6. SUMMARY
Cancer is one of the most widespread and feared diseases in the world today because it is known to be difficult to cure. Currently, the majority of drugs used for cancer treatment are limited and cytotoxic. As most of the developed anticancer drugs have lack of target specificity and cause more side effects, a major challenge is to design new drugs that will be more selective for cancer cells, and thus have lesser side effects.

Triterpenes represent a varied class of natural products. Betulinic acid [3β-hydroxy-lup-20(29)-en-28-oic acid] is a naturally occurring pentacyclic lupane type triterpenes isolated from the external bark of White Birch Tree Betula alba Linn (Betulaceae) (Newman et al., 2003). Betulinic acid (BA) is known for its anticancer and anti-HIV activities (Singh et al., 2002; Fluda et al., 1998; Evers et al., 1996; Walker, 2001). This research was undertaken at Dabur Research Foundation, in an effort to explore the potential of the BA derivatives to be developed as an anticancer drug.

The main objective of our research effort was to select novel BA derivative(s) for further preclinical ADME evaluation by using in vitro studies as a filter prior to carrying out in vivo animal studies and develop as a potential anticancer drug candidate.

**In vitro Cytotoxicity Screening in Human Cancer Cell Lines**

*Anti-cancer activity of betulinic acid and derivatives*

*(Derivatives selected based on Activity, Specificity and Purity)*

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.

Various modifications of substituent at positions 2, 3, 20 and 28 of betulinic acid have reported as potent lead compounds (Kvasnica et al., 2005; You et al., 2003; Urban et al., 2004; Deng and Synder, 2002; Kim et al., 2001; Jeong et al., 1999). The halo-substituted heterocyclic ring (indolo) at C-2 and C-3 positions in betulinic acid offered highly potent cytotoxic compounds (Fulda et al., 1998). The cytotoxicity of betulinic acid against melanoma cell lines was reported previously (Pisha et al., 1995).
In this thesis, about 500 (five hundred) novel derivatives with modifications in C\textsubscript{2}, C\textsubscript{3}, C\textsubscript{20}, and C\textsubscript{28} position of betulinic acid were primary screened for cytotoxicity. Out of these, 25 (twenty five) derivatives were better than betulinic acid selected in terms of broad-spectrum cytotoxicity based on mean cytotoxicity values towards various cancer cell lines like colon (PTC), ovary (PA1), lung (A549), pancreas (MIAPaCa), prostate (DU145), and human leukemia (K562) cells. The cancer specificity [normal/tumor (N/T) > 2] results showed that derivatives 4012, 4015, 807, 829 and 1065 had cancer specificity to two or more cell lines were selected in second screening. From cytotoxicity and cancer specificity data certain structure activity relationships were observed for selected derivatives. It was found that modification of heterocyclic ring like indole at C-2 and C-3 positions, 2, 3-didehydroindolo amide linkage and C-3 hydroxyl to keto, oxime, phenylhydrazine etc. groups significantly enhanced the activity and resulted in more potent derivatives when compared to betulinic acid. Since broad-spectrum activity was a criterion for selection, derivatives 4012, 4015, 1065, 829 and 807, selective to at least two cancer cell lines and have purity greater than 95% were “short-listed” for further development. The five short listed derivatives were dihydrobetulinic acid derivatives with free carboxylic acid group at C-28 (R\textsubscript{3}) and functionalized at C-2 and C-3 (R\textsubscript{2}) with the following functional groups i.e. 4012 (5’-Chloro-2, 3-didehydroindolo), 4015 (2, 3-didehydroindolo amide linkage), 829 (4-fluorophenyl-hydrazone), 807 (benzoyl-hydrazone) and 1065 (2, 4, difluoro-benzylidene-amino). The other compounds screened had narrow spectrum activity and specificity to only one out of the six sensitive cell lines.
were not selected for further studies. The high specificity of these compounds provides a wide therapeutic window thereby reducing dose-limiting toxicities.

The anti-cancer studies of betulinic acid derivatives have resulted in the selection of five new and novel derivatives, based on their better in vitro anti-cancer activity as compared to betulinic acid. The next step was to carry out development studies in order select one or two derivatives which have favorable drug-like ADME profile. 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. This further lead to the selection and evaluation of potential derivatives in animal pharmacokinetics (PK) and tumor xenograft studies.

