1. INTRODUCTION

1.1 Drug Discovery and Development

Drug research is a unique multi-disciplinary process heading towards the development of therapeutic agents in the area of currently unmet medical need. The process of drug discovery applies rigorous selection pressures. The past 30 years has seen an accelerating increase in our understanding of the mechanisms that underlie disease processes. It has a fundamental impact on the process of drug discovery, and most of modern pharmaceutical research is based on target-focused discovery, where the goal is to affect the biological activity of a particular target to provide a cure or treatment for a disease. In a target-focused approach, the cycle of discovery is very similar with or without a structure for the target. Initial-hit compounds are found that bind to the target and enter a medicinal chemistry cycle of making compound analogues and testing in suitable biological models.

The drug research can be divided functionally into two stages: discovery and development (Panchagnula et al., 2000). Drug discovery is a long, arduous process broadly grouped into disease target identification, target validation, high-throughput identification of hits and leads, Lead optimization, preclinical and clinical evaluation (Gombar et al., 2003). Drug development focuses on evaluation of safety/toxicity and efficacy of new drug molecules. The key objective of drug development is generation of a scientific database that supports effectiveness and safety profile of a drug and its dosage regimen(s) intended for marketing (Panchagnula et al., 2000).

**DISCOVERY PHASE**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Duration</th>
</tr>
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<tbody>
<tr>
<td>Target Identification</td>
<td>1 - 2 years</td>
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<tr>
<td>Target Validation</td>
<td>1 - 2 years</td>
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<tr>
<td>Hit Identification</td>
<td>~1 year</td>
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<tr>
<td>Lead Optimization</td>
<td>2 - 4 years</td>
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<td>SoPD Candidate</td>
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**DEVELOPMENT PHASE**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Duration</th>
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<tbody>
<tr>
<td>Pre-Clinical Development</td>
<td>~ 1 year</td>
</tr>
<tr>
<td>Clinical Development: Phase I to III</td>
<td>5 - 7 years</td>
</tr>
<tr>
<td>New Drug Approval</td>
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**Figure 1.1:** Overview of the process of drug discovery and development
The discovery and development process of therapeutically useful drug requires an enormous amount of money and time due to high attrition rates of new chemical entities (NCEs)/drug candidate. According to recent literature, it is estimated that only one in ten of the agents that enter clinical development are successful, with an average cost of US $500-800 million and a typical time scale of 10-15 years from pre-clinical discovery research to regulatory approval (Workman, 2003). Typically, the whole process is fragmented into ‘Discovery’, ‘Development’ and ‘Registration’ phases. Figure 1.2 depicts different phase of drug discovery, development and registration.

![Diagram of drug discovery, development and registration phases](image)

**Figure 1.2:** Typical phases of drug discovery, development and registration

The lead identification phase in drug discovery involves identification and characterization of drug targets, synthesis of new lead molecules together with traditional chemistry, computer aided drug design and/or combinatorial chemistry and further characterization of physiochemical characters of promising candidate (Panchagnula et al., 2000). The hits are identified using in vitro screening assays together with high throughput screening for ‘hit’ generation.

In lead optimization step, chemists use empirical and semi-empirical structure-activity relationship (SAR) to modify the chemical structure of a compound to improve the in
vitro activity of the hits. However, good in vitro activity cannot be extrapolated to good in vivo activity unless a drug has good bioavailability and a desirable duration of action. The lead optimization phase typically includes ADME screening to improve upon the degree of potency, which has already been achieved (Roberts, 2001). Further, in vivo screening is performed involving testing of the candidate drug in suitable animal models, their pharmacokinetic and toxicokinetic profile. This whole process from high throughput screening to in vivo screening constitutes preclinical phase in drug discovery and development. Once the candidate drug reaches this phase, an investigation IND is filed to the drug regulating authorities. After IND application approval, the clinical phase starts which involves testing in human subjects, and is divided into 4 phases. Each phase involves process scale-up, pharmacokinetics, drug delivery, and drug safety activities (Lee et al., 1999).

In past, researchers typically turned their attention to studying and optimizing ADME/TOX properties of lead compounds only after optimizing for potency and selectivity which involved multiple rounds of re-synthesis and re-testing (Serial-Cyclical model of lead optimization, Figure 1.3). An intrinsic problem with this approach is the large expenditures of time and resources are needed to produce a suitably potent and specific lead compound and yet little if any consideration will have been given to the compound’s bioavailability or toxicity. This approach yields low throughput, is expensive, time consuming and marginally predictive. Consequently, efforts were on to enable increased parallelism in lead optimization by eliminating troublesome compounds before they plug up the pipeline at the lead optimization stage. This model is termed as parallel model of lead optimization (Figure 1.4) (Rubenstein, 2003). It is high throughput (only quality molecules are re-synthesized) rapid, inexpensive and selective.

