Pharmacokinetics in Drug Discovery

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ABSTRACT: The aim of this current review is to summarize the present status of pharmacokinetics in Drug Discovery. The review is structured into four sections. The first section is a general overview of what we understand by pharmacokinetics and the different LADMET aspects: Liberation, Absorption, Distribution, Metabolism, Excretion, and Toxicity. The second section highlights the different computational or *in silico* approaches to estimate/predict one or several aspects of the pharmacokinetic profile of a discovery lead compound. The third section discusses the most commonly used *in vitro* methodologies. The fourth and last section examines the various approaches employed towards the pharmacokinetic assessment of discovery molecules; including all the LADME processes, discussing the different mathematical methodologies available to establish the PK profile of a test compound; what the main differences are and what should be the criteria for using one or another mathematical approach. The major conclusion of this review is that the use of the appropriate preclinical assays has a key role in the long-term viability of a pharmaceutical company since applying the right tools early in discovery will play a key role in determining the company's ability to discover novel safe and effective therapeutics to patients as quickly as possible. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:654–690, 2008

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REVIEW OF BASIC PHARMACOKINETIC CONCEPTS

Pharmacokinetics (PK) is the study of a drug and/or its metabolite kinetics in the body. It refers to the temporary evolution of a drug and its metabolites in serum, plasma, or whole blood, tissue target and target organs over time.[1] The body is a very complex system and a drug undergoes many steps as it is being absorbed, distributed through the body, metabolised, and/or excreted (ADME). Pharmacokinetics has been broadly divided into two categories of study: absorption and disposition. Disposition is further subdivided into the study of distribution and elimination. The term elimination includes metabolism and excretion since, from the PK perspective, we consider that the drug has been eliminated when it is no longer in its original

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chemical structure. Restated, when any biotransformation of the parent compound takes place and even if the resulting metabolites remain in the body, it has been eliminated.

LADMET-R and Pharmacokinetics

When the studies are focused solely in one specific pharmacokinetic aspect (Absorption, Distribution, Metabolism, or Excretion) by *in vitro*, *in situ*, *in vivo*, *or in silico* techniques it is usually referred to as ADME studies whereas the name Pharmacokinetics is normally reserved to *in vivo* studies where an integrated approach of all the ADME processes together is taken. For either ADME or Pharmacokinetics, the truth of the matter is that under both approaches, it is necessary to command a more or less sophisticated knowledge of algebra and calculus to correctly interpret the dataset. Although ADME assays have been the gold standards in PK, there are additional tests that should be incorporated, since they play a key role in Drug Discovery and further development. Liberation of the drug from the pharmaceutical form is a key parameter in bioequivalence studies (e.g., a sustained release versus immediate release formulation)[2–7] or, for intravenous formulations, where the rate of release from the formulation (liposomes, micellar solutions. . .etc) determines the disposition of the drug.[8–12] Response and toxic effects are the other two key aspects to consider since they are the main reasons for Drug Discovery failure (see Fig. 1). In summary, when we refer to the different individual assays that should be performed to characterize the PK profile of a new drug *in vitro*, *in situ, in vivo*, or *in silico* we should also consider, besides the "gold standard" ADME, (1) release from the pharmaceutical form, (2) toxicity, and (3) activity/response in the target site (LADMET-R).

DISCOVERY AND DEVELOPMENT

New drug development can be divided in two different stages: discovery and development. Recently, Kola and Landis[13] reviewed the major causes of attrition in development (see Fig. 1). In their review, they showed how the root causes of drug failure have evolved over time (1991–2000). In 1991, PK and bioavailability were the major reasons for drug failure (40%) dropping dramatically to 10% in 2000. This significant change is mainly due to the time and effort that Industry has invested in the last decade toward a deeper and better understanding of PK, partially in an attempt to overcome poor bioavailability but also trying to look into more predictive kinetic