**In vitro and In vivo evaluation of C-2 and C-3 modified Betulinic acid derivative**

In vitro and in vivo pharmacological screening of Betulinic acid (BA) derivatives was carried out using ADME, animal PK and tumor uptake studies.

**Early ADME findings of short-listed betulinic acid derivatives**

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 can be evaluated in order to reduce compound drop-out at a later stage of development. In this thesis several in silico and in vitro studies have been standardized and validated using known drugs. Thus, in silico screening using PreADME software would give a preliminary idea about the absorption and distribution characteristic of compounds without the need for setting-up real time experimental data. From the in silico screening, it could be inferred that betulinic acid derivatives are highly lipophilic compounds with poor solubility and absorption properties showing lack of correlation between Caco-2 and MDCK. Additionally, these compounds were predicted to have poor intestinal absorption, except for two compounds 4012 and 4015. These properties need to be further characterized using in vitro ADME models.
The in vitro ADME studies of five short listed derivatives were tested using well standardized rapid assays. Evaluation of ADME parameters of selected derivatives included

1. Solubility
2. Log P & Log D
3. Permeability
4. Metabolic stability
5. Plasma protein binding
6. Plasma stability
7. Cytochrome P450 inhibition

The high throughput solubility assay using DMSO precipitation method was used to evaluate aqueous solubility of test compounds. Results of solubility assay showed poor aqueous solubility of these derivatives. Solubility of betulinic acid could not be determined as this was below the Limit of Quantitation and highest solubility 102.3 µM was reported with 4012. Suitable formulation approaches need to be developed to make them clinically useful.

The octanol/water partition coefficient was used to measure the lipophilicity or partition coefficient (Log P & log D). Log P and log D values of Betulinic acid and short-listed derivatives were found to be greater than 5. Derivatives 4012 and 807 showed Log D value of 4.92 ± 0.58 and 4.74 ± 0.79, respectively which are showing comparatively more distribution in buffer at pH 7.4 compared to other BA derivatives. Higher Log P and log D values of Betulinic acid and derivatives suggested that these compounds are highly lipophilic.

The permeability of betulinic acid and short listed compounds was determined using the parallel artificial membrane permeability assay (PAMPA). Log $P_e$ values obtained for the lower permeability compounds 4015 and 4012 were more variable than those obtained for the other drugs due to analytical imprecision associated with being at or near the LOQ (around 0.1 µg/ml). Based on permeability the short listed compounds may be ranked as 1065 > 807 > 829 > 4012 > 4015.
Metabolic stability of these compounds was evaluated using pooled liver microsome preparations comprising of a pool of 22 human livers. After incubation with pooled human liver microsomes for 60 min, derivatives 4012, 4015 and 1065 remained un-metabolized. Though derivative 807 metabolized to some extent (about 15%), while 829 metabolized by more than 60%. About 100% of the compounds remained un-metabolized in the case of 4012 and 4015. The compounds 4012 and 4015 are more stable and may be expected to remain in plasma and tissues and elicit good activity for a longer time. Compound 829 do not have enough metabolic stability to be developed for clinical use.

The plasma protein binding of short listed derivatives were determined by the ultra-filtration method using a 10-kD membrane filter. All the short listed compounds showed high protein binding (>98%). The high protein binding of short listed derivatives reflected that high doses need to be administered for desired activity.

Stability of the selected derivatives was evaluated in human plasma by incubation the derivatives with human plasma at 37°C for 60 min. None of the derivatives showed any instability in plasma. All the derivatives were found to be stable in human plasma and no degradation was observed.

Assessment of CYP inhibition potential of the compounds at early stage has become very important now a day, especially in the cancer therapy where the combination therapy is very common. These studies assess the potential of drug-drug interaction of the compound if given together with other compounds. 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 (CYP1A2, CYP2C9, CYP2D6 and CYP3A4) at a clinical relevant concentration of 10 µM. Although these compounds did not have any significant effect on CYP enzymes at clinically relevant concentrations, 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.

