml)**
1	*Leukemia & Lymphoma*	HL 60	2.80 ± 0.32
2		K 562	3.25 ± 0.49
3		MOLT-4	1.23 ± 0.70
4		Jurkat E6.1	0.65 ± 0.04
5		CEM.CM3	0.98 ± 0.03
6		U937	0.69 ± 0.01
7		BRISTOL-8	0.84 ± 0.05
8	*Melanoma*	Malme 3M	2.20 ± 0.70
9	*CNS cancer*	U87MG	> 10
10		U373MG	> 10
11	*Cervical cancer*	HeLa	> 10
12	*Breast cancer*	MDA.MB.453	> 10
13		T47D	> 10
14	*Ovarian cancer*	PA-1	10 ± 4.35
15	*Colon cancer*	HT29	1.8 ± 0.0
16		SW 620	> 10
17		CoLo 205	> 10
18	*Lung cancer*	A549	> 10
19		L132	1.30 ± 0.55
20	*Oral cancer*	KB	> 10
21	*Prostate cancer*	DU145	9.80 ± 3.35
22	*Pancreatic cancer*	MiaPaCa2	> 10
23	*Myeloma*	RPMI 8226	>10

Cytotoxicity was assessed by MTT assay as described in Methods. Data shown are IC_{50} ± SEM of three independent experiments. If IC_{50} was not achieved it was represented as greater than highest concentration tested i.e.10 µg/ml.

Table-2
Cytotoxicity of potent derivatives in a panel of sensitive human tumor cell lines.
(Secondary screening data)

S.No.	Deriv.	IC_{50} (50% growth inhibitory concentration, µg/ml)						Mean Cytotoxicity
		Leukemia	Lymphoma	Prostate	Lung	Ovary	Colon	
		MOLT-4	U 937	DU145	L132	PA-1	HT-29	
1	**1098**	0.5	0.4	1.5	1.3	0.9	2.6	1.20 ± 0.3*
2	**937**	0.9	1.2	2.5	1.1	1.6	1.7	1.50 ± 0.2*
3	**1161**	0.34	0.3	0.4	3.5	1.2	3.5	1.54 ± 0.6
4	**998**	1.9	1.6	2.2	2.5	1.2	4.0	2.23 ± 0.4
5	**463**	2.5	2.4	2.5	3.2	0.7	ND	2.26 ± 0.4
6	**542**	0.12	1.3	5.2	3.6	3.4	0.41	2.34 ± 0.8
7	**1065**	1.0	1.9	2.5	3.4	1.4	4.9	2.52 ± 0.6
8	**347**	1.1	1.0	>10	1.4	1.3	0.48	2.55 ± 1.5
9	**455**	1.7	2.6	3.8	3.0	1.6	3.4	2.69 ± 0.4
10	**458**	1.0	1.7	>10	1.5	0.6	1.8	2.77 ± 1.5
11	**829**	0.4	0.5	>10	>4	0.5	1.3	2.78 ± 1.5
12	**878**	1.6	0.4	9.9	0.8	3.5	1.75	2.99 ± 1.4
13	**1104**	1.5	1.1	2	5.9	>4	3.5	3.00 ± 0.7
14	**586**	1.7	0.8	>10	2.0	1.1	2.4	3.00 ± 1.4
15	**912**	1.0	1.2	8.5	>4	ND	0.35	3.01 ± 1.5
16	**617**	1.5	1.9	>10	0.9	3.5	1.6	3.23 ± 1.4
17	**940**	0.4	0.8	>4	2.6	1.6	>10	3.23 ± 1.5
18	**807**	1.0	ND	>10	<0.5	1.7	ND	3.30 ± 2.2
19	**568**	2.3	0.5	>10	2.7	2.0	ND	3.51 ± 1.7
20	**935**	0.6	2.6	3.2	1.2	ND	>10	3.52 ± 1.7
21	**1103**	1.9	1.0	>4	4.0	ND	>10	4.18 ± 1.6
22	**1108**	1.5	1.0	>4	4.6	ND	>10	4.22 ± 1.6
23	**789**	2.0	1.4	>10	6.5	>4	1.4	4.22 ± 1.4
24	**909**	1.2	2.2	10	7	1	5.8	4.53 ± 1.5
25	**943**	1.6	3.2	>4	4.0	ND	>10	4.56 ± 1.4
Betulinic acid		1.9	0.7	9.8	3.2	10.0	1.8	4.57 ± 1.7

IC_{50} (50% growth inhibitory concentration, µg/ml) of each cell line is mean of three experiments. Cytotoxicity was assessed by MTT method. The mean cytotoxicity is expressed as IC_{50} ± SEM. Based on mean cytotoxicity value the derivatives have been arranged in ascending order. Wherever IC_{50} was not obtained it is expressed as greater than highest concentration tested. * represents $p < 0.05$ as compared to betulinic acid. ND=not done.

Table - 3

Cancer cell specificity of potent derivatives of betulinic acid to the panel of sensitive human tumor cell lines

S.No.	Deriv.	Cancer cell specificity (N/T) (IC_{50} spleenocytes/IC_{50} cancer cell line)						
		MOLT-4 (Leukemia)	U-937 (Lymphoma)	DU-145 (Prostate)	HT-29 (Colon)	L-132 (Lung)	PA-1 (Ovary)	Specificity to no. of cell lines
1	**1098**	>13.3	>13.3	9.0	>2.1	>13.3	>10.0	6
2	**937**	>22.2	>15.3	7.1	-	18.2	-	4
3	**998**	-	-	2.1	-	-	3.8	2
4	**455**	-	-	5.2	-	2.5	-	2
5	**1065**	-	-	3.5	-	-	3.7	2
6	**829**	>5.0	-	-	-	-	2.7	2
7	**807**	-	-	-	-	2.5	3.2	2
8	**940**	4.8	7.6	-	-	-	-	2
9	**789**	>8.0	-	-	2.4	-	-	2
10	**1161**	24.3	-	-	-	-	-	1
11	**542**	3.2	-	-	-	-	-	1
12	**347**	3.1	-	-	-	-	-	1
13	**1104**	-	-	5.7	-	-	-	1
14	**878**	-	2.1	-	-	-	-	1
15	**935**	>15.4	-	-	-	-	-	1
16	**1103**	-	-	-	-	-	>2.0	1
17	**909**	-	-	-	-	-	3.1	1
18	**463**	-	-	-	-	-	-	0
19	**458**	-	-	-	-	-	-	0
20	**586**	-	-	-	-	-	-	0
21	**912**	-	-	-	-	-	-	0
22	**617**	-	-	-	-	-	-	0
23	**568**	-	-	-	-	-	-	0
24	**1108**	-	-	-	-	-	-	0
25	**943**	-	-	-	-	-	-	0
Betulinic acid		>13.3	>14.2	-	-	>8.0	-	3

Values represent ratio of IC_{50} spleenocytes/IC_{50} cancer cell line (N/T). IC_{50} values were mean of three independent experiments. "-" represent N/T < 2. Based on specificity to number of cancer cell lines which have N/T > 2, the derivatives have been arranged in descending order.

Table - 4

Cancer cell specificity of potent derivatives based on cytotoxicity to normal fibroblast cell line (CHO)

S.No.	Cancer type	Cell line	Cancer cell specificity (N/T) (IC_{50} normal cell line [CHO] / IC_{50} cancer cell line)					
			1098	**937**	**1065**	**829**	**807**	**BA**
1	**Breast**	**HBL100**	4.9	>6.9	-	2.1	-	>3.98
2	**Prostate**	**DU145**	17.0	>8.4	2.2	-	-	-
3	**Oral**	**KB**	-	-	-	-	-	-
4	**Colon**	**HT-29**	-	-	-	-	-	-
5		**SW620**	4.1	>2.0	-	3.1	2.3	-
6	**Duodenum**	**Hs294T**	4.5	-	-	-	2.6	-
7	**Stomach**	**HuTu-80**	-	-	-	-	-	-
8	**Pancreas**	**MiaPaCa-2**	4.4	>5.1	2.3	-	3.4	-
9	**Glioblastoma**	**U87MG**	-	-	-	-	-	-
10	**Larynx**	**Hep-2**	-	>2.1	-	-	-	-
11	**Ovary**	**PA-1**	13.8	>2.6	2.1	2.1	-	-
12	**Lung**	**A549**	11.9	-	-	-	-	>6.6
Specific to number of cell lines			7	6	3	3	3	2

Values represent ratio of IC_{50} CHO cell line/IC_{50} cancer cell line (N/T). IC_{50} values are mean of three independent experiments. "-" represent N/T < 2. BA = betulinic acid.

1.4.3 Structure activity relationship of betulinic acid derivatives

Based on the cytoxicity and cancer specificity data of more potent derivatives from secondary screening (structures shown in Table - 5) the structure activity relationships are described below. The findings were published by us [Mukherjee R et al., 2004c].

Structural requirements at C-3 position

It was reported earlier that additional studies are needed to probe the potential for further C-3 (R_2) modifications that may alter the cytotoxicity and selectivity of betulinic acid. [Kim et al.,

1998a]. In the present study it was found that modification of C-3 hydroxyl to keto, oxime, o-acyl, o-benzoyl, benzyloxime, benzylidene, phenylhydrazine and o-sulfonylmethane groups significantly enhanced the activity and resulted in more potent derivatives when compared to betulinic acid. All the derivatives which were more potent than betulinic acid in secondary screening had one of the above substitutions at C-3 position. Some derivatives (1161, 542, 347, 935, 942, 878, 1138, 940, 1103, 606, 909, 936, and 1104) that were better than betulinic acid in secondary screening were selective to only one cancer type i.e. narrow spectrum compounds. When the structure of such compounds was analyzed, it was found that most of these compounds had either keto or benzoyloxy substitution at C-3 position.

Structural requirements at C-20 position

Earlier studies have shown that C-20 (R_4) position is not good for structural modifications (Cichewicz RH, 2004). On the contrary, it was interesting to note that 20 (twenty) out of 23 (twenty three) derivatives that were better than betulinic acid in secondary screening, had hydrogenation of the double bond at C-20 in betulinic acid.

Structural requirements at C-28 position

It was reported that C-28 (R_3) carbonyl was essential for preserving cytotoxicity of betulinic acid [Kim et al., 1998a; Jeong et al., 1999; Hata et al., 2002]. The present study confirms this fact. It was found that C-28 carboxylic acid group in betulinic acid and its derivatives was found essential for cytotoxic activity. 23 (twenty three) out of 24 (twenty four) derivatives that were better than betulinic acid in secondary screening had free acid group at C-28 position.

Presence of halogen atoms

The presence of halogen atoms (F, Br) in the aromatic groups at C-3 side chain position resulted in the compounds being more specific to cancer cells than normal cells. 10 (ten) derivatives (1161, 542, 935, 942, 940, 936, 937, 998, 1065, and 829) that were better than betulinic acid in secondary screening, had halogen atoms at C-3 side chain position. 5 (five) of these compounds (1161, 542, 935, 942, 940) were specific to either leukemia or lymphoma. This shows the importance of the presence of electron withdrawing group i.e. halogen atom for anti-leukemia or lymphoma activity.

Table-5

Structural modifications in potent derivatives of betulinic acid

S.No.	Derivative	R_1 (C-2)	R_2 (C-3)	R_3 (C-28)	R_4 (C-20)
1	**1098**	H	$=NOCH_2C_6H_4NO_2$	H	$CH(CH_3)_2$
2	**937**	H	$-OCOC_6H_3F_2$	H	$CH(CH_3)_2$
3	**1161**	Br	=O	X	$CH(CH_3)_2$
4	**463**	H	=NOH	H	$CH(CH_3)_2$
5	**542**	Br	=O	H	$CH(CH_3)_2$
6	**998**	H	$=NCHC_6H_3F_2(3,4)$	H	$CH(CH_3)_2$
7	**347**	H	=O	H	CH_2CCH_3
8	**455**	H	-COCH3	H	$CH(CH_3)_2$
9	**1065**	H	$-NCHC_6H_3F_2(2,4)$	H	$CH(CH_3)_2$
10	**458**	H	-OH	H	$CH(CH_3)_2$
11	**829**	H	$=NNHC_6H_4F(4)$	H	$CH(CH_3)_2$
12	**1104**	H	$-OCOC_6H_4(C_5H_{11})(4)$	H	CH_2CCH_3
13	**878**	H	$=NHNHC_6H_5$	H	$CH(CH_3)_2$
14	**586**	H	$=NNHC_6H_2Cl_3$	H	$CH(CH_3)_2$
15	**912**	H	$=NHNHC_6H_4OCH_3$	H	$CH(CH_3)_2$
16	**617**	H	$=NNHC_6H_4\ OCH_3$	H	$CH(CH_3)_2$
17	**807**	H	$=NNHCOC_6H_5$	H	$CH(CH_3)_2$
18	**568**	Br	-OH	H	$CH(CH_3)_2$
19	**935**	H	$-OCOC_6H_3F_2(3,5)$	H	CH_2CCH_3
20	**940**	H	$-OCOC_6H_4CF_3(3)$	H	$CH(CH_3)_2$
21	**1103**	H	-OH	$-COCHCH_2$	$CH(CH_3)_2$
22	**1108**	H	$-OCOCH_2C_6H_3(OCH_3)_2$	H	CH_2CCH_3
23	**789**	H	$-OSO_2CH_3$	H	$CH(CH_3)_2$
24	**943**	H	$-OCOC_6H_4CF_3(2)$	H	$CH(CH_3)_2$
25	**Betulinic acid**	H	-OH	H	CH_2CCH_3

R_1, R_2, R_3, and R_4 represent C_2, C_3, C_{28}, and C_{20} positions of betulinic acid. X=3-deoxy dihydro betulinic acid.

1.4.4 Effect on induction of apoptosis in cancer cells

The effect of betulinic and derivatives on the growth of cancer cells suggested a potential apoptotic or cytotoxic action, hence the effect of these compounds on apoptosis were examined.

Betulinic acid and derivatives caused morphological changes like cell shrinking, granularity and formation of apoptotic bodies as early as 24 hrs. This effect is characteristic of cells undergoing apoptosis. The effect of the most potent derivative 1098 on leukemia (MOLT-4) cells is illustrative of this effect and is comparable to that shown by Camptothecin, which is known to induce cell death by apoptosis (Figure - 5).

1.4.5 Effect on Annexin V binding

Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages. Cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose Phosphatidyl serine (PS) which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. Annexin V has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+} [Vermes et al., 1995].

Betulinic acid and 1098 caused significantly increased Annexin V binding in treated lung carcinoma (A549) cells as compared to untreated cells ($p < 0.01$) (Figure - 6). Further, the effect of 1098 was significantly better than betulinic acid ($p < 0.05$). These results show that betulinic acid and 1098 cause cell death by apoptosis. The data further shows that 1098 is more potent than betulinic acid which corroborates with the cytotoxicity data.

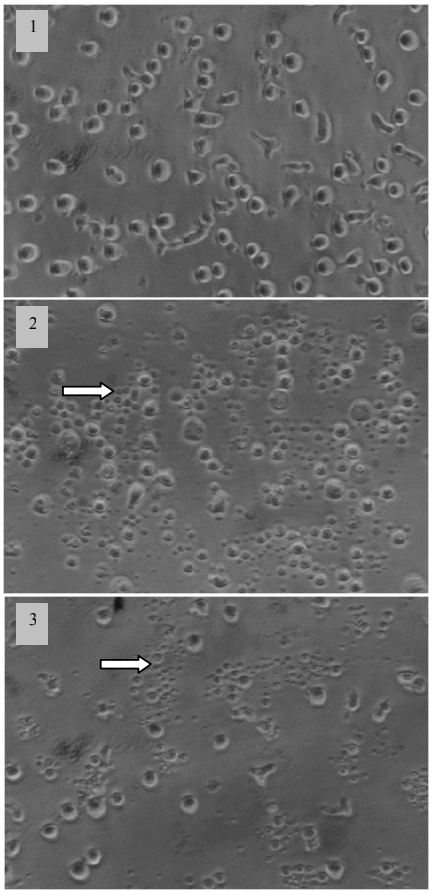

Figure - 5

Morphological changes in MOLT-4 cell line in response to betulinic acid derivative 1098. Cells were treated by 1098 for 24 hrs. Phase contrast microscopy shows increased cell granularity and formation of apoptotic bodies (arrow) in 1098 and camptothecin treated cells. 1: normal control; 2: 1098 treated cells; 3: camptothecin treated cells.

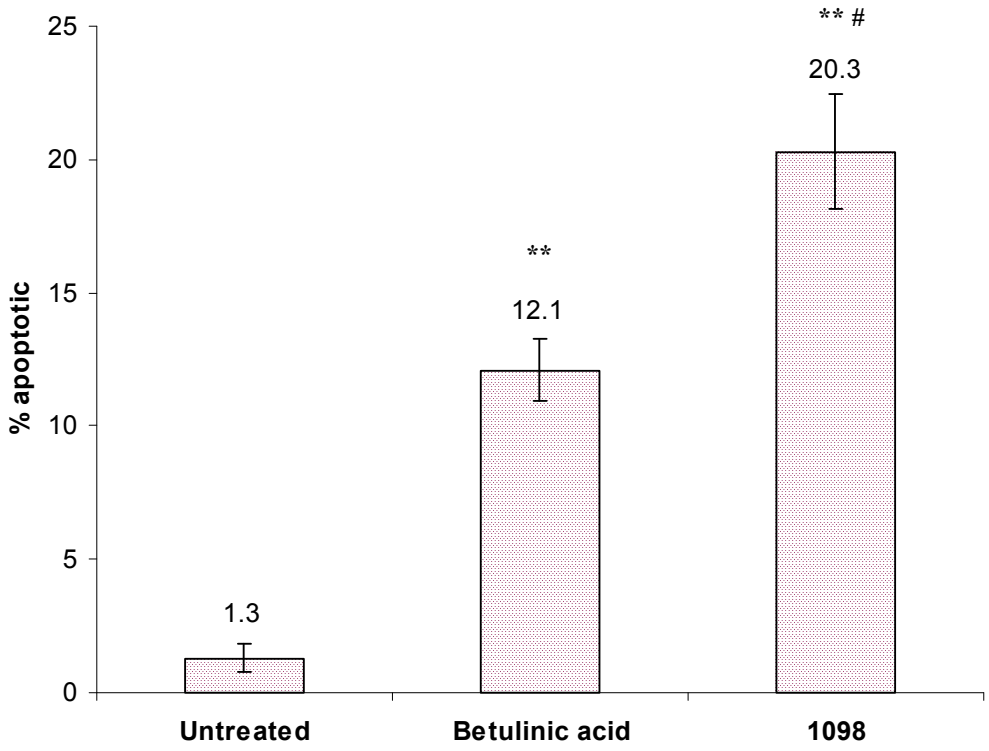

Figure - 6

Apoptosis detection by Annexin V assay

A549 (lung carcinoma) cells were treated with either betulinic acid or 1098 for 18 hrs at 10 µM then assayed for annexin V binding to externalized membrane phospholipid phosphatidylserine with annexin V propidium iodide assay kit. Data shown are mean ± SEM of three independent experiments. ** represent $P < 0.01$ compared to untreated. # represents $p < 0.05$ compared to betulinic acid.

1.4.6 Effect on induction of active caspase-3 in cancer cells

The caspase family of cysteine proteases plays a key role in apoptosis. Caspase-3 (CPP32, Yama, apopain) is a key protease that is activated during the early stages of apoptosis and, like other members of the caspase family, is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. Active caspase-3 proteolytically cleaves and activates other caspases, as well as relevant targets in the cytoplasm, e.g., D4-GDI and Bcl-2, and in the nucleus, e.g. PARP [Thornberry et al., 1998].

Leukemia cells (MOLT-4) are known to express active caspase-3 in apoptosis caused by external agents [Inanami et al., 1999]. It was found that when MOLT-4 cells were treated

with a known apoptosis inducing agent, camptothecin, it led to levels of active caspase-3 in cell lysate which was 2.2 times more than that found in untreated control cells as early as 5 hrs (Figure - 7). The effect was slightly lower at 17 hrs and 24 hrs. Betulinic acid marginally induced active caspase-3 (1.2 fold compared to control) while 1098 induced active caspase-3 by 1.4 fold and 1.6 fold at 17 hrs and 24 hrs. It was found that the effect of 1098 was significantly better than betulinic acid at 17 hrs ($p < 0.05$).

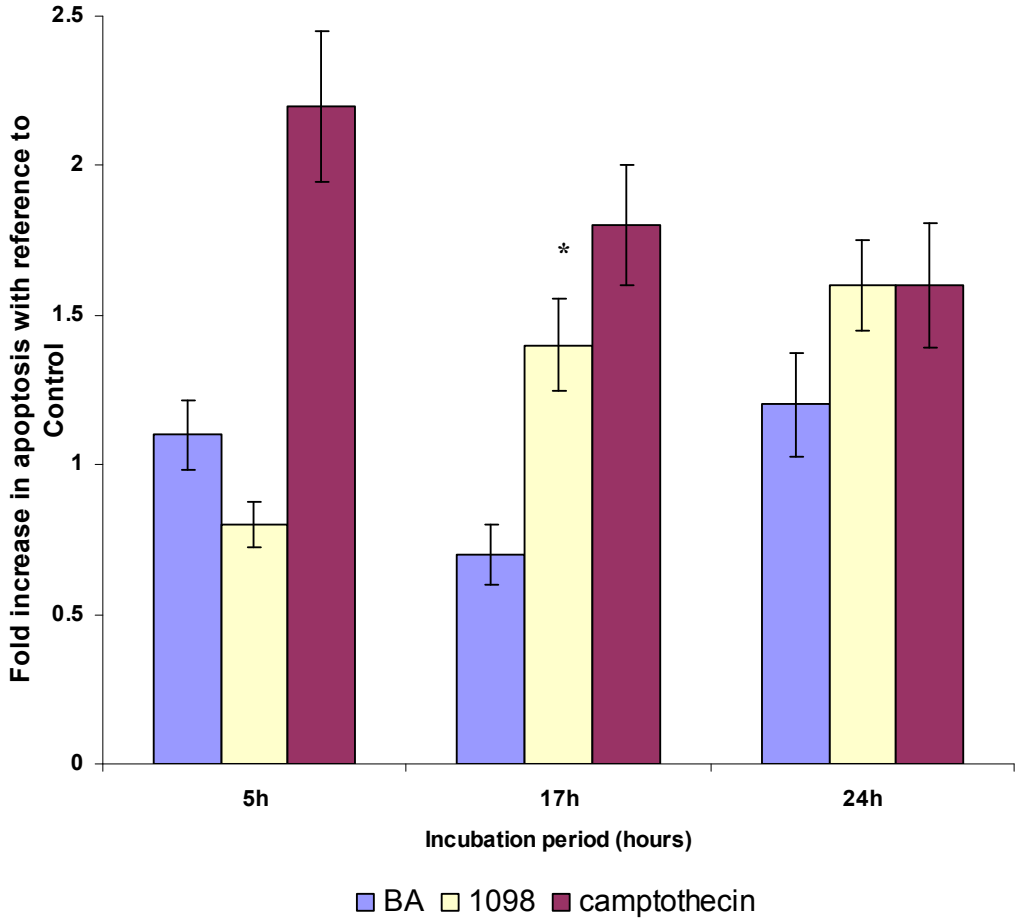

Figure - 7

Effect on caspase-3 activity

The effect of betulinic acid, 1098 and positive control camptothecin on caspase-3 activity in Molt-4 (leukemia) cell line. The cells were treated with the compounds for 24 hrs. Data shown are mean ± SEM of three independent experiments. * represents $P < 0.05$ compared to betulinic acid (BA) at 17 hrs.

1.4.7 *In vivo* efficacy in established tumor xenograft models.

Betulinic acid was reported to have good anti-tumor effect in melanoma xenograft model [Pisha et al., 1995]. This effect was confirmed by studying the effect of betulinic acid in an established melanoma tumor xenograft model (B16F10).

In melanoma xenografts, when betulinic acid was dosed at an early stage (tumor volume ≈ 100 mm^3), by intra-peritoneal route, it significantly inhibited the growth of xenografts ($p < 0.05$) on days 11 and 18 and very significantly ($p < 0.01$) inhibited growth on days 22 and 27 post inoculation (p.i.) (Figure - 8). Treatment by the oral route had a delayed effect and significantly inhibited growth of xenografts on day 27 p.i. ($p < 0.05$). The treatment/control (T/C) value on day 27 p.i., before control animals were sacrificed, was 57.33% for the oral treatment group and 73.65% for the i.p. treatment group. The results show that betulinic acid has good anti-tumor efficacy when treatment is initiated early and better activity was seen for treatment by intra-peritoneal route as compared to oral route. Further, the anti-tumor effects were seen very within 4 to 5 days of treatment.

In melanoma xenografts, when betulinic acid was dosed at a later stage (tumor volume ≈ 1000 mm^3) it does not show any effect till day 22 p.i. (Figure - 9). However, it showed a delayed effect on day 27 p.i. where it significantly inhibited the growth of xenografts ($p < 0.05$) both by intra-peritoneal and oral routes. The treatment/control (T/C) value on day 27 p.i., before control animals were sacrificed, was 59.64% for the oral treatment group and 64.35% for the i.p. treatment group. The results show that late treatment by betulinic acid shows a delayed effect as compared to early treatment study, but nevertheless shows anti-tumor efficacy in melanoma both by intra-peritoneal route and oral routes.

In addition to its anti-tumor activity in melanoma xenografts, betulinic acid was evaluated in the colon tumor xenograft model (PTC).

In colon xenografts, when betulinic acid was dosed at an early stage (tumor volume ≈ 100 mm^3), by intra-peritoneal route, it significantly inhibited the growth of xenografts ($p < 0.05$)

on days 12, 14 and 19 p.i. (Figure - 10). Treatment by the oral route significantly inhibited growth on day 19 p.i. ($p < 0.05$). At day 19, before control animals were sacrificed, the treatment/control (T/C) value was 73.34% for the oral treatment group and 79.3% for the i.p. treatment group. The results show that betulinic acid has good anti-tumor efficacy when treatment is initiated early and better activity was seen for treatment by intra-peritoneal route as compared to oral route. Further, the anti-tumor effects were seen within 4 to 5 days of treatment.

In colon xenografts, when betulinic acid was dosed at a later stage (tumor volume ≈ 1000 mm^3) it does not significantly inhibit the growth of xenografts either by intra-peritoneal or by the oral routes (Figure - 11). The treatment/control (T/C) value on day 19, before control animals were sacrificed, was 31.67% for the oral treatment group and 60.09% for the i.p. treatment group. The results show that betulinic acid shows anti-tumor efficacy in colon tumor xenografts only when treated early. To our knowledge, this is the first report of the anti-tumor efficacy of betulinic acid in colon cancer.

There are no reports yet on the anti-tumor efficacy of betulinic acid derivatives. Studies were carried out to determine the anti-tumor potential of the most potent compound selected from *in vitro* studies i.e. 1098. These studies were carried out in colon (PTC) xenografts by administering the test compound to large tumors where betulinic acid did not show any activity.

In colon xenografts, treatment with 1098 was initiated when tumors had reached a mean tumor volume of over 1000 mm^3. 1098 caused the formation of crater at the centre of the tumor by day 18 followed by tumor shrinkage and regression as illustrated (Figure-12). 1098 treatment significantly inhibited the growth of xenografts on days 15 and 22 p.i ($p < 0.05$) and very significantly on day 27 ($p < 0.01$) (Figure - 13). The anti-tumor effect was characterized by At day 27, before control animals were sacrificed the treated/control (T/C) value was 81.63%. From earlier studies, it was found that betulinic acid does not have any significant anti-tumor activity when treatment was initiated in large tumors (tumor volume ≈ 1000 mm^3). It may be concluded that 1098 is better than betulinic acid for the treatment of colon xenografts.

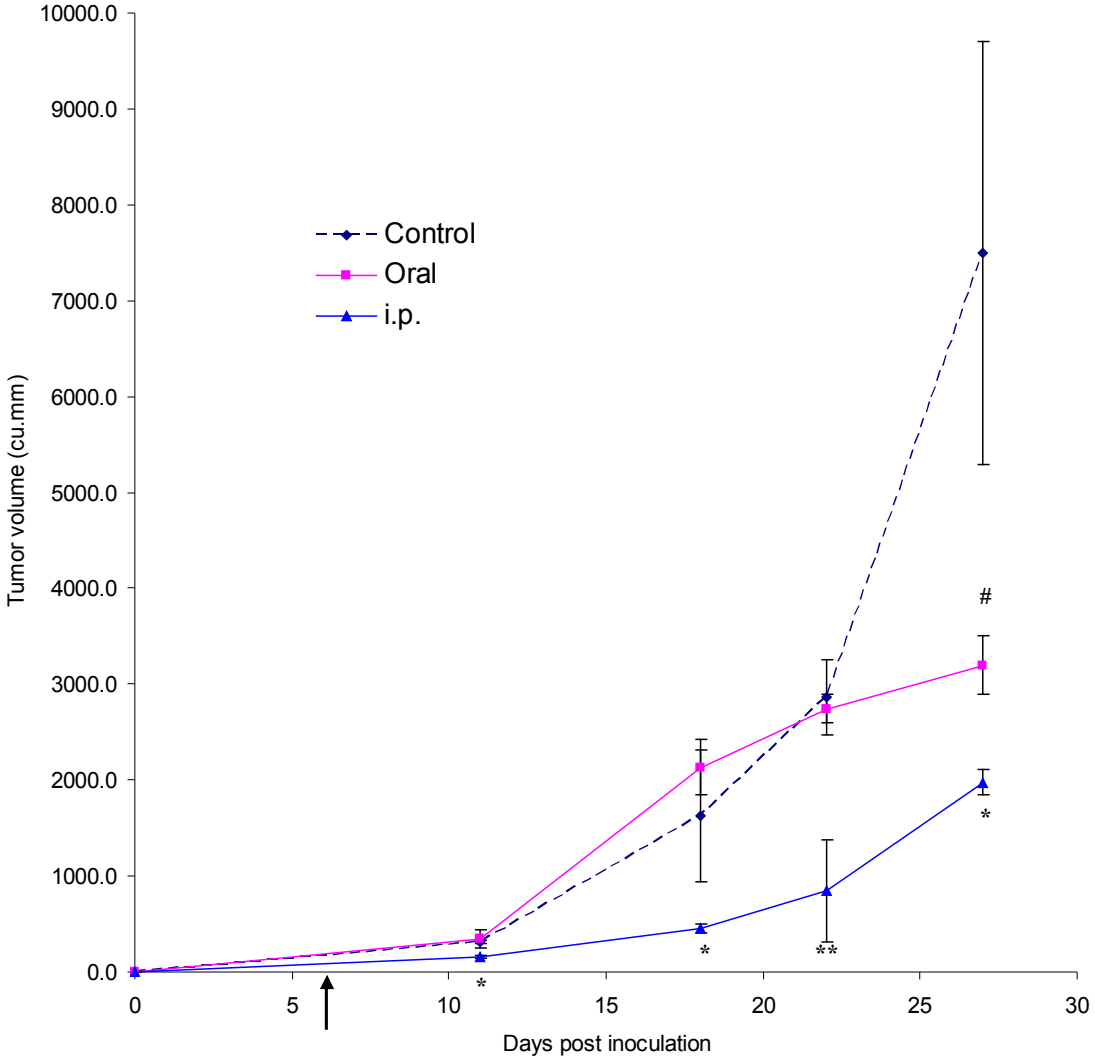

Figure - 8

Early treatment of B16F10 (melanoma) xenografts

Tumor bearing mice were treated with betulinic acid when mean tumor volume was around 100 mm^3 (6 days after tumor implantation) at a dose of 33 mg/kg b.wt. every day for a period of 2 weeks. Data shown are mean ± SD, n=6. * and ** represent $p < 0.05$ and $p < 0.01$ for i.p treatment and # represent $p < 0.05$ for oral treatment respectively, compared with untreated group. Arrow indicates start of treatment.