The common ADME issues encountered during lead optimization are systemic exposure and the potential for drug-drug interactions. Problem with systemic exposure typically become apparent through the failure of molecules with in vitro potency to show subsequent efficacy in vivo. The various problems with systemic exposure are related to absorption, distribution, and clearance, while issues related to drug-drug interaction related to enzyme induction and inhibition (Bertz et al., 1997). Hence, it is now more common for initial ADME studies to be conducted prior to in vivo efficacy testing to evaluate systemic exposure and make best use of animal model. The ability to relate
systemic drug concentrations to a pharmacodynamic effect can ensure that rational decisions are made in context of the target product profile (Krzyzanski et al., 1998; Sharma et al., 1998). After establishing that ADME findings are relevant to compound progression, it is necessary to investigate the underlying causes of any problems that might be encountered. A good understanding of these problems is the cornerstone of a rational ADME lead optimization strategy because it ensures that the appropriate tests are conducted and that the synthetic program is directed accordingly (Rodrigues, 2003).

**Figure 1.3:** Serial-cyclical model of lead optimization

**Figure 1.4:** Parallel model of lead optimization
The drug development process involves a series of specialized events performed to satisfy criteria, internal (i.e. competitive industry benchmarks) and external (i.e. regulatory compliance), to yield a novel drug (Lee et al., 1999). The preclinical stage of drug development focuses on activities that are necessary for filling an IND. The IND contains animal toxicity data, protocols for early clinical phase trials, and an outline of specific details and plans for evaluation. Process research, formulation, metabolism and toxicity are the major areas of responsibility in this development stage. In this context, ADME studies provide supportive information to augment the interpretation of toxicological finding. Of primary importance among them are drug exposure, expressed as AUC (area under the concentration-time curve of drug) and $C_{\text{max}}$ (maximum drug concentration in plasma), which are then related to dose levels and toxicological outcomes (Cayen, 1995). Based on the toxicokinetic data at no-observed toxic effect dose, an acceptable exposure limit in humans can be defined. To assist in putting the toxicokinetic data into a broader perspective, the basis pharmacokinetic behavior of the NCE is assessed in the toxicology species. In later development, the focus of ADME work is shifted to human studies, which define the disposition of the drug in humans, particularly in target therapeutic population. Ultimately these data are integrated to a NDA to secure approval to market the drug. ADME data gathered during the full course of development are incorporated into drug labeling, which is intended to optimize therapeutic utilization of the drug in the target population (Cayen, 1995). Hence, it is true that ADME is critical in all phases of a fully integrated drug development program.

The increased costs in the discovery and development of new drugs, is because of the high failure rate of drug candidates in development, this has led to a new strategy to introduce early, parallel evaluation of efficacy and biopharmaceutical properties of drug candidates. Having a look at the closed projects revealed that the basic cause for drug failure in the development phase was the poor pharmacokinetic and ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties rather than unsatisfactory efficacy. In addition, the applications of parallel synthesis and combinatorial chemistry to expedite lead finding and lead optimization processes has shifted the chemical libraries towards poorer biopharmaceutical properties. Establishments of high throughput and fast ADMET profiling assays allow for the prioritization of leads or drug candidates by their biopharmaceutical properties in parallel with optimization of their efficacy at early discovery phases. This is expected to not only improve the overall
quality of drug candidates and therefore the increased chances of their success, and also decreases the drug discovery and development process.

The reason for failing discovery of NCEs can be attributed to:-

- The selection of improper targets in early phases that lack proof of concept in human.
- High attrition rate during development phases due to poor pharmacokinetics.
- Poor toxicological and safety related pharmacological Properties.
- Elongated discovery and development time course. Attempts to address these challenges embrace:
  - Pursuit of physiologically viable targets using Genomics and proteomics strategies

**Future Trends**

Combination chemistry and high throughput screening for lead in drug discovery has resulted in the need for higher throughput methods of screening for ADME properties (Tarbit et al., 1998). The solution to this problem by the introduction of concepts such as ‘N-in-One’ dosing or Cassette Dosing, Sample Pooling approaches, use of automation for sample preparation and bioanalysis and use of new experimental approaches, for the evaluation of many substances in parallel, have shown useful ways to increase the speed with which compounds are studied (Frick et al., 1998a; Frick et al., 1998b). For absorption studies, in vitro models involving use of cultured, immortalized cells like Caco-2 and MDCK cells have given improved throughput (Stewart et al., 1995; Gan et al., 1997; Delie et al., 1997; Artursson et al., 1996). Use of in vitro liver preparations like S-9, microsomes, cultured hepatocytes have yielded in high throughput for establishing metabolic stability of promising molecules at an early stage (Lave et al., 1997). The technological advances in liquid chromatography–mass spectrometry (LC/MS) have given further impetus to high throughput pharmacokinetics. A key technique of LC/MS is the use of multiple reactions monitoring (MRM) which allows quantitation of multiple compounds simultaneously (Berman et al., 1997). These advances in the field of bioanalysis have given birth to the possibilities of novel concepts of increasing the throughput of pharmacokinetic screening.

More recent technologies such as micro titre plate, robotic instrumentation coupled to MS sufficient to provide a platform on which to build a more fully automated approach to
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perform analysis of biological matrix samples, thereby promoting High Throughput Pharmacokinetic (HTPK).

1.2 Pharmacokinetics and Its Role in Drug Discovery and Development

The discovery and development process of therapeutically useful drug requires an enormous amount of money and time due to high attrition rates of new chemical entities (NCEs)/drug candidate (Prentis et al., 1988). Lack of efficacy and toxicity are considered to be major reasons for drug failures and pharmacokinetics (PK) governs them to large extent (Ruiz-Garcia et al., 2007). PK is the study of the time course of absorption, distribution, metabolism and elimination (ADME) of the drugs in biological system and helps to understand the relationship between pharmacological and toxicological effects and concentration of a drug and its metabolites in the body fluid.