The in vitro ADME results show that BA and derivatives have poor solubility and permeability. Also, we found that derivatization at C-3 did not improve the solubility much, except that solubility increased in presence of DMSO for some derivatives. The poor aqueous solubility may explain some of the variability in the results of in vitro
biological assays of BA reported in literature. Similarly, derivatization of BA did not improve the passive permeability of these compounds. The metabolic stability of BA and short-listed derivatives was determined using pooled “Human” liver microsome preparations. The high metabolic stability of derivative DRF-4012 (more than 90% of the parent compound remaining un metabolized) provide a good filter for the selection of these derivatives as it was expected that these compound may have longer circulation half-lives in the absence of rapid metabolism and additionally may not produce unexpected toxic metabolites. Some of these compounds may however form phase-II conjugations, for example, with glucuronides as reported earlier (Wen et al., 2006), due to presence of free carboxylic acid groups.

In mouse, rat or dog plasma, BA was reported to be 99.99% bound to serum protein (Cheng et al., 2003). Our results correlated well with earlier reports as our derivatives too exhibited high protein binding (>98%) similar to the reported values of BA in rats. Thus these compounds may lead to low plasma concentration and higher doses may need to be administrated to attain therapeutic concentrations.

Since combination chemotherapy is the standard in anti-cancer treatment, the importance of drug-drug interactions is significant in cancer drug development. Inhibition of these enzymes by co-administered drugs has led to the removal of several drugs from the market during the past years (Friedman et al., 1999; Lasser et al., 2002). None of the other major CYPs were inhibited by BA and derivatives. We confirmed these results in CYP inhibition assays using fluorescent substrate too (data not shown). The lack of inhibition of CYP3A4 enzyme activity, in particularly, by BA and derivatives show that these compounds may be given in combination chemotherapy with several known anticancer drugs, which are know inhibitors of CYP3A4 enzyme e.g. ifosfamide, etoposide, cyclophosphamide, tamoxifen, docetaxel, teniposide, irinotecan, paclitaxel, vinca alkaloids, retinoic acid etc.

Although, all the derivatives had poor solubility, permeability, and high protein binding. DRF-4012 and DRF 4015 were metabolically stable and in addition did not inhibit any key CYP enzymes. Based on our in vitro anticancer activity, specificity, purity and ADME results, we found that derivative 4012 and 4015 had more or less comparable ADME characteristics and taken up for further development work.
Betulinic acid derivative 4012 was found to possess significant antitumor activity against established tumor of colon in nude mice when administered by i.v. route. Conventional chemotherapy has several shortfalls in that the drugs are toxic to normal cells leading to dose-limiting toxicities like myelosuppression, neuropathy, alopecia etc. The selection of betulinic acid derivatives is based on enhanced cytotoxicity and more importantly on being specific to cancer cells with wide therapeutic window. Hence it was expected that the selected derivatives would show good anti-tumor activity while sparing normal cells. The in vivo activity in tumor xenograft model is further proof that these compounds may be both effective and safe for human use.

**In vivo Pharmacokinetic Screening of Selected LEAD molecules 4012 and 4015**

After in vitro Cytotoxicity and ADME screening, potential drug likeness short listed LEAD molecules 4012 and 4015 were subjected to in vivo preliminary pharmacokinetic evaluation in wistar rats \((n = 6)\) each. The study was done at 10 mg/kg body weight, administered by both oral and intravenous routes. Plasma levels of 4012 and 4015 were below detection limits at all time points measured following oral administration at a dose of 100 mg/kg. This shows the poor intestinal absorption of this compound.

Non-compartmental analysis of 4012 and 4015 data using WinNonlin v5.0.1 revealed that longer elimination half life, significant higher plasma concentration and AUC \((p < 0.01)\) of 4012 compared with 4015, indicated slow elimination of 4012 over longer periods of time and higher exposure in vivo, resulting in the maintenance of plasma concentrations over a longer period of time. From PK screening parameters evaluation it was concluded that more favorable PK was seen with 4012 compared to 4015. So, 4012 was selected as a LEAD molecule for further in vivo development.

In terms of data, we find that 4012 has been extensively tested both in vitro and in vivo. 4012 has also shown evidence of pro-apoptotic activity. These studies need to be carried out for 4015 as well.