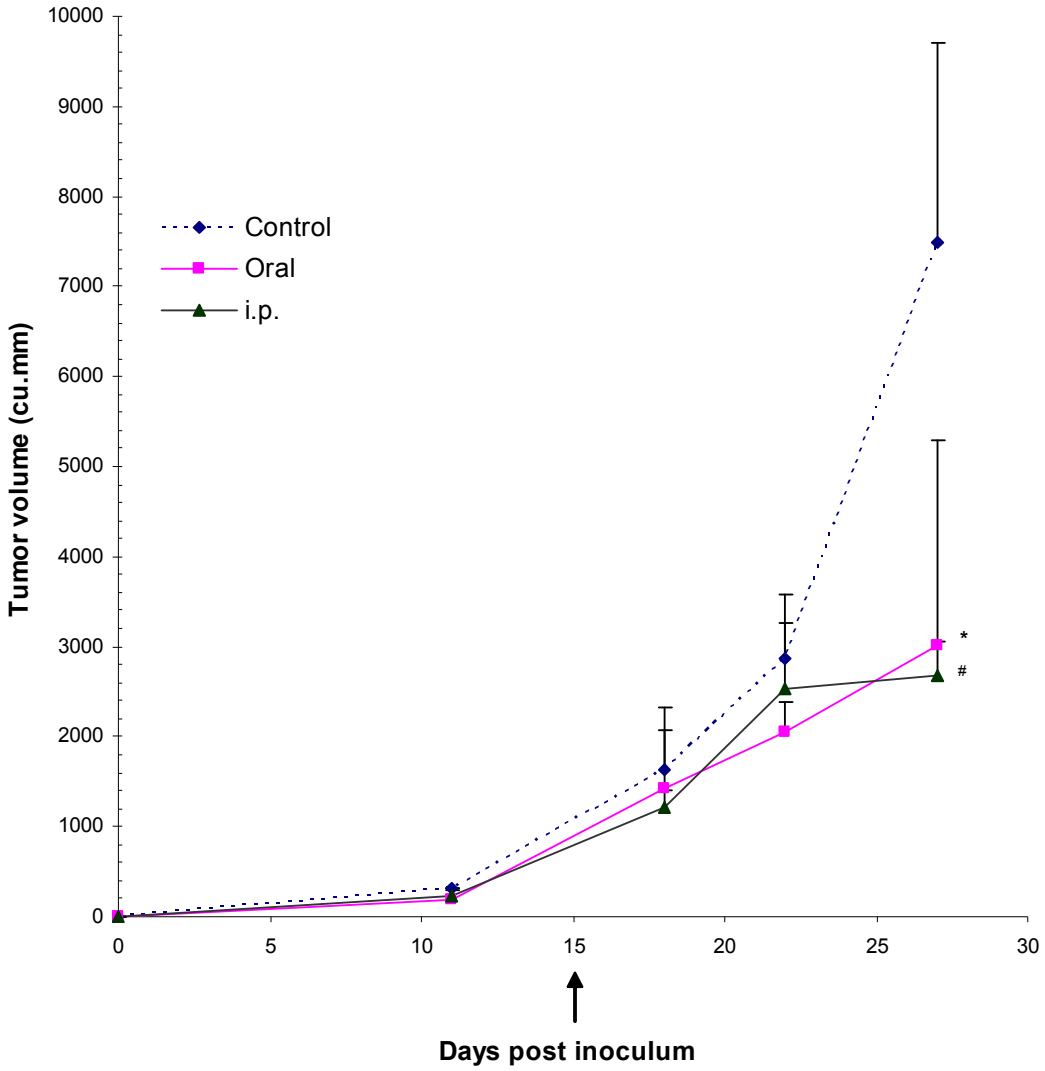

Figure - 9

Late treatment of B16F10 (melanoma) xenografts

Tumor bearing mice were treated with betulinic acid when mean tumor volume was over 1000 mm^3 (15 days after tumor implantation) at a dose of 33 mg/kg b.wt. every day for a period of 2 weeks. Data shown are mean ± SD, n=6. *, # represent $p < 0.05$ of i.p and oral treatment groups respectively, compared with untreated group. Arrow indicates start of treatment.

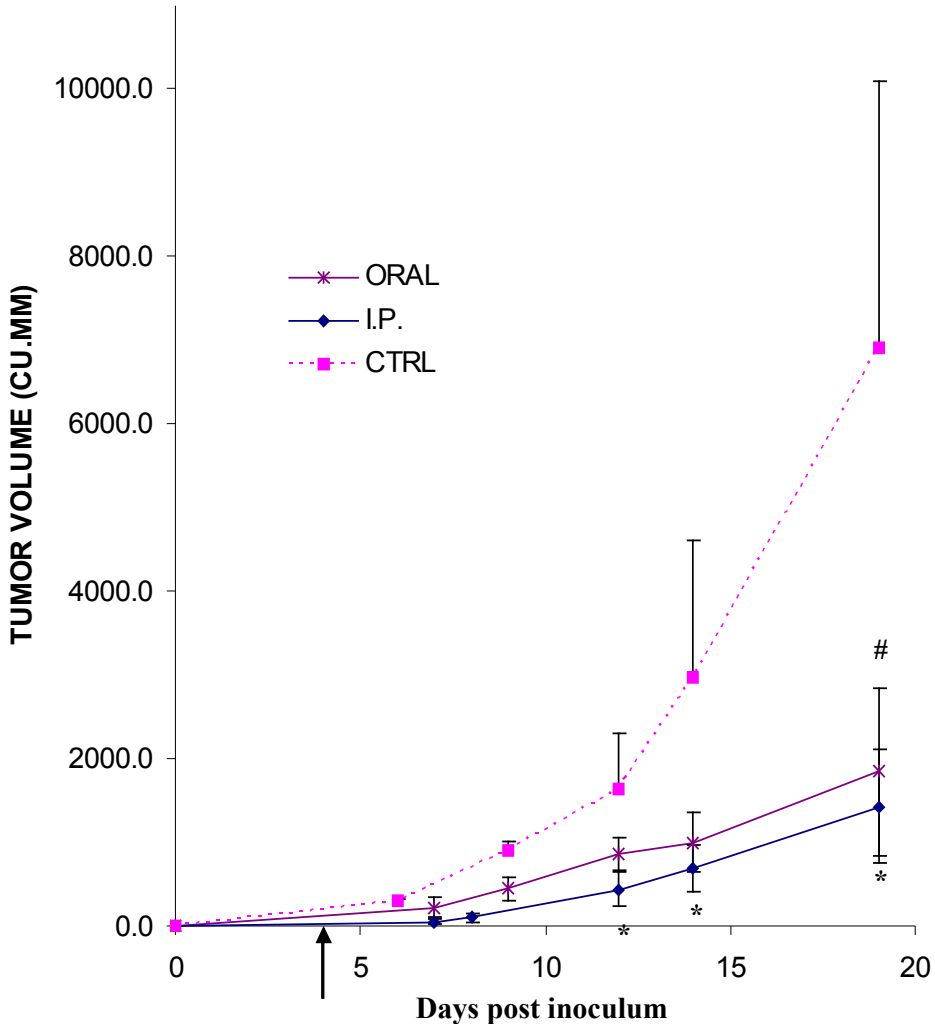

Figure - 10

Early treatment of PTC (colon) xenografts

Tumor bearing mice were treated with betulinic acid when mean tumor volume was around 100 mm^3 (4 days after tumor implantation) at a dose of 33 mg/kg b.wt. every day for a period of 2 weeks. Data shown are mean ± SD, n=6. *, # represent $p < 0.05$ of i.p treatment and oral treatment respectively, compared with untreated group. Arrow indicates start of treatment.

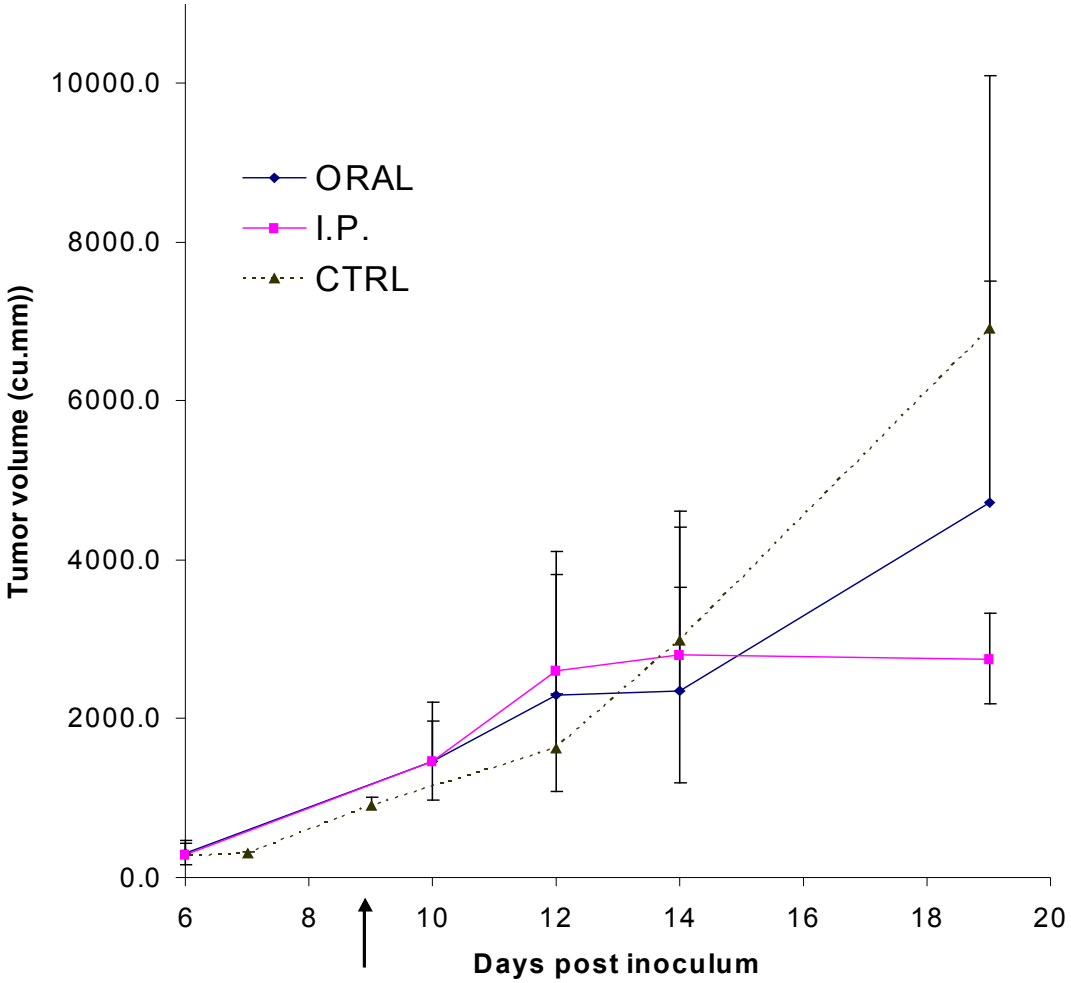

Figure - 11

Late treatment of PTC (colon) xenografts

Tumor bearing mice were treated with betulinic acid when mean tumor volume was over1000 mm^3 (9 days after tumor implantation) at a dose of 33 mg/kg b.wt. every day for a period of 2 weeks. The treated groups were not significantly different from untreated control at any time during treatment. Data shown are mean ± SD, n=6. Arrow indicates start of treatment.

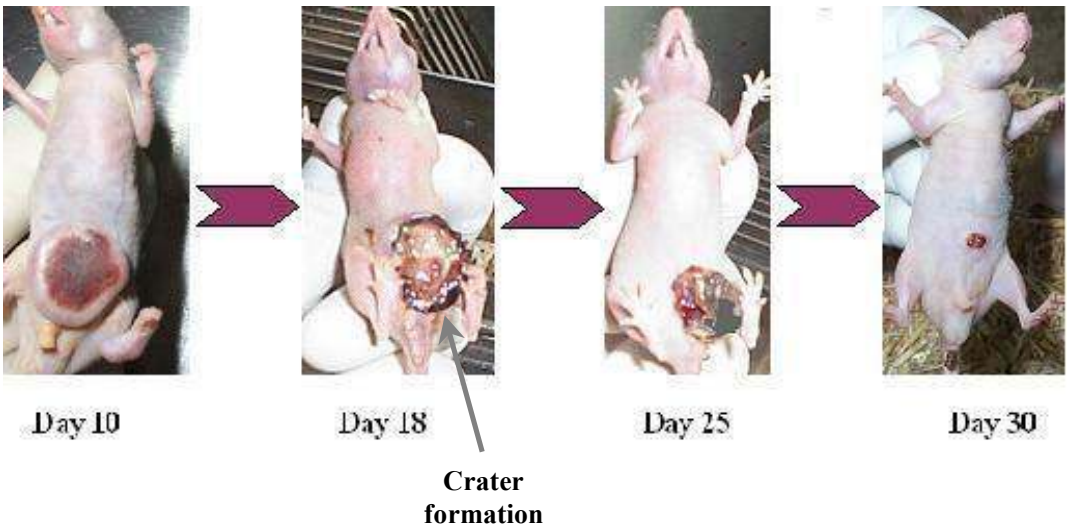

Figure - 12

An illustrative photograph of nude mouse showing stages of tumor regression of colon (PTC) xenograft at different days following treatment with betulinic acid derivative 1098.

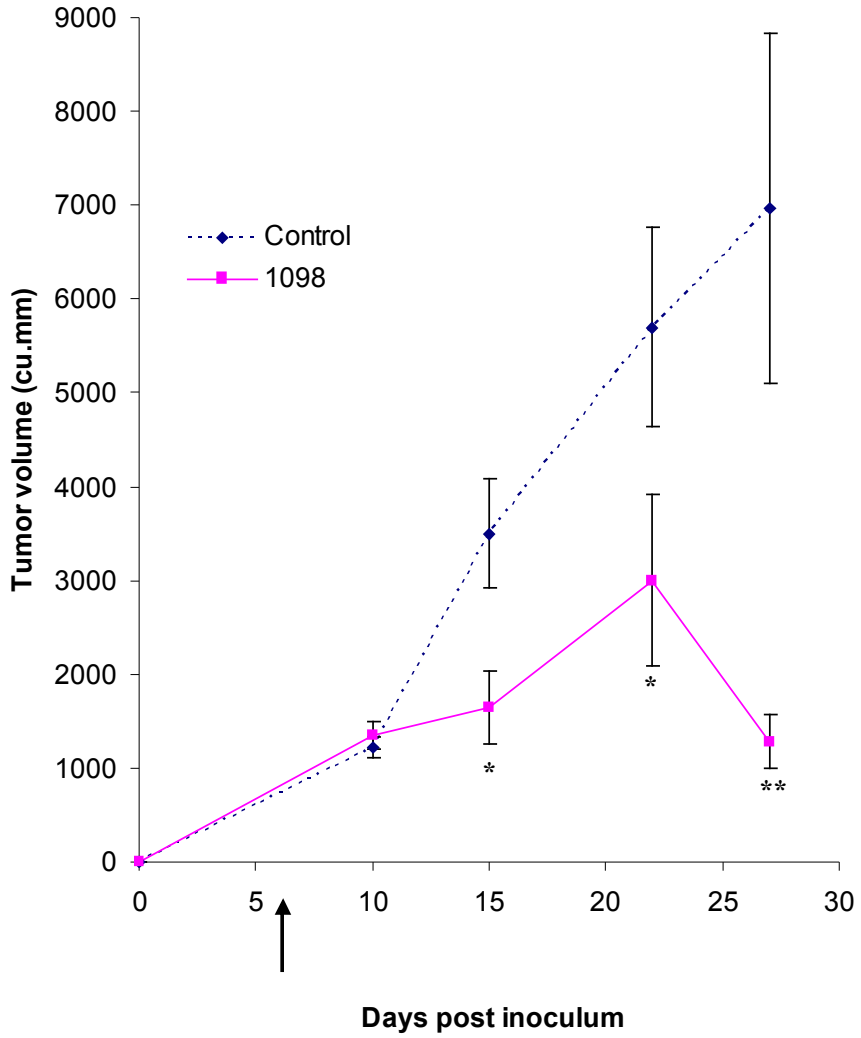

Figure - 13

Effect of 1098 formulation on PTC (colon) xenograft

Tumor bearing mice were treated with betulinic acid derivative 1098 when mean tumor volume was over 1000 mm^3 (6 days after tumor implantation) at a dose of 10 mg/kg b.wt. by i.v. route every day for a period of 2 weeks. Data shown are mean ± SD, n=6. * and ** represent $p < 0.05$ and $p < 0.01$ respectively compared with untreated group. Arrow indicates start of treatment.

1.5 CONCLUSION

The cytotoxicity of betulinic acid against melanoma cell lines was reported previously [Pisha et al., 1995], whereas the broad-spectrum activity against human leukemia, lymphoma, melanoma, colon, lung, ovary and prostate has been demonstrated here for the first time. Betulinic acid was most potent in leukemia and lymphoma cell lines and was non-cytotoxic to cell lines of pancreas, cervix, breast, oral, myeloma and glioblastoma. These results show that betulinic acid possesses broad-spectrum anti-cancer activity in different types of cancers.

About 500 (five hundred) novel derivatives with modifications in C_2, C_3, C_{20}, and C_{28} position of betulinic acid were screened for cytotoxicity. Out of these, 182 (one hundred eight two) derivatives were selected from the primary screening and tested in a panel of cell lines in the secondary screening. Among these, 24 (twenty four) derivatives were better than betulinic acid in terms of mean cytotoxicity to the panel of sensitive cell lines.

Based on the cytoxicity and cancer specificity data certain structure activity relationships were observed. It was found that modification of C-3 hydroxyl to keto, oxime, o-acyl, o-benzoyl, benzyloxime, benzylidene, phenylhydrazine and o-sulfonylmethane groups significantly enhanced the activity and resulted in more potent derivatives when compared to betulinic acid. It was found that compounds having either keto or benzoyloxy substitution at C-3 position had narrow spectrum activity i.e. selective to only one cancer type. Derivatives that were better than betulinic acid had hydrogenation of the double bond at C-20 in betulinic acid. It was found that C-28 carboxylic acid group in betulinic acid and its derivatives was found essential for cytotoxic activity. The introduction of halogen atoms (F, Br) in the aromatic ring at C-3 resulted in the compounds being more specific to cancer cells, particularly leukemia and lymphoma. The five short listed derivatives were dihydrobetulinic acid derivatives with free carboxylic acid group at C-28 (R_3) and functionalized at C-3 (R_2) with the following functional groups i.e. 1098 (4-nitrobenzyl-oximino), 937 (2, 4, difluoro-benzoyloxy), 829 (4-fluorophenyl-hydrazono), 807 (benzoyl-hydrazono) and 1065 (2, 4, difluoro-benzylidene-amino) were short-listed for further development.

Betulinic acid and derivatives caused characteristic morphological changes like cell shrinking, granularity and formation of apoptotic bodies as early as 24 hrs which were comparable to the effects of camptothecin.

Betulinic acid and 1098 significantly increased Annexin V binding, used in detecting apoptotic cells. Both betulinic acid and 1098 increased Annexin V binding to lung carcinoma (A549) cells. Further, the effect of 1098 was significantly better in causing early changes in exposing phosphatidyl serine on the surface of the cytoplasm of cells as compared to betulinic acid.

Betulinic acid and 1098 induced active caspase-3, which plays a key role in apoptosis. In leukemia (MOLT-4) both betulinic acid and 1098 induced active caspase-3 at 24 hrs post incubation, while at 17 hrs 1098 was significantly better in caspase-3 induction as compared to betulinic acid.

Betulinic acid was found to possess significant anti-tumor activity against established tumors of colon and melanoma xenografts in nude mice when administered by oral and intra-peritoneal routes. The results show that betulinic acid had good anti-tumor efficacy in melanoma xenografts when treatment is initiated early and better activity by intra-peritoneal route as compared to oral route. Further, the anti-tumor effects were seen very early in treatment. Late treatment by betulinic acid had a delayed effect as compared to early treatment study, but nevertheless shows anti-tumor efficacy both by intra-peritoneal route and oral routes.

In colon xenografts, betulinic acid had good anti-tumor efficacy when treatment was initiated early and better activity by intra-peritoneal route as compared to oral route. Further, the anti-tumor effects were seen very early in treatment. Further it was seen that betulinic acid had anti-tumor efficacy in colon tumor xenografts only when treated early. To our knowledge, this is the first report of the anti-tumor activity of betulinic acid in colon tumor xenografts.

Studies were carried out to determine the anti-tumor potential of the most potent compounds selected from *in vitro* studies i.e. 1098. In large colon xenografts, treatment with 1098 significantly inhibited the growth of xenografts. The anti-tumor effect was characterized by

the formation of crater at the centre of the tumor, followed by tumor shrinkage and regression. It may be concluded that 1098 is better than betulinic acid in its anti-tumor activity to colon xenografts.

In this chapter, based on cytotoxicity and specificity to cancer cells five betulinic derivatives (1098, 937, 807, 829 and 1065) which were better than betulinic acid were selected for further studies. The most potent derivative, 1098 was further found to possess pro-apoptotic activity and remarkable anti-tumor activity.

****

CHAPTER - 2

ANTI-ANGIOGENIC ACTIVITY OF BETULINIC ACID AND DERIVATIVES

2.1 INTRODUCTION

Early pioneers of angiogenic research observed over a century ago that the growth of human tumors is often accompanied by increased vascularity. They suggested that a key aspect of the cancer process is a disease of the vasculature in the whole area affected [Ferrara, 2002]. The existence of tumor-derived factors responsible for promoting new vessel growth was postulated over 65 years ago [Ide et al., 1939], and a few years later it was proposed that tumor growth is crucially dependent on the development of a neovascular supply [Algire et al., 1945]. In 1971, it was hypothesized that inhibition of angiogenesis (anti-angiogenesis) would be an effective strategy to treat human cancer, and an active search for angiogenesis inducers and inhibitors began [Folkman, 1971]. Extensive research has led to the identification and isolation of several regulators of angiogenesis, some of which represent therapeutic targets. Despite some initial setbacks and negative clinical trial results, major progress has been made over the past few years in targeting angiogenesis for human therapy. In February 2004, the US Food and Drug Administration (FDA) approved bevacizumab, a humanized anti-VEGF-A monoclonal antibody, for the treatment of metastatic colorectal cancer in combination with 5-fluorouracil (5-FU)-based chemotherapy regimens. This followed from a phase III study showing a survival benefit [Hurwitz et al., 2004]. In December 2004, the FDA approved pegaptinib, an aptamer that blocks the 165 aminoacid isoform of VEGF-A, for the treatment of the wet (neovascular) form of age-related macular degeneration (AMD) [Gragoudas et al., 2004]. These achievements have validated the notion that angiogenesis is an important target for cancer and other diseases. These advances not withstanding, much progress is needed on a variety of important issues; for example, how do we achieve the most effective combinations of antiangiogenic agents with chemotherapy or other biological agents and how do we select patients that are most likely to respond to the treatment? Another issue is that resistance to antiangiogenic therapy is emerging [Kerbel et al., 2002] and thus a better understanding of pathways that may mediate tumor angiogenesis in various circumstances is necessary. Furthermore, the hope that ‘therapeutic angiogenesis’ will provide a treatment for ischemic disorders still remains unfulfilled, in spite of considerable preclinical and clinical efforts [Ferrara et al., 2005].

2.2 REVIEW OF LITERATURE

2.2.1 The process of angiogenesis

Angiogenesis is a multi-step process. The cascade of events involved in angiogenesis is shown in Figure 13 [Folkman, 1997].

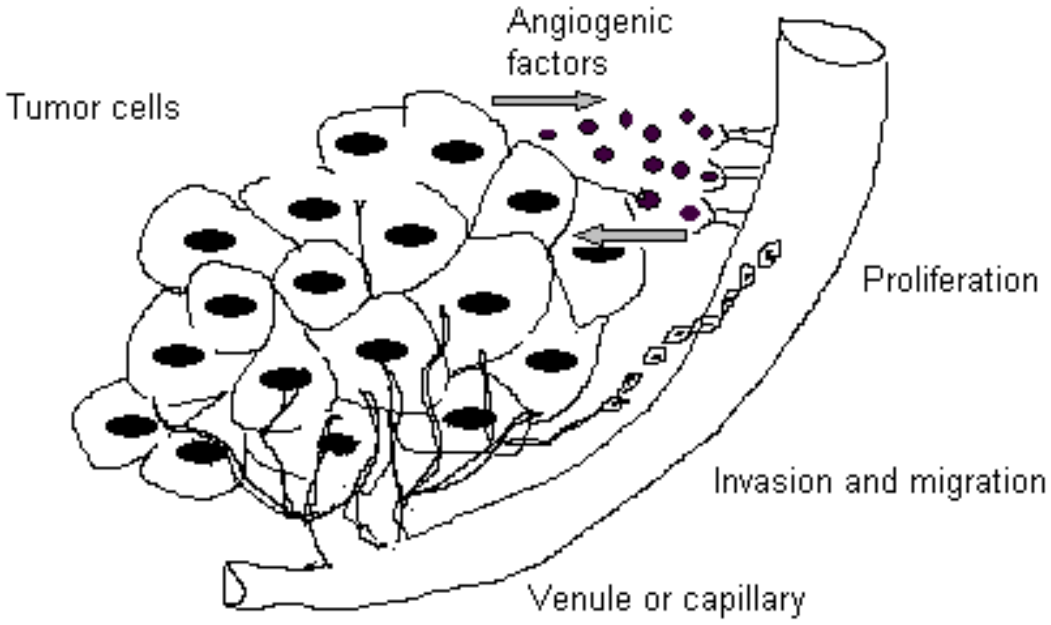

Figure - 14

The process of angiogenesis

The tumor cells secrete angiogenic factors which bind to the endothelial cells of the venules and capillaries by receptor ligand interaction. These factors cause the endothelial cells to proliferate, migrate and invade the extracellular matrix thus reaching the tumor and proving necessary nutrition and other growth factors for the growth and sustenance of the tumor

2.2.2 Angiogenic targets for novel cancer chemotherapy

Recent reports of the highly effective elimination of tumors in mice by the anti-angiogenic molecules angiostatin and endostatin, peptidyl compounds that antagonize the angiogenic actions of angiogenin, have resulted in an increased attention on angiogenic targets for novel cancer chemotherapy [Folkman, 1998; Nelson, 1998]. In addition to pursuing the anti-angiogenic polypeptides angiostatin and endostatin [Folkman, 1998], considerable anti-angiogenesis cancer drug discovery has been directed at growth factors and growth factor

receptors involved in endothelial cell proliferation. The most prominent of these are VEGF and its receptor VEGFR-2 (flk-1) and bFGF and its receptor. One other important angiogenic factor is angiogenin, a polypeptide that can both induce and suppress angiogenesis, but does not appear to be mitogenic towards endothelial cells. Many small-molecule angiogenesis inhibitors have been discovered [Powell et al., 1997]. They include suramin and its analogs, which are nonspecific agents that block growth factor binding to their cognate receptors, selective inhibitors affecting receptor kinase activity of VEGFR-2 (flk-1), bFGFR, or PDGFβ receptor and other small molecules of diverse structural classes with yet unclear mechanisms of action such as thalidomide and fumagilins, as well as monoclonal antibodies [Mordenti et al., 1999]. The X-ray crystal structure of the bFGFR tyrosine kinase domain in complex with inhibitors was solved recently [Mohammadi et al., 1997] and may pave the way for structure-based design of novel bFGF RTK inhibitors. Some of these anti-angiogenic agents are now undergoing clinical evaluation, such as SU5416 (SUGEN, San Diego, CA, USA).

Extracellular matrix proteinases particularly matrix metalloproteinases (MMPs), urokinase (uPA) and cell adhesion molecules are also the targets of much anticancer drug discovery activity because of their involvement in tumor invasion and angiogenesis (which culminate in cancer progression and metastasis) [Rabbani, 1998, Weidle et al., 1998]. MMPs are a large family of zinc-binding proteins that can be divided into five classes, on the basis of substrate preference as follows: type 1 collagenases, comprising MMP-1 and MMP-8, MMP-13; type IV collagenases, MMP-2 and MMP-9; stromelysins, MMP-3, MMP-7, MMP-10 and MMP-11; elastases, MMP-12; and membrane-type MMPs, MT-MMPs, which are regulated by endogenous inhibitors known as TIMPs (tissue inhibitors of metalloproteinases) [Rabbani, 1998]. uPA is a serine protease formed initially as high molecular weight uPA that is cleaved into an amino terminal fragment (ATF) and low molecular weight uPA. uPA and the uPA receptor have been shown to cooperate with MMPs, especially MMP- 9, to cause tumor cell intravasation [Edwards et al., 1998]. Many small-molecule potent MMP inhibitors have been discovered with nanomolar to picomolar IC_{50} values, as reviewed recently [Summers et al., 1998]. Notable among these are the hydroxamate-based inhibitors batimastat and its more water-soluble analog marimastat, which are now under advanced clinical evaluation against many human cancers [Rothenberg et al., 1998].

Another important class of extracellular matrix targets in connection with cancer progression are the cell adhesion molecules, integrins. These are transmembrane heterodimeric proteins comprising α and β subunits that function as receptors for matrix proteins such as fibronectin, vitronectin, laminin and collagen. Synthetic peptides designed to antagonize adhesion interactions, especially those incorporating a RGD (Arg-Gly-Asp) motif, are being investigated with some success in preventing metastasis [El-Hariry et al., 1997].

2.2.3 Assays for studying angiogenesis

One of the most important technical challenges in studies of angiogenesis is selection of the appropriate assay. There are increasing numbers of angiogenesis assays being described both *in vitro* and *in vivo*. It has been proved that it is necessary to use a combination of assays for identification of the cellular and molecular events in angiogenesis and the full range of effects of a given test protein. Although the endothelial cell whose migration, proliferation, differentiation and structural rearrangement is central to the angiogenic process, it is not the only cell type involved in angiogenesis. The supporting cells (e.g. tumour cells, pericytes, smooth muscle cells and fibroblasts), the extracellular matrix produced by endothelial cells and their apposed mesenchymal cells, and the circulating blood with its cellular and humoral components are also involved. No *in vitro* assay exists currently to model/simulate this complex process. Whilst *in vivo* the components of the process are all present, disparate results and limitations also exist depending on specific microenvironments, organ sites, species used and manner of administration of test substances [Carolyn et al., 2004].

Current methods for assaying *in vitro* and *in vivo* angiogenesis [Carolyn et al., 2004] are outlined in Table-6:

Table - 6

***In vitro* and *In vivo* methods to study angiogenesis**

Type of assay	Specific assay	Advantages	Disadvantages
Proliferation	MTT	Measures cell number.	Cells not necessarily proliferating. Does not measure toxicity of drug.
Proliferation	Tritiated thymidine	Measures DNA replication.	Uses radiation. Does not measure toxicity of drug.
Proliferation	BrdU	Measures DNA replication. No radiation	Does not measure toxicity of drug.
Proliferation	Cell cycle analysis	Measures apoptosis and therefore toxicity of drug. Measures DNA replication. Measures percentage of cells proliferating.	Cells have to be in suspension for analysis.
Migration	Boyden chamber	Measures migration in response to a gradient. Extremely sensitive to small changes in concentration.	Technically difficult to set up. Problems in maintaining trans filter gradients. Difficult to obtain accurate cell counts. Time consuming to analyze.
Migration	Phagokinetic track	Measures total cell movement. Measures directional effects of drug.	Only a small number of cells studied. Unnatural substrate to migrate on.
Migration	'Wound healing'	Measures rate of endothelial cell migration	Quantification is somewhat arbitrary. Technical problems in achieving identical conditions of confluence.
Differentiation	Matrix assays	Endothelial cells pushed down differentiation pathway. Formation of tube-like structures. Quick.	Lumen formation is under debate. Non-endothelial cells also form tubes. Homogeneous pattern of tubule lengths.