Pharmacokinetic data is not only important in drug development stages but also important in early discovery phase. According to Center for Medicines Research report, nearly 40% of the drugs were terminated from further development due to unsatisfactory pharmacokinetic data, whereas poor pharmacokinetic were solely responsible for nearly all (90%) terminations of anti-infective drugs (Prentis et al., 1998) (Figure 1.5). It is therefore, becoming apparent that in addition to pharmacological properties, ADME/Tox properties are crucial determinates of ultimate clinical success of a drug. This realization has leads to early introduction of ADME/Tox screening during the drug discovery process, in an effort to select against drugs with problematic ADME/Tox profiles (Li, 2001). Considering pharmacokinetics to be the weakest link in the drug development chain was also a major influence in expanding ADME/Tox from its traditional role as a preclinical safety support function toward the earlier stages of drug discovery (Lin et al., 1997). The systematic application of PK can considerably reduce the cost and time involved in new drug development (Roberts, 2003; Lin et al., 2003).

Today PK and metabolism are among the most highly interactive disciplines in the pharmaceutical research and development and intimately involved in the design of new chemical entities (Caldwell et al., 2001; Stoner et al., 2004; Singh, 2006).
Fundamentally, ADME/PK information is critical in all phases of a fully integrated drug development program (Table 1.1). The goal of ADME-guided synthesis is to maximize the ability of NCEs to access the therapeutic target in vivo. Once a lead compound is identified it is subjected to preclinical PK/ADME studies.

Preclinical pharmacokinetics is referred to PK in vivo and in vitro activities supporting drug development program. It can be broadly divided into three areas: (i) PK at the discovery levels that enables selection of lead chemical, (ii) toxicokinetics or PK in toxicology studies, and (iii) preclinical studies in animals that support the clinical studies and regulatory obligations. Preliminary ADME studies of lead compounds in animal species also provide information on routes of clearance (such as renal, biliary, or metabolic), which is helpful in guiding the selection of compounds that exhibit a balance between elimination pathways and thus would not be unduly dependent on a single organ for excretion. Furthermore, preclinical studies in animals help in selecting the first dose in clinical studies and appropriate dosing regimen.

The drug candidate succeeds in passing the preclinical drug development stage (drug metabolism, PK, toxicity testing, etc) (Heykants & Meuldermans, 1994), is submitted as an investigational new drug application (IND) and may enter full development: phase I (safety and tolerability in healthy volunteers), phase II (efficacy and dose- effect relationship using a small number of patients), and phase III (efficacy studies using a large number of patients) (Charles, 1992). If the IND passes all three clinical phases, it is submitted as a new drug application (NDA) and upon Food and drug administration (FDA) approval, eventually enters for the clinical use/marketplace. Thus PK/ADME play an increasingly important role in drug development starting with drug discovery and lead

![Figure 1.5: Reasons for failure in drug development](image)
optimization, pharmacological and safety evaluation continuing into clinical development and finally helping to position the NCEs as the drug (Balani et al., 2005).

**Table 1.1: Role of Pharmacokinetics in the Development of New Drug**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Stage</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preclinical PK</td>
<td>Discovery</td>
<td>To chose the optimum candidate</td>
</tr>
<tr>
<td></td>
<td>Development</td>
<td>To understand the pharmacology in experimental animals</td>
</tr>
<tr>
<td></td>
<td>Toxicity testing</td>
<td>To determine exposure in two nonhuman species</td>
</tr>
<tr>
<td>Phase I</td>
<td>Dose ranging</td>
<td>Tolerability over range of doses</td>
</tr>
<tr>
<td></td>
<td>Dose linearity</td>
<td>Establishing whether plasma concentration increase in proportion to dose administered in humans</td>
</tr>
<tr>
<td></td>
<td>Definitive kinetics</td>
<td>Measurement of half-life, C_{max} and AUC using single and multiple doses</td>
</tr>
<tr>
<td>Phase II</td>
<td>Sex differences</td>
<td>Assessment of the influence of gender on PK profile</td>
</tr>
<tr>
<td></td>
<td>Food interaction</td>
<td>Assessment of the influence of food on PK profile</td>
</tr>
<tr>
<td></td>
<td>Absolute bioavailability</td>
<td>Quantitative determination of the distribution of a compound in bodily fluids and tissues</td>
</tr>
<tr>
<td></td>
<td>PK-PD relationships</td>
<td>Establishing clear dose-response relationship avoids drugs being marketed at excessive doses</td>
</tr>
<tr>
<td></td>
<td>Genetic polymorphisms</td>
<td>Assessment of the influence of genetic differences in drug metabolizing enzyme on PK profile</td>
</tr>
<tr>
<td>Phase III</td>
<td>Effect of disease</td>
<td>Determination of the PK profile in the target population</td>
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<tr>
<td></td>
<td>Subgroup analysis</td>
<td>Determination of the PK profile in the final subgroup of the target population</td>
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<tr>
<td></td>
<td>Final dosage form PK</td>
<td>Determination of the PK profile of the final formulation of the drug candidate</td>
</tr>
<tr>
<td></td>
<td>Dose response</td>
<td>Determination of a clear dose-response relationship in the target population. Response can be determined on the basis of a biochemical, imaging or clinical surrogate or the explicit demonstration of therapeutic efficacy.</td>
</tr>
<tr>
<td>Phase IV</td>
<td>Dosage form improvements</td>
<td>Determination of the PK profile of new formulations</td>
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<td></td>
<td>Line extensions</td>
<td>Determination of the PK profile of modified designed to extend patent life</td>
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<tr>
<td></td>
<td>Drug interactions</td>
<td>Determination of the possible influence of the other drugs on the PK profile</td>
</tr>
<tr>
<td></td>
<td>Pharmacovigilence</td>
<td>Continual assessment of competitor and potential competitor compounds</td>
</tr>
</tbody>
</table>
1.3 Preclinical Paradigm/Strategies
Pharmacokinetics at preclinical level is basically of two types: in vivo and in vitro. Studies of drug databases showed that successful drug candidates tend to have ‘drug like properties’ (Matter et al., 2001). Drug likeness, when viewed at the in vivo level, is thought of in term of PK and safety. Complex in vivo properties result from an interaction of physiochemical and structural properties, such as solubility, permeability and stability, which are studied in vitro. These properties are, in turn, dictated by fundamental molecular properties, such as molecular weight, hydrogen bonding and polarity. As a result of the importance of these properties, a new strategy emerged: testing the ‘drug like’ properties of compounds during early discovery using high-throughput property screening methods in silico, in vitro and in vivo (often termed ‘pharmaceutical profiling) (Van, 2005; Selick et al., 2002).