Above findings suggested that 4012 can be considered as the LEAD compound and subjected to the detailed in vivo pharmacokinetic and preliminary efficacy study. After final selection from in vivo screening, the LEAD compound 4012 was coded as DRF-4012.
The poor permeability coupled with poor aqueous solubility suggests that these compounds may be unsuitable for oral administration. Even for systemic administration in animals, novel cremophor-free nanoparticle formulation was designed better formulation strategies to overcome the problem of compound precipitation, reduced target specificity and low bioavailability in animal models.

The poor solubility and permeability of DRF-4012 resulted in poor bioavailability by oral route as shown in the rat oral PK study. However, favorable PK was seen by i.v. route. Apart from the thesis work, in vivo efficacy studies were carried out by i.v. route in colon cancer to determine the anti-tumor potential of the most potent compounds selected from in vitro studies i.e. DRF-4012. We found that DRF-4012 was most sensitive and selective in colon cancer cell line among the tested cancer cell lines and also the in vivo treatment with DRF-4012 significantly inhibited the growth of large colon 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 DRF-4012 is better than betulinic acid in its anti-tumor activity to colon xenografts. DRF-4012 appears to function by a rapid increase in production of reactive oxidative species (ROS) and concomitant dissipation of mitochondrial membrane potential in a dose and time dependent manner, resulted in cell apoptosis.

**Preliminary in vivo efficacy evaluation**

The LEAD derivative 4012 showed acceptable in vitro ADME and in vivo pharmacokinetics. It also showed the excellent in vitro cytotoxicity in PTC (Primary tumor cells of colon adenocarcinoma) cell line. These finding suggests that it should show
the in vivo efficacy. In order to validate this, a preliminary efficacy study was conducted in colon xenograft. Co-solvent formulation of 4012 was dosed continuously for 14 days PTC xenograft at an early stage (tumor volume ≈ 1000 mm$^3$), by intravenous route along with vehicle control group. All the animals were observed daily for 22 days for tumor reduction and body weight.

Data showed that 4012 significantly inhibited the growth of tumor volume in comparison with vehicle control. The anti-tumor effect was characterized by at day 22. Before control animals were sacrificed, the treated/control (T/C) value was 64.8%. Tested dose level of 10 mg/kg did not cause significant body weight loss and mortality during dosing period which indicates there was no 4012 related systemic toxicity.

**Bioanalytical method development and validation of DRF-4012**

Bioanalytical method can be defined as an analysis of sample or analyte or drug in biological fluid like Plasma, serum, urine, blood and tissue etc. Quantitative determinations of drugs in biological samples, such as blood or plasma, play a significant role in evaluation and interpretation of bioequivalence data.

Full scale bioanalytical method development and validation for PK and Biodistribution studies was performed according to USFDA guidance (Guidance for Industry, 2001).

**Method I: Analysis of rat plasma by HPLC**

The simple and sensitive bio-analytical methodology using HPLC was developed and validated for the determination of novel betulinic acid DRF-4012 in rat plasma. Method showed excellent sensitivity, a wide linearity range of 0.040-75.0 µg/ml, with very good intra-and inter-day accuracy and precision. The method was successfully applied for the evaluation of pharmacokinetic profiles of DRF-4012 in rats at 5 mg/kg intravenous dose by using small sample volume (100 µl). This validated method also successfully applied on pharmacokinetic linearity study of DRF-4012 at higher doses because of wide linear range and accurate and precise dilution integrity.

**Method II: Analysis in rat plasma, urine, tumor and tissues homogenates of athymic nude mice by LC/MS**

A liquid chromatography-electrospray mass spectrometric (LC/MS) method assay has been developed which is highly sensitive and selective, shows good linearity of response
and high precision for the quantification of DRF-4012 in microsize (100 µl) biological specimens including rat plasma, mice plasma, urine, feces and tissues (liver, brain, lungs, heart, spleen, stomach, thigh muscle, kidneys, urinary bladder, small intestine and tumor etc) of nude mice. This LC/MS method has been successfully applied for the determination of DRF-4012 in oral bioavailability study in rats, tumor uptake and biodistribution study after administration of 30 mg/kg dose of DRF-4012 nanoparticle formulation in athymic nude mice bearing human tumor.