Table - 6 (contd.) ***In vitro* and *In vivo* methods to study angiogenesis**			
Differentiation	3D gel	More closely mimics the *in vivo* situation. Tubules form in all three dimensions.	Long time period. Problems of quantifying a 3D structure.
Differentiation	Co-culture	Tubules form lumen. More heterogeneous pattern of tubule lengths. Closer to *in vivo* situation.	Long time period. Undefined interactions between endothelial and other cells.
Organ Culture	All	Mimic the *in vivo* situation. Include surrounding cells and matrix.	Difficult to quantify. Growth requirements differ between explant and cell out-growth.
In vivo	Sponge implant	Endothelial cells are not proliferating at start of assay. Inexpensive. Technically simple.	Time consuming. Nonspecific immune responses may lead to an angiogenic response. Sponge composition varies, making inter experimental comparisons difficult.
In vivo	Matrigel plug	Nonartificial, providing a more natural environment for angiogenesis.	Expensive. Analysis is time consuming.
In vivo	CAM assay	Technically simple. Inexpensive. Suitable for large-scale screening.	Very sensitive to oxygen tension. Due to the pre-existing vascular network, visualization of new capillaries can be difficult. Immune response can mask new vasculature.
In vivo	Corneal Angiogenesis assay	Reliable	Expensive. Technically Difficult. Ethically Questionable.

Table – 6 (contd.) ***In vitro* and *In vivo* methods to study angiogenesis**			
In vivo	Dorsal air sac model	Technically simple. Natural environment in which to study blood vessels.	Invasive. Visualization of new capillaries can be difficult due to pre-existing ones.
In vivo	Chamber Assays	Can follow 3D vessel growth over a relatively long period. Minimizes number of mice used.	Invasive. Technically difficult. Expensive (in rabbits). Can get surgery associated angiogenesis.
In vivo	Tumor models	Can follow pharmacokinetics of drug as well as anti-angiogenic effects. Long-term studies possible.	Tumour environment depends on tumour growth site (orthotopic vs. subcutaneous). Real-time studies not possible.
In vivo	Angiomouse®	Visualization is noninvasive. Allows for real-time imaging of angiogenesis.	Sensitivity can be limited by quenching due to surrounding tissue, especially skin. Hypoxia can decrease GFP gene expression and hence, the degree of fluorescence.
In vivo	Zebrafish	Relatively fast assay (6–12 h). Fully quantitative. Disruption to vasculature does not damage embryo.	Does not indicate exact point in angiogenic cascade specifically disrupted. Expensive to maintain in breeding condition. Does not distinguish between cytotoxic effects and genuine inhibition.

2.2.4 Anti-angiogenic activity of betulinic acid

Previous studies have revealed that betulinic acid inhibits endothelial cell growth, invasion and tube formation of endothelial cells at non-cytotoxic concentrations through a modulation of mitochondrial function. [Kwon et al., 2002a]. Betulinic acid potently inhibited basic fibroblast growth factor (bFGF)-induced invasion and tube formation of bovine aortic endothelial cells (BAECs) at a concentration, which had no effect on the cell viability. To access whether the anti-angiogenic nature of betulinic acid originates from its inhibitory action against aminopeptidase N (APN) activity, the effect of betulinic acid on APN was investigated. Surprisingly, betulinic acid did not inhibit *in vivo* APN activity in endothelial cells or APN-positive tumor cells. On the other hand, betulinic acid significantly decreased the mitochondrial reducing potential, and treatment with mitochondrial permeability transition (MPT) inhibitors attenuated betulinic acid-induced inhibition of endothelial cell invasion. These results imply that anti-angiogenic activity of betulinic acid occurs through a modulation of mitochondrial function rather than APN activity in endothelial cells [Kwon et al., 2002a]. It also inhibits *in vitro* enzymatic activity of aminopeptidase N, which is known to play an important role in angiogenesis [Melzig et al., 1998].

Certain cancer cells are known to adhere to E-Selectin via E-Selectin ligands on their cell surface and this event is one component of the metastasis process. Betulinic acid and its derivatives interfere with Selectin binding. Betulinic acid inhibited P-Selectin binding to 2, 3, sLex, a chemical known to bind to P-Selectin, with an IC_{50} of 125 μM. It also inhibited P-Selectin binding to HL-60 cells in a dose-dependent way with an IC_{50} of 0.75 mM. Betulinic acid and derivatives also significantly interfere with the binding to colon cancer cells, LS 174T to E-Selectin [Anderson et al., 1995].

The human umbilical vein endothelial cells (HUVECs) have been used extensively to study the biology and pathobiology of the human endothelial cells including their role in angiogenesis. Due to the functional and structural endothelial cell heterogeneity between and within species, and the time consuming and difficult isolation and culture methods, a transformed endothelial cell line has been used that provides standardization of observations. The spontaneously transformed and fully characterized human umbilical vein endothelial cell line ECV304 provides a reproducible and biologically relevant experimental model system

for *in vitro* angiogenesis. ECV304 cells maintain a stable functional phenotype throughout serial cultivations and exhibit consistent response in *in vitro* angiogenesis assays [Hughes, 1996].

2.3 MATERIALS AND METHODS

2.3.1 Materials

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma, USA), Matrigel^{TM} (Becton Dickinson, USA), DMEM (Dulbeccos modified Eagles medium), and Fetal bovine serum, FBS (Gibco BRL, USA), DMSO (Merck, India). Chemicals used in synthesis were purchased from Sigma, USA. Sterile disposable tissue-culture grade plastic-ware was from Nunc, Denmark or Tarsons, India.

2.3.2 Cell culture

ECV304 (human umbilical vein endothelial cells) cell line was generously gifted by Dr. Takahashi (Tokyo University, Tokyo, Japan). Human tumor cell lines MOLT-4 (leukemia), U937 (lymphoma), DU145 (prostate), L132 (lung), PA-1 (ovary) and HT-29 (colon) were purchased either from American Type Culture Collection [ATCC, USA] or National Cell Science Centre [NCCS, Pune, India]. Cells were cultured in Dulbecco's Modified Eagles Medium [DMEM, Gibco BRL, USA], containing L-glutamine, 25mM HEPES and supplemented with 10% heat-inactivated fetal bovine serum [Gibco BRL, USA], penicillin (100 units/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL) in the form of antibiotic solution [Hyclone, USA]. Cultures were maintained in a humidified incubator at 37 ^{0}C and 5% CO_2 [BB16, Heraeus, Germany]. Tissue culture grade, sterile plastic-ware (Nunc, Denmark) was used.

2.3.3 Endothelial cell cytotoxicity assay

Cells were collected from 70-80% confluent cultures by trypsinization (0.25% trypsin and 0.02% EDTA) and seeded in 96-well plates at 5000 to 30,000 cells/well in cell culture

medium for 24 hours in a CO_2 incubator. The test substance was dissolved in DMSO (Merck, India) and further dilutions were made in cell culture medium such that the final DMSO concentration in the well even at the highest concentration is less than 1%. After 24 hours the cells were incubated with the above test substance to obtain drug concentrations in the range of 0.5 to 20 μg/mL. After 72 hours of incubation in a CO_2 incubator cytotoxicity was measured by the tetrazolium-based MTT assay adapted from previously published methods [Mosmann, 1983; Carmichael et al., 1987; Cole, 1986; Park et al., 1987]. Briefly, 25 uL of MTT (5 mg/ml, Sigma, USA) was added to each well of the 96-well plate and incubated at 37^0C for 3 hours. MTT was converted to greenish-brown colored formazan by mitochondrial dehydrogenase enzyme present in viable cells. For adherent cells, the medium in the wells was gently pipetted out and replaced with 150 µL of DMSO and kept with gentle shaking for 15 minutes to dissolve formazan crystals. For suspension cultures, formazan was dissolved by direct addition of 50 µL of sodium dodecyl sulfate (SDS) acidified with 1N HCl, added to the wells followed by incubation for one hour and mixing the contents using a pipetman. The optical density (O.D.) in the wells was measured at 540 nm (for adherent cells) or 570 nm (for suspension cells) using a Multi-well spectrophotometer. Percentage cytotoxicity was calculated using the formula:

$$\text{Percent cytotoxicity} = 1 - \frac{X}{R_1} * 100$$

where X= O.D. of wells containing the test substance and R_1= O.D. of control wells. Each experiment was repeated thrice and IC_{50} values (half-maximal cytotoxicity) were calculated by employing non-linear regression analysis using Prism® software.

2.3.4 Cell migration/Chemotaxis assay

The Boyden chamber migration assay was adapted from previously described methods [Kamath, 2003; Adatia et al., 1997]. A Boyden chamber type apparatus was prepared by placing 8µm pore size polycarbonate filter plate inserts with 12 mm diameter (Nunc, Denmark) inside the wells of 24-well tissue culture plate (Nunc, Denmark). ECV304 cells were harvested with trypsin, resuspended in cell growth medium with 0.2% BSA (Bovine serum albumin) and placed in the upper compartment of the boyden chamber (1 x 10^5 cells/200 µL/chamber) in the absence (control) or presence of test compounds. The bottom compartment was filled with 600µL of medium containing chemo-attractant. The chemoattractant consisted of ECV304 cell culture supernatant that was collected from

confluent flasks of ECV304 cells cultured in cell growth medium with 0.2% BSA for 24 hrs. Test compounds were dissolved in DMSO at a concentration of 2 mg/ml and diluted in cell culture medium with 0.2% BSA to obtain a concentration of 5 µg/mL in triplicate wells. After 4 – 6 hrs of incubation at 37^0C, 5% CO_2, number of cells that migrated to the lower compartment was counted manually under a phase-contrast microscope (Diaphot 300, Nikon, Japan). The percentage inhibition of migration caused by the test compound was calculated as a percentage of cells that migrated in the control wells. Assays were performed in triplicate and repeated three times.

2.3.5 Tube formation assay

This protocol was adapted to 96-well plate format from previously published method [Adatia et al., 1997]. Matrigel™ (BD Biosciences, USA) was thawed at 4^0C in an ice-water bath, and 70 µL/well was carefully added to a 96-well plate, pre-chilled at 4^0C using a cold pipette. Matrigel was allowed to polymerize for 30 minutes at 37^0C. After polymerization, 1 x 10^4 ECV304 cells/well in growth medium (DMEM containing 10% FBS) was seeded on top of polymerized Matrigel in the absence (control) or presence of compounds solubilized in DMSO at non-cytotoxic concentrations previously determined using MTT assay. Test compounds were added in triplicates. Plates were then incubated in a humidified incubator at 37 ^{0}C and 5% CO_2 [BB16, Heraeus, Germany]. Tube formation by the cells was evident after few hours and after overnight incubation control cells started to form an intense network of tube-like structures. Wells were photographed at 18 hours in a phase contrast microscope (Diaphot 300, Nikon, Japan) connected to an image analysis system (VideoPro®, Australia). The total area covered by the tubes was measured using the image analyzer and percentage inhibition in terms of tube area compared to control was calculated.

2.3.6 Measurement of VEGF, bFGF and Endostatin levels

Leukemia (K562) cells were plated at the density of 8-10 x10^5 cells in RPMI 1640 media in a six well plate. After an overnight incubation of cells at 37^oC, 5% CO_2 the test compounds were added to the treated wells at previously determined non-cytotoxic concentrations. The untreated wells were controls. The plate was incubated at 37^oC, 5% CO_2 incubator. After 6 hours of incubation cell culture supernatant was collected from treated and untreated wells

and centrifuged at 2000 rpm for 10 minutes to remove any cellular material and stored at -20^{0}C till further use. The culture supernatant was analyzed for the levels of VEGF, bFGF and Endostatin using commercially available ELISA kits by following kit instructions. [Quantikine™ human VEGF kit, R&D Systems, USA, catalogue no. DVE00, Quantikine™ human bFGF kit, R&D Systems, USA, catalogue no. DFB50, Human Endostatin Protein Accucyte™ EIA kit, Oncogene Research Products, catalogue no. QIA65, USA). The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF, bFGF or Endostatin respectively has been pre-coated onto a micro plate. Standards and samples are pipetted into the wells and any VEGF, bFGF or Endostatin respectively, present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the respective protein is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of protein bound in the initial step. The color development is stopped and the intensity of the color is measured using a multi-well spectrophotometer.

2.3.7 Lung nodule assay

A method as described previously was used [Adatia et al., 1997]. A highly metastatic murine melanoma cell line (B16F10, 1 - 3 million cells/100 µl in culture medium) was injected into the tail vein of athymic (nu/nu) mice 6-8 weeks old. The mice were randomly divided in to two groups (control, treated) of four animals each and housed independently. Betulinic acid derivative (1098) was prepared at a concentration of 1.25 mg/ml in a suitable injectable cosolvent formulation. Treatment was initiated in the treatment group animals by injecting 100 µL of test formulation i.e. dose of 5 mg/kg immediately after injection by i.v. route and thereafter every alternate day for 3 weeks. The untreated group served as control. After three weeks of treatment all the animals were sacrificed and lungs were dissected out and fixed in formalin. The lungs were dried using blotting paper and weighed in a balance. The extent of metastasis in the form of lung nodule formation correlated well with weight of the lungs.

2.3.8 Statistical Analysis

The results were expressed as mean ± SEM. The student's unpaired t-test was used to compare the mean of two groups. Difference were considered significant when $p < 0.05$. Results of animal studies were expressed as mean ± SD.

2.4 RESULTS AND OBSERVATIONS

2.4.1 Anti-angiogenic activity of betulinic acid

To determine the effect of betulinic acid in angiogenesis, the effect on growth of endothelial cells was measured *in vitro* using the MTT method. These experiments were carried out at different time points in order to identify doses which were cytotoxic as well as non-cytotoxic, in order to select concentrations for other angiogenesis assays. Endothelial cells (ECV304) were incubated with various doses of betulinic acid for 72 hrs. Based on the cytotoxicity at different concentrations IC_{50} value was calculated.

Betulinic acid was cytotoxic to endothelial cells (ECV304) in a concentration-dependant manner. The half-maximal cytotoxic concentration (IC_{50}) was 1.26 ± 0.44 μg/ml (Figure - 15). The IC_{50} value in endothelial cells is in line with that observed for cancer cell lines (0.65 – 10 μg/ml), particularly with leukemia cell line (MOLT, IC_{50} = 1.23 μg/ml) and lung cancer cell line (L132, IC_{50} = 1.3 μg/ml). This activity of betulinic acid to endothelial cells prompted us to study its role in other activities related to angiogenesis.

Since the growth of endothelial cells is regulated by several known stimulatory and inhibitory molecules, studies were performed to explain the effect on the growth of cells by studying angiogenic growth factors and inhibitors. One of the best studied and the most potent pro-angiogenic factor is VEGF (Vascular endothelial growth factor), discovered in the early eighties [Senger et al., 1983]. FGF (Fibroblast growth factor) is also a potent inducer of angiogenesis. Cellular responses mediated by FGF include cell migration, proliferation, and differentiation [Kanda et al., 1997].

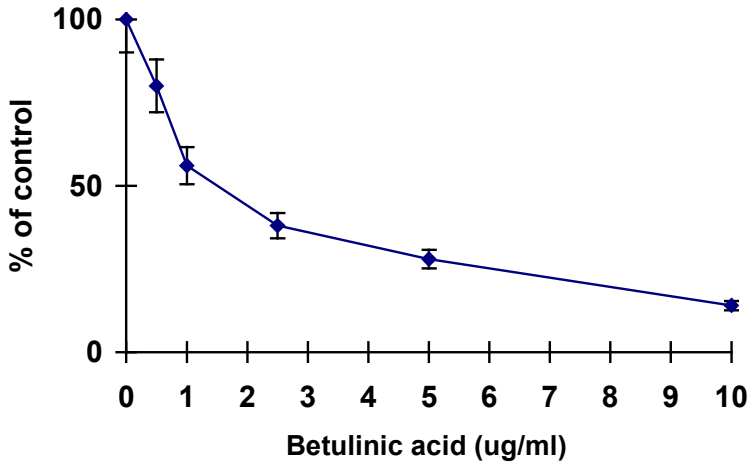

Figure - 15

Dose response curve of betulinic acid on ECV304 cells

Effect of treatment with betulinic acid on the growth of ECV304 (endothelial) cells. The cells were grown in 96-well plates with 0.5, 1, 2.5, 5 and 10 µg/ml of betulinic acid for 72 hours. Viability was determined by MTT assay. The data shown are mean ± SEM from three independent experiments. The IC_{50} (50% growth inhibitory concentration) was determined by non-linear regression using Prism™ software.

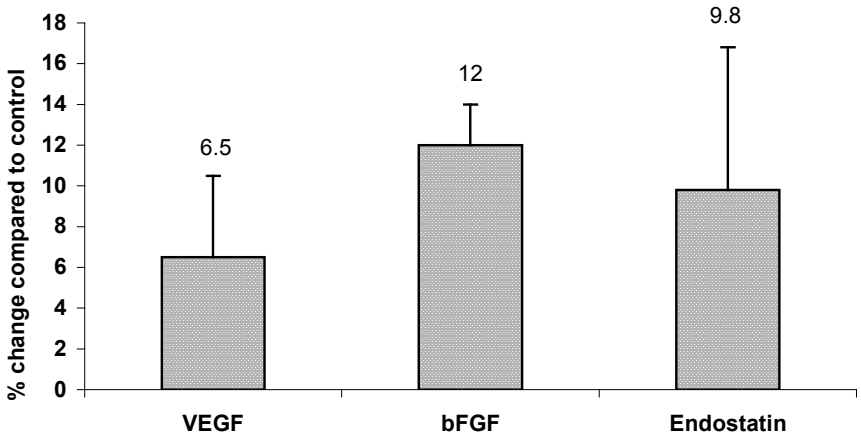

Figure - 16

Effect of betulinic acid on pro-angiogenic factors

K562 (Chronic myelogenous leukemia), cells were incubated with test compounds at 1 µg/ml. After 6 hrs incubation the culture supernatant was analyzed for levels of the pro-angiogenic molecules VEGF, bFGF and Endostatin using commercially available Elisa kits. Data represent mean ± SEM of three independent experiments.

In later years, several endogenous molecules with angiostatic activity were described. Among these molecules is Endostatin, which is a proteolytic fragment of collagen XVIII that affects endothelial cell survival via the induction of an imbalance between the antiapoptotic proteins Bcl-2 and Bcl-XL and the pro-apoptotic protein Bax [Dhanabal et al., 1999]. Endostatin is well known for its anti-growth and anti-migratory effects on endothelial cells. In this capacity, it has received much attention for its potential use as an angiogenesis inhibitor capable of reducing the blood supply necessary for the maintenance and growth of tumors [O'Reilly et al., 1997].

Betulinic acid was incubated with endothelial cells (ECV304) and levels of angiogenic factors VEGF, bFGF and Endostatin was measured using specific Elisa kits. It was found that betulinic acid alters the levels of angiogenic factors (VEGF, bFGF and Endostatin) in endothelial cells (ECV304) by 6.6%, 12.2% and 9.8% respectively (Figure - 16). These results show that betulinic acid has marginal effects on these angiogenic factors and this level of activity does not completely explain the very significant cytotoxicity that is seen on endothelial cells.

2.4.2 Anti-angiogenic activity of short listed betulinic acid derivatives

The in vitro anti-angiogenic activity of short listed betulinic acid derivatives was assessed by carrying out the following studies. The activity of some derivatives was published by us [Mukherjee et al., 2004a, b].

2.4.2.1 Effect on growth of endothelial cells *in vitro*.
2.4.2.2 Effect on Migration / Chemotaxis of endothelial cells *in vitro*.
2.4.2.3 Effect on tube formation by endothelial cells *in vitro*, and
2.4.2.4 Effect on metastasis of tumor cells in mice.

2.4.2.1 Cytotoxicity and specificity of betulinic acid and derivatives to endothelial cells

The short listed derivatives of betulinic acid were assessed for cytotoxicity based on MTT assay. These derivatives were found to be cytotoxic to endothelial cell line ECV304 with IC_{50} values in the range of 0.35 - 2.4 µg/mL (Table - 7). It was found that derivatives 807, 829,

1098 and 937 were significantly better than betulinic acid ($p < 0.01$) with IC_{50} values less than 1 µg/mL.

These derivatives were assessed for specificity to endothelial cells by comparing the IC_{50} values in cancer cells using the endothelial cell specificity (ECS) values calculated using the formula:

$$\text{Endothelial cell specificity (ECS)} = \frac{IC_{50\ \text{tumor cell}}}{IC_{50\ \text{endothelial cell}}}$$

It was found that derivative 807 and 829 had high endothelial specificity (ECS > 20) to endothelial cells when tested against prostate cancer cells (DU145) and 829 had moderate endothelial specificity (ECS between 10 and 20) when tested against lung cancer cells (L132). All the other derivatives have low endothelial specificity i.e. ECS < 10 (Table - 8). It may be concluded that compounds 807 and 829 may have the potential to be developed as specific anti-angiogenic agents while 1098, 937 and 1065 would affect the growth of both cancer cells and endothelial cells equally and hence may have a double-pronged effect in inhibiting tumor growth.

Table - 7

Cytotoxicity of short listed betulinic acid derivatives to endothelial cells

S.No.	Derivative	ECV304 IC_{50} (µg/mL)
1	**829**	0.35 ± 0.03 **
2	**807**	0.39 ± 0.04 **
3	**1098**	0.60 ± 0.07 **
4	**937**	0.70 ± 0.05 **
5	**1065**	2.40 ± 0.08

Data shown are mean ± SEM of three independent experiments. ** represent $p < 0.01$.

Table - 8

Specificity of short-listed betulinic acid derivatives to endothelial cells

S.No.	Derivative	Endothelial cell specificity (ECS) ECS = IC_{50} (Tumor cell growth)/IC_{50} (Endothelial cell growth					
		MOLT-4 (Leukemia)	U-937 (Lymphoma)	DU-145 (Prostate)	L-132 (Lung)	PA-1 (Ovary)	HT-29 (Colon)
1	**829**	1.14	1.43	28.57	11.43	1.43	3.71
2	**807**	2.56	ND	25.64	1.28	4.36	ND
3	**1098**	0.83	0.67	2.50	2.17	1.50	4.33
4	**937**	1.29	1.71	3.57	1.57	2.29	2.43
5	**1065**	0.42	0.79	1.04	1.42	0.58	2.04

Table shows endothelial cell specificity (ECS) values when tested against the cancer cell lines (MOLT-4, U-937, DU-145, L-132, PA-1 and HT-29). ECS less than 10 = Low ECS; ECS between 10 - 20 = Moderate ECS; ECS greater than 20 = High ECS. ND = not done.

2.4.2.2 Effect on migration/chemotaxis of endothelial cells

A key step in the process of angiogenesis and tumor metastasis is migration of endothelial cells towards a stimulus. Chemotaxis assays were used to measure the effects of betulinic acid and shortlisted derivatives on cell responses to strong angiogenc factors contained in conditioned medium. For in vitro chemotaxis assay, ECV304 cells were starved for 24 hrs and stimulated with a chemotactic factor. The migration of starved endothelial cells (ECV304) toward various stimuli were studied using the transwell assay. In order to fnd out the approprate stimulus for ECV304 cells to migrate, the effects of known angiogenic factors like bovine serum albumin (BSA) and fetal calf serum (FCS) and additonally ECV304 conditioned medium were tested in the transwell migration assay.

It was found that fetal calf serum (FCS) and endothelial cell supernatant stimulated the starved cells significantly better ($p < 0.01$) than unstimulated cells. 0.2% bovine serum

albumin (BSA) was not stimulatory to ECV304 cells (Table - 9). ECV304 conditioned medium was chosen in future experiments to study the effect of test compounds.

The test compounds along with ECV304 cells were added to the filter wells at previously determined non-cytotoxic concentrations. The number of cells that migrated to the bottom well were counted under a phase contrast microscope. Betulinic acid inhibited chemotaxis of endothelial cells only marginally whereas derivatives 937 and 1065 very significantly ($p < 0.01$) and derivative 1098 significantly ($p < 0.05$) inhibited chemotaxis as compared to untreated cells (Figure - 17).

These results suggest that betulinic acid and derivatives inhibit angiogenesis *in vitro*, and the anti-migratory activity of these compounds may have an effect on the *in vitro* tube formation of endothelial cells representative of formation of capillaries *in vivo*.

Table - 9

Chemotaxis of ECV304 (endothelial) cells towards different chemo-attractants

S.No.	Chemo-attractant	Number of cells migrated to the lower well
1	Untreated (control) cells	5.0 ± 2.0
2	0.2% Bovine serum albumin (BSA)	6.0 ± 2.0
3	10% Fetal calf serum (FCS)	23.0 ± 1.0**
4	ECV304 cell supernatant	27.5 ± 1.0 **

Data shown are mean ± SEM of three independent experiments. ** $p < 0.01$ compared with BSA.

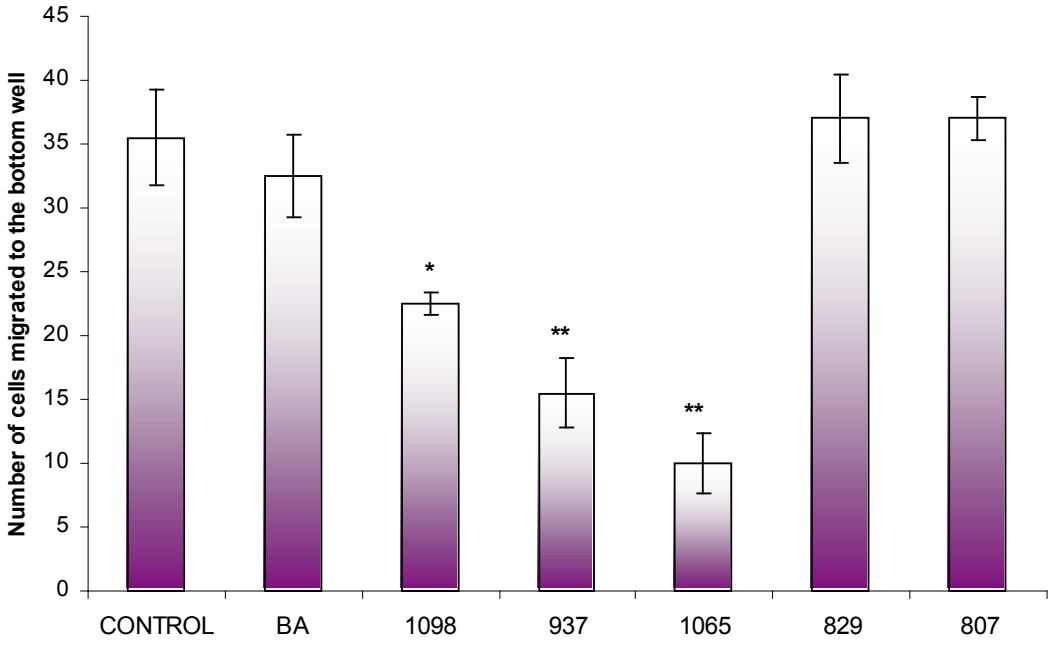

Figure - 17

Inhibition of chemotaxis by betulinic acid and short-listed derivatives.

Effect of betulinic acid and short-listed compounds at non-cytotoxic concentrations on the migration/chemotaxis of ECV304 (endothelial) cells towards factors in the conditioned medium. Data shows mean ± SEM. * and ** represent $p < 0.05$ and $p < 0.01$ respectively as compared to control.

2.4.2.3 Effect on tube formation of endothelial cells

Migration, invasion and matrix remodeling are all required for the morphogenesis of endothelial cells in to capillaries. The tube formation on Matrigel assay provides an indication of the ability of endothelial cells to reorganize and differentiate in to capillary-like structures. Untreated ECV304 cells showed formation of the typical capillary network 16 - 18 hrs after plating. Betulinic acid had little effect on the tube formation of ECV304 cells. In contrast positive control camptothecin and the short listed derivatives caused breakdown of capillary tubes and inhibited of capillary network formation (Figure - 18).

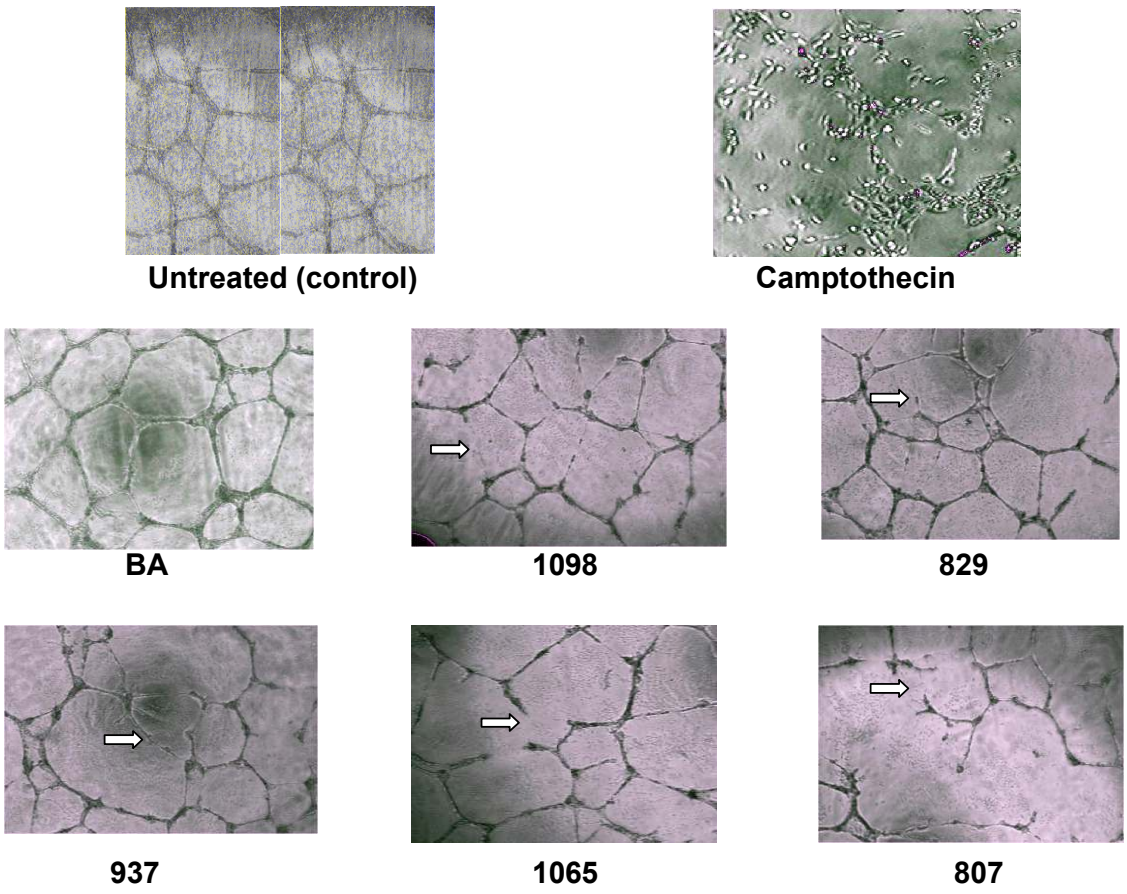

Figure - 18

Inhibition of tube formation

Phase contrast microscopy images of tube formation by ECV304 (endothelial) cells at 18 hrs. The total area covered by the tubes was measured by Image analysis (VideoPro®, Australia). The short-listed compounds inhibited tube formation characterized by the breakdown of capillary tubes (arrow) and inhibition of capillary network formation as seen in the images.