1.3.1 In silico strategies
The high throughput screening (HTS) of large proprietary compound collections and combinatorial libraries have increased the pressure on gathering pharmacokinetics and drug metabolism data as early as possible. Properties related to ADME can be estimated by a range of in vivo and in vitro methods, most of which are now available or under development in high(er) throughput modus (Kassel, 2004; Roberts, 2001; Kariv et al., 2002). In addition, progress has been made in in silico methods using various quantitative structure activity relationships (QSAR) and molecular modeling techniques that employ a range of recently introduced descriptors tailored to ADME in silico (e-ADME) (Ekins et al., 2000). The computational approach is one of the newest and fastest developing techniques in PK, ADME evaluation, drug discovery and toxicity (Darvas et al., 2002; Van & Gifford, 2003, Yamashita & Hashida, 2004; Van, 2002). However, to date, the software packages devoted to ADME prediction, especially of metabolism, have not yet been adequately validated and still require improvements to be effective (Boobis et al., 2002; Subramanian et al., 2006). Most are ‘open’ systems, under constant evolution and able to incorporate rapidly, and often easily new information from user or developer databases. Quantitative in silico predictions are now possible for several PK parameters, particularly absorption and distribution (Vedani at al., 2007; Lee et al., 2007; Dokoumetzidis et al., 2007). The emerging consensus is that the predictions are no worse than those made using in vitro tests, with the decisive advantage that much less investment in technology, resources and time is needed. In addition, and of critical
importance, it is possible to screen virtual compounds. Some packages are able to handle thousands of molecules in a few hours.

However, common experience shows that, in part at least for essentially irrational reasons, there is currently a lack of confidence in these approaches. An effort is required by the software producers towards more transparency, in order to improve the confidence of their consumers. It seems highly probable that in silico approaches will evolve rapidly, as did in vitro methods during the last decade. Past experience with the latter should be helpful in avoiding repetition of similar errors and in taking the necessary steps to ensure effective implementation. A general concern is the lack of access to the large amounts of data on compounds no longer in development, but still kept secret by the pharmaceutical industry. Controlled access to these data could be particularly helpful in validating new in silico approaches. These in silico approaches are promising filters for virtual libraries to aid synthesis as well as the selection of compounds for acquisition and screening in the early stages of drug discovery.

1.3.2 In vitro strategies
Assessment of ADME properties is now conducted at very early stages of drug discovery for the purpose of accelerating the conversion of Hits and Leads into qualified developmental candidates. To meet this need, high throughput in vitro tests have been developed that can profile NCEs in a model for each of the major barriers to good bioavailability post oral dose, and are often able to screen hundreds of compounds per week with better projections for PK properties of compounds in humans (Li, 2004; Chaturvedi et al., 2001; Lahoz et al., 2006). A number of new in vitro techniques are available to screen compounds for key ADME characteristics such as absorption and metabolic stability, which, when applied within a rational strategy, can make a major contribution to the design and selection of successful NCEs (Bohets et al., 2001; Sun et al., 2004). One or all of them will have a major impact on the exposure of individual to orally administered drugs and to their efficacy. These assay models have been applied not only to screening and ranking of potential drugs candidate but also to the understanding of the mechanisms leading to in vitro pharmacokinetic outcomes.
1.3.2.1 Absorption