In vivo Pharmacokinetic (ADME) Evaluation of LEAD molecule DRF-4012

Despite that valuable insight is obtained from in vitro ADME (absorption, metabolism, distribution and excretion) screening assays, in vivo drug exposure is still emphasized by drug discovery teams when making decisions about molecules. In vivo animal PK studies provide a reality check which guides the medicinal chemists to optimize the chemical structure of compounds. In vivo animal PK information also assists pharmacologists to design effectively in vivo efficacy studies and accurately interpret pharmacodynamic (PD) observations. There is no substitute for actual in vivo data in assessing pharmacokinetic profiles of drug candidates.

The in vivo pharmacokinetic study of the lead molecule DRF-4012 was conducted to assess the bioavailability, dose linearity and gender effect. Following set of pharmacokinetic study was conducted in order to evaluate complete pharmacokinetic profile of 4012.

In vivo pharmacokinetic (ADME) evaluation included
1. To evaluate the single dose i.v. and oral bioavailability of Lead molecule in wistar rat.
2. Regression analysis of mean AUC_{inf} versus dose was performed to gain an appreciation of pharmacokinetic linearity of Lead molecule at low, mid and high doses.
3. Excretion study in urine and feces to determine the fate of Lead molecule.
4. Gender difference pharmacokinetic study.
5. To evaluate and compare the biodistribution pattern of Lead molecule in tumorogenic and non- tumorogenic athymic nude mice i.e., control group.
6. To study the pharmacokinetic and tumor uptake of Lead molecule in human tumor xenograft induced athymic nude mice.

**Single dose pharmacokinetic and pharmacokinetic dose linearity/ dose escalation study**

The pharmacokinetics of DRF-4012 nanoparticle formulation was carried out in rats by single intravenous route at a dose of 5 mg/kg via the catheter to a group of six male rats to generate pharmacokinetic data. DRF-4012 is widely distributed and has a moderate clearance, high volume of distribution and long elimination half-life in rats. Pharmacokinetics dose linearity was carried out by i.v. route at 2, 5 and 10 mg/kg. Linear relationship between the i.v. administered dose and initial concentration extrapolated to time zero ($C_0$) ($r^2=0.98$) and/or area under the curve (AUC) ($r^2=0.98$) were observed.

**Bioavailability study**

Absolute bioavailability was carried out by the oral route at 50 and 100 mg/kg. Furthermore, disproportionate change in $C_{max}$ and AUC suggest nonlinear pharmacokinetics in rats. Absolute bioavailability of DRF-4012 was very poor with a value being 0.03% and 0.02% at oral dose 50 and 100 mg/kg, respectively when compared with at 2 mg/kg i.v. dose. The potential hydrolysis in the gastrointestinal tract, poor solubility and permeability though the intestinal epithelial membrane and first-pass effect in the liver might be responsible for the low bioavailability of this compound. It exhibits very poor absolute bioavailability in rats. This shows the poor intestinal absorption of DRF-4012, nevertheless the derivative was found to have favorable characteristics of a systemically administered drug based.

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.

**Pharmacokinetic study of gender effect**

Estimated pharmacokinetic parameters after intravenous administration of DRF-4012 from male and female wistar rats at dose 5 mg/kg suggested similar PK profile and no significant difference in PK parameters were observed. Hence, gender difference has no effect on pharmacokinetic of DRF-4012.
Excretion studies
The excretion study in rats indicated that DRF-4012 was poorly excreted in urine after i.v. administration and major portion of dose administered recovered unchanged from feces. The contribution of the urinary routes for excretion of unchanged DRF-4012 was not significant. Although, greater % recovery was obtained after alkaline hydrolysis of urine and feces samples suggesting that role of phase II metabolism in excretion of DRF-4012. There was insignificant excretion of DRF-4012 in unchanged form though urine, so it is necessary to carry out further studies to determine the fate of compound in the body.

Pharmacokinetics in tumor bearing mice
Plasma concentration profile of DRF-4012 nanoparticle formulation in male human tumor bearing athymic nude mice was performed at i.v. dose of 30 mg/kg. High elimination half-life, large Vz and moderate CL suggest that DRF-4012 undergoes more extensive distribution. As DRF-4012 has a long elimination half life and resulted nanoparticle formulation leads to pro-longed circulation time in blood and enhance accumulation by tumors.

WinNonlin calculated PK parameters in tumor concluded that DRF-4012 has long elimination half life, high mean residence time (MRT\text{last}) and apparent volume of distribution with low clearance.