In order to quantitate the effect of these compounds in ECV304 tube formation, the total area covered by the tubes was measured using an image analysis software. Betulinic acid and shortlisted molecules inhibited tube structure formation of endothelial cells by 5.5 to 49.2% in terms of reduction of total tube area (Figure - 19). Betulinic acid had only little effect on tube formation while derivatives 807 and 1065 very significantly ($p < 0.01$) inhibited tube formation as compared to betulinic acid. The derivatives can be arranged as per their ability to inhibit tube formation as follows: 807 > 1065 > 937 > 829 > 1098.

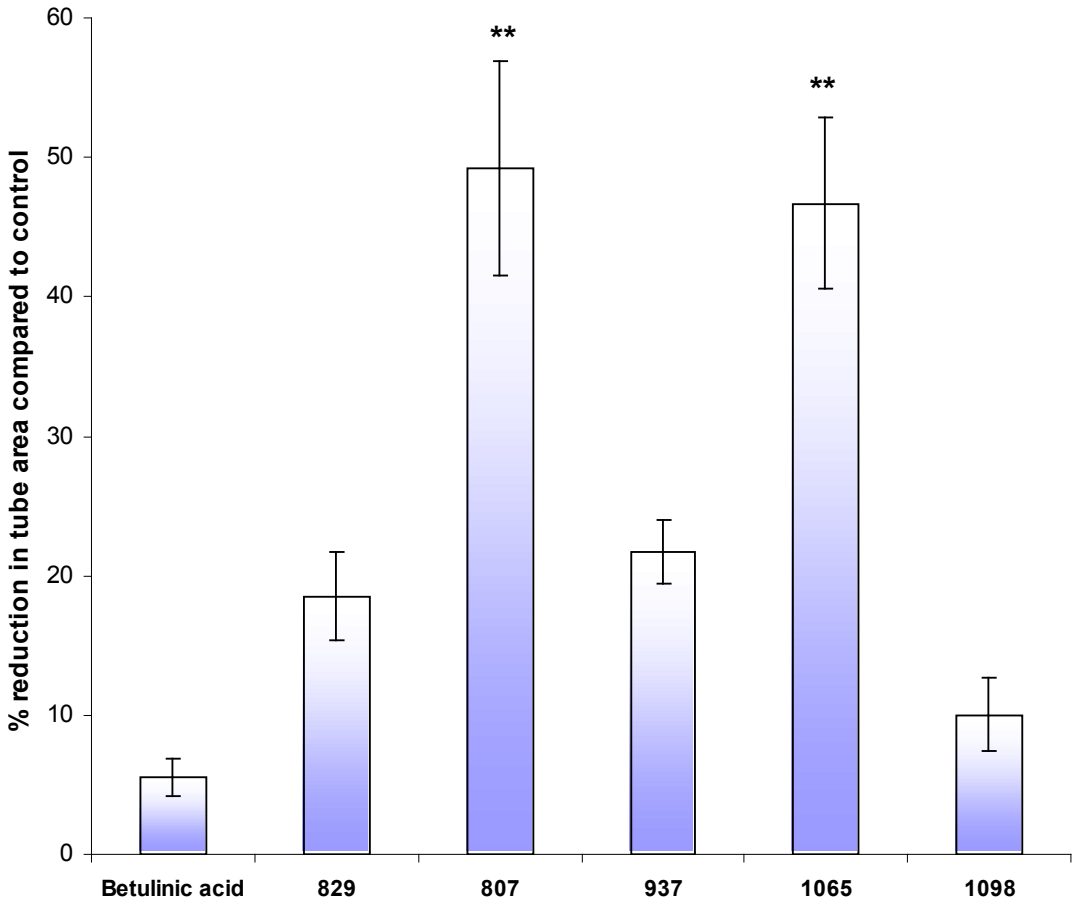

Figure - 19

Effect of betulinic acid and short-listed derivatives on tube formation by endothelial cells

ECV304 (endothelial) cells were plated on Matrigel™ coated 96-well plates and treated with the compounds for 18 hrs at non-cytotoxic concentrations. Data shown are mean ± SEM of three independent experiments. ** represent $p < 0.01$ compared with betulinic acid.

2.4.2.4 Effect on formation of lung nodules

Anti-angiogenic therapy could be applied to all forms of solid tumors to inhibit the growth of both the primary lesion and any metastatic disease. Anti-angiogenesis therapy may represent a universal treatment for cancer because all solid tumors require neovascularization to grow. The role of angiogenesis is more relevant to metastatic disease since metastasis involves cancer cell migration form primary tumor via the blood vessel to the secondary site of metastasis. The process includes several key steps involved in angiogenesis. The reduction in cancer metastasis in the lung is an example of how an anti-angiogenic agent can reduce tumor burden by inhibiting metastasis.

A model of metastasis to the lung was established in athymic nude mice. Mice were injected in the tail vein with 1 - 3 x 10^6 melanoma cells (B16F10) and preliminary evidence of nodules on the lung surface appear at 14 to 15 days post injection. On day 21, all the mice were subsequently sacrificed at which time the lungs were weighed.

Betulinic acid derivative 1098 was administered intravenously at a dose of 5 mg/kg soon after melanoma cells were injected in to mice. Treatment was continued daily for a period of 21 days. Treatment with 1098 inhibited the growth of metastatic tumors in the lung as quantified by measuring weight of the lungs.

Figure - 20 shows photographic images of 1098 treated and untreated lungs. Gross examination of the lungs revealed numerous black colored lung nodules. However, the animals in the control treated group had significantly more surface nodules than those mice which had received treatment. The number of nodules could not, however, be counted as they were too numerous. Instead, the lungs were weighed and based on the weight of the lungs the efficacy of drug treatment was assessed. The average weight of normal mouse lungs was 0.23 ± 0.03 g, whereas control lungs bearing lung nodules had a mean lung weight of 0.60 ± 0.20 g. Treatment with 1098 reduced the mean lung weight of treated animals to 0.27 ± 0.14 g (Figure - 21). This difference was significant ($p < 0.05$). 1098-treated lungs contained lesions that were small and isolated but retained most of the normal alveolar architecture of a non-diseased lung.

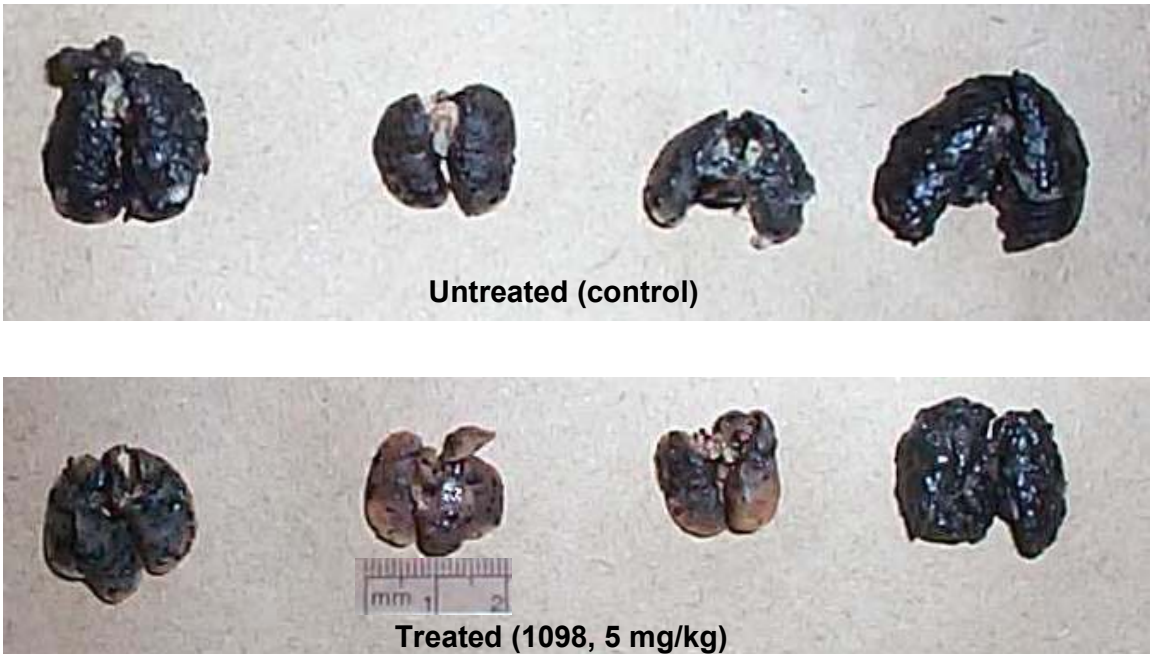

Figure - 20

Lung nodule formation

Top panel shows photographs of lungs of untreated animals on day 21 post i.v. inoculation of B16F10 (melanoma) cells. The lungs appear dark in color due to numerous nodules formed by the cells and excessive melanin production. Bottom panel show lungs of animals treated with 1098.

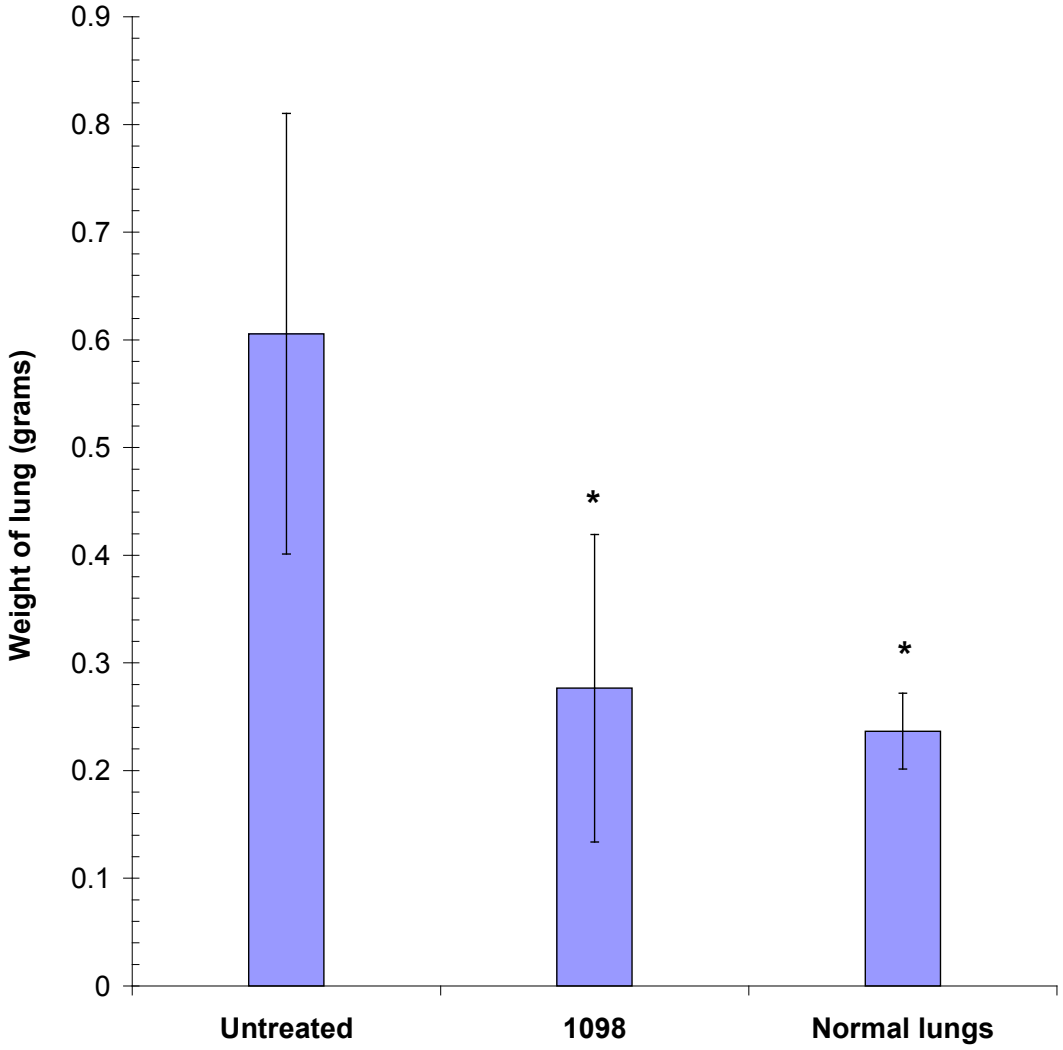

Figure - 21

Anti-metastatic activity of 1098 in a mouse lung nodule assay

B16F10 melanoma cells ($3x10^6$/animal) were injected through the lateral tail vein. Test compounds (5 mg/kg b.wt.) was injected intravenously continuously for 21 days. Animals were sacrificed 21 days after tumor inoculation; lungs were dissected, dried and weighed. Data shown is mean lung weight ± SD, n=4 animals per group. * represents $p < 0.05$ compared to untreated control lungs. There was no significant difference between normal lungs and 1098 treated lungs.

2.5 CONCLUSION

The *de novo* development of endothelial cells into organized vessels occurs only in early embryonic development, and is known as vasculogenesis. Angiogenesis, the process of continued expansion of endothelial cells from pre-existing blood vessels in the embryo, is highly regulated in the mature animal and plays a role in many physiological functions [Cines et al., 1998]. Angiogenesis is necessary for tissue growth, wound healing, and the menstrual cycle of females. However, uncontrolled angiogenesis is seen in many disease processes including retinal neovascularization, rheumatoid arthritis, hemangiomas, and psoriasis. The growth of new blood vessels is especially significant to the support of tumor growth and metastasis [Folkman, 1995].

Angiogenesis is a key process for the outgrowth of cancer cells and their spread into other tissues. Therefore, the specific inhibition of angiogenesis may be a powerful means to suppress angiogenesis-related diseases including cancer. Extensive studies have been carried out to identify the cellular target proteins for angiogenesis and several of these target proteins have been identified, i.e., matrix metalloproteinases [Moses et al., 1991], vascular endothelial growth factor receptors [Terman et al., 1992; Millauer et al., 1993], methionine aminopeptidase [Sin et al., 1997; Griffith et al., 1997] and histone deacetylases [Kim et al., 2001b; Kwon et al., 2002b].

Application of chemotherapeutics for anti-angiogenesis cancer therapy was proposed in 1991 [Kerbel, 1991] and has been reviewed [Kerbel, 2000]. Recent studies have shown continuous low-dose application of chemotherapeutics has resulted in regression of large established tumors and inhibition of angiogenesis in animal models, without major toxicities or signs of acquired drug resistance during the course of treatment in mice [Browder et al., 2000; Klement et al., 2000]. Epirubicin, doxorubicin, mitoxantrone, vinblastine, vincristine, and paclitaxel have been shown to have some antiangiogenic activity [Steiner, 1992, Dordunoo, 1995, Burt, 1995]. Therefore, further analysis and evaluation of cytotoxic compounds like betulinic acid as antiangiogenic agents should uncover more benefits of these agents in cancer therapy.

The present data show that betulinic acid inhibits key processes of angiogenesis *in vitro*. viz. growth of endothelial cells, migration and tube formation. This result is consistent with the previous observation [Kwon et al., 2002a]. However, previous studies done using bovine aortic endothelial cells (BAEC) found that betulinic acid reduced BAEC viability by almost 50% at 20 μM (around 10 μg/mL). Similarly, the authors reported that betulinic acid inhibited MTT reduction by 70% at 10 μM. We have used ECV304 cells which are human umbilical vein endothelial cells and have shown very significant cytotoxicity (IC_{50} = 2.8 μM i.e.1.26 μg/mL) to these cells by the MTT reduction assay. The human origin of these cells makes our results more applicable to the clinical situation. Further, it was found that betulinic acid had very little effect on the levels of angiogenic factors like VEGF, FGF and Endostatin. Therefore, the molecular mechanism to explain the significant effect of betulinic acid on the growth of endothelial cells needs to be further examined.

The short listed derivatives of betulinic acid were assessed for cytotoxicity in the MTT assay, and were found to be cytotoxic to endothelial cell line ECV304 with IC_{50} values in the range of 0.35 - 2.4 μg/mL and some derivatives were significantly better than betulinic acid with IC_{50} values less than 1 μg/mL. Two derivatives 807 and 829 had high endothelial specificity; these compounds may have the potential to be developed as specific anti-angiogenic agents, while the other compounds (1098, 937 and 1065) affect the growth of both cancer cells and endothelial cells equally and hence may have a double-pronged effect in inhibiting tumor growth.

It was found that fetal calf serum (FCS) and endothelial cell supernatant stimulated the starved ECV304 cells better than unstimulated cells. ECV304 conditioned medium was tested in an Elisa assay and was found to contain very high levels of Vascular endothelial growth factor (VEGF) (data not shown). The results suggest that VEGF may have played a major role in stimulating the chemotaxis of ECV304 cells. It was earlier reported that betulinc acid inhibited FGF-stimulated invasion of BAEC cells significantly [Kwon et al., 2002a]. In our experiments, betulinic acid inhibited chemotaxis toward ECV304 conditioned medium of endothelial cells (ECV304) only marginally whereas derivatives 937, 1065 and 1098 significantly inhibited chemotaxis as compared to untreated cells.

The effects of betulinic acid on tube formation by endothelial cells were known [Kwon et al., 2002]. In our experiments, betulinic acid had little effect on the tube formation of ECV304 cells. In contrast, short-listed derivatives 807 and 1065 very significantly ($p < 0.01$) inhibited tube formation as compared to betulinic acid causing breakdown of capillary tubes and inhibiting capillary network formation.

The role of angiogenesis is more relevant to metastatic disease since metastasis involves cancer cell migration from primary tumor via the blood vessel to the secondary site of metastasis. Betulinic acid derivative 1098 was selected for anti-metastatic studies based on the potent *in vitro* and *in vivo* anti-cancer activity, described in Chapter-1 and its significant inhibitory effects on endothelial cell proliferation and migration as well as some activity in the tube formation assay. Betulinic acid derivative 1098 treated mice had significantly lesser growth of metastatic tumors in the lung when administered at a dose of 5 mg/kg for 21 days. 1098-treated lungs contained lesions that were small and isolated but retained most of the normal alveolar architecture of a non-diseased lung. The anti-metastatic effect of betulinic acid derivative has been reported here for the first time.

In conclusion, we find that betulinic acid shows good anti-angiogenic potential. The derivatives which were tested for anti-angiogenic activity based on better cytotoxicity and specificity to tumor cells than betulinic acid have shown very potent anti-angiogenic effects too which are better than betulinic acid. It has been suggested, that antiangiogenic compounds given along with standard cytotoxic drugs in combination chemotherapy regimens are more effective than cytotoxic drugs alone (Folkman, J, 1997). Since long duration combination chemotherapy regimens have the drawbacks of adverse toxic effects, compounds such as these betulinic acid derivatives, which possess both anti-tumor and antiangiogenic potential could be envisioned to act more effectively and with acceptable toxicity profiles [Mukherjee et al., 2004a, b]. The recognition of the anti-angiogenic potential of betulinic acid and its novel derivatives may help in designing strategies to tackle angiogenesis-dependant tumor growth.

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CHAPTER - 3

ABSORPTION, DISTRIBUTION, METABOLISM, ELIMINATION AND TOXICITY (ADMET) STUDIES OF BETULINIC ACID AND DERIVATIVES

3.1 INTRODUCTION

The term ADMET (Absorption, Metabolism, Distribution, Elimination and Toxicity) is typically used in reference to nonclinical studies. Fundamentally, ADMET information is critical in all phases of a fully integrated drug development program. The initial charge of early development programs is to file the Investigational New Drug (IND) application such that approval may be secured to investigate a new chemical entity (NCE) in humans.

Animal toxicology studies comprise the foundation of the IND. The toxicology program is designed to identify toxicities that may potentially be encountered in humans, helping ensure that the initial human studies are conducted safely and ethically. In this context, ADME studies provide supportive information to augment the interpretation of toxicology findings. Of primary importance among them are toxicokinetic studies, in which systemic drug and/or metabolite exposure in toxicology animals is evaluated. Drug exposure, expressed in terms of AUC (area under the drug plasma concentration-time curve), Cmax (maximum drug concentration in plasma), or an alternative parameter, is then related to dose level and toxicological outcomes [Cayen, 1995]. Based on toxicokinetic data at the no-observed toxic effect dose, an acceptable exposure limit in humans can be defined.

The basic pharmacokinetic behavior of the NCE is assessed in the toxicology species. Some typical studies that may be conducted in this stage of development are Toxicokinetics, Pharmacokinetics/absolute bioavailability in toxicology species (male/female), Protein binding, Erythrocyte/plasma distribution, Whole body autoradiography/tissue distribution, Mass balance in toxicology species, Metabolite profile in toxicology species, Pharmacodynamics and Allometric scaling.

In discovery stage of drug development, ADME assays can be incorporated to act as filters for better selection of compounds for advanced pre-clinical development. Physico-chemical parameters of the drugs and basic ADME parameters like permeability, metabolic stability etc. can be evaluated in order to reduce compound drop-out at a later stage of development.

3.2 REVIEW OF LITERATURE

3.2.1 DMPK methods in the drug discovery process

Absorption

The ability of an NCE to provide activity by the oral route (i.e. have good bioavailability) is imperative. Whereas oral bioavailability can be determined relatively easily in animal pharmacokinetic studies, it is not reasonable to expect that this approach can function optimally in a large scale screening environment. Fortunately a number of *in vitro* and cell culture techniques have evolved in recent years that have facilitated the assessment of intestinal permeability.

It has been demonstrated that membrane permeability can be predicted for some compounds with reasonable accuracy based solely on physicochemical parameters. Therefore, close scrutiny of the chemical structure may provide valuable basic information about an NCE prior to the commencement of laboratory experiments. It is well established, for instance, that efficient oral absorption will occur only after drug has dissolved and presented itself to the intestinal mucosal surface from whence it can traverse the epithelium. Dissolution is determined by the highly interdependent influences of aqueous solubility, ionizability (pKa), and lipophilicity (octanol/water log P or log D7.4). Furthermore, log P is a crucial factor governing passive membrane partitioning, influencing permeability opposite to its effect on solubility (i.e. increasing log P enhances permeability while reducing solubility). In light of this counter dependence, it has been suggested that oral absorption may be optimal within a log P range of 0.5 to 2.0 [Austel, 1989].

A number of *in vitro* tools have been adapted, with notable efficiency, to high-throughput assessments of membrane permeability and potential oral bioavailability. Most notable among them are CaCO-2 cells. Derived from a human colon carcinoma cell line, these cells are grown in a confluent monolayer on porous membrane filters which are mounted in diffusion chambers. Permeability measurements are based on the rate of appearance of test compound in the receiver compartment. The apical (donor) surface of the monolayer contains microvilli and thus retains many characteristics of the intestinal brush border. The cells also express functional transport proteins [Inui et al., 1992; Lu et al., 1994] and metabolic

enzymes [Bjorge et al., 1991], the degree of expression being dependent upon the post-seeding age of the cells.

Everted intestinal rings and brush-border membrane vesicles (BBMV) are also commonly used systems for assessing membrane permeability. The former technique is a refinement of one of the earliest *in vitro* absorption systems in which an everted intestinal segment was suspended in a buffer system to measure mucosal-to-serosal transfer. The current methodology involves isolating a rat intestinal segment, everting, and slicing into rings which are suspended in buffer. BBMV are prepared by removing the brush-border surface from rat or rabbit intestine and molding it into vesicles by homogenization and differential centrifugation. Both everted intestinal rings and BBMV are most useful for determining drug uptake rates rather than transepithelial flux.

In the *in situ* intestinal perfusion system, an intestinal segment is exposed in an anesthetized rat and drug solution is perfused through the lumen in a single-pass or recirculating fashion. Drug permeability is derived from the rate of disappearance of drug from the perfusate. Though more labor-intensive, in situ intestinal perfusions remain popular owing to the perceived clinical relevance of permeability data derived there from. In a recent study, it was demonstrated with a series of small organic molecules as well as a series of peptidomimetics that CaCO-2 cells, everted intestinal rings, and in situ perfusions have strong potential for predicting fraction absorbed in humans [Stewart et al., 1995].

One of the most appealing attributes which these experimental systems possess is their capability to perform relatively high throughput screening. This is particularly relevant for CaCO-2 cells, everted intestinal rings, and BBMV. An imposing rate-limiting step, once the systems are established and optimized, is the development of assay methods (usually HPLC) to quantify the analytes of interest. On the positive side, as these experiments are conducted in aqueous buffers, the pre-analytical sample purification requirement is minimal. These analytical burdens can be even further reduced by immobilized artificial membranes (IAM), a recently developed system which has been greeted with considerable enthusiasm [Pidgeon et al., 1995].

Assays that predict passive absorption of orally administered drugs have become increasingly important in the drug discovery process. As previously described such assays provide rapid, low cost and automation friendly methods to measure a compound's passive permeability. The Lipid-PAMPA method is a non-cell based assay designed to predict passive, transcellular permeability of drugs in early drug discovery. The assay is carried out in a 96-well MultiScreen Permeability plate and measures the ability of compounds to diffuse from a Donor to an Acceptor compartment separated by a PVDF membrane filter pretreated with a lipid-containing organic solvent [Kansy et al., 2001; Schmidt et al., 2003].

Metabolism

The systems described above have the greatest utility when absorption is rate-limiting to systemic availability. For many compounds, even if absorption is optimized bioavailability may be limited by extensive metabolism. Indeed, metabolism can complicate the *in vivo* activity profile irrespective of route of administration.

The majority of drug biotransformation processes appear to typically result in increasingly water soluble metabolic products which can be more readily excreted in urine. Thus one may surmise that within a group of compounds, highly lipophilic drugs would be metabolized most actively. More explicitly, the metabolic potential of a compound containing a chemical moiety known to be avidly biotransformed may be predicted reasonably accurately on the basis of abundant historical data. For example, being cognizant that unhindered phenolic hydroxyl groups are exquisitely good substrates for conjugating enzymes, NCEs which contain this moiety can be eliminated from further consideration if a predilection to rapid metabolism is felt to be detrimental. As we gain information about the specific conformational constraints imposed by the catalytic sites of major metabolic enzymes, our ability to predict *in vivo* metabolic events based solely on chemical structure is enhanced commensurately.

Various *in vitro* methods are available which are being increasingly incorporated into drug discovery strategies. Among the most popular and widely utilized systems in use today are hepatic microsomes. These preparations retain activity of those enzymes which reside in the smooth endoplasmic reticulum, such as cytochromes P450 (CYP), flavin monooxygenases, and glucuronosyltransferases. Isolated hepatocytes appear to retain a broader spectrum of

enzymatic activities, including not only reticular systems, but cytosolic and mitochondrial enzymes as well. Because of a rapid loss of hepatocyte-specific functions, it has been possible to generate useful data only with short term hepatocyte incubations or cultures [Hawksworth, 1994]. However, significant strides have been made toward maximizing the viability of hepatocytes in culture. Liver slices, which like hepatocytes retain a wide array of enzyme activities, are also increasing in popularity. Furthermore, both hepatocytes and liver slices are capable of assessing of enzyme induction *in vitro*. The choice of which system to employ in a drug discovery screening program will depend on many factors, not the least of which is availability of tissue for large-scale implementation. In addition, historical information about a particular chemical series is invaluable. Information gathered from *in vitro* metabolism studies is especially useful in choosing drug candidates for future development. The potential utility of *in vitro* metabolism data in predicting *in vivo* intrinsic clearance has been touted [Hoener, 1994; Houston, 1994].

The metabolic stability of a test compound, e.g. drug substance in liver microsomes of different species is determined in order to assess the potential of this compound to form undesired potentially toxic or pharmacologically inactive metabolites due to phase I metabolism or to accumulate in the body due to lacking or negligible metabolic degradation. The determination of the metabolic stability is therefore a measure to describe the metabolic fate. The determination of the metabolic stability in liver microsomes summarizes all the possible reactions.

Isolated heterologous human CYP enzymes have been available for several years, being expressed from cDNA in yeast (*Saccharomyces cerevisiae*), bacterial (*Escherichia coli*), and mammalian (B-lymphoblastoid) cell lines [Ohgiya et al., 1989; Winters et al., 1992; Crespi et al., 1991]. These systems can be used to ascertain whether a compound is a substrate for a particular CYP isozyme and, if so, what metabolite is generated by that enzyme. Moreover, these enzymes, in sufficient quantity, may possibly be used as bioreactors to generate usable amounts of a metabolic product that may be difficult to chemically synthesize in the laboratory. Isozyme-specific antibodies and isozyme-specific inhibitory substrates, by selectively abolishing the activity of a particular CYP isoform, may be used to determine the relative importance of that enzyme in the turnover of an NCE. Conversely, the ability of an

NCE to interact with a particular CYP isozyme may be determined by metabolic cross-inhibition studies against prototypical CYP substrates.

Cytochromes P450 are the principal enzymes for the oxidative metabolism of drugs and other xenobiotics. Among the xenobiotic-metabolizing cytochromes P450, five forms, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 appear to be most commonly responsible for the metabolism of drugs. Inhibition of cytochrome P450-mediated metabolism is often the mechanism for drug-drug interactions. The potential for enzyme inhibition is routinely assessed by performing *in vitro* inhibition studies using cDNA-expressed enzymes or human liver microsomes. These types of studies are becoming a routine part of the drug registration data package. Combinatorial chemistry and high throughput screening for pharmacological activity can lead to the identification of relatively large numbers of compounds which have potential as human therapeutics (drug candidates). The availability of high throughput assays for cytochrome P450 inhibition would facilitate the identification of those drug candidates which have a lower potential for drug-drug interactions (i.e. weak enzyme inhibitors).

In vitro metabolism systems are not limited to those derived from the liver. Most pharmaceutical companies have been building liver and tissue banks to permit a cross-comparison of metabolic turnover rates in various tissues from various species. It is not uncommon now for liver banks to house tissues from a variety of species, including those from animals treated with enzyme inducers or inhibitors. Therefore, cross-species in vitro metabolism comparisons are becoming more feasible and commonplace. In addition to providing information on potential rates and routes of metabolism, interspecies comparisons may help in choosing species to be used in toxicology studies.

Pharmacokinetics

Unfortunately, there is no substitute for actual *in vivo* data in assessing pharmacokinetic profiles of drug candidates. While insight into various aspects of the pharmacokinetic profile (absorption, metabolism, protein binding) can be gleaned from *in vitro* techniques, there are as yet no methods available for accurately predicting what will happen to a drug when it is put into a whole animal.

For a useful assessment of pharmacokinetics and bioavailability, it is necessary to administer the drug to selected animal species both intravenously and by the intended route of

administration (usually oral). Blood samples are collected over a predetermined time course after dosing and the drug is quantified in serum or plasma by a suitable bioanalytical method (e.g. HPLC). Alternatively or concurrently it may be possible to collect plasma samples from animals used in whole animal pharmacologic models and, based on the concentration/effect relationship established, make a link between *in vitro* pharmacologic activity and the behavior of a compound *in vivo* [Guttendorf et al., 1992].

In any case, the most significant impediment to providing pharmacokinetic input to a high-throughput discovery team is the time and labor-intensiveness of the bioanalytical methods available. To assess biochemical or pharmacologic activity *in vitro* or *in vivo*, a standardized screening method is established with a common assay endpoint that can be applied to test all compounds that are available. In contrast, a separate bioanalytical method for pharmacokinetic assessment must be developed for each compound. For chromatographic assays, which comprise the vast majority of the methods employed in pharmacokinetic studies, remarkable improvements in assay detection limits and sample cleanup have been realized. However, little has been done to shorten the time required for assay development and implementation. Pharmacokinetic characterization is therefore often relegated to end-stage discovery, being utilized to select which of 2 or 3 potential lead candidates has the most "acceptable" pharmacokinetic profile. Clearly, if pharmacokinetic input is to be available for early discovery decisions, more efficient methodologies are necessary.