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. While the promise of predicting membrane permeability from chemical structure alone is enticing, these methods do not yet enjoy the level of sophistication required to supplant experimental methods. In that regard, a number of in vitro tools have been adopted, 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 system for assessing membrane permeability. In the in situ intestinal perfusion system, an intestinal segment is exposed in an anesthetized rat and drugs solution is perfused through the lumen in a single-pass or recirculating fashion. Drugs permeability is derived from the rates of disappearance of drug from the perfusate. Though more labor-intensive, in situ intestinal perfusion 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 perfusion have strong potential for predicting fraction absorbed in human (Stewart et al., 1995). One of the most appealing attributes which these experimental systems possess is the capability to perform relatively high throughput screening. This is particularly relevant for Caco-2 cells, everted intestinal rings, and BBMV. An imposing rate-limiting state once the system are established and optimized and 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 preanalytical 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 & Ong, 1995). Briefly, the analyte is injected onto specialized IAM HPLC column packed with a phosphatidylcholine (PC) stationary phase selected to closely mimic biological membranes. Theoretically, the chromatography capacity factor for the analyte should correlate with the water–to-PC membrane partition coefficient, a useful parameter for calculating membrane permeability. The ability IAM technology to predict membrane permeability is currently being evaluated. Elegant in its simplicity, it is well suited to a rapid screening environment, although it is recognized that its applicability will be limited to those compounds, which are absorbed by passive processes.

1.3.2.2 Metabolism

The systems described above have the greatest utility when absorption is rate–limiting to system 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. As with absorption, valuable metabolic input can be imparted to the drug discovery team based on information gleaned from in vitro metabolic techniques such as hepatic microsomes, S9 fractions, hepatocytes and recombinant CYP-450 enzymes (Yengi et al., 2007).

In vitro metabolic stability profile is qualitative as well as quantitative comparison of metabolism of a compound in human and animal models. It helps in identifying the right model for toxicity studies. Extensive metabolism is generally considered a liability as it limits the systemic exposure and shortens the half–life of a compound. Metabolic stability results are usually reported as measure of intrinsic clearance, from which secondary pharmacokinetic parameters such as bioavailability and half–life can be calculated when other data on volume of distribution and fraction absorbed are available. Since these parameters are very important in defining the pharmacological and toxicological profile of drugs as well as patient compliance, the pharmaceutical industry has a particular interest in optimizing for metabolic stability during the drug discovery and development process (Thompson, 2000). Several strategies such as reduction of lipophilicity, modification and blocking of metabolically soft spots and use of enzyme inhibitors; have been developed to combat metabolism. In spite of several concerns, the fact that active metabolites of several marketed drugs have been developed as drugs with better efficacy,
safety and pharmacokinetics profile; cannot be denied (Masimirembwa et al., 2003; Baranczewski et al., 2006). Therefore, instead of considering metabolic instability a liability it can be exploited as a tool for discovering better drugs. It is equally important to identify the metabolic pathway of the drugs candidates by conducting in vitro CYP450 reaction phenotyping assays. The identification of drug metabolizing enzymes involved in the major metabolic pathways of a compound helps in predicting the probable drug-drug interactions in human (Ekins et al., 2000). Compounds with more than one metabolic pathway have less likelihood of clinically significant drug interactions. In vitro CYP450 inhibition and induction screens are used to evaluate the potential of compound towards drug-drug interactions and the most prone candidates may either be discarded or taken ahead with a caution. It is known that only unbound drug is pharmacologically active and therefore the assessment of bound fraction by the estimation of plasma proteins binding of a compound is another important parameter to be explored in vitro.

Available in vitro metabolism technologies are considerably more efficient than traditional in vivo methods. With these systems, it is possible to assess the relative rates and routes of biotransformation of a handful of compounds in the time required to comparably characterize one compound using in vivo approaches. In the past, a relative paucity of tissues was the most notable impediment to conducting large-scale screening efforts with in vitro tools (Ansed & Thakker, 2004; Gombar et al., 2003). However the continued growth of in–house tissue banks, in concert with the optimization of isolated enzyme expression systems, has made high-throughput metabolism assessment in the discovery arena a reality.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) have played an invaluable role in the structural characterization and quantification of drug metabolites. Indeed, liquid chromatography (LC) coupled with atmospheric pressure ionization (API) MS has now become the most powerful tool for the rapid detection, structure elucidation, and quantification of drug–derived material within various biological fluids (Nassar et al., 2006; Pedraglio et al., 2007; Chu & Nomeir, 2006). Often however, MS alone is insufficient to elucidate unambiguously the structure of metabolite in terms of stereochemistry and exact position of functional groups. In such cases, multiple analytical and wet chemistry techniques, such as LC-NMR enzyme hydrolysis, chemical derivatization, and hydrogen/deuterium-exchange (H/D-exchange) combined with MS are
used to characterize the novel and isomeric metabolites of drug candidates (Prakash et al., 2007).

1.3.3 In vivo strategies
Unfortunately, there is no substitute for actual in vivo data in assessing pharmacokinetics profiles of drug candidates. While insight into various aspects of the pharmacokinetic (ADME) can be gleaned from in vitro techniques, there are as yet no method available for accurately predicting what will happen to a drug when it administered into a animal. The primary information is needed about drug candidate before it can be taken to clinical studies are: whether it can be given orally, its rates of uptake, distribution elimination. The first such information is generally generated in rat model. Where the rat body is treated as black box: compound enters and leaves the body in quantities and at rat that can be precisely quantitate, but what goes in the body can only be inferred through first order kinetics.