An improved kinetics with better safety profile and dose linearity was observed with DRF-4012 nanoparticle formulation (Mishra et al., 2011). Nanoparticle formulation changed the pharmacokinetic behaviors of DRF-4012 by prolonging the half-life of systemic circulation and releases at a sustained rate or in an environmentally responsive manner. The compound showed good tumor growth inhibition (%T/C, TGI) in colon xenografts compared to betulinic acid.

As DRF-4012 has showed the potent anticancer activity and favorable pharmacokinetic. Hence, the compound selected for preclinical evaluation of biodistribution and excretion studies in human tumor-bearing nude mice to understand the target efficiency, assessment of off-target accumulation and prediction of potential sites of adverse reactions for safe biomedical application.
Biodistribution study of DRF-4012

Biodistribution and elimination studies of LEAD molecule DRF-4012 was performed in in tumor and non-tumor bearing mice at i.v. dose of 30 mg/kg with nanoparticle formulation. In liver, DRF-4012 concentrations were maximal at 0.5 h after drug administration and declined with the time thereafter. Interestingly, initially after 0.5 h, tumor showed the second highest uptake, which was nearly half the level observed in liver. After 4 h, tumor showed almost same 0.5 h concentration, which indicates the good retention properties of DRF-4012 nanoparticle in tumor. After 24 h, the highest concentration of DRF-4012 was found in the tumor with virtually none in the blood and a significant portion found in the liver. At 0.5 h a mean percentage 43.89% of the DRF-4012 were localized in the liver, 22.37% in the tumor, 2.0% in the kidney, 2.57% in the spleen and 2.77% in lungs. The lower percentage was observed in the spleen after 24 h. At all time points, the lower uptake of DRF-4012 were observed in brain, small intestine, stomach, thigh muscle and urinary bladder. The accumulation at 0.5 and 4 h in other non-reticulo endothelial tissue (RES) (tumor, lungs, kidneys and heart) was 27.47% and 27.18% ID, respectively. The sum of biodistribution to liver and spleen (RES tissues) accounted 46.47% and 26.29% ID at 0.5 and 4 h, respectively. Biodistribution data revealed that substantial improvement in the pattern of DRF-4012 biodistribution was the combined result of an absolute decrease of liver uptake and increase in tumor uptake with time. Although the liver uptake was still 1.8-fold higher at 0.5 h than that of tumor, this ratio may be therapeutically acceptable, since the liver is the usual metabolizing organ for most of the drugs. As DRF-4012 has a long elimination half life and resulted nanoparticle formulation leads to pro-longed circulation time in blood and enhance accumulation by tumors.

Plasma concentration of DRF-4012 after i.v. administration 30 mg/kg dose in tumor bearing and control mice were found to be same and not significantly observed. The biodistribution profile of DRF-4012 nanoparticle of both tumor bearing and control was found to be similar in all the organs except in spleen.

This factor may help to explain the manner in which DRF-4012 can inhibit tumor growth without apparent toxicity to other organs.
Elimination study of DRF-4012 in tumor bearing and control mice

The elimination profile was similar, as same %ID content of DRF-4012 was found in tumor bearing and control (non-tumor bearing mice) groups. Elimination studies revealed that very low elimination of unchanged DRF-4012 in urine was observed. Major portion of dose administered recovered unchanged from feces. Although, greater % recovery was obtained after alkaline hydrolysis of feces sample suggesting that role of phase II metabolism in excretion of DRF-4012. There was insignificant excretion of DRF-4012 in unchanged form through urine, so it is necessary to carry out further studies to determine the fate of compound in the body.

We summarized biodistribution and elimination study of DRF-4012, as described herein, should be useful for preclinical or clinical evaluation of this potential antitumor agent.

In vivo metabolism study

For the registration of any potential drug candidate it is essential to provide evidence of general metabolism in the animal species utilized for toxicological evaluation. To obtain this information, a number of studies are routinely performed using both radiolabelled and unlabelled compounds, thus enabling the analyst, as far as is reasonably practicable, to determine the metabolic fate of the drug candidate. Drugs are generally metabolized to generate more polar entities, which are more readily excreted. Drug metabolism can most simply be divided into two main phases: Phase I, fictionalization type reactions such as oxidation, reduction and hydrolysis; and Phase II, conjugative reactions such as formation of glucuronic acid (glucuronidation) and amino acid conjugates, sulphation, methylation and acetylation. In some instances, Phase III reactions also exist.