Semi-simultaneous bioavailability estimation is a screening method which has been successfully utilized in drug discovery [Karlsson et al., 1989]. With this technique, an intravenous dose of drug is administered and at a suitable time post dose (i.e. post distributional) an oral dose is administered to the same animal. Pharmacokinetic parameters such as clearance and volume of distribution can be determined from the intravenous concentration-time curve. Oral pharmacokinetic parameters, including bioavailability, are subsequently extricated from the combined i.v. and Oral concentration-time data by deconvolution. By reducing inter- and intra-animal variability, accurate pharmacokinetic data can be gathered with fewer animals. In addition, the total number of plasma samples from both routes of administration is reduced by the overlapping dosing regimes. On the other hand, a prior knowledge of the pharmacokinetic characteristics of the NCE is needed to optimize the administration paradigm. In that regard, it may be possible to extrapolate from

studies with structurally analogous predecessors from the same chemical series. More critical, however, is that compound-specific analytical methods are still required for each NCE.

3.2.2 Metabolism, disposition and toxicity of betulinic acid

Absorption, Distribution, and Pharmacokinetics

Because of its potential clinical application for the treatment of cancer and HIV infection, studies aimed at determining the fate of betulinic acid in mammals have been initiated. The poor solubility of betulinic acid in aqueous systems generated a great deal of interest in investigating several formulation schemes. [Son et al., 1998; Rusmawati et al., 2001] In one study, [Udeani et al., 1999] a formulation of polyvinylpyrrolidone (PVP)–betulinic acid complex was administered to CD-1 mice as an intraperitoneal dose of 250 or 500 mg/kg. These high dosage levels were selected because they were previously found to be effective *in vivo* for the treatment of cancer in experimental mice. [Pisha et al., 1995] The authors concluded that, under these experimental conditions, the pharmacokinetic data of betulinic acid were best described fitting a standard two compartment first-order model. Peak serum concentrations of betulinic acid were observed at 0.146 and 0.228 hr for the 250 and 500 mg/kg doses, respectively, by LC–MS analysis. At the 250 and 500 mg/kg doses, betulinic acid exhibited distribution volumes of 106 and 108 L/kg, respectively, and half-lives of 11.5 and 11.8 hr, respectively. The distribution of betulinic acid, administered at 500 mg/kg, was found to vary considerably among the various tissues over the course of 24 hr. High concentrations of betulinic acid were noted in fat tissues peaking after 24 hr at 2,260±850 μg/g. Other high peak concentrations of betulinic acid were found in the bladder (3,523±744 μg/g, 8 hr), lymph node (4,218±2,809 μg/g, 4 hr), mammary gland (1,184±904 μg/g, 24 hr), ovary (3,055±1,421 μg/g, 4 hr), spleen (1,287±162 μg/g, 24 hr), and uterus (908±165 μg/g, 24 hr).

Additional LC–ESMS (negative ion mode) analyses were performed by Shin and co-workers [Shin et al., 1999] to detect betulinic acid in nude mice bearing human melanoma following a single 500 mg/kg intraperitoneal dose. Concentrations of betulinic acid in the blood, liver, lung, kidney, and tumor were determined 24 hr post-injection. Interestingly, the highest concentration of betulinic acid was found in the tumor (452.2±261.2 μg/g) with virtually none in the blood (1.8±0.5 μg/mL). A significant portion of betulinic acid was also found in the liver (223.9±80.3 μg/g).

Metabolism

In an effort to elucidate its metabolic fate in humans, microorganisms were utilized as *in vitro* model systems to predict and prepare the potential mammalian metabolites of betulinic acid. Microorganisms have long been recognized as appropriate tools for studying mammalian drug metabolism based on the extensive homology between microbial and mammalian metabolic pathways. [Clark et al., 1991] In a series of pertinent experiments, [Kouzi et al., 2000; Chatterjee et al., 1999; Chatterjee et al., 2000] betulinic acid was incubated with resting-cell suspensions of Cunninghamella spp. (NRRL 5695), Cunninghamella elegans (ATCC 9244), Bacillus megaterium (ATCC 13368 and 14581), and Mucor mucedo (UI-4605). From these studies, a series of oxidized and conjugated metabolites of betulinic acid were obtained. All biotransformation products of betulinic acid were evaluated for anti-melanoma activity. Microbial transformations of the structurally related betulin and betulonic acid have also been investigated. [Akihisa et al., 2002]

In addition to the use of microorganisms as models of its mammalian metabolism, molecular modeling studies have been conducted to predict the sites of metabolism mediated by human cytochrome P450 enzyme systems in betulinic acid. One such system is the human CYP2C9. Human CYP2C9 exhibits selectivity for substrates containing an ionizable carboxylic acid group or an analogous group/isostere, and the usual site of metabolism lies at a fairly well-defined distance from this structural feature. However, there are additional criteria associated with substrate selectivity for CYP2C9 which relate to various other contacts with active site residues, including hydrogen bonding, p-p stacking, and certain hydrophobic interactions. Many of the key regions (substrate recognition sites) governing substrate selectivity within the CYP2 family have been probed using site directed mutagenesis, and a number of particularly important contact points with potential substrates have been identified. Based on the accumulating evidence from mutagenesis and other experimental data, a homology model for human CYP2C9 has been constructed and shown to be consistent with the known substrate selectivity characteristics exhibited by CYP2C9. [Lewis et al., 1998]

Toxicity of betulinic acid

Studies have shown that betulinic acid exhibits greatly reduced *in vitro* cytotoxicity against normal dermal fibroblast and peripheral blood lymphocytes.[Zuco et al., 2002] However, i.p. injections of betulinic acid administered to mice pre-infected with Plasmodium berghei, at a

dose of 250 mg/kg/day for 4 days, resulted in the death of one of the experimental mice. [Steele et al., 1999] In contrast, a Hippocratic screen of betulinic acid and some of its derivatives, administered i.p. to rats at doses of 200 and 400 mg/kg provided no evidence of toxicity. [Sandberg et al., 1987] Likewise, Pisha and co-workers [Pisha et al., 1995] reported no toxicity associated with the intraperitoneal administration of six doses (500 mg/kg each) of betulinic acid to mice on every fourth day. Further tests using a similar treatment regimen of six doses (250 mg/kg each) of betulinic acid on every third day to mice were also non-toxic. *In vitro* studies utilizing Madin Darby kidney cells did reveal that betulinic acid is responsible for a significant increase in the concentration of intracellular-free calcium; however, the increase in free calcium levels was associated with only a slight decrease in cell viability. [Chou et al., 2000] Based on these findings, it appears that betulinic acid possesses very little broad cytotoxicity, if any, at relatively high therapeutic doses.

3.3 MATERIALS AND METHODS

3.3.1 *In silico* screening

The 2D structures of the betulinic acid and derivatives was drawn using Chem 3D® software and saved in .MOL format. The structures were analyzed and predicted for physicochemical, absorption and distribution parameters using PreADMET® version 1.0 [BMD, Research Institute of Bioinformatics and Molecular Design, Seoul, Korea]. The software uses artificial neural network with back-propagation method to determine the permeability across Caco-2 cells, MDCK cells, human intestine, blood brain barrier (BBB) and skin, protein binding, solubility and log P.

3.3.2 Solubility assay

The method (Figure - 22) as reported earlier [Onofrey et al., 2003] was followed.

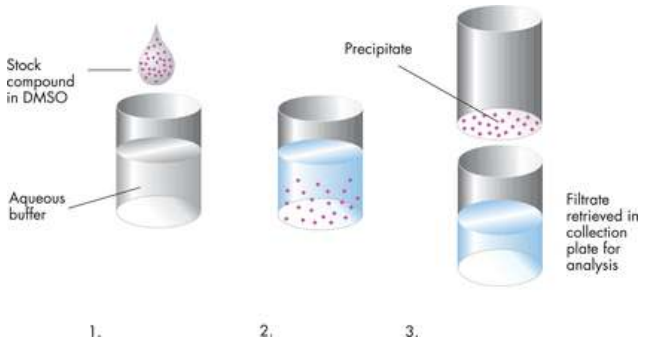


Figure - 22

A diagrammatic illustration of the methodology used in solubility determination by DMSO precipitation method

The assay was carried out as follows:

a. An aqueous buffer solution (500 mL) of pH 7.4 was prepared as given below:

Milli-Q water	250 mL
(45 mM) ethanolamine	1.36 mL
(45 mM) potassium dihydrogen phosphate	3.08 g
(45 mM) potassium acetate	2.21 g
0.15 M KCl	q.s. to 500 mL

The above reagents were thoroughly mixed and pH was adjusted to 7.4 ± 0.05 with 1.0 N HCl. The buffer was filtered with a 0.22 µm filter unit to remove any particulates, and stored at 4° C (stable for up to one month prior to use).

b. Test compounds were dissolved in DMSO at a concentration of 10 mM.

c. Standards of each test compound were prepared in buffer: ACN solution (80:20) in a deep well plate as shown below:

Reagents	**500 µM**	**200 µM**	**50 µM**	**12.5 µM**	**3.13 µM**	**0 (blank)**
Buffer:ACN Solution	285 µL	380 µL	285 µL	285 µL	285 µL	285 µL
DMSO	----	12 µL	15 µL	15 µL	15 µL	15 µL
10 mM DMSO Stock	15 µL	8 µL	----	----	----	----
200 µM Standard	----	----	100 µL	----	----	----
50 µM Standard	----	----	----	100 µL	----	----
12.5 µM Standard	----	----	----	----	100 µL	----

d. 200 µl of each standard was transferred in to a UV Star 96-well plate (Cat. No. 655801, Greiner Bio-One, Germany).

e. 190 µL/well of pH 7.4 buffer at room temperature was dispensed into a 0.5 ml centrifuge tube (Tarsons, India).

f. 10 µL of stock compound was dispensed at 10 mM in DMSO, directly into the buffer to the centrifuge tube. The final concentration of test compound in each well was 500 µM.

g. The centrifuge tube was covered and mixed with gentle shaking (100-300 rpm, orbital shaker) at room temperature for 1.5 hours.

h. After mixing for 1.5 hours, the contents were withdrawn in individual syringes and filtered using a syringe filter (0.45 µM, Millex, Millipore, USA). After filtration, 160 µL/well of filtrate was transferred to the above UV Star 96-well plate and diluted with 40 µL/well of acetonitrile and mixed with gentle shaking at room temperature for 10 minutes.

i. After mixing, the UV-Star analysis plate was scanned in a micro plate spectrometer (Varioskan, Thermo Corporation, USA) from 260 nm to 500 nm at 10 nm increments. The absorbance for each well of the UV-Star analysis plate was determined at each wavelength of the scan.

j. Each compound's aqueous solubility spectral scan was superimposed with calibration scan to determine if the solubility spectra matched the calibration spectra. If the absorbance of the aqueous solubility filtrate sample coincided with the absorbance range of the calibration spectra, it was used for quantification of solubility.

k. One or more wavelengths > 260 nm was identified, at which the maximum absorbance for the highest concentration was > 0.1 absorbance units (AU). Selected the wavelength to use for standard curve if the optical density (OD) at 260 µM and 500 µM were less than 2.0 AU; the OD for the 50 µM standard was significantly greater than the OD for the 12.5 µM standard and the relationship between concentration and OD for three or more standards was linear.

l. The final drug concentration in the filtrate was determined from the standard curve using the spectrophotometer (SkanIt software v2.2 for Varioskan, Thermo Corporation, USA) and multiplying by a factor of 1.25 to account for dilution with acetonitrile.

3.3.3 Permeability assay

Permeability was determined as described previously [Kansy et al., 2001; Schmidt et al., 2003] (Figure - 23).

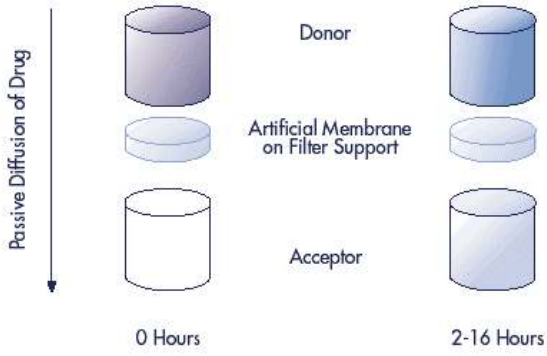


Figure – 23

A diagrammatic illustration of the methodology used in PAMPA (parallel artificial membrane permeability assay) method

1% w/v lecithin in dodecane was prepared by sonication and 5 µL of the lecithin/dodecane solution was pipetted into each donor plate well of a drug filtration plate (Elispot IP, Cat. No. ELIIP10SSP, Millipore, USA), avoiding pipette tip contact with the membrane. Immediately after the application of lecithin (within 10 minutes maximum), 150 µL of test compound-containing test compounds dissolved in phosphate buffered saline (PBS) containing 5% DMSO was pipetted to each well of the donor plate. 300 µL of buffer containing 5% DMSO was pipetted to each well of the acceptor plate (MSSACCEPTOR, Millipore, USA). The donor plate was placed gently into the acceptor plate, making sure the underside of the membrane is in contact with the buffer in all wells. Plate lid was replaced and incubated, at room temperature in a sealed container, for 16 hours. After incubation, UV/Vis absorption was measured from 250 to 500 nm for 100 µL/well of the donor solution and 250 µL/well of the acceptor solution. Solutions at the theoretical equilibrium concentration (i.e., the resulting concentration if the donor and acceptor solutions were simply combined) were prepared and measured by UV/Vis absorption (Varioskan, Thermo Corporation, USA) from 250 to 500 nm for 250 µL/well of each.

Permeability value was calculated as mean of 6 samples/wells using formula given below:

$$\text{Log Pe} = \log\left[-2.303*1.19*10^{-5}* \log \frac{1- OD_{acceptor}}{OD_{equilibrium}} \right] \qquad \text{[Sugano et al., 2001]}$$

3.3.4 Metabolic stability assay

Metabolic stability of the molecules was determined by HPLC by adapting a previously published method [Rodrigues, 1994] by calculating the amount of the compound remaining un-metabolized following incubation with pooled human liver microsomes (Cat. No. 452161, BD Gentest, USA). The pool comprised of 22 human specimens with a protein content of 20 mg/ml. DMSO stock solutions of test compound were prepared at a concentration of 1 mg/ml and further diluted with 0.1M phosphate buffer, pH 7.4, to a concentration of 100 µg/ml. The test compounds (20 µM) were incubated with the microsomes and cofactors as shown below:

Solution A (451220, Gentest, USA) *(25 mM NADP+, 66 mM glucose-6-phosphate, 66 mM $MgCl_2$ in water)*	= 10 µL
Solution B (451200, Gentest, USA) *(0.4 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium citrate),*	= 2 µL
Test compound (100 µg/ml)	= 20 µL
Pooled human liver microsomes	= 10 µL
0.1 M phosphate buffer	= q.s. to 200 µL

The mixture of cofactors and test compound in buffer was warmed to 37^0C in a water bath followed by addition of cold liver microsomes. Immediately after addition of the microsomes 100 µl of sample (0 min) was withdrawn from the above reaction mixture and terminated by the addition of equal volume of cold acetonitrile, kept in ice for 30 minutes, centrifuged at 10,000 rpm for 10 min in a refrigerated centrifuge. The supernatant was collected and stored at -20^0C for analysis. The remaining mixture was incubated at 37^0C in a water bath with shaking and terminated similarly after 60 minutes. The samples were analyzed by HPLC (L2010, Shimadzu, Japan), using a previously standardized analytical method. Stability was assessed by the disappearance of test compound peak based on the change in test compound

to internal standard peak height ratio. Metabolic stability was defined as the amount of test compound metabolized by the incubation with human liver microsomes and expressed as a percentage of the initial amount of the test compound (0 min). The conversion of diclofenac to 4-hydroxy diclofenac was used as the standard reaction.

3.3.5 Plasma protein binding assay

Plasma protein binding was determined in rat plasma using a previously reported method [Millipore user guide, 2002]. Test solutions were prepared by adding an aliquot of 5 µL of test compound (2 mM in DMSO) to 495 µL of rat plasma affording a final concentration of 20 µM (i.e. within LOQ = 2 µM). The test solution was mixed well and incubated for 1 hr at 37 °C. After incubation the test solution was loaded onto 1.5 ml centrifuge tubes fitted with YMC membrane with 10 KD cut-off (Microcon YM-10, Cat. No. 42407, Millipore, USA) and centrifuged for 45 minutes at 2000 x g at 37 °C. The filter was removed and the sample ultra-filtrate was recovered from the receiver tube. Desired volume was pipetted from the receiver tube for analysis. The amount of the test compound in the ulltrafiltrate and retentate was measured by HPLC using standard curves. The percentage protein binding was calculated using the formula:

$$\text{Plasma protein binding} = 1 - \left(\frac{[\text{drug}_{\text{ultrafiltrate}}]}{[\text{drug}_{\text{total}}]} \right) * 100$$

3.3.6 Cytochrome P450 inhibition assay

HPLC method

Phenacetin (ICN Biomedicals Inc. USA), Diclofenac (Aarti drugs, India), Ketokonazole (ICN Biomedicals Inc. USA), Paracetamol (Dabur Research Foundation, India), 4-hydroxy-diclofenac (451443, Gentest, USA), 6α-hydroxy-paclitaxel (451656, Gentest, USA), Testosterone (T1500,, Sigma or UC-339, Natutec, Germany), Quinidine (Q3625, Sigma), 6β-hydroxy-testosterone (UC-282, Natutec, Germany), Bufuralol (UC-168, Natutec, Germany), Sulfaphenazole (S0758, Sigma), Furafylline (F124, Sigma). The analytical chromatographic system (HPLC) was from Merck Hitachi. Acetonitrile used was HPLC grade (Glaxo) and trifluoroacetic acid (TFA) was purchased from Sigma Chemicals Co. Ltd. All other chemicals

were of the highest grade available commercially and were used as received. Water used was Milli Q grade (Milli Q System, Waters).

Pooled human liver microsomes (452161, BD Gentest, USA). The pool comprised of 22 human specimens with a protein content of 20 mg/ml. The microsomes were well characterized for their enzyme activity based on metabolism of specific substrates. Microsomes were stored at -70^{0}C. NADPH regenerating system- Solution A (451220, BD Gentest, USA), NADPH regenerating system- Solution B (451200, BD Gentest, USA).

The following model substrates were used:

Phenacetin O-deethylase assay [Tassaneeyakul et al., 1993], Diclofenac 4'-hydroxylase assay [Schmitz et al., 1993], Bufuralol 1'-hydroxylase assay [Kronbach et al., 1987], Testosterone 6β-hydroxylase assay [Buters et al., 1994]. The incubation and analysis conditions are summarized in Table - 10 and Table – 11, respectively. Test compounds were added from stock solutions at 10μM (clinically relevant concentration) in the incubation mixture in a small volume of solvent (DMSO, Methanol, Acetonitrile etc.). Solvent concentration was <1% in incubation mixture. Analysis of the substrate concentration was done by HPLC. Percent inhibition was expressed as inhibition relative to inhibitor-free data. The assay was performed as follows:

The reaction mixture was incubated, in the presence or absence of cofactors, at 37^{0}C in a shaker water bath. All the ingredients in the reaction, except the enzyme, were maintained at 37^{0}C and the reaction was initiated by adding cold enzyme.

The reaction was terminated at the specified time point by adding equal volume of cold acetonitrile or ethanol and kept in ice for 15 minutes.

It was then centrifuged at 13,000 rpm for 10 minutes and supernatant was collected and analyzed using HPLC.

Percentage metabolism was calculated using the formula given below:

$$\text{Percentage substrate remaining} = \frac{AUC_{-cofactor}}{AUC_{+cofactor}} * 100$$

Table-10

Assay conditions for Cytochrome P450 (CYP450) enzyme reactions

Reagents	**CYP1A2**	**CYP2C9**	**CYP2D6**	**CYP3A4**
NADP+ and Glucose-6-phosphate	5 µl	5 µl	5 µl	5 µl
Glucose-6-phosphate dehydrogenase	1 µl	1 µl	1 µl	1 µl
Substrate	2.5 uL of 180 µg/mL Phenacetin in 5% MeoH (50 µM)	25 uL of 118 µg/ml Diclofenac in buffer (50 µM)	2.5 uL of 1mM Bufuralol in buffer (100 µM)	5 uL of 1 mg/mL Testosterone in 10% DMSO/buffer (200 µM)
Pooled human liver microsomes	5 µl	5 µl	5 µl	5 µl
Phosphate buffer (pH7.4)	86.5 µl	64 µl	86.5 uL	84 uL
Total volume	100 µl	100 µl	100 µl	100 µl
Incubation time	60	30	60	30
Stop solution	Acetonitrile	Acetonitrile + 6% glacial acetic acid	70% Perchloric acid	Acetonitrile

Table - 11

Incubation and analysis conditions to determine inhibition of

Cytochrome P450 (CYP450) enzymes

<table>
<tr><th>CYP450</th><th>Substrate</th><th>Protein (mg/ml)</th><th>Co-factor system</th><th>Determination (λ)</th><th>HPLC conditions</th></tr>
<tr><td>CYP1A2</td><td>Phenacetin</td><td>1</td><td>NADPH-regeneratin g</td><td>UV-HPLC, 244</td><td rowspan="3">Column: YMC C18 250 x 4.6, 5µm.
Flow: 1 ml/min.
Gradient:
<u>Time % ACN % 0.05M NH_4COCH_3 (pH 5.0)</u>
0 20 80
5 20 80
25 100 0
30 20 80
40 20 80
For Phenacetin O-dethylase the gradient was ACN 10% to 100% from 5 to 25 min.</td></tr>
<tr><td>CYP2C9</td><td>Diclofenac</td><td>1</td><td>NADPH-regeneratin g</td><td>UV-HPLC, 280</td></tr>
<tr><td>CYP3A4</td><td>Testo sterone</td><td>1</td><td>NADPH-regeneratin g</td><td>UV-HPLC, 254</td></tr>
<tr><td>CYP2D6</td><td>Bufuralol</td><td>1</td><td>NADPH-regeneratin g</td><td>Fluoro-HPLC, Ex 280, Em 310</td><td>Column: Phenyl-Hypersil 250 x 4.6 , 5µm.
Flow: 1 ml/min.
Isocratic:
ACN/Methanol : H_3PO_4, pH 3.0 (30:70)</td></tr>
</table>

Fluorescence method

Compounds were screened for their ability to inhibit CYP2C9, 2D6, and 3A4 marker substrates using an *in vitro* fluorometric microtiter plate assay described previously [Crespi et al., 1997; GENTEST Technical Bulletin, 1998]. All reagents were purchased in the form of a kit [CYP450 inhibition assay, GENTEST, USA]. The assay was performed as per manufacturer's instructions.

1. With a multi-channel pipette 0.144 mL of cofactor/serial dilution buffer was dispensed into the wells in column 1.
2. With a multi-channel pipette 0.1 mL of cofactor/serial dilution buffer with acetonitrile was dispensed into the wells in columns 2 - 12.

3. Test compound or positive control (0.006 mL) was dispensed to the desired well(s) in column 1.
4. With a multi-channel pipette 0.05 mL from the wells in column 1 was dispensed to the wells in columns 2 through 8. Mixed by pipetting 3 to 5 times in each well. The (extra) 0.05 mL in the wells in column 8 was discarded.
5. The lid was placed on the plate and plate incubated at 37° C incubator for at least 10 minutes (to prewarm the buffer and the plate).
6. With a multi-channel pipette 0.1 mL enzyme/substrate mix was dispensed to columns 1 through 10. The liquid was dispensed in a stream, not drop wise. Mixing of the components in the wells was dependent upon dispensing rapidly.
7. The lid was replaced and incubated at 37° C for the desired time.
8. With a multi-channel pipette 0.075 mL of 'STOP' solution was dispensed to all wells. The liquid was dispensed in a stream, not drop-wise.
9. With a multi-channel pipette 0.1 mL of the respective enzyme/substrate mix as shown below was dispensed to the wells in columns 11 and 12.
10. The plate was scanned with a spectrophotometer (Varioskan, Thermo Corporation, USA) at the recommended excitation/emission filters as shown below for the specific assays.
11. The $IC_{50.}$was calculated using the software contained in the spectrophotometer.
12. Positive inhibitors i.e. Ketoconazole, Quinidine and Sulfaphenazole were used for CYP3A4, CYP2D6 and CYP2C9 respectively as shown in Table - 12.

Table-12

Incubation conditions for positive inhibitors of CYP activity in the fluorescence assay

Enzyme	Positive Control (Inhibitor Stock)	Enzyme substrate Mix	Incubation Time	Excitation (bandwidth)	Emission (bandwidth)
2C9	0.5 mM Sulfaphenazole	2C9-MFC-E/S mix	45 min	409 nm (20 nm)	530 nm (25 nm)
2D6	0.025 mM Quinidine	2D6-AMMC-E/S mix	30 min	390 nm (20 nm)	460 nm (40 nm)
3A4	0.25 mM Ketoconazole	3A4-BFC-E/S mix,	30 min	409 nm (20 nm)	530 nm (25 nm)

3.3.7 Pharmacokinetics

Animals

Male Wistar Rats, age 6-10 weeks, weighing between 100-150 gms were obtained from National Centre for Laboratory Animals Sciences (NCLAS, NIN, Hyderabad, India) and maintained in Small animal facility, Dabur Research Foundation. Animal experiments were carried out as per guidelines of Institutional Animal Ethics Committee (IAEC), Dabur Research Foundation. These animals were quarantined for 1 week prior to beginning the study. The rats were divided in to two groups of four each and numbered for with picric acid dye for identification. Each group was kept in acrylic cages. The standard food pellets (Gold feed, Delhi) and water (Aquaguard pure) through a glass bottle fitted with a nozzle, was available for 24 h. The animals were kept in an air-conditioned laboratory (room temperature maintained between 22-26^{o}C) with 12h light and dark cycle.

Test compounds

Derivative 1098 were provided by Medicinal chemistry lab, Dabur Research Foundation. For intravenous administration, it was dissolved in co-solvents Dimethylacetamide (20%), PEG400 (20%), Tween 80 (20%) and water for injection (q.s. to 100%) at a concentration of 5 mg/ml. For oral administration, compound was suspended in 0.5% carboxy methyl cellulose (CMC) at a concentration of 15 mg/ml.

Study design

1098 doses were selected based on preliminary toxicity studies conducted in rats using a range of doses. The pharmacokinetics studies were carried out by either route at a time. For intravenous study, four rats each was administered a single intravenous dose of 10 mg/kg in the peripheral tail vein using 26G sterile needle. For oral study, four rats each was administered an oral dose of 150 mg/kg using appropriate gavage needle directly in to the mouth. After the dose administration, blood samples (500 µL) were collected by orbital bleeding under mild ether anesthesia in tubes containing 20 µL of saturated EDTA solution in water at time points of 3 min, 10 min, 30 min, 1 hr, 2hr, 4hr, 6hr, 8hr, and 24 hr (n=4 each time point) for intravenous group and 30 min, 1 hr, 2hr, 4hr, 6hr, 8hr, and 24 hr for oral group. Blood samples were collected prior to dosing (0 min). The blood samples were

centrifuged at 10,000 rpm for 10 minutes. A 200 µL plasma sample was subsequently collected and stored at -20^{0}C.

Analytical procedure

High performance liquid chromatography (L2010, Shimadzu, Japan) was used to determine plasma concentrations of betulinic acid and 1098. The mobile phase consisted of acidified Milli-Q water (pH 3.0 ± 0.5 using phosphoric acid) (30%) and acetonitrile (70%), with gradient elution from 70% to 100% acetonitrile over 20 min. The flow rate was 1 ml/min. A C18, 250 X 4.6 mm column (YMC-Pack ODS-A), internal diameter 5 µm was used for the separation of the sample components. Betulinic acid derivative 1097 was used as the internal standard. The retention times for 1098 and internal standard were 35.1, and 31.3 min, respectively.

Standard solutions of the test compound in plasma were prepared by spiking with an appropriate volume (10 µL) of stock solution in DMSO (1 mg/ml) giving a final concentration of 200, 100, 10, 5, 1 and 0.1 µg/mL. These solutions were treated with the same procedure outline below and used for the generation of standard calibration curve.

90 µL sample of plasma was thawed prior to analysis, with 10 µL of internal standard and 500 µl of a mixture of ethyl acetate: methanol (75:25) was added to each sample for this analytical procedure. Each sample was vortexed until thoroughly mixed and then centrifuged at 10,000 rpm for 5 min.. The organic phase of each sample was collected and allowed to dry for 30 min by N_2 gas. The residue containing test compound and internal standard was reconstituted with 200 µl of 10% DMSO in methanol for HPLC analysis.

The concentration of the test compound was determined by calculating the ratio of its peak to the internal standard peak and then comparing the ratio to a simultaneously performed standard curve. The limit of Quantitation (LOQ) of 1098 was 0.3 µg/ml (10 µL injection). The linear range for 1098 was 0.3 - 100 µg/mL, and the value of r^2 for the regression line was

0.9996. The intra day coefficient of variation was 0.5 – 5.8%. The inter day coefficient of variation was 3.2 - 12.5%. The recovery of extraction procedure was 69%.

Pharmacokinetic analysis

The pharmacokinetic parameters were determined using WinNonlin v5.0.1 software [Pharsight Corporation, USA] package. Non-compartmental analysis (NCA Model 201 - IV-bolus input for plasma data) was selected for pharmacokinetic modeling. The linear trapezoidal with linear interpolation method was selected for calculation with uniform weighting for lambda z calculations.

3.3.8 Toxicity/Safety studies

Female Swiss albino mice, age 6-8 wks, weighing between 20-25gms bred in Small animal facility, Dabur Research Foundation were used in the study. Animal experiments were carried out as per guidelines of Institutional Animal Ethics Committee (IAEC), Dabur Research Foundation.

The mice were divided in two groups of five each. Each group was kept in acrylic cages. The standard food pellets and water (through a glass bottle fitted with a nozzle) was available for 24 h. The animals were kept in an air-conditioned laboratory (room temperature maintained between 22-26^{o}C) with 12h light and dark cycle.

The test substance was administered by intravenous routes in the lateral tail vein of mice using a 30G needle in separate groups.

Solution of test compound (10 mg/ml) was prepared in a cosolvent formulation comprising Dimethylacetamide (20%), PEG400 (20%), Tween 20 (20%) and volume made up with water for injection. The doses used were 10, 20, 40, 100 and 200 mg/kg. Doses of test substance could not be exceeded because of increased volume.

The animals were observed daily for mortality and gross behavioral effects. Body weights were recorded prior to dosing and weekly thereafter. These observations continued for 14 consecutive days. The minimum lethal dose (MLD) was defined as the dose at which at least one death was recorded. If MLD was not achieved it was represented as greater than the highest dose tested i.e. > 200 mg/kg.

3.3.9 Statistical Analysis

The results were expressed as mean ± SEM. The student's unpaired t-test and ANOVA was used to compare between groups. Difference were considered significant when $p < 0.05$. Results of animal studies were expressed as mean ± SD.

3.4 RESULTS AND OBSERVATIONS

3.4.1 *In silico* screening

The structures of known anti-cancer drugs were analyzed using a predictive software (PreADME™) to re-ascertain the value of the software in terms of its accuracy in prediction when compared to published experimental values.

Two parameters i.e. human intestinal absorption and protein binding were studied. It was found that the predictive values very well compared with those already published [DRUGDEX, 2005] (Figure - 24). The plasma protein binding values of some drugs viz. imatinib, capecitabine and gemcitabine had some deviations. The predictive value of the software was extended to study the ADME characteristics of betulinic acid and derivatives.

Betulinic acid and several different classes of derivatives (more than 100) were screened using the predictive software to understand the general ADME characteristics and specific structural requirements, if any, that could be identified for this class of compounds before testing them using experimental models.. The compounds were classified as high or low based on the prediction of the software and values that were given for high and low activity. The results and general conclusions are summarized in Table - 13.