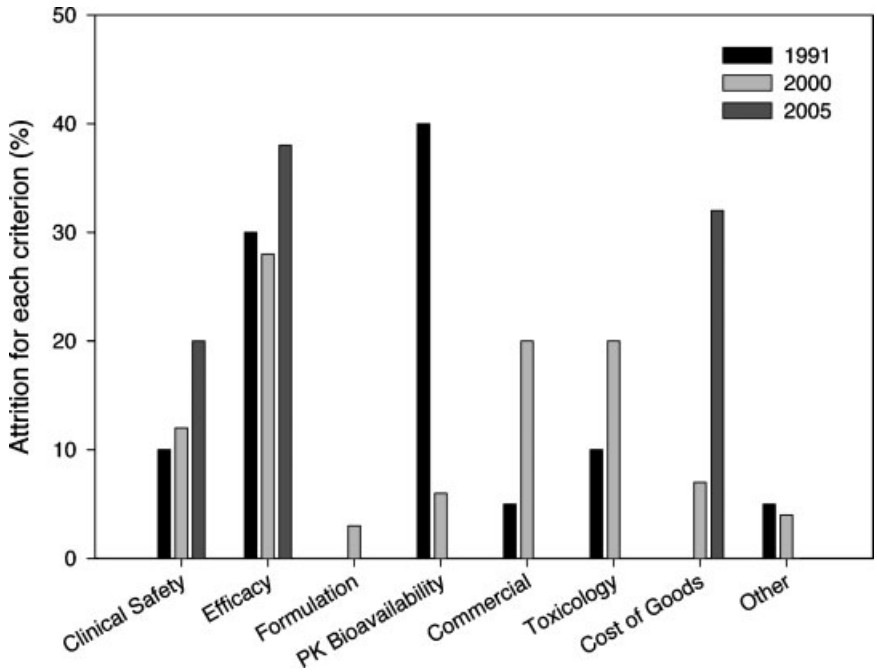

Figure 1. Main reasons for drug development failure. Adapted from Kola 2000[13] and Tufts Center for the Study of drug Development.

behavior of the drug candidates to allow for more efficient dose regimens. Lack of efficacy and safety were reported as the most relevant causes of why compounds undergo attrition in the clinic in 2000 (30%). The Tufts Center for the Study of Drug Development[14] published in 2005 the three main reasons for terminating unpromising new drugs. Again, safety and efficacy were listed among the main three. In summary, the identified issues in that report have been the main focus of study in recent years as well as a driving force determining which strategy to follow in Drug Discovery. The composite of activity, safety, and acceptable LADME properties, rather than a specific attribute, will dictate the success of the drug program. In order to identify potential liabilities in discovery and eliminate those molecules from further consideration, high throughput screening (HTS) of reliable and appropriate *in vitro*, and/or *in situ* assays seem to be the fastest and more efficient way to proceed,[15–17] as shown in Figure 2.

Generally, when a drug is granted to progress into development, a project team is formed with members of different areas of expertise (i.e., including, but not restricted to, toxicology, pharmacokinetics, clinical development, medicinal chemistry, formulation, marketing and regulatory affairs), with the goal of establishing an early development plan. Successful drug development is a result of getting to this stage with enough information about the previously mentioned processes (LADMET-R) in conjunction with a worthwhile investment that provides value to the sponsor. The project team needs to be aware of the target product profile in order to make educated decisions about the direction that the project needs to evolve in order to reach the next milestone in development. Components of the target product profile are: disease indication, minimum efficacy requirements, required safety profile, desirable dose regimen, dosage form, maximum cost of goods, planned date of regulatory submission and expected approval date.[18]

In summary, when planning exploratory studies in humans, under an Investigational New Drug (IND) application, there is some preclinical data as well as chemistry, manufacturing and controls information that need to be generated. The approaches taken in generating this data can be optimized expediting the progress into development and increasing the chances of success of the IND filing by compiling a good quality dataset in an efficient manner. Depending on the goals of the proposed investigation, the amount of data that need to be submitted can vary.[19]

DRUG DISCOVERY: HOW DOES THIS WORK?