The preclinical PK work group first requires development and validation of an assay for measurement of the drug in rat plasma and serum. While many analytical techniques may lie used for this purpose, HPLC-UV, LC/MS or LC-MS/MS are most used methods. Often the plasma assay developed at this stage is used to measure drug level in plasma/ serum during clinical PK studies. The concentration time data are generally analysed using compartmental or non-compartmental approach and various PK characteristics such as half life ($t_{1/2}$), volume of distribution ($V_d$), clearance ($CL$),area under curve ($AUC$) (Jang et al., 2001). If both oral and intravenous formulations are available bioavailability ($%F$) i.e. fraction of an orally administered dose that reaches the blood stream is also calculated.

1.4 Key Pharmacokinetic Parameters
1.4.1 $C_{max}$ and $t_{max}$
Following intravenous (i.v.) or extravascular drug administration, the maximum observed concentration in the concentration time profile ($C_{max}$) and the time to reach that concentration ($t_{max}$, which equal 0 for i.v. bolus dosing) are important descriptors of the extent and nature of drug exposure. $C_{max}$, an indicator of maximum drug exposure, may sometimes relate better to pharmacological or toxicological effects other measures of exposure.
1.4.2 Area under the curve (AUC)

When blood, plasma or serum drug concentrations are plotted versus time, the \( AUC \) is the primary measure of overall exposure following i.v. or extravascular administration of a drug. \( AUC \) is most commonly determined using the linear trapezoidal method. \( AUC \) is expressed in unit of concentration * time (e.g. ng.h/ml).

\( AUC \) is used to calculate clearance and bioavailability which, along with volume of distribution, constitute the most important primary PK parameters. In the drug discovery setting however, secondary or derived parameter such as \( t_{1/2} \) or \( AUC \) have impractical importance.

1.4.3 Clearance (CL)

Clearance is proportionality constant that relates the rate of drug elimination (in mass/time) to blood or plasma concentrations. It is assumed that all drugs entering the body is eventually eliminated equals the amount administered (for an i.v. dose).

Therefore,

\[
\text{Dose}_{i.v.} = CL \times AUC_{i.v.} \quad \text{or} \quad CL = \frac{\text{Dose}_{i.v.}}{AUC_{i.v.}}
\]

Clearance is expressed in units of volume/time and can be thought of as the volume of blood or plasma that must be cleared of drug per unit time to produce the observed rate of elimination. For drugs that are eliminated only by liver, maximum blood \( CL \) is hepatic blood flow (\(~1.2\) l/h per kg in humans); for drugs cleared by the kidney, maximum blood \( CL \) is the glomerular filtration rates (0.1 l/h per kg) for those excreted passively or renal blood flow (\(~1.1\) l/h per kg) if transport mechanism is involved.

1.4.4 Bioavailability (f)

Bioavailability is the fraction of extravascular administered dose that react systemic circulation. Absolute bioavailability is determined by calculating the ratio of dose normalized \( AUC \) following i.v. and extravascular administration.

\[
\%F_{\text{absolute}} = \frac{AUC_{\text{ext}} \times \text{Dose}_{i.v.}}{AUC_{i.v.} \times \text{Dose}_{\text{ext}}} \times 100
\]

Relative bioavailability between two dose routes, forms or formulations is calculated

\[
\%F_{\text{relative}} = \frac{(AUC \times \text{Dose})_{\text{test}}}{(AUC \times \text{Dose})_{\text{ref}}} \times 100
\]
1.4.5 Volume of distribution ($V_z$)

Volume of distribution is an indicator of how extensively a molecule distributes in the body. The volume of distribution ($V_z$) is a function of plasma protein and tissue binding.

$$V_z = V_p + V_e \times (f_u/f_{ut})$$

Where $V_z$ is the apparent volume of distribution, $V_p$ is the volume of plasma, $V_e$ is the extra vascular tissue space volume, $f_u$ is the fraction unbound in plasma and $f_{ut}$ is the fraction unbound in tissues. Thus as $f_u$ increases, $V_z$ increases; conversely as $f_{ut}$ increases, $V_z$ decreases. It is often assumed that unbound plasma concentrations determined in vivo efficacy, if $V_z$ approximates plasma volume (~0.04 l/kg in humans), this suggests that the molecule distributes in the vasculature. On the other hand, a $V_z$ ~0.6 l/kg indicates distribution into total body water. A volume greater than ~2 l/kg implies extensive distribution into tissues.

1.4.6 Volume of distribution at steady state ($V_{ss}$)

A useful volume of distribution term, $V_{ss}$ can be calculated from intravascular PK data using the following equation

$$V_{ss} = CL \times MRT$$

Where, MRT is the mean residence time. Therefore, a steady state parameter can be calculated without steady-state data.