Based on the results it was predicted that betulinic acid and derivatives were poorly soluble compounds, their solubility was less than 0.01 mg/L. Based on permeability through CaCo-2 and MDCK cell lines, some betulinic acid derivatives were predicted to permeate the CaCo-2 barrier and some others were predicted to permeate the MDCK barrier, but none of the compounds were predicted to permeate both these cell barriers. Additionally, these compounds were predicted to have poor intestinal absorption, except for two compounds 1097 and 1098.

The partition coefficient values (lop P) of betulinic acid and derivatives was predicted to be greater than 4, which was well above the optimal value of 0.5 to 2.0 [Austel, 1989]. This meant that all these compounds were highly lipophilic, may be poorly soluble and may not readily cross intestinal membranes, as predicted by Caco-2 and MDCK permeability predictions. The blood brain barrier (BBB) permeability predictions, however, show that betulinic acid and derivatives have the ability to cross the BBB and enter the brain. This shows that these compounds are prone to Central nervous system (CNS) effects and may show better activity in brain tumor or in cancers like neuroblastoma. Betulinic acid and derivatives were predicted to have high protein binding (more than 70%). This leads to low free drug concentrations and lower plasma concentrations.

The findings of the predictive software show that betulinic acid and derivatives are highly lipophilic compounds with poor solubility and absorption properties. They might however be active in the brain, but may have less than optimum plasma concentrations. Based on these findings, betulinic acid and short listed derivatives were tested in suitable experimental models of solubility, permeability, metabolic stability, protein binding and cytochrome P450 inhibition studies.

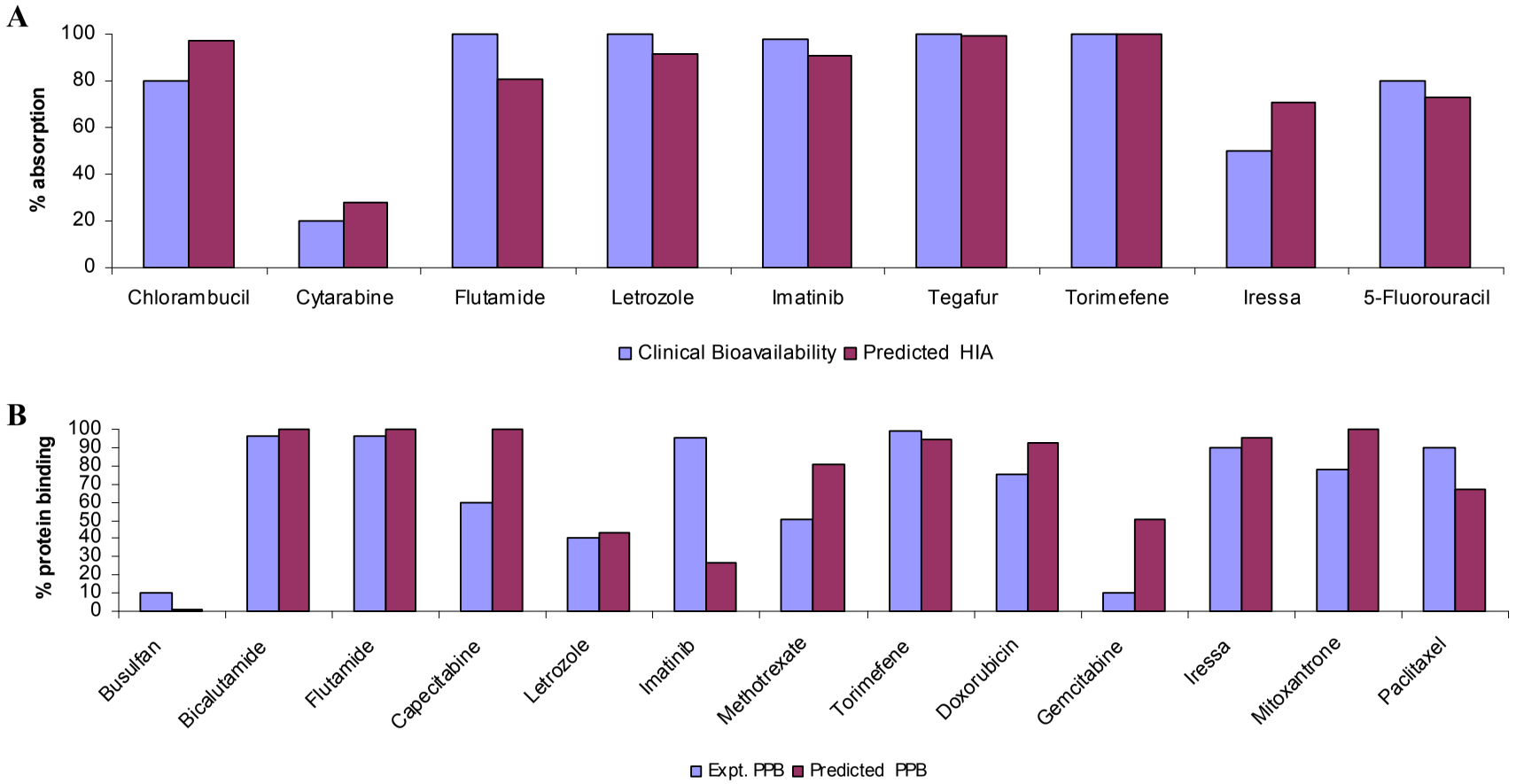


Figure - 24
ADME predictions of known drugs

Panel A shows the comparison of oral bioavailability and predicted Human intestinal absorption (HIA) for known drugs. Similarly, Panel B shows the comparison of reported plasma protein binding and predicted protein binding values. The reported values were from (Cheryl L Vogt, 1974 – 2005) and the predictive values were generated using Preadme™ software. The figure shows that there is good comparison between experimental and predicted values.

Table-13

Summary of predictive ADME characteristics of betulinic acid and derivatives

Parameter	• **Results**	**Conclusions**
Solubility	• less than 0.01 mg/L	Poor solubility
Permeability (Caco-2)	• Most compounds had low permeability (P < 7 *10^6 cm/sec). • Some compounds had high permeability permeability (P > 7 *10^6 cm/sec). i.e. betulinic acid, 347, 351, 352, 417, 443, 458, 463, 542, 577, 677, 692, 790, 830, 878, 912, 1002, 1022, 1027, 1068, 1073, 1101, 1103, 1138, 1155, 1161, 1264 and 1469.	Poor intestinal permeability
Permeability (MDCK)	• Most compounds had low permeability (P < 50 *10^6 cm/sec). • Two compounds had high permeability (P > 50 *10^6 cm/sec) i.e. 548 and 874.	
Permeability (Blood brain barrier)	• All compounds had high permeability i.e. concentrations in brain up to 70 times the concentrations in plasma.	Very high permeability through blood brain barrier
Human intestinal absorption	• Most compounds had 0% absorption • Two compounds 1097 and 1098 had 50.2%, 23.1% absorption, respectively.	Poor human intestinal absorption
Partition coefficient (log P)	• Log P > 4	Very lipophilic
Plasma protein binding	• percent protein binding > 70%	High protein binding

3.4.2 Solubility studies

In order to evaluate the potential clinical use and ease of formulating betulinic acid and short listed derivatives, studies to evaluate aqueous solubility of test compounds was undertaken using the DMSO precipitation method. This method was known to give similar results as compared to traditional shake-flask method [ASTM: E 1148-02, 2002] and is compatible to high throughput drug development.

The high throughput solubility assay using DMSO precipitation method, as described in materials and methods, was tested using known drugs testosterone, diclofenac, ketokonazole, nifedipine and beta-estradiol. The results obtained were compared with published results for these compounds [Onofrey et al., 2003]. It was found that aqueous solubility of these compounds was in conformance with these published values (Table - 14). Testosterone and Diclofenac were highly soluble compounds, whereas ketokonazole and β-estradiol were poorly soluble compounds. One compound, Nifedipine, showed higher solubility than reported by other methods, due to the effect of DMSO. Further, it was found that this assay was fully automatable and can be performed in the plate format. Some limitations of the assay were that test compounds and standards must remain in solution over the duration of the assay, impure compounds give faulty results, and compounds need to have UV spectroscopic absorbance.

Table - 14

Solubility of some known drugs

S.No.	Drugs	Solubility (µM)	
		Experimental	Reported
1	Testosterone	445.9 ± 17.4	365
2	Diclofenac	551.5 ± 17.2	500
3	Ketoconazole	134.6 ± 13.7	141
4	Nifedipine	223.9 ± 34.1	380
5	Beta-estradiol	51.8 ± 9.9	34

The in-house solubility was measured by UV spectroscopy method as described in Material and Methods section. The experimental data shown are mean ± SEM of three independent experiments. The reported values were from published data. Data shows that the experimental values are comparable with the reported solubility values.

Using the standardized solubility assay, the solubility of betulinic acid and short listed derivatives was determined. The analysis of betulinic acid with UV detection has been described [Bae, 1996], but this method has poor sensitivity (LOQ < 3 µg/mL) and cannot be used to measure trace amounts of betulinic acid. The short listed derivatives however had better sensitivity (LOQ = 0.1 µg/mL). The solubility of betulinic acid and short listed derivatives was in the range of 9.9 to 203.5 µM (i.e. < 100 µg/ml). The thermodynamic solubility, using the traditional shake-flask method, however, could not be determined as they were below the Limit of Quantitation (LOQ) (Table - 15). These results are in conformance with solubility predictions made by PreADME software. With these experiments it was clear that these compounds were poorly water soluble, but solubility could be enhanced by the addition of amphoteric solvents like DMSO. Suitable formulation approaches need to be developed to make them clinically useful.

Table - 15

Solubility of betulinic acid and short-listed derivatives

S.No.	Compound	Solubility at pH 7.4 (µM) (by shake-flask method)	Solubility (µM) at pH 7.4 (by precipitation method)
1	Betulinic acid	< LOQ	<LOQ
2	1098	< LOQ	9.9
3	807	< LOQ	17.4
4	829	< LOQ	124.7
5	937	< LOQ	203.5
6	1065	< LOQ	102.3

LOQ (Limit of Quantitation = 0.1 µg/mL).

3.4.3 Permeability studies

The permeability of betulinic acid and short listed compounds was determined using the parallel artificial membrane permeability assay (PAMPA) as described in the Methods. This assay measures the transport of test compounds across a lipid layer that mimics the intestinal lipid bi-layer and determines the passive, transcellular compound permeability over a period

of 16 to 18 hrs. The assay was standardized by measuring the permeability of known drugs and comparing with published values [Kansy et al., 2001]. Based on the amount of drug in the lower compartment of the filter, the log of the effective permeability, log Pe, was calculated. The log Pe values of the known drugs were in conformance with the published data (Table - 16) [Schmidt et al., 2003]. The PAMPA assays were found to be robust, reproducible automation compatible assays and relatively fast. The results correlated with human drug absorption values from published methods. The PAMPA assay provides the benefits of a more biologically relevant system. It is also possible to tailor the lipophilic constituents so that they mimic specific membranes such as the blood–brain barrier. Optimization of incubation time, lipid mixture and lipid concentration will also enhance the assay's ability to predict compound permeability.

Table - 16

Permeability of known drugs

S.No.	Drugs	Permeability (log Pe)	
		Experimental	Reported
1	Testosterone	-4.893 ± 0.18	-4.8
2	Propranolol	-5.103 ± 0.04	-5.0
3	Methotrexate	-6.593 ± 0.12	-7.2

The experimental data shown are mean ± SEM of three independent experiments. The reported values were from published data.

The permeability of betulinic acid and short listed compounds was evaluated using above validated PAMPA assay. For these compounds, the donor plate concentration was reduced to 100 μM after initial testing revealed their marginal solubility at the higher concentration. It was found that the short listed compounds had moderate to poor permeability (Log Pe < -5.0) (Table - 17). Betulinic acid could not be detected using this assay since values were less than LOQ (3 μg/mL). Log P_e values obtained for the lower permeability compounds 937 and 1098 were more variable than those obtained for the other drugs due to analytical imprecision associated with being at or near the LOQ(around 1 μg/mL). Based on permeability the short listed compounds may be ranked as given below:

Permeability ranking of short listed derivatives: 1065 > 807 > 829 > 937 > 1098

Table - 17

Permeability of betulinic acid and short-listed derivatives

S.No.	Compound	Permeability (%)	Log Pe	Remarks
1	1065	32.7 ± 5.4	-5.33 ± 0.06	Moderate
2	807	18.6 ± 2.1	-5.61 ± 0.13	Low
3	829	15.6 ± 3.3	-5.70 ± 0.05	Low
4	937	7.0 ± 1.2	-6.06 ± 0.68	Low
5	1098	4.9 ± 1.7	-6.22 ± 0.59	Low

Data shown are mean ± SEM of three independent experiments.

3.4.4 Metabolic stability screening

The first pass through the liver is a significant source for inactivation of therapeutic agents. Using human liver microsomes, we can assess a drug's metabolic stability. This allows for early *in vitro* derived insight into a potential drug's duration of action and metabolic fate.

The metabolic stability assay was standardized by studying the metabolic stability of known drugs Phenacetin, Diclofenac and Diazepam using pooled liver microsome preparations comprising of a pool of 22 human livers. The percentage of drug remaining un-metabolized is given in Table - 18. These values were compared with reported values (Charles River Laboratories, 2003). It was seen that the observed values were in conformance with the reported values.

Table - 18

Metabolic stability of known drugs

S.No.	Drugs	Percentage un-metabolized (%)	
		Experimental (Human microsomes)	**Reported** (Rat microsomes)
1	Phenacetin	55.3 ± 5.1	75
2	Diclofenac	54.1 ± 7.3	60
3	Diazepam	53.3 ± 12.1	35

The metabolic stability of the compounds was determined using human liver microsomes as described in Material and Methods section. The experimental data shown are mean ± SEM of three independent experiments. The reported values were from published data [Charles River Laboratories, 2003].

The fate of betulinic acid and short listed derivatives after incubation with pooled human liver microsomes for 60 min in the presence of cofactor NADPH is shown in Figure - 25. More than 90% of the parent compounds remained un-metabolized in the case of 1098, 937 and 1065 as measured by monitoring the area of the parent peak using HPLC. Compound 807 metabolized to some extent (about 15%), while betulinic acid and 829 metabolized by more than 60%. No metabolites were detected in the adopted HPLC method. This clearly shows that betulinic acid and 807 do not have enough metabolic stability to be developed for clinical use. The other compounds are more stable and may be expected to remain in plasma and tissues and elicit good activity for a longer time. The fate of these compounds, however, needs to be determined by performing a complete pharmacokinetic and elimination study, so as to ascertain their elimination pathway.

3.4.5 Plasma protein binding studies

The plasma protein binding experiments were carried out by the ultra-filtration method across a 10-kD membrane as describe in Methods. The method was first tested by studying the plasma protein binding of known drugs. Known drugs Methotrexate, Testosterone and Paclitaxel were tested for protein binding (PPB) to rat plasma using the ultra filtration method. The PPB values observed (Table-19) are in conformance to published data done by other methods (Goodman LS et al., 2001).

Using this standardized protein binding assay, the plasma protein binding of betulinic acid and derivatives was studied. Compounds 937, 1065, 1098 and 807 show high protein binding (>98%) (Table - 20). Compound 829 was not measured due to poor recovery. The high protein binding of 937, 1065, 1098 and 807 shows that the free drug concentration of these compounds will be less, therefore high doses need to be administered for sustained activity.

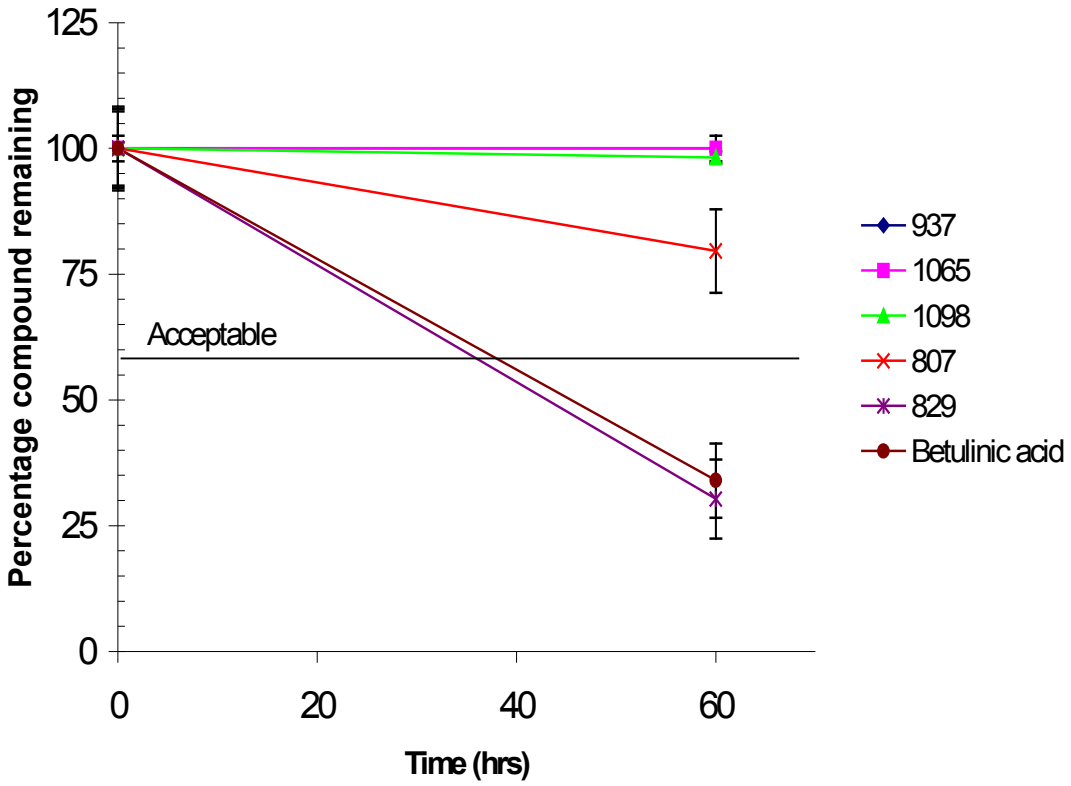


Figure - 25

Metabolic stability of betulinic acid and short-listed derivatives

The data shown are mean ± SEM of three independent experiments. Compounds 937, 1098, 807 and 937 were metabolically stable as compared to betulinic acid, $p < 0.01$.

Table - 19

Plasma protein binding of known drugs

S.No.	Drug	% Protein binding	
		Experimental	Reported
1	Methotrexate	35.0 ± 7.8	34-57
2	Testosterone	85.5 ± 10.2	85-95
3	Paclitaxel	96.1 ± 9.5	88-98

The protein binding of the compounds was determined as described in Material and Methods section. The experimental data was reported in Goodman LS et al., 2001. Experimental PPB data are mean ± SEM of three independent experiments. The data shows that the experimental values are comparable with the published data [Goodman et al., 2001].

Table - 20

Percentage plasma protein binding of betulinic acid and short-listed derivatives

S.No.	Compounds	Plasma protein binding (% binding)
1	937	>99
2	1065	>99
3	1098	98.5 ± 8.8
4	807	98.5 ± 15.1
5	829	ND
6	Betulinic acid	>99

Data are mean ± SEM of three independent experiments. ND = not done, due to poor recovery.

3.4.6 Cytochrome P450 inhibition studies

3.4.6.1 Effect on the enzyme activity of Cytochrome P450 (CYP) enzymes (HPLC method)

The effect of betulinic acid and short listed derivatives was determined on four most common CYP enzyme isoforms (CYP1A2, CYP2C9, CYP2D6 and CYP3A4). The assay tests the effect of a test compound when incubated along with CYP enzyme isoforms and the substrate as illustrated by the effect on CYP2C9 on diclofenac (Figure - 26). Diclofenac is metabolized by CYP2C9 to 4-hydroxy-diclofenac. Positive inhibitors (eg. Sulfaphenazole) stop or reduce the metabolism of the substrate.

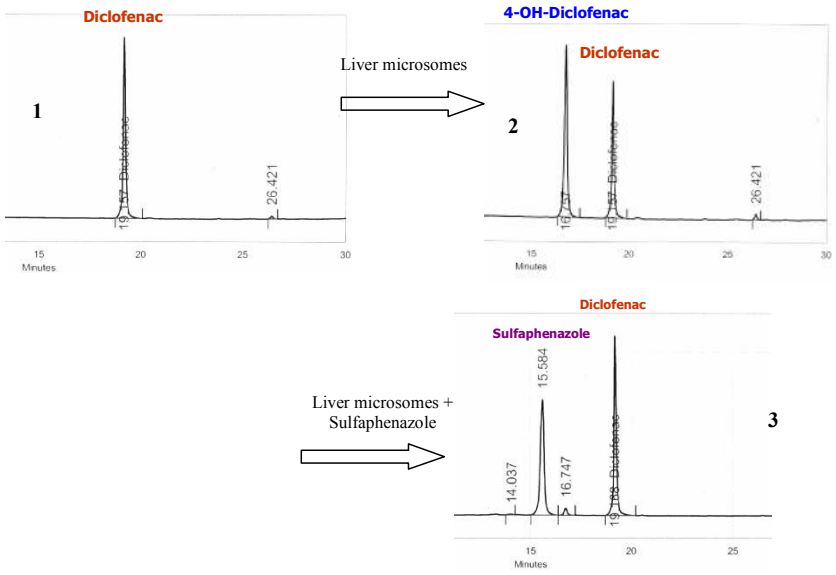


Figure - 26

Representative HPLC chromatogram of CYP2C9 reaction

1: Diclofenac, 2: Diclofenac metabolism by liver microsomes, 3: Diclofenac metabolism inhibited by positive inhibitor sulfaphenazole. Diclofenac The retention time of diclofenac was 19.157 min and 4-hydroxy diclofenac was 16.757 min.

Betulinic acid and derivatives were tested for CYP inhibition at 10 µM, as this concentration was known to be clinically relevant for initial studies. If the compounds inhibited CYP enzymes by more than 50%, then the IC_{50} values would be determined. Known inhibitors of CYP enzymes were used as positive control i.e. Ketokonazole (for CYP1A2 and CYP3A4), Sulfaphenazole (for CYP2C9) and Quinidine (for CYP2D6).

Effect on CYP1A2 activity

The effect of betulinic acid and short listed derivatives on CYP1A2 enzyme activity was determined by studying the effect on Phenacetin metabolism by human liver microsomes, characterized for the presence of CYP 1A2 activity. Figure - 27 shows the metabolism of phenacetin (RT = 19.7 min) to acetamidophenol (RT ≈ 4 min) by liver microsomes in different incubations. Figure - 31 shows the percentage inhibition of CYP enzymes, calculated based on area of phenacetin peak calculated in the presence and absence of test compound (10 μM). Compound 1065 inhibited CYP1A2 by 58% which was significantly ($p < 0.01$) more than the other derivatives. None of the other derivatives inhibited CYP1A2 by greater than 50%. The positive inhibitor Ketokonazole inhibited CYP1A2 activity by more than 80% which was very significant ($p < 0.01$) inhibition compared to betulinic acid and derivatives. Further studies need to be carried out to determine the IC_{50} of 1065, if it is selected for further development.

Effect on CYP2C9 activity

The effect of betulinic acid and short listed derivatives on CYP2C9 enzyme activity was determined by studying the effect on Diclofenac metabolism by human liver microsomes, characterized for the presence of CYP 2C9 activity. Figure - 28 shows the metabolism of diclofenac (RT = 17.3 min) to 4-hydroxy diclofenac (RT = 14.8 min) by liver microsomes in different incubations. Figure - 32 shows the percentage inhibition of CYP enzymes, calculated based on the area of diclofenac peak calculated in the presence and absence of test compound (10 μM). Betulinic acid and short listed derivatives did not inhibit CYP2C9 activity. The positive inhibitor Sulfaphenazole inhibited CYP2C9 activity by more than 80% which was very significant ($p < 0.01$) inhibition compared to betulinic acid and derivatives. It was earlier reported that CYP2C9 may be involved in the metabolism of betulinic acid-like compounds [Lewis et al., 1998].

Effect on CYP2D6 activity

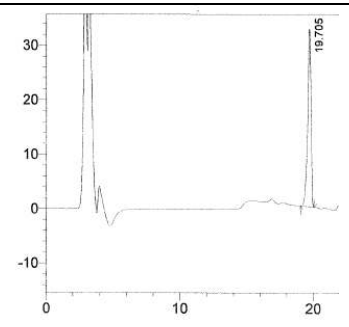
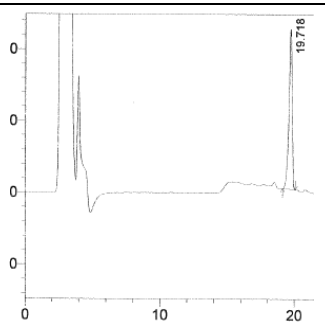
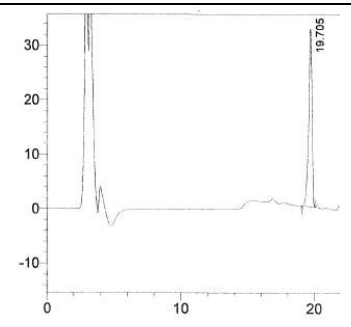
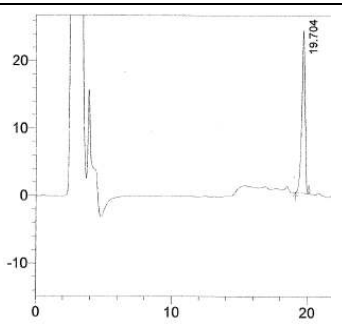
The effect of betulinic acid and short listed derivatives on CYP2D6 enzyme activity was determined by studying the effect on Bufuralol metabolism by human liver microsomes, characterized for the presence of CYP2D6 activity. Figure - 29 shows the metabolism of bufuralol (RT = 21.5 min) to 1-hydroxy bufuralol (RT = 7.3 min) by liver microsomes in different incubations. Figure - 33 shows the percentage inhibition of CYP enzymes, calculated based on area of bufuralol peak calculated in the presence and absence of test

compound (10 μM). Betulinic acid and short listed derivatives inhibited CYP2D6 activity in the range of 35 – 45%, but none of the compounds inhibited CYP2D6 activity by 50%, whereas the positive inhibitor inhibited CYP2D6 activity by about 60% which was significant inhibition ($p < 0.05$) as compared to betulinic acid derivatives. The results show that betulinic acid and derivatives do not inhibit CYP2D6 activity very significantly but may have some effect for which further studies need to be carried out at higher concentrations of test compounds or by testing against different substrates.

Effect on CYP3A4 activity

CYP3A4 is the most commonly implication CYP enzyme in drug interactions. The effect of betulinic acid and short listed derivatives on CYP3A4 enzyme activity was determined by studying the effect on testosterone metabolism by human liver microsomes, characterized for the presence of CYP 3A4 activity. Figure - 30 shows the metabolism of testosterone (RT = 18.9 min) to 6-β-hydroxy testosterone (RT = 13.8 min) by liver microsomes in different incubations. Figure - 34 shows the percentage inhibition of CYP enzymes, calculated based on area of testosterone peak calculated in the presence and absence of test compound (10 μM). Betulinic acid and short listed derivatives inhibited CYP2D6 activity in the range of 20 – 40%, but none of the compounds inhibited CYP2D6 activity by 50%, whereas the positive inhibitor inhibited CYP2D6 activity by about 90% which was very significant inhibition compared to betulinic acid derivatives. The lack of inhibition of CYP3A4 enzyme activity by betulinic acid and short listed compounds shows that these compounds may be given in combination chemotherapy with several known anti-cancer drugs, which are known inhibitors of CYP3A4 enzyme e.g. ifosfamide, etoposide, cyclophosphamide, tamoxifen, docetaxel, teniposide, irinotecan, paclitaxel, vinca alkaloids, retinoic acid etc.

In conclusion, it may be said that betulinic acid and short listed derivatives do not significantly inhibit common CYP enzymes at clinically relevant concentrations. But, there was some inhibition of CYP activity in CYP2D6 by all the test compounds. Similarly, CYP1A2 was inhibited by by 1065. Hence further studies may be carried out for these enzyme systems, before considering betulinic acid and the short listed compounds for combination chemotherapy.

Without cofactor	With cofactor	With cofactor + positive inhibitor	With cofactor + betulinic acid
			

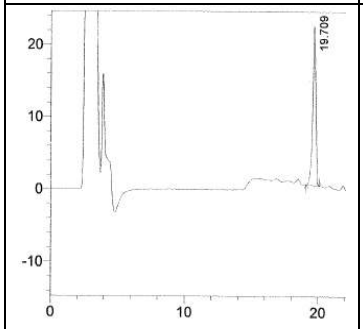
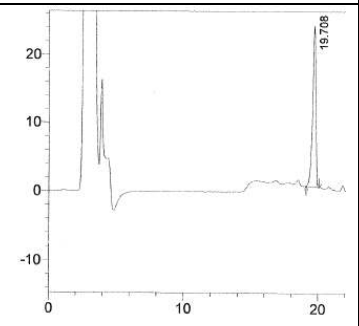
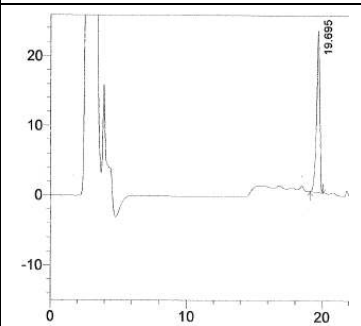
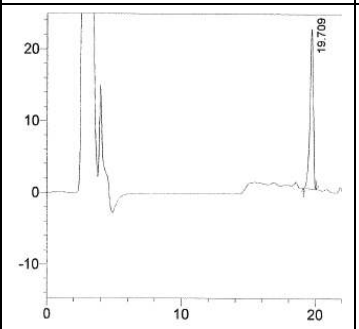
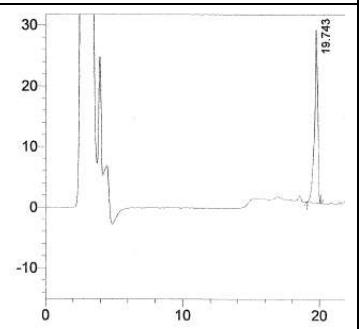
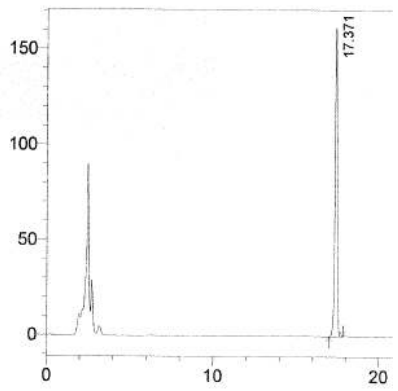
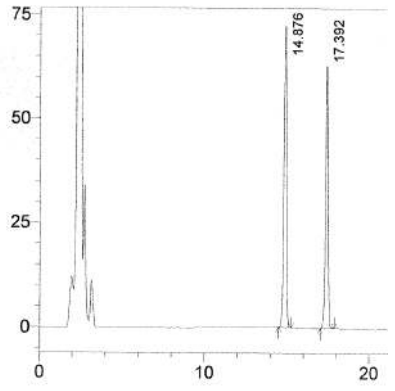
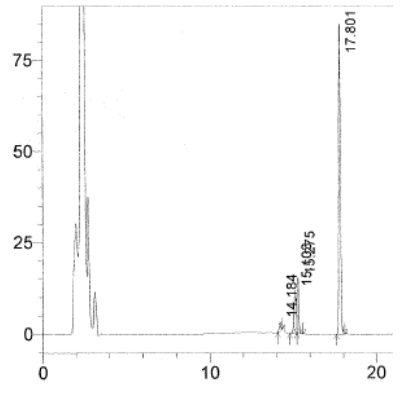
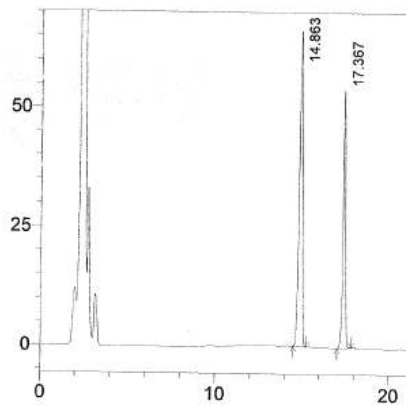
With cofactor + 1098	With cofactor + 937	With cofactor + 807	With cofactor + 829	With cofactor + 1065
				

Figure – 27

HPLC traces showing effect on the metabolism of CYP1A2 substrate Phenacetin by liver microsomes.