Conventionally, drug metabolite identification in the past has usually been based on the comparison of ultraviolet (UV) spectral data and high-performance liquid chromatography (HPLC) retention times of isolated ‘unknown’ metabolites with those of synthesized standards. Such a method of detecting and characterizing drug metabolites is an uncertain, time-consuming and expensive process, as well as affording very limited structural information. Furthermore, Phase I metabolism of a drug candidate often results in only minor structural modification of the parent compound; these minor changes can make it particularly difficult to determine suitable chromatographic conditions to effect HPLC separation of metabolites. This study describes contemporary approach to
identification and characterization of xenobiotic metabolites in complex biological fluids derived from drug metabolism studies.

To identify and characterize the in vivo metabolite formation of DRF-4012, a suitable chromatographic and liquid chromatography-mass spectrometric method was developed. The peaks obtained were characterized by their molecular mass on LC/MS. Two metabolites are obtained with DRF 4012 in urine during in vivo metabolism study. The proposed metabolites can be hydroxy and di-hydroxy DRF 4012. Five metabolites are obtained with DRF 4012 in feces during in vivo metabolism study. Glucuronide conjugate was present as major metabolite in feces with other unknown metabolites. During characterization it is concluded that DRF 4012 was recovered as unchanged form in feces and urine. Minor hydroxy metabolites are obtained in urine with major glucuronide metabolite was observed in feces (due to presence of free carboxyl group) and excreted as phase-II elimination reaction.

From the above studies it can be concluded that betulinic acid derivative DRF 4012 have the potential to be developed as therapeutics for the treatment of various cancers when administered by systemic route.

**Specific contributions of this thesis work**

Consequently, there is an increasing trend to optimize pharmacokinetics, enhance antitumor activity and reduce toxicity. Hence present works aims at defining to choose the optimum candidate with drug-like properties after in vitro assays and the selected candidates are subjected to various aspect of in vivo pharmacokinetic evaluation (ADME). The strategy was to set up in vitro and in vivo models logically and systematically as applicable during various phases of lead selection and optimization. These insights are expected to help in identifying potent anticancer agent with improved pharmacokinetic and better safety profile versus existing anticancer drugs in the market.

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-2 and 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. The comprehensive studies carried out on betulinic acid derivatives has enable the selection of five compounds from a library of about 500 new and novel
betulinic acid derivatives with modifications at C-2 and C-3 position and successfully selected more potent and selective derivatives for further development.

Based on the preliminary ADME and toxicity profiles two derivatives 4012 and 4015 were found to have the potential to be taken up for development work. DRF-4012 (5’-chloro-2, 3-didehydroindolo [2’, 3’: 2, 3] betulinic acid) is a synthetic novel betulinic acid derivative, identified as a potential compound based on the superior in vitro cytotoxicity and efficacy towards various cancer cell lines and can be developed as anti-cancer drugs for the treatment of human leukemia, lungs, breast, ovarian, pancreas, prostate, and colorectal cells.

Further, this study is a complete characterization of the in vitro ADME properties and pharmacokinetics of potent betulinic acid derivatives. The results indicate that 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. Tumor uptake results in human tumor bearing athymic nude mice indicated that DRF-4012 achieved an effective concentration in the tumor. Biodistribution data revealed that DRF-4012 is more target specific to tumor and not accumulated in other organs which resulted in lesser side effect. As most of the developed anticancer drugs have lack of target specificity and causes more side effects. So this study in human tumor bearing nude mice may be helpful in guiding DRF-4012 develop as a potential anticancer agent in human.

Betulinic acid derivative 4012 was found to possess significant antitumor activity against established tumor of colon in nude mice when administered by i.v. route. Conventional chemotherapy has several shortfalls in that the drugs are toxic to normal cells leading to dose-limiting toxicities like myelosuppression, neuropathy, alopecia etc. The selection of betulinic acid derivatives is based on enhanced cytotoxicity and more importantly on being specific to cancer cells with wide therapeutic window. Hence it was expected that the selected derivatives would show good anti-tumor activity while sparing normal cells. The in vivo activity in tumor xenograft model is further proof that these compounds may be both effective and safe for human use.