1.4.7 Half life ($t_{1/2}$)

As implied, the half life of a drug is the time it takes for its concentration in blood or plasma to decrease by half. By examining the log drug concentration versus time profile, one can determine how many half life best describe drug loss. Accordingly, when only one phase is observed, there is a single elimination rate constant $K_e$ and half life is described by,

$$t_{1/2} = \frac{0.693}{K_e} = \frac{V_z}{CL} \times 0.693$$

As $CL$ increases, $t_{1/2}$ decreases; as $V_z$ increases, $t_{1/2}$ increases. Thus half life is secondary parameter that is a function of clearance and distribution of the drug. For the drugs that display multiple half lives, an important consideration is determining which half life is more important from pharmacological, on PK standpoint. This can be assessed by
comparing the \( AUC \) of each phase relative to total \( AUC \) i.e., determining under which half-life the majority of exposure occurs.

The other important information at preclinical stage are generated include tissue distribution and some information about its in vitro/in vivo metabolism. Tissue distribution studies often require radiolabelled compound preferable \( ^{14}C \) and reveal the information that where the drug candidate goes in the body. Does it has affinity for a particular tissue and thus accumulates there; does it penetrate the blood brain barrier, how much drug is eliminated in the feces or urine? Plasma, urine and feces sample are also provide an opportunity to search for metabolites.

Due to in vivo constraints, classical PK methods are not high throughput and have often impeded the quick development of new drug candidates. To overcome this problem significant progress has been made in throughput of in vivo pharmacokinetics studies, with the introduction of cassette, or multiple in one protocols. Cassette dosing has been widely applied to PK screening using LC-MS/MS. This technology has proven to be an effective method to improve the throughput of PK screening. Cassette dosing advantage of testing more compounds in a shorter time using fewer animals, on the other hand misleading PK results may be obtained due to problems such as drug-drug interactions. Therefore, the error potentially incurred with cassette dosing for any single compound may be well within the variability encountered in discrete PK studies relative few animals.

1.5 Predicting human pharmacokinetics from preclinical data

The primary objective of preclinical PK is to generate information describing ADME process in animal that can be used for extrapolation to humans ADME process (Poggesi, 2004). Approaches used for prediction of PK in relevant population of human mostly rely on in vivo data from animal using allometric scaling or time invariant methods (Lin, 1995). The growth of in vitro and more recently in silico screens for evaluating pharmaceuticals, PK and toxicity properties has been used to predict complex in vivo behavior in humans. In most cases, careful and educated application of available approaches provides predictions of PK parameters within 2 or 3 fold of that observed. Attention has now been directed towards integrating information from different sources to
increase the precision and accuracy of these PK predictions and to enable a better understanding of the process underlying ADME behavior in humans.

1.6 Cancer facts and figure
Cancer is a devastating disease but largely preventable. Its impact can be reduced through basic research and improvements in treatment and care. It is estimated that there would be over 12 million people diagnosed with cancer in 2008. The global cancer burden doubled in the last thirty years of the twentieth century, and it is estimated that this would double again between 2000 and 2020 and nearly triple by 2030 according to the World health organization (WHO) world cancer report (Boyle et al., 2008). 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 (Stewart et al., 2003).

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 are associated with chronic infections, the most significant ones being hepatitis B and C viruses (liver cancer), human papilloma viruses (cervical 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 percent 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).

### 1.7 Need for the development of anti-cancer drugs
There still is a great need for the identification of novel biologically active compounds. Achieving the difficult goal of identifying novel compound 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. At present number of clinically active anticancer 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 (Andrew et al., 1997).

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 antitumor activity (Indap et al., 1991). 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.

### 1.8 Natural products and their significance
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)).

1.9 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 (Lee, 1999; Cragg et al., 2005) (Figure 1.6).

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.
(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.
4. Molecular modeling studies including determination of three-dimensional pharmacophores.

Toxicological, production, and formulation concerns are addressed before clinical trials can begin (Lee, 1999).

1.10 Research Envisaged and Plan of Work

Now the plants products are seen as best source of anticancer drugs as they provide the lead molecule which can be further converted to the most potent and targetable anticancer drug. Drug discovery from medicinal plants has played an important role in the
treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives have been applied towards combating cancer, over the last half-century (Newman et al., 2000). Of all available anticancer drugs between 1940 and 2002, 60% were natural product or natural product-derived (Newman et al., 2003).

Betulinic acid, 3β-hydroxy-lup-20(29)-en-28-oic acid, Figure 1.7a a naturally occurring pentacyclic lupane type triterpene, is widely distributed throughout tropics. Betulinic acid was isolated from the external bark of White Birch Tree (Betula alba) Figure 1.7b. A variety of biological properties are ascribed to betulinic acid (Figure 1.8), but betulinic acid is recognized for its anticancer and anti-HIV activities (Singh et al., 2002; Fluda et al., 1998; Evers et al., 1996; Walker, 2001).

Previous reports revealed that betulinic acid is a melanoma-specific cytotoxic agent (Pischa et al., 1995), but recent evidence has indicated that betulinic acid also possesses a
broader spectrum of cytotoxic activity against other cancer cell line. A need therefore exists for novel betulinic acid derivatives, which are not only possess potent antitumor activity, but also clinically safe or lack of toxicity and moreover, to have improve pharmacokinetic properties than betulinic acid.