Retention times of phenacetin and its metabolite were 20 min and 4 min respectively.

Without cofactor	With cofactor	With cofactor + positive inhibitor	With cofactor + betulinic acid
			

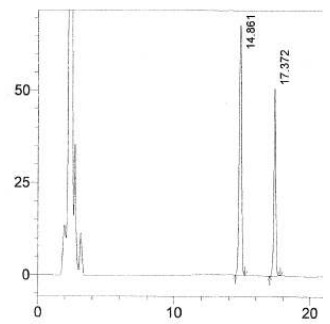
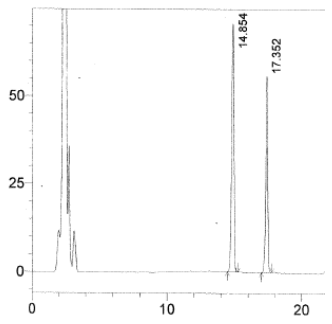
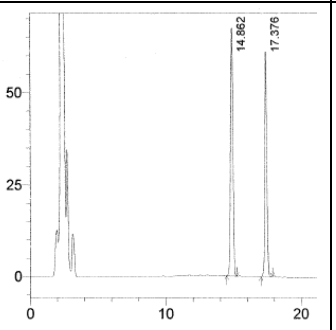
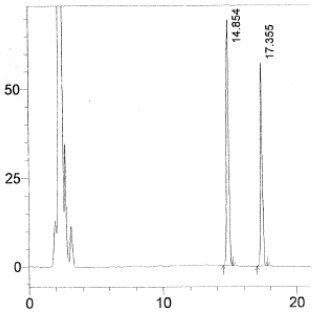
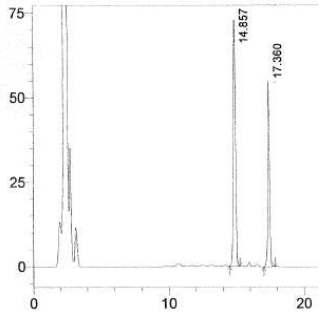
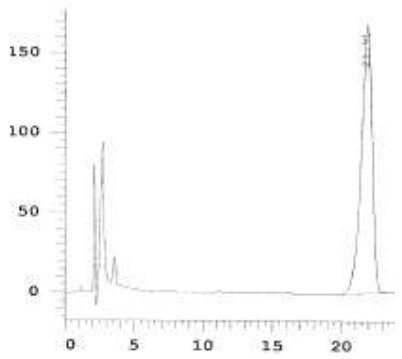
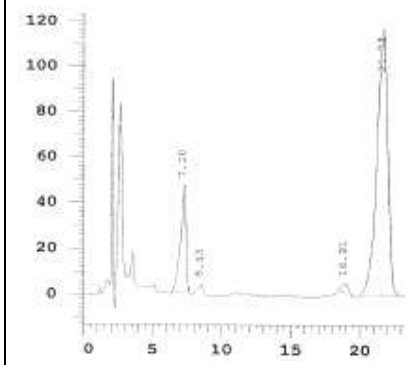
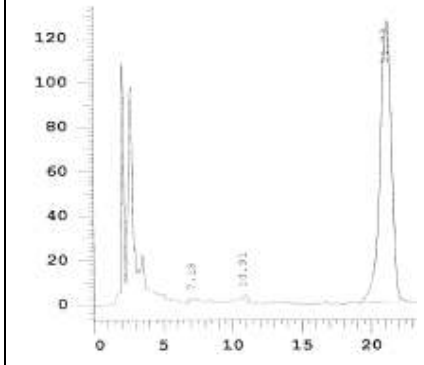
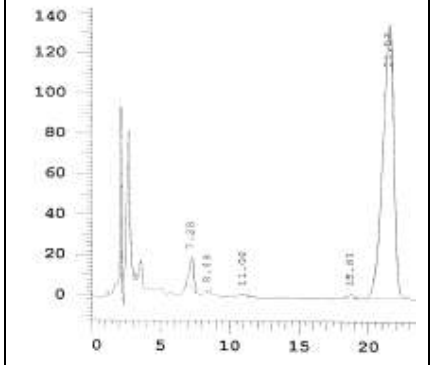
With cofactor + 1098	With cofactor + 937	With cofactor + 807	With cofactor + 829	With cofactor + 1065
				

Figure – 28

HPLC traces showing effect on the metabolism of CYP2C9 substrate Diclofenac by liver microsomes.

Retention times of diclofenac and its metabolite were 17.3 min and 14.8 min respectively.

Without cofactor	With cofactor	With cofactor + positive inhibitor	With cofactor + betulinic acid
			

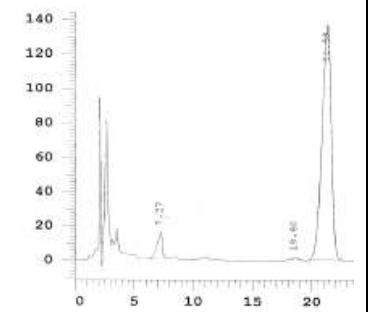
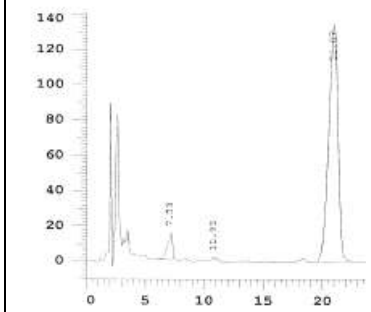
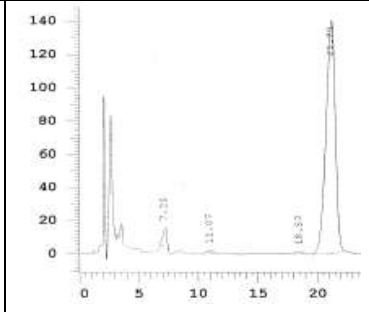
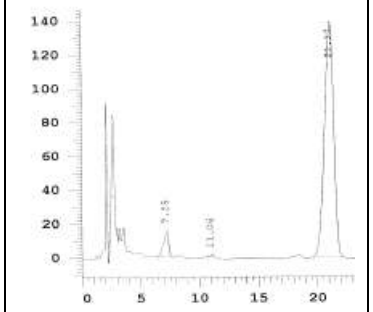
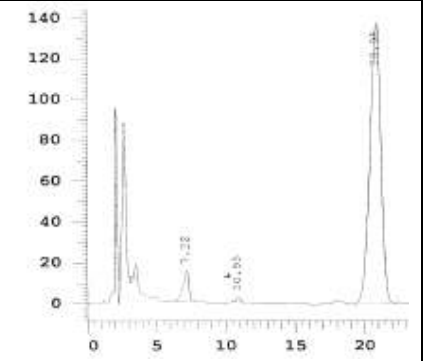
With cofactor + 1098	With cofactor + 937	With cofactor + 807	With cofactor + 829	With cofactor + 1065
				

Figure – 29

HPLC traces showing effect on the metabolism of CYP2D6 substrate Bufuralol by liver microsomes.

Retention times of bufuralol and its metabolite were 21.5 min and 7.3 min respectively

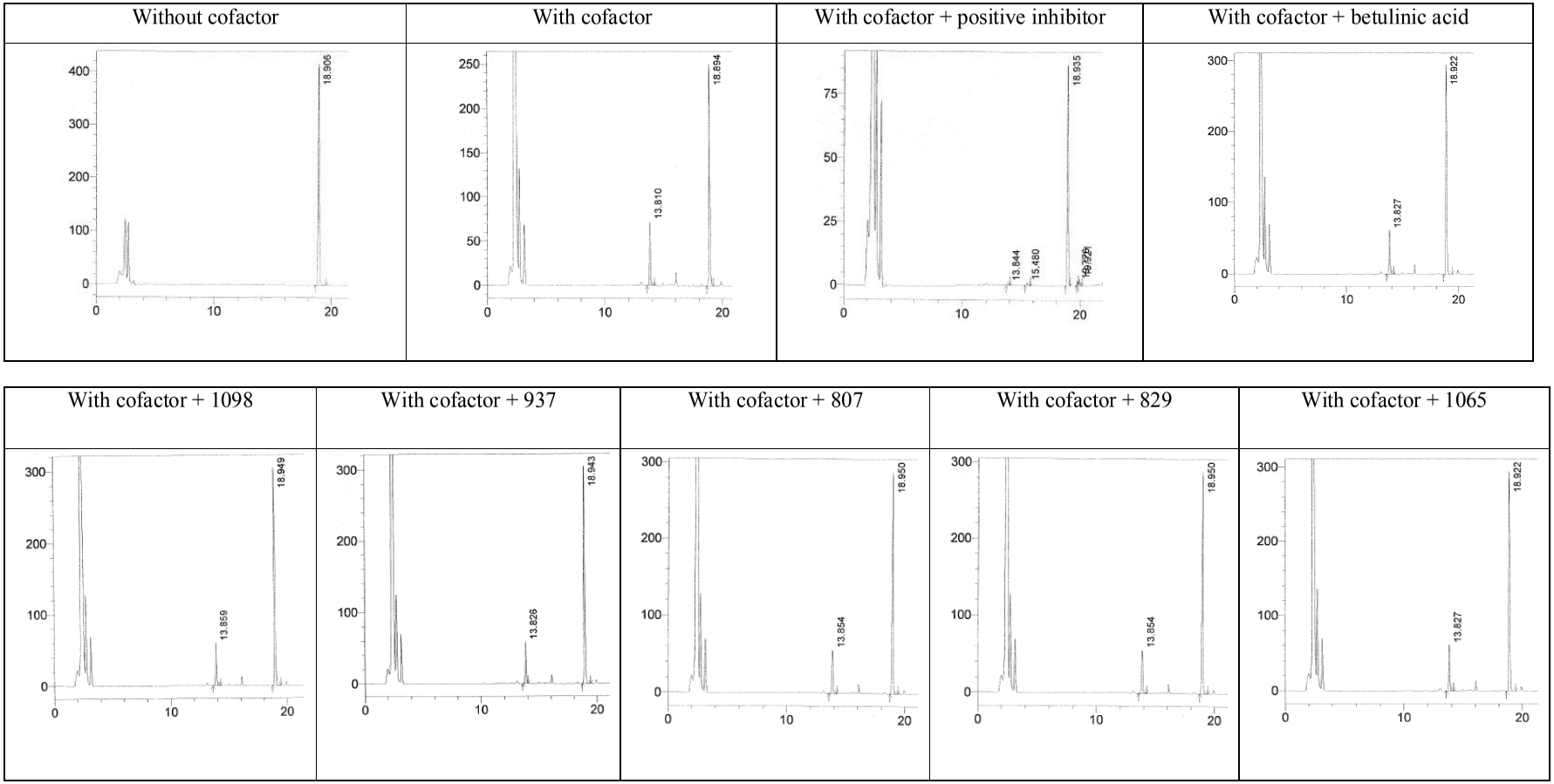


Figure – 30

HPLC traces showing effect on the metabolism of CYP3A4 substrate Testosterone by liver microsomes.

Retention times of testosterone and its metabolite were 18.8 min and 13.8 min respectively.

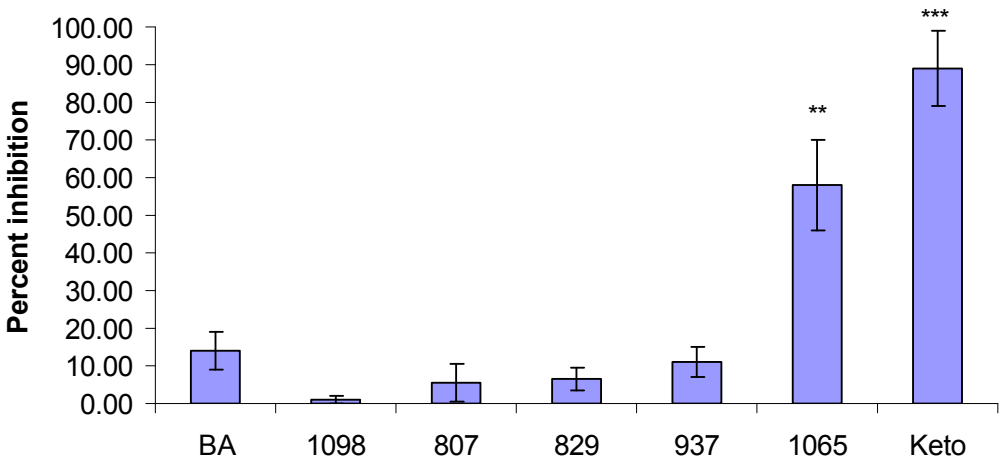


Figure - 31

Inhibition of Cytochrome P450 1A2 isoform enzyme

The ability of compounds at 10 µM to inhibit Phenacetin metabolism by human liver microsomes was analyzed by HPLC. Data shown are mean ± SEM of three independent experiments. Keto (Ketokonazole) was used as positive inhibitor. *** and ** represents $p < 0.01$ and $p < 0.001$ when compared with others in the study. BA = betulinic acid

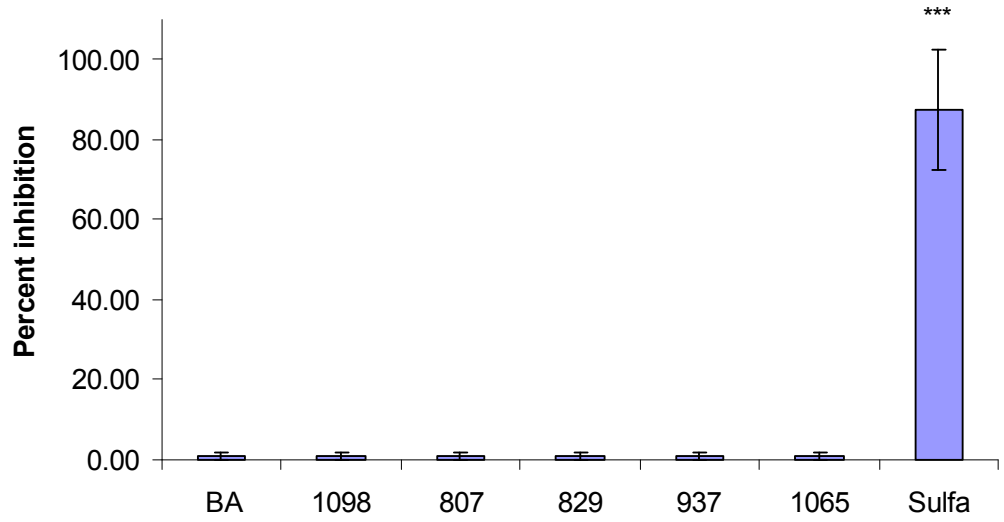


Figure - 32

Inhibition of Cytochrome P450 2C9 isoform enzyme

The ability of compounds at 10 µM to inhibit Diclofenac metabolism by human liver microsomes was analyzed by HPLC. Data shown are mean ± SEM of three independent experiments. Sulfa (Sulfaphenazole) was used as positive inhibitor. *** represents $p < 0.001$ when compared with others in the study. BA = betulinic acid

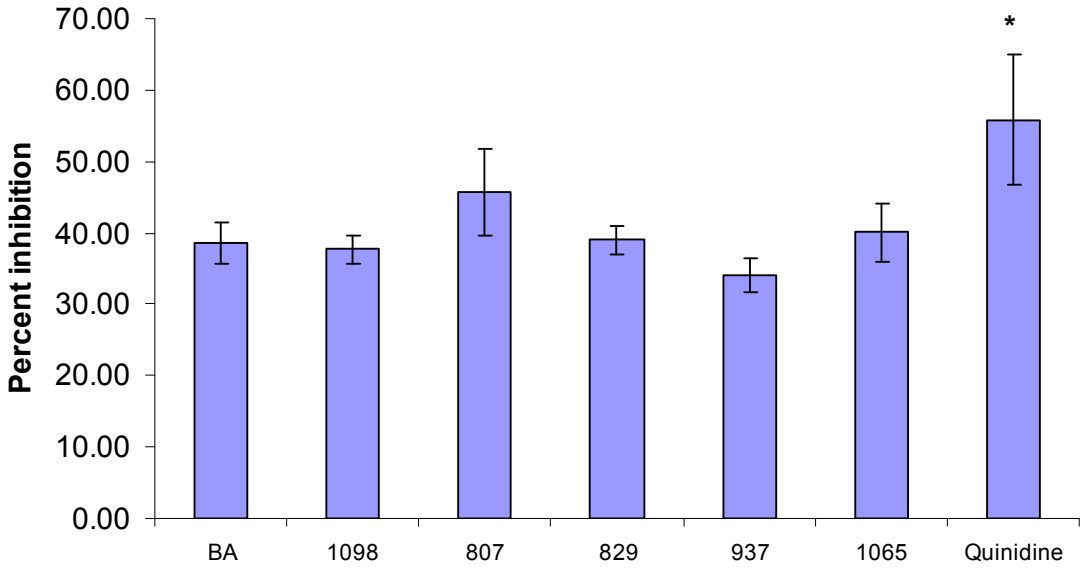


Figure - 33

Inhibition of Cytochrome P450 2D6 isoform enzyme

The ability of compounds at 10 µM to inhibit Bufuralol metabolism by human liver microsomes was analyzed by HPLC. Data shown are mean ± SEM of three independent experiments. Quinidine was used as positive inhibitor. * represents $p < 0.05$ when compared with others in the study. BA = betulinic acid

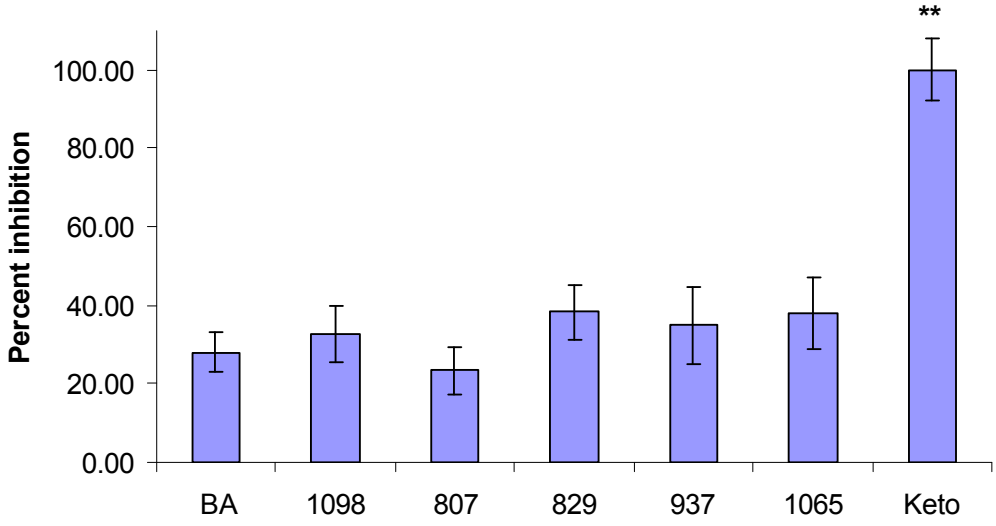


Figure - 34

Inhibition of Cytochrome P450 3A4 isoform enzyme

The ability of compounds at 10 µM to inhibit Testosterone metabolism by human liver microsomes was analyzed by HPLC. Data shown are mean ± SEM of three independent experiments. (Keto) Ketokonazole was used as positive inhibitor. ** represents $p < 0.01$ when compared with others in the study. BA = betulinic acid

3.4.6.2 Effect on the enzyme activity of Cytochrome P450 (Fluorescence method)

CYP inhibition assays based on metabolism of fluorescent substrates using a commercially available kit [CYP450 inhibition assay, GENTEST, USA] in the 96-well format was standardized. This assay enables high throughput estimation of cytochrome P450 inhibition studies. It is based on measuring the decrease in fluorescence of the substrate using a fluorescence spectrophotometer. Betulinic acid and derivatives were tested for CYP inhibition of CYP2C9, CYP2D6 and CYP3A4 enzymes using this assay at a concentration of 2.5 µM, since higher concentrations could not be tested in this assay due to solubility problems.

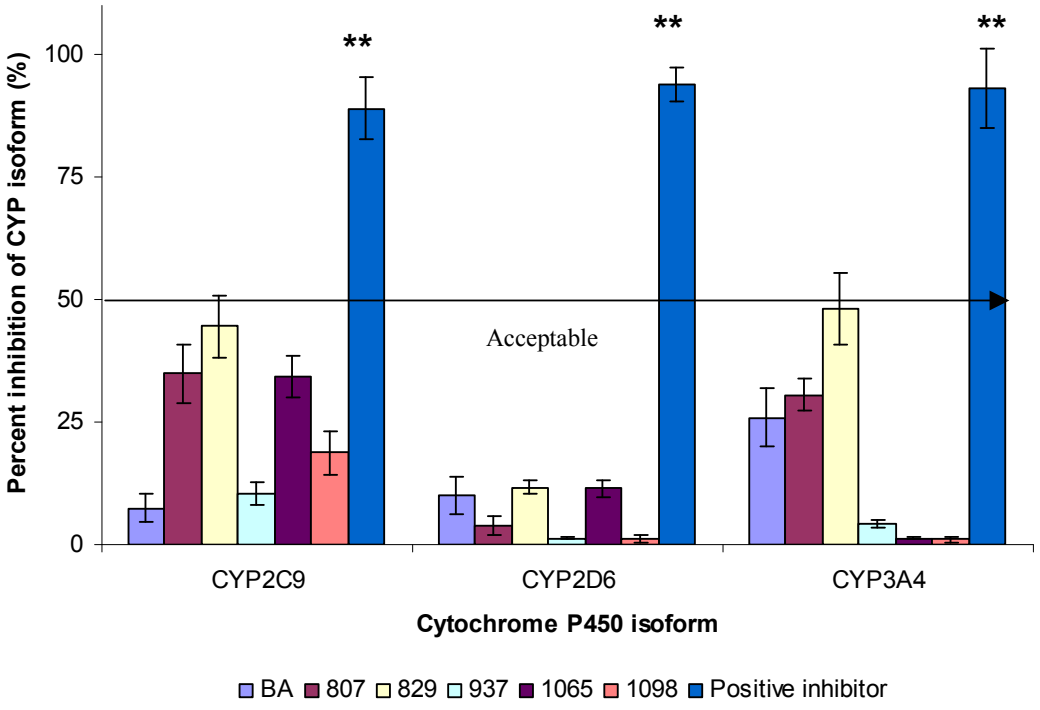


Figure - 35

Inhibition of Cytochrome P450 (CYP450) betulinic acid and short-listed derivatives

Data shown are mean ± SEM of three independent experiments. Sulfaphenazole (2C9), Quinidine (2D6) and Ketokonazole (3A4) were positive inhibitors. Higher concentrations could not be tested as the drugs were insoluble in this assay system. Known drugs were tested at 0.1 - 1 µM.

Betulinic acid and short listed compounds inhibited CYP enzymes by less than 50% (Figure – 35). Therefore, the IC_{50} values could not be calculated. The results were comparable to the CYP inhibition assays carried out using non-fluorescent assays by HPLC method, in that none of the compounds had any significant effect on each of these CYP isoforms i.e.

CYP2C9, CYP2D6 and CYP3A4. However, the positive controls used for each enzyme very significantly ($p < 0.01$) inhibited the respective CYP enzyme. The inhibition was seen to be greater than 90% at less than 1 μM. However, the percent inhibition of the enzyme activity by certain short-listed derivatives of betulinic needs to be addressed. Dose response studies need to be carried out with different concentrations, so as to rule any effect of these key CYP enzymes.

3.4.7 Pharmacokinetics of short-listed derivative 1098 in rats

The fate of the most potent short-listed derivative 1098 was tested by carrying out a pharmacokinetic study in rats ($n = 4$) each. The study was done to determine the absorption, distribution, metabolism and elimination profile. 1098 were administered by both oral and intravenous routes.

Intravenous dosing

The plasma concentration profile of 1098 is shown in Figure - 36. Non-compartmental analysis of 1098 data using WinNonlin v5.0.1 (Table - 21) revealed lengthy elimination half-life of 10.3 hrs. The extrapolated concentration at 0 min was 101.5 μg/mL, which was well above the required cytotoxic concentration of around 5 μg/ml. The AUC was 43.6 hr.μg/mL, volume of distribution (V_d) and clearance values (CL) values were 760.3 mL and 49.9 mL/hr respectively.

Oral dosing

Plasma levels of 1098 were below detection limits at all time points measured following oral administration at a dose of 150 mg/kg. The LOQ was 0.3 μg/mL. This shows the poor intestinal absorption of this compound.

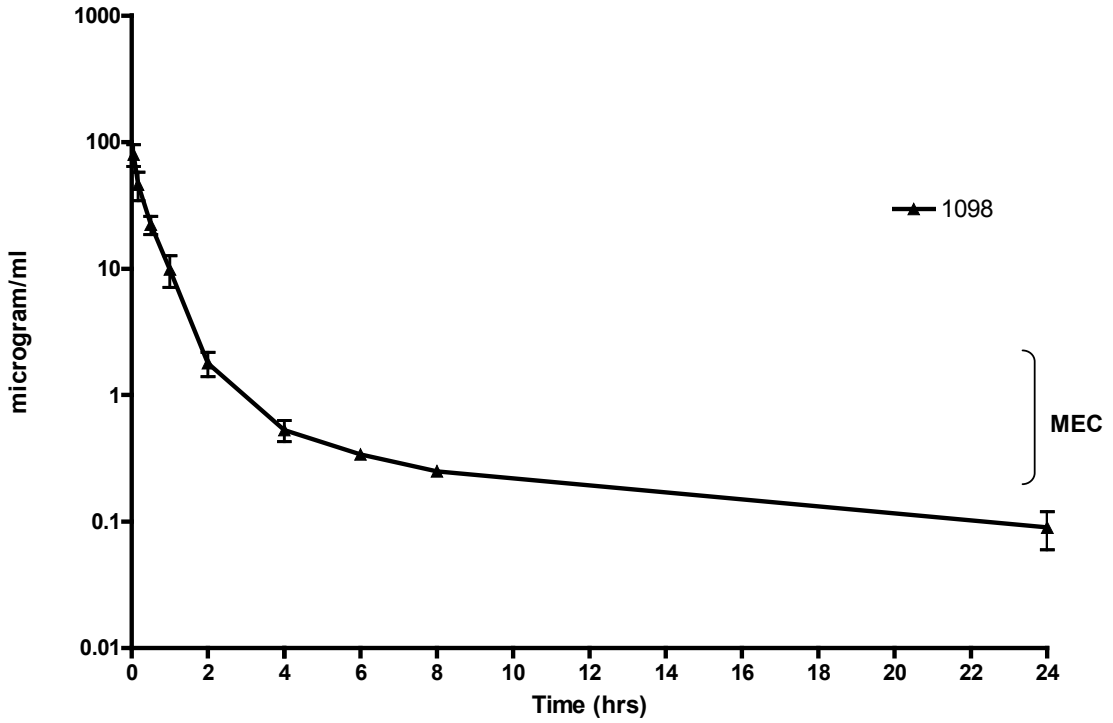


Figure - 36

Plasma concentration profile of 1098

Data shown is mean ± SD (n=4). Rats were dosed at 10 mg/kg intravenously and plasma concentration (µg/ml) was determined by HPLC as described in Methods.

Table - 21

Pharmacokinetic parameters of 1098 in rats (n=4)

Terminal half-life (hr)	T_{max} (hr)	C_0 (µg/mL)	**AUC** (hr.µg/mL)	V_d (mL)	**CL** (ml/hr)
10.3 ± 2.2	0.05 ± 0.0	101.5 ± 21.7	43.6 ± 6.3	760.3 ± 294.2	49.9 ± 8.9

Data shown is mean ± SD, n=4. The plasma time concentration data was analyzed using Winnonlin v5.0.1 using non-compartmental model.

3.4.8 Preliminary Toxicity of betulinic acid and short-listed derivatives

The toxicity of betulinic acid and short listed derivatives was tested in a modified acute toxicity model. Since LD_{50} determinations, are not longer practiced due to ethical issues, minimum lethal concentration (MLD) was used to assess the toxicity. The test compounds were formulated in co-solvents and administered to the six mice per group per dose intravenously at doses in the range of 10 mg/kg up to a maximum of 200 mg/kg. The results have been summarized in Table-22.

The vehicle treated animals did not show any toxic signs or symptoms and did not cause any mortality when administered at a volume equivalent to the highest dose tested i.e. 200 mg/kg i.v. All the treated animals experienced sedation and somnolence immediately after injection, but recovered thereafter. No mortality was recorded even at 200 mg/kg in the betulinic acid and 1065 treated groups. At least one mortality was seen in 1098 treated group at 150 mg/kg), 937 treated group at 200 mg/kg, 807 treated group at 50 mg/kg and 829 treated group at 100 mg/kg. However, no abnormal salivation, piloerection or skin irritation was observed after administration of any of surviving animals in any treatment group.

Table - 22

Toxicity of betulinic acid and short-listed derivatives in mice

S. No	Compound	Minimum Lethal Dose (MLD)	Clinical findings
1	937	200 mg/kg	Mild to moderate sedation, mortality at 200 mg/kg
2	1098	150 mg/kg	Mild to moderate sedation, mortality at 150 mg/kg
3	1065	>200 mg/kg	Mild to moderate sedation, no mortality
4	807	50 mg/kg	Mild to moderate sedation, mortality at 50 mg/kg
5	829	100 mg/kg	Mild to moderate sedation, mortality at 100 mg/kg
6	Betulinic Acid	>200 mg/kg	Mild to moderate sedation,, no mortality
7	Vehicle	>200 mg/kg	No significant effect, no mortality

All doses were given by intravenous route. The highest dose tested was 200 mg/kg. Higher doses could not be administered due to increased volume. If mortality was not recorded it is shown as > 200 mg/kg. Vehicle was administered at the volume equivalent to 200 mg/kg.

3.5 CONCLUSION

Based on anti-cancer and anti-angiogenic potential, five betulinic acid derivatives were selected for further pre-clinical development work. In order to select the best derivative (s) for further development, a preliminary ADMET comparison of these compounds was carried out. Betulinic acid was also included in all the studies, to compare with published reports of ADMET activity, if any. The efforts in this chapter were mainly directed towards setting up rapid ADME screening methods which could be used as filters in the discovery stage so as to reduce 'drop-outs' at later stages of development, which consume more time, money and man power. In this chapter, relevant *in silico* and *in vitro* models were selected, followed by *in vivo* studies.

Betulinic acid and several different classes of derivatives (more than 100) were screened using the predictive software to understand the general ADME characteristics and specific structural requirements, if any, that could be identified for this class of compounds before testing them using experimental models.. It was predicted that betulinic acid and derivatives were poorly soluble compounds. Although some betulinic acid derivatives were predicted to permeate the CaCo-2 barrier and some others were predicted to permeate the MDCK barrier, none of them were predicted to permeate both these cell barriers. Therefore, these compounds were predicted to have moderate to poor intestinal absorption. The partition coefficient values (lop P) of betulinic acid and derivatives was predicted to be greater than 4, i.e. these compounds were highly lipophilic, and were predicted to have the ability to cross the BBB and enter the brain. Betulinic acid and derivatives were predicted to have high protein binding (more than 70%) which may lead to low free drug concentration.