The quality and quantity of preclinical data provided by discovery groups to support the development of a new drug has considerably improved in the last few years. This is due to the acknowledgement from Industry of the relevance of this information to the success of the drug as

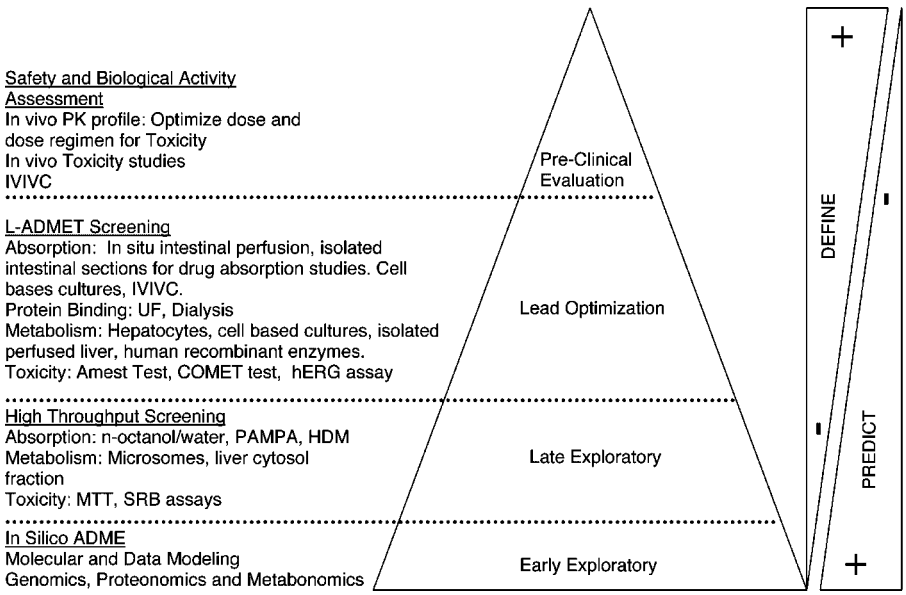

Figure 2. Drug discovery.

mentioned above. A variety of *in vitro* assays have been automated through the use of robotics. *In silico* models are being used to assist in the selection of the right assay and the set of compounds undergoing further *in vitro* screening. With the emerging new computational models (*in silico*), a deeper understanding of the relationship between important LADME-T parameters and molecular descriptors and/or *in vitro* parameters has been achieved, allowing for an early estimation of several LADME-T properties (see Tab. 1).

HTS facilitates a researcher to effectively conduct some biological test to a large number of potential therapeutic moieties.[20] Through this process, rapid discrimination of active ingredients versus undesirable compounds based on the results of the particular assay can be achieved. The main difference of this strategy versus the traditional pharmaceutical screening is that less rigorous results are needed. There are few samples from many compounds as opposed to a very rich database from few compounds. HTS screening is, in essence, a single goal to which all the data may subsequently be applied. HTS is used at early stages of discovery to gather LADME-T information that serve as key factors for candidate selection. As a result, there has been a recent focus on enhancing the efficiency of obtaining absorption, disposition, and toxicity data, which has permitted LADME-T scientists to contribute more effectively to the drug discovery process.

Since the oral route is the preferred administration route for patients, and this fact assures compliance of the drug therapy, a lot of time and effort has been invested toward a good understanding of the physicochemical properties that play a key role in bioavailability. Bioavailability has been defined by the FDA as *the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action*.[21] Thus, for oral pharmaceutical forms, systemic exposure is going to be highly dependent on the extent of absorption in the gastrointestinal tract (GI). The Biopharmaceutics Classification System (BCS)[22] as a drug development tool allows for the estimation of contributions of the three major factors that affect drug absorption: dissolution, solubility, and intestinal permeability.[22–24] Based on *in vitro* solubility and *in vivo* permeability values, drugs can be divided into four groups: class 1 (high permeability, high solubility, HP:HS), class 2 (high permeability, low solubility, HP:LS), class 3 (low permeability, high solubility, LP:HS), and class 4 (low permeability, low solubility, LP:LS), see Figure 3.