In light of the tremendous interest generated with respect to the chemistry and pharmacological properties of these type of compounds, this research was undertaken at Dabur Research Foundation, in an effort to explore the potential of the Pentacyclic triterpene Betulinic acid found in abundance in the plant kingdom. More than 600 derivatives of Betulinic acid with various modifications of substituent at positions C2, C3, C20 and C28 of betulinic acid were proposed and screened using a panel of human tumor cell lines. Potent derivatives were selected and further studies were carried out to determine the potential of these derivatives for the development as anticancer drugs.

1.10.1 Lead Evaluation and Lead Profiling
In vitro bioassays that are used in lead optimization can be divided into lead evaluation and lead profiling assays. The lead evaluation assays assess potency and selectivity for a given lead compound. The assays used for lead profiling is to provide ADMET liability data that gives the researcher confidence in chemical series as they progress toward drug development. Together, these data direct to identify the most promising preclinical drug candidates for in vivo assays.

1.10.2 Work Plan of Project
The main objective of our research effort was to select novel BA derivative(s) for further preclinical development. Primary in-vitro screening was performed using a diverse panel of human tumor cell lines. Potent derivatives were selected which shows Cytotoxicity activity, specificity and purity. Based on good in vitro anti-cancer activity, purity and established SAR, selected molecules were evaluated using in vitro ADME screening assay for lead optimization.

After in vitro ADME screening, potential drug likeness short listed Lead molecules were subjected to in vivo pilot pharmacokinetic screening in wistar rat (n=6). Finally successful Lead molecule was selected based on favorable or better in vivo pharmacokinetic profile. Full in vivo evaluation of Lead molecule like single dose
pharmacokinetic, relative bioavailability, dose range finding, Gender effect, excretion, tumor uptake, biodistribution and metabolism studies were performed.

Biodistribution study would allow the quantitative determination of Lead molecule in tumor against the normal cells. This factor may help to explain the manner in which Lead molecule can inhibit the tumor growth without apparent toxicity. Therefore, biodistribution, and tumor uptake studies were carried out in human tumor bearing Athymic nude mice. These studies should be useful for preclinical or clinical evaluation of this potential antitumor agent.

**Need for bioanalytical method development and validation**

It is essential to employ well characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily interpreted. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study.

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies.

Applications of bioanalytical studies

- Pharmacokinetic and bioavailability study
- Excretion and metabolism study
- Biodistribution and Tumor uptake study

**1.11 Objectives and Specific Aims**

In the light of above discussion the project was conceptualized to choose the optimum candidate with drug-like properties after in vitro assays and the selected candidate was subjected to various aspect of in vivo pharmacokinetic evaluation (ADME). The objective is:

“**Estimation of New Chemical Entities in Biological Fluids for Bioavailability and Its Pharmacokinetic Evaluation to Screen the Suitable Lead Candidate**”
In vitro Screening

- To study the potential for anti-cancer activity of Betulinic acid and its synthetic derivatives by in vitro Cytotoxicity, Specificity and Purity assay.
- To study the early Absorption, Distribution, Metabolism, Elimination (ADME) of the potential derivative(s) of Betulinic acid derivatives to enable selection of Lead candidate for further development.

In vivo Pharmacokinetic Screening

- After in vitro Cytotoxicity and ADME screening, potential drug likeness short listed LEAD molecules were subjected to in vivo preliminary pharmacokinetic evaluation in wistar rat.

In vivo Pharmacokinetic (ADME) Evaluation

- To evaluate the single dose i.v. and oral bioavailability of Lead molecule in wistar rat.
- 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.
- Excretion study in urine and feces to determine the fate of Lead molecule.
- Gender difference pharmacokinetic study.
- To evaluate and compare the biodistribution pattern of Lead molecule in tumorogenic and non-tumorogenic athymic nude mice i.e., control group.
- To study the pharmacokinetic and tumor uptake of Lead molecule in human tumor xenograft induced athymic nude mice.
- In vivo metabolism study of Lead molecule
The general strategy adopted for screening and selection of Betulinic acid derivatives is shown in the flow chart below:

1. **Design/Synthesize of Novel Betulinic Acid derivatives by Medicinal Chemistry Dept.**
2. **In vitro Cytotoxicity Screening in Human Cancer Cell Lines**
   - Based on Activity, Specificity and Purity
3. **Selection of HIT molecules based on Superior in vitro Cytotoxicity**
4. **Pre-clinical ADME evaluation of selected derivative**
   - Solubility
   - Log P & Log D
   - Permeability
   - Metabolic stability
   - Plasma protein binding
   - Plasma stability
   - Cytochrome P450 inhibition
5. **In vivo pilot pharmacokinetic screening of potential Lead molecules in wistar rat (n=6)**
6. **Selection and full in vivo evaluation of one Lead molecule**

**Full scale bioanalytical method development and validation for PK and Biodistribution studies**

**In vivo Absorption and Distribution Study**
- Single Dose PK Study in Wistar Rat (n=6)
- Relative bioavailability determination (n=6)
- Pharmacokinetic dose linearity of LEAD molecule at low, mid and high doses (n=6)
- Gender difference study (n=6)
- Biodistribution study in tumor bearing and non-tumor bearing nude mice (nu/nu) (n=4/group/time point)
- Tumor uptake study (n=4/group/time point)

**In vivo Excretion and Metabolism Study**
- Fate of Lead molecule
- Major route of elimination (Urine or feces)
- Possible metabolite identification