It has been demonstrated that membrane permeability can be predicted for some compounds with reasonable accuracy based solely on physicochemical parameters. It is well established, for instance, that efficient oral absorption will occur only after drug has dissolved and presented itself to the intestinal mucosal surface from whence it can traverse the epithelium. Dissolution is determined by the highly interdependent influences of aqueous solubility, ionizability (pKa), and lipophilicity (octanol/water log P or log D7.4). [Austel, 1989].

The thermodynamic solubility of betulinic acid and short listed derivatives was found to be less than 0.1 μg/mL, whereas DMSO precipitation method showed increased solubility. These results were in conformance with solubility predictions made by the PreADME™ software as well as published results [Son et al., 1998; Rusmawati et al., 2001]. The potential complications arising from low aqueous solubility are compound precipitation, reduced target specificity and low bioavailability in animal studies.

Based on the transport across a lipid layer that mimics the intestinal lipid bi-layer, the permeability of betulinic acid and short listed compounds were evaluated in a PAMPA assay. It was found that the short listed compounds had moderate to poor permeability (Log Pe < -5.0). Based on permeability the short listed compounds were ranked based on log Pe values as: 1065 > 807 > 829 > 937 > 1098.

As a result of the enormous increase in the number of lead compounds being identifed during drug discovery, information on drug metabolism and in particular, metabolic stability is needed as early as possible to help predict clearance and oral bioavailability [Walker et al., 1994, Kuhnz et al., 1998]. Screening of new chemical entities for susceptibility to metabolic degradation is becoming standard practice during early drug discovery instead of belonging exclusively to the drug development process. Several approaches have been developed to increase the throughput of measuring the metabolic stability of new chemical entities. These include assays using cryopreserved human hepatocytes [Li et al., 1999; Caldwell et al., 1999], liver microsomes in 96-well plates with automated liquid chromatography-mass spectrometry [Korfmacher et al., 1999], or enzymatic incubations (microsomes, cytosol or plasma) with an automated liquid handler linked to HPLC [Linget et al., 1999].

The metabolic fate of betulinic by microbial transformation was previously reported. A series of oxidized and conjugated metabolites of betulinic acid were obtained [Akihisa et al., 2002]. The metabolic stability of betulinic acid and short-listed derivatives was determined using pooled liver microsome preparations comprising of a pool of 22 human livers. It was found that more than 90% of the parent compounds remained un-metabolized in the case of 1098, 937 and 1065. Compound 807 metabolized to some extent (about 15%), while betulinic acid and 829 metabolized by more than 60%. These results provided a filter for the selection of 1098, 937 and 1065 based on their better metabolic stability.

By studying the plasma protein binding of betulinic acid and short listed derivatives by the ultra-filtration method across a 10-kD membrane, most of the compounds 937, 1065, 1098 and 807, barring 829 which had poor recovery from plasma, exhibited high protein binding (>98%).

Since combination chemotherapy is the standard in anti-cancer treatment, the importance of drug-drug interactions is significant. Cytochromes P450 are the principal enzymes for the oxidative metabolism of drugs and other xenobiotics. Among the xenobiotic-metabolizing cytochromes P450, five forms, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 appear to be most commonly responsible for the metabolism of drugs. Inhibition of cytochrome P450-mediated metabolism is often the mechanism for drug-drug interactions. The potential for enzyme inhibition is routinely assessed by performing *in vitro* inhibition studies using cDNA-expressed enzymes or human liver microsomes. These types of studies are becoming a routine part of the drug registration data package.

The effect of betulinic acid and short listed derivatives was determined on four most common CYP enzyme isoforms (CYP1A2, CYP2C9, CYP2D6 and CYP3A4) at the therapeutically active concentration of 10 μM. The test compounds were assessed by their ability to inhibit the CYP enzyme activity leading to substrate conversion. Compound 1065 inhibited CYP1A2 by 58%. None of the other molecules inhibited CYP1A2 by greater than 50%. Betulinic acid and short listed derivatives did not inhibit CYP2C9 activity. It was earlier reported that CYP2C9 may be involved in the metabolism of betulinic acid-like compounds [Lewis et al., 1998]. But our findings do not support any interaction with CYP2C9 enzyme. Betulinic acid and short listed derivatives inhibited CYP2D6 activity in the range of 35 – 45%, but none of the compounds inhibited CYP2D6 activity by 50%. The results show that these compounds may have some effect on CP2D6 enzyme activity. Betulinic acid and short listed derivatives inhibited CYP3A4 activity in the range of 20 – 40%, but none of the compounds inhibited the enzyme activity by more than 50%. In conclusion, it may be said that although betulinic acid and short listed derivatives do not have any significant effect on CYP enzymes at clinically relevant concentrations, further studies may be carried out for these enzyme systems, before considering betulinic acid and the short listed compounds for combination chemotherapy The results were reproduced in a CYP inhibition assays using fluorescent substrates.

Betulinic acid was reported to be safe at doses up to 500 mg/kg in rats when administered intra-peritoneally [Sandberg et al., 1987]. The toxicity of betulinic acid and short listed derivatives was tested in a modified acute toxicity model. Since LD_{50} determinations, are no longer practiced due to ethical issues, minimum lethal concentration (MLD) was used to assess the toxicity. All the treated animals experienced sedation and somnolence immediately after injection, but recovered thereafter. No mortality was recorded even at 200 mg/kg in the betulinic acid, as reported earlier. No mortality was seen in the 1065 treated group. At least one mortality was seen in 1098 treated group at 150 mg/kg, 937 treated group at 200 mg/kg, 807 treated group at 50 mg/kg and 829 treated group at 100 mg/kg. However, no abnormal salivation, piloerection or skin irritation was observed after administration of any of surviving animals in any treatment group.

The pharmacokinetics of betulinic acid was reported previously [Udeani et al., 1999]. It was reported that betulinic acid had long elimination half life and high apparent volume of distribution with substantial accumulation in fatty tissues. The pharmacokinetics kinetics of 1098 was carried out in rats by intravenous route at a dose of 10 mg/kg. Additionally pharmacokinetics was carried out by the oral route at 150 mg/kg. Non-compartmental analysis of 1098 data using WinNonlin v5.0.1 (Table-23) revealed lengthy elimination half-life (10.3 hrs), high initial plasma concentration (C_0 =101.5 µg/mL) and maintenance of minimum effective concentration (1 µg/ml) for longer time periods. The AUC was 43.6 hr.µg/mL, volume of distribution (V_d) and clearance values (CL) values were 760.3 mL and 49.9 mL/hr respectively. Plasma levels were below detection limits following oral administration at a dose of 150 mg/kg. This shows the poor intestinal absorption of 1098, nevertheless the derivative was found to have favorable characteristics of a systemically administered drug based.

Betulinic acid and short listed compounds were found to have poor solubility and permeability. Some short listed compounds had better metabolic stability as compared to betulinic acid. All the compounds exhibited high protein binding and none significantly inhibited any of the major CYP enzymes. The pharmacokinetics of the most potent compound 1098 was found to have favorable characteristics of a systemically administered drug based. It was found that the short listed compounds were more toxic to mice as compared to betulinic acid.

Based on the above findings, compounds 807 and 829 had poor metabolic stability, and were removed from further evaluation. Compound 1065 was the only short listed compound that caused any significant CYP inhibition, inhibiting CYP1A2 by more than 50%. Since drug interactions play a very important role in cancer treatment when administered in combination chemotherapy, 1065 was removed at this stage. Compounds 1098 and 937 had more or less comparable ADMET characteristics. Both compounds were metabolically stable, did not inhibit any CYP enzymes, and had high protein binding, poor solubility and permeability. They were almost equally toxic to mice at doses between 150 -200 mg/kg. Among the two compounds, 1098 was selected for further studies based on its better efficacy data in cytotoxicity and proven in vivo anti-tumor activity.

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CHAPTER - 4

DISCUSSION

In terms of cancer treatment, there are serious limitations in chemotherapy, namely the lack of selectivity of active ingredients and the development of resistance by cancer cells to these chemicals [Setzer et al., 2003]. Current chemotherapeutic agents destroy both cancerous and non-cancerous cells. Thus, there is an urgent need to find new chemical agents that can differentiate between normal and cancerous cells in order to selectively kill the cancerous cells and drug resistant tumor with reduced toxicity [Tian et al., 2006].

Betulinic acid is a remarkable natural product. It is obtained from a variety of botanical sources and can be easily prepared from the more plentiful precursor betulin in a simple two-step process. Betulinic acid and many of its derivatives exhibit great promise as bioactive agents for the treatment of HIV infection and cancer. Betulinic acid has shown great promise as an agent for the treatment of multiple forms of cancer. The anti-melanoma activity of betulinic acid was first discovered in 1995 at the University of Illinois at Chicago as part of a National Collaborative Drug Discovery Group supported by the National Cancer Institute. Remarkably, betulinic acid exhibits little or no in vivo toxicity at very high doses. Mechanistic studies have provided a great deal of information as to the mode of action of betulinic acid; however, further research is needed to validate its activity in humans [Cichewicz et al., 2004]. The National Cancer Institute (NCI, Bethesda, USA) has taken up betulinic acid in its RAID (Rapid Access to Intervention and Development) program for the clinical development for topical & systemic treatment of melanoma. The FDA has approved 3, 3 - dimethyl succinyl derivative (PA-457, Panacos Pharmaceuticals, USA) in Jan 2005. Currently the molecule is in Phase II trial as anti-HIV. It is given orally once-a-day as single dose of 200 mg. It has a half life of 2-3 days and no dose-limiting toxicities have been identified. The final FDA approval is expected in 2008.

4.1 General conclusions

Anti-cancer activity of betulinic acid and derivatives

Previous investigation demonstrated that simple modifications of the parent structure of betulinic acid can produce a number of potentially important derivatives, which may improve the selective toxicity profile or introduce general toxic effects. However, results from a more extensive investigation using a greater number of derivatives was required for structure activity relationship (SAR) study for the design and ultimate synthesis of a more effective

betulinic acid-derived anti-tumor agent. Limited derivatives of betulinic acid were earlier reported but role of derivatization could not be deduced. Ketone, oxime and benzyl derivatives at C-3 lost activity while acetyl derivative retained activity. It was suggested that additional studies needed to be carried out to determine the structure activity relationship [Kim et al., 1998a]. Further it was found that the C-20 side chain was not useful for structural modifications except that C-20 hydrogenation does not adversely affect cytotoxicity [Kim et al., 2001a]. The C-28 carboxylic acid was found to be essential for preserving cytotoxicity. Some amino acid conjugates were found to retain activity and increase solubility, but glucoside conjugates did not have any activity. The C-28 position was found to have a variety of structural constraints [Kim et al., 1998a; Jeong et al., 1999; Hata et al., 2002]. Few other derivatives i.e. hydroxylation at 6,7, and 23 positions either had same activity or reduced activity as compared to betulinic acid [Kouzi et al., 2000; Ye et al., 2001].

In this thesis, about five hundred (500) novel betulinic acid or dihydro-betulinic acid derivatives with modifications in C_2, C_3, C_{20}, and C_{28} positions were screened for cytotoxicity. Out of these, one hundred eight two (182) derivatives were selected from the primary screening and tested in a panel of cell lines in the secondary screening. Among these, (twenty four) 24 derivatives were better than betulinic acid in terms of mean cytotoxicity to the panel of sensitive cell lines. Based on specificity to cancer cells, derivatives 1098, 937, 1065, 829 and 807 which had cancer specificity to three or more cell lines were selected for further studies. It was found that modification of C-3 hydroxyl to keto, oxime, o-acyl, o-benzoyl, benzyloxime, benzylidene, phenylhydrazine and o-sulfonylmethane groups significantly enhanced the activity and resulted in more potent derivatives when compared to betulinic acid. Most of derivatives that were better than betulinic acid had hydrogenation of the double bond at C-20 position of betulinic acid. Further, it was found that C-28 carboxylic acid group in betulinic acid and its derivatives was found essential for cytotoxic activity. Compound 1098 (Figure - 37) was identified as the most potent derivative with selectivity to a large number of cancer cell lines.

Betulinic acid was shown to act through induction of apoptosis [Pisha et al., 1995]. In our studies, betulinic acid and short listed derivatives were found to cause apoptosis as seen by the morphological changes of surface blebbing and cell shrinking that are characteristic of apoptosis. Both betulinic acid and 1098 induced active caspase-3 and significantly increased

Annexin V binding. In these studies it was found that the pro-apoptotic effect of 1098 was significantly better than betulinic acid.

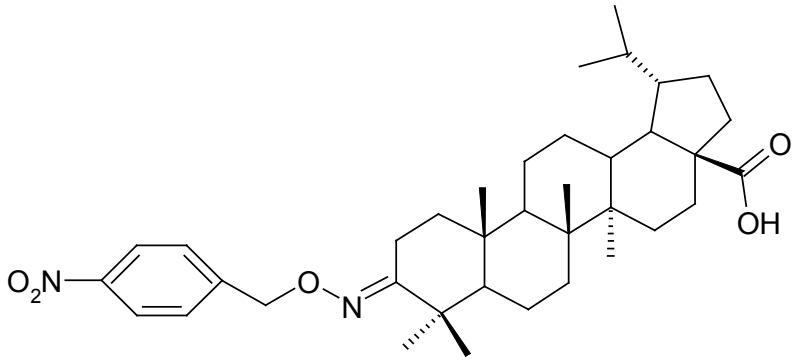

Figure-37

Structure of 1098 [3, (4-nitrobenzyl-oximino)-betulinic acid]

Betulinic acid was reported to be active *in vivo* against TPA-induced tumors [Yasukawa et al., 1991; Yasukawa et al., 1995], ovarian [Zuco et al., 2002] and melanoma [Pisha et al., 1995] xenografts in mice. The *in vitro* studies carried out in this thesis show that betulinic acid was active in seven different cancer types (leukemia, lymphoma, melanoma, prostate, lung, colon and ovarian cancer). Based on these results, tumor xenograft studies were carried out for two cancer types for which xenograft models were established in our lab i.e. melanoma (B16F10) and colon (PTC). Betulinic acid was found to inhibit the growth of both colon and melanoma xenografts at a dose of 33 mg/kg. The results also revealed that betulinic acid had better anti-tumor efficacy when the treatment was initiated early and when the treatment was by systemic (intra-peritoneal) route.

It is known that, except for certain cancers of breast or cervix, cancer diagnosis is still in its early days, as a result of which, most cancer patients present for treatment at a late stage. Betulinic acid did not have any significant activity in large colon tumors. So, in order to test the better *in vitro* activity of 1098 and prove its superior *in vitro* efficacy by *in vivo* studies, mice carrying large colon (PTC) tumors (> 1000 cu.mm) were treated with 1098 at 10 mg/kg by intravenous route. 1098 treatment significantly inhibited the growth of large colon xenografts, characterized by the formation of crater at the centre of the tumor, followed by tumor shrinkage and regression. This study provided good *in vitro- in vivo* correlation in

terms of anti-cancer activity and also proved the superior *in vivo* efficacy of 1098 as compared to betulinic acid, in colon tumor xenografts.

Anti-angiogenic activity of betulinic acid and short-listed derivatives

Angiogenesis is a key process for the outgrowth of cancer cells and their spread into other tissues. Therefore, the specific inhibition of angiogenesis may be a powerful means to suppress angiogenesis-related diseases including cancer. Many natural products which have shown anti-angiogenic effects include angiostatic steroid, calphostin C, castanospermine, cytogenin, 15-deoxyspergualin, eponemycin, erbstatin, fisetin, fumagillin, ginsenosides, herbimycin A, isoliquiritin, krestin, lovendustin A, magnosalin, radicicol, staurosporin, sulphated carbohydrates, protamine, tecogalan, cytotoxic agents of natural origin like doxorubicin, bleomycin, colchicine, paclitaxel, vincristine and vinblastine, the triterpenes oleanolic acid and ursolic acid [Paper, 1998], and betulinic acid [Kwon et al., 2002a].

A detailed investigation was carried out to study the effect of betulinic acid and the short listed derivatives in angiogenesis. The present data show that betulinic acid inhibited key processes of angiogenesis *in vitro*. viz. growth of endothelial cells, migration and tube formation. Further, it was found that betulinic acid had little effect on the migration and tube formation by endothelial cells and on the levels of angiogenic factors like VEGF, FGF and Endostatin. The molecular mechanism of action of betulinic acid could be a direct effect on the mitochondria of endothelial cells based on its inhibition of MTT reduction by mitochondrial enzymes (cytotoxicity data). The same effect was also suggested by an earlier study [Kwon et al., 2002a].

The short listed derivatives of betulinic acid were found to be cytotoxic to endothelial cells and some derivatives (829, 807, 1098 and 937) were significantly better than betulinic acid with IC_{50} values less than 1 μg/mL. Two derivatives 807 and 829 had high endothelial cell specificity. Derivatives 937, 1065 and 1098 significantly inhibited chemotaxis to conditioned medium (known to contain high level of VEGF) as compared to untreated cells at non-cytotoxic concentrations. Derivatives 807 and 1065 significantly inhibited tube formation by endothelial cells at non-cytotoxic concentrations by causing breakdown of capillary tubes and inhibiting capillary network formation. One compound i.e. 1098, which was known to have significant anti-tumor activity, was selected for testing the anti-metastatic effect as it

inhibited both growth and migration of endothelial cells in the angiogenesis assays. The role of angiogenesis is more relevant to metastatic disease since metastasis involves cancer cell migration from primary tumor via the blood vessel to the secondary site of metastasis. 1098 treated mice had significantly lesser growth of metastatic tumors in the lung when administered at a dose of 5 mg/kg for 21 days. 1098-treated lungs contained lesions that were small and isolated but retained most of the normal alveolar architecture of a non-diseased lung. The anti-metastatic effect of betulinic acid derivative was reported here for the first time through this work. As shown in these studies betulinic acid and derivatives will be a good source to provide new leads for the development of new or improved angiogenesis inhibitors. The recognition of their anti-angiogenic potential may help in designing strategies to tackle angiogenesis-dependant tumor growth.

Early ADME and Toxicity findings of betulinic acid and short-listed derivatives

The anti-cancer and anti-angiogenic studies of betulinic acid and derivatives have resulted in the selection of five new and novel derivatives, which were short listed based on their better biological activity as compared to betulinic acid and favorable structural characteristics. The next step was to carry out development studies in order select one or two derivatives which have favorable drug-like ADMET profile. In recent years several ADMET screening assays have been incorporated earlier in the drug discovery pipeline as part of the lead optimization process. The synergistic use of predictive models, automation, 96-well filter based assays and flexible analytical detection techniques have greatly enabled this process, allowing scientists to screen large numbers of compounds with relative ease, increased throughput, decreased time commitments and precious sample quantities. Results have shown correlation with manual testing methods as well as a decrease in variability through the use of automation.

Based on screening several different classes of betulinic acid derivatives using ADME predictive software, it was predicted that betulinic acid and derivatives would have poor solubility, permeability and poor intestinal absorption. The lop P was predicted to be greater than 4, protein binding was predicted to be more than 70% and these compounds were predicted to cross the BBB. Very similar results were obtained when *in vitro* ADME studies of betulinic acid and five short listed derivatives were carried out. The solubility, permeability, metabolic stability, protein binding and CYP inhibition were tested using well standardized plate-based assays. Betulinic acid and five short listed derivatives were found to

have poor solubility (less than 0.1 μg/mL), permeability (log Pe < -5.0) with high protein binding (% binding > 70%). However 1098, 1065 and 937 were found to have good metabolic stability. As far as the potential for drug interaction was concerned, barring one derivative i.e. 1065, none of the other short listed derivatives, including betulinic acid inhibited any of the key CYP enzymes, at tested clinically relevant concentration of 10 μM. But they do show some inhibition of CY2D6 activity. Therefore, further studies need to be carried out before considering betulinic acid and the short listed compounds for combination chemotherapy.

Betulinic acid and short listed derivatives were tested for toxicity in a modified acute toxicity model. It was earlier reported that betulinic acid was non-toxic up to doses of 500 mg/kg i.p. Our data shows that betulinic acid and short-listed derivatives caused mild sedation and somnolence at doses higher than 50 mg/kg as compared to the co-solvent vehicle. No mortality was seen in betulinic acid and 1065 treated animals while at least one mortality was seen with the other short listed compounds (1098 treated group at 150 mg/kg, 937 treated group at 200 mg/kg, 807 treated group at 50 mg/kg and 829 treated group at 100 mg/kg).

Based on *in vitro* ADME and toxicity studies, compounds 1098 and 937 were were metabolically stable and did not inhibit CYP enzymes. Among the two compounds, 1098 was selected for further studies to determine the pharmacokinetic profile. Intravenous administration of 1098 in rats revealed linear pharmacokinetics, achieving minimum effective concentration.

Based on the ADME and toxicity studies of betulinic acid and short-listed derivatives we find two derivatives 1098 and 937 which could be taken up for further development work. In terms of data, we find that 1098 has been extensively tested both in vitro and in vivo. 1098 has also shown evidence of pro-apoptotic activity. These studies need to be carried out for 937 as well. At the moment, for detailed pre-clinical development, derivative 1098 could be subjected to formulation development, detailed efficacy and toxicity studies.

4.2 Specific contributions of this thesis work

Broad-spectrum anti-cancer activity

Earlier reports indicated that betulinic acid is active against melanoma, neuroblastoma and brain cancers. This thesis work demonstrates for the first time that betulinic acid has broad spectrum anti-cancer activity against leukemia, lymphoma, prostate, lung, ovary and colon cancer.

More potent derivatives of betulinic acid

In the search for better and more potent molecules several structurally modified derivatives of betulinic acid have been reported in literature. In summary it has been reported that C-3 position is important for activity but since only few derivatives have been reported till date the exact role of structural modifications at this position is not well understood. This thesis work is the first study that has comprehensively examined more than 1000 derivatives with modifications at C-3 position and successfully selected more potent and selective derivatives for further development.

Anti-angiogenic activity

This study further demonstrates the potential anti-angiogenic activity of betulinic acid and potent derivatives with better anti-angiogenic potential than betulinic acid. The identification of betulinic acid derivatives having both cytotoxicity and anti-angiogenic effect has demonstrated the two-pronged anti-cancer effect of these classes of molecules.

Characterization of ADMET properties

Further, this study is a complete characterization of the *in vitro* ADME properties and pharmacokinetics of betulinic acid and more potent derivatives. The results indicate that betulinic acid and derivatives have poor solubility and permeability and hence poor bioavailability. But these compounds can be administered systemically with desired elimination and plasma concentration which is higher than the minimum effective concentration required for activity.

Selection of a potential anti-cancer compound for clinical development

The comprehensive studies carried out on betulinic acid derivatives has enabled the selection of five compounds from a library of about 500 new and novel betulinic acid derivatives.

Based on the preliminary ADME and toxicity profiles two derivatives 1098 and 937 were found to have the potential to be taken up for development work. At the moment, based on detailed *in vivo* and mechanism of action studies, one derivative 1098 could be selected for formulation development, detailed efficacy and toxicity studies.

4.3 Future scope of work

An increasing number of experimental anticancer drugs, including lonidamine, arsenite, betulinic acid, CD437, and several amphipathic cationic a-helical peptides, act directly on mitochondrial membranes and/or on the PTPC. Such agents may induce apoptosis in circumstances in which conventional drugs fail to act because endogenous apoptosis induction pathways, such as those involving p53, death receptors, or apical caspase activation, are disrupted. Thus, the design of mitochondrion-targeted cytotoxic drugs may constitute a novel strategy for overcoming apoptosis resistance. [Costantini et al., 2000]. Betulinic acid, a pentacyclic triterpene, is a novel experimental anticancer drug. It possesses an anti-tumoral activity *in vitro* and *in vivo* in melanoma, neuroectodermal tumors, and glioma cell lines. It has been shown that betulinic acid induces apoptosis via direct mitochondrial alterations [Fulda et al, 1998]. All of these effects have been observed in intact cells and in cell-free systems. The potent derivatives that have been identified in this study could be further studied for their direct effect on mitochondria of cancer cell lines and endothelial cell lines.

Betulinic acid when combined with anticancer drugs like taxol, doxorubicin, cisplatin etc. was found to induce apoptosis in different human tumor cell lines, including P53 mutant cells, and also in primary tumor cells, but not in normal human fibroblasts [Fulda et al., 2005]. These findings indicate that betulinic acid acts as a sensitizer in chemotherapy-based combination regimens may be a novel strategy to enhance the efficacy of anticancer therapy. Further studies may be carried out to test whether any of the potent derivatives identified as a result of this study could be used in combination regimens.

It was reported that betulinic acid exhibited cytotoxicity to small lung cancer doxorubicin-resistant cells (POGB/DX) and the parent cell (POGB) to the same extent [Zuco et al., 2002]. Future studies of the potent derivatives identified in this study could be investigated in drug-resistant leukemia, lymphoma, prostate, lung, ovary and colon cells.

Within the worldwide research of betulinic acid and betulin in the field of anti-tumor agents, number of structural modifications and derivatization has been studied. Some studies demonstrate that derivatization of 3-β-hydroxy group with lower diacids or amino acids that resulted in highly polar compounds. Future derivatives could be synthesized that make more polar derivatives of betulinic acid.

Since betulinic and short listed derivatives, including 1098, have poor solubility and permeability, better formulation strategies like liposomes, nanoparticles and pegylation could be used to make these molecules more soluble and improve the pharmacokinetic profile. A nanosome formulation of betulinic acid was reported with improved therapeutic efficacy, lower toxicity and prolonged circulation time [Castor, 2005]

Finally, for the development of betulinic acid derivatives for clinical assessment, detailed pharmacology and toxicology, including genotoxicity and reproductive toxicology studies need to be performed in order to generate data on the potential short and long term toxicities, other pharmacological actions etc. for submission to the regulatory authorities for initiation of Phase-I clinical trials.

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LIST OF PUBLICATIONS AND PRESENTATIONS

International Publications

1. Rama Mukherjee, Manu Jaggi, **Praveen Rajendran**, Mohammad J.A. Siddiqui, Sanjay K. Srivastava, Anand Vardhan and Anand C. Burman. "Betulinic acid and its derivatives as anti-angiogenic agents". Bioorg Med Chem Lett. (2004), Vol. 14, pp. 2181-2184.

2. Rama Mukherjee, Manu Jaggi, **Praveen Rajendran**, Sanjay K. Srivastava, Mohammad J.A. Siddiqui, Anand Vardhan and Anand C. Burman. "Synthesis of 3-O-acyl/3-benzylidene/3-hydrazone/3-hydrazine/17-carboxyacryloyl ester derivatives of betulinic acid as anti-angiogenic agents". Bioorg Med Chem Lett. (2004), Vol. 14, pp. 3169-72.

3. Rama Mukherjee, Manu Jaggi, Mohammad J.A. Siddiqui, Sanjay K. Srivastava, **Praveen Rajendran**, Anand Vardhan and Anand C. Burman. "Synthesis and cytotoxic activity of 3-O-acyl/3-hydrazine /2-bromo/20,29-dibromo betulinic acid derivatives". Bioorg Med Chem Lett. (2004), Vol. 14, pp. 4087-91.

Posters

1. Mukherjee R, Jaggi M, **Rajendran P**, Srivastava SK, Siddiqui MJ, Vardhan A, Burman AC. "Betulinic acid derivatives as anti-angiogenic agents" presented at CTDDR, CDRI, Lucknow, Feb, 2004.

2. Mukherjee R, Jaggi M, Siddiqui MJ, **Rajendran P**, Srivastava SK, Vardhan A, Singh M, Jajoo H, Burman AC. "Discovery and development of Betulinic acid derivatives" presented at International Workshop on cervical cancer, NCBS, Bangalore. Nov, 2004.

3. Manu Jaggi, Anu T. Singh, Sudhanand Prasad, **Praveen Rajendran**, Sarjana Dutt, Anand C. Burman, Rama Mukherjee. "Anticancer activity of DRF7295: A peptide

combination targeting multiple neuropeptide receptors in colorectal cancer" presented at AACR cancer conference, USA, 2005.

4. Anu T. Singh, Manu Jaggi, Sudhanand Prasad, Gurvinder Singh, Kakali Dutta, **Praveen Rajendran**, Anand C. Burman, Rama Mukherjee. "DRF7295: A Novel peptide based Signal Transduction Modulator for the Treatment of Gastrointestinal Carcinomas" presented at AACR cancer conference, USA, 2005.

5. Rama Mukherjee, Manu Jaggi, Mohammad J. A. Siddiqui, Sanjay K. Srivastava, **Praveen Rajendran**, Anand Vardhan and Anand C. Burman. "Synthesis and cytotoxic activity of C-2/C-3/C-19/C-28 modified betulinic acid derivatives". Presented at ISCB-2005, Jan 8-10, 2005, Rajkot, India.

Patents

- Jaggi Manu, Ramadoss Sunder, **Rajendran Praveen**, Siddiqui MJ."Betulinic acid derivatives having anti-angiogenic activity, processes for producing such derivatives and their use for treating tumor associated angiogenesis". US Patent No. 6,403,816.

- Jaggi Manu, Ramadoss Sunder, **Rajendran Praveen**, Siddiqui MJ. "Anti-angiogenic activity of betulinic acid and its derivatives". US Patent No. 6,228,850.

- Mukherjee Rama, Jaggi Manu, Prasad Sudhanand, Burman Anand C, **Rajendran Praveen**, Mathur Archana, Singh Anu T. "Anti-angiogenic drugs". US Patent No. 6,492,330.

VIII) BRIEF BIOGRAPHY OF THE CANDIDATE

Praveen. R is a pharmacologist with more than 12 years of working experience in the field of drug development, specifically in the field of cancer. He is presently working as Assistant Manager, Experimental Oncology at Dabur Research Foundation, 22, Site-4, Sahibabad, Ghaziabad, UP-201010. He is part of a team responsible for conducting pre-clinical anti-cancer drug discovery and development of New Chemical Entities being developed by the Experimental oncology lab. He is specifically responsible for conducting lead development studies including ADME, toxicity and *in vivo* animal studies that will help in the identification of potential 'leads' and will further aid efforts in lead optimization.

He has B.Pharm (Hons) degree from BITS, Pilani and PGDBM, equivalent to MBA with specialization in Marketing, from IMT, Ghaziabad. His research work in the field of pre-clinical oncology and drug development has earned him 3 US patents. He has co-authored 3 International publications and given 5 poster presentations. He is a member of Indian Association of Cancer Research and Indian Pharmaceutical Association.

IX) BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. Manu Jaggi is M.Pharm (Gold Medalist) form Delhi University, and Ph.D. in cancer biology from National Institute of Immunology, Delhi. His research work at the National Institute of Immunology, New Delhi proposed a new concept of autocrine mechanism in cancer involving key neuropeptides as growth factors.

He is presently Joint Director, Experimental Oncology, Dabur Research Foundation. He is working on new drug discovery and preclinical development of NCEs, herbal extracts, peptides, radiopharmaceuticals, drug delivery systems and adjuvant therapies. One of the peptide based anticancer drugs that he started working in the year 1990 is now in Phase II clinical trials in India where it is being investigated as a first line therapy for the treatment of colorectal and peri-ampulary cancer. He has contributed significantly in the development of a novel drug delivery system for cancer therapy based on nanoparticle technology, which is also currently undergoing Phase II clinical trials.

He has over 20 US patents and has published his work in over 40 International publications/posters/presentations. He has been actively collaborating with national and international research and academic institutions in various ongoing projects where the major focus is on developing new anticancer molecules using state-of-the art technologies like HTS, Combichem, Computational biology and QSAR. He has traveled extensively and attended several Indian and International workshops and conferences. He is a member of Indian Association of Cancer Research, American Association for Cancer Research, Academy of Sciences (New York), European Tissue Culture Society (U.K.), Indian Immunology Society, Indian Pharmaceutical Association and Association of Cell Biologists (India).