This analysis points out conditions under which no *in vitro–in vivo* correlation may be expected for example, rapidly dissolving low permeability drugs. Furthermore, it is suggested that for very rapidly dissolving high solubility drugs, for example, 85% dissolution in less than 15 min, a simple one-point dissolution test is all that may be needed to insure bioavailability. For slowly dissolving drugs, a dissolution profile is required with multiple time points in systems which would include low pH, physiological pH, and surfactants, where the *in vitro* conditions should mimic the *in vivo* processes. The draft guidance document entitled "*Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate Release Solid Oral Dosage Forms Containing Certain Active Moieties/Active Ingredients Based on a Biopharmaceutics Classification System*" proposes to further expand the regulatory applications of BCS and also recommends methods for classifying drugs and immediate release formulations.[25] However, Wu and Benet[26] suggest an alternative classification attending to solubility values and metabolism rather than permeability values, The Biopharmaceutics Drug Disposition Classification System (BDDCS). The authors, while recognizing that drug metabolism can differ depending on the drug's solubility and permeability characteristics, consider that switching permeability values to extent of elimination would be less restrictive, expanding the Class I drugs eligible for waiver of bioequivalence (BE).[27]

For drugs with low permeability, the rate at which they are being actively carried through the GI and reaching the systemic circulation is highly dependent on the carrier-mediated systems involved. Both influx and efflux under these circumstances will play an active role in the oral bioavailability of the compound. In light of this, Klopman et al.[28] discussed the importance of lipophilicity in membrane transport models.

Several experimental techniques have been described to evaluate intestinal absorption including physicochemical measurements (e.g., solubility, lipophilicity, partition coefficients), subcellular fractions (brush border membrane vesicles, basolateral membrane vesicles), cell culture-based models, artificial membranes, isolated tissues, and organ preparations. These techniques are briefly described in this review

Table 1. *In Silico* LADMET Studies

	Descriptor Involved/Substrate Used	Modeling Approach	Comments	Reference
Elimination	CYP1A2	Pharmacophore		73
				91
	CYP2C9	3-D QSAR		66
		Pharmacophore		74,75,9
	CYP2D6	Pharmacophore		76
		Neuronal network and Bayesian statistics	Characterizing the sensitivity of a compound to metabolism predicting its potential elimination profile	17
	CYP3A4	Pharmacophore		77–80
	CYP450s	Homology models		88–90
	MAO A,B	QSAR		92
		3D-QSAR and CoMFA		67
	Phenol sulfotransferase (PST)	QSAR		93
	Glucuronidation UDP-glucuronosyltransferases	QSAR(DARC/PELCO)		94,95
Distribution	Bile acid carrier	3-D-QSAR and CoMFA		68
	PEPT	Correlation		245
		CoMFA		70
	P-gp	CoMFA	Characterizing the sensitivity of a compound to be substrate of protein carriers which will affect its distribution	71
		Catalyst Pharmacophore		81
		Pharmacophore		82
		3-D-QSAR/PLS		69

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Absorption	Partition coefficient and Molecular Weight	Biophysical models	Absorption/permeability predictions	246,124
		PATQSAR		100
		QSAR		28
	Liposome partitioning	Correlation		128
	Polar surface data	Correlation		142,247
	Polar surface data	Correlation	Solubility predictions	142
	Molecular descriptors	ANN		101,103
	Aminoacid sequence	SVM		248
	Everted intestinal rings	Correlation	Drug accumulation predictions	139
	Partition coefficient, Molecular weight	Biophysical models	Bioavailability Predictions	249
	In situ absorption rate constant values (Rat small intestine)			249,250
	Physico-chemical properties (HDM, Caco-2 and 2/4/A1)	Non linear regression		142
Liberation	*In vitro* dissolution test	IVIVC	Bioavailability predictions	2–7,105,106
	Physicochemical and structural factors	QSAR/ORMUCS		29
Response	Partition coefficient, Molecular weight	PATQSAR	*In vitro* evaluation for prediction of *in vivo* response	100
	Atom typing	Naive Bayes classifier		25
	Physicochemical descriptors	2D-QSAR		64
Toxicity	Chemical structure (Ames test)	Correlation	*In vitro* evaluation for prediction of toxic effects *in vivo*	43
	Identification of potential hERG channel blockers (Dofetidine DisplacementTest)	Neuronal Networks and Bayesian statistics		17
	Subcutaneous and ocular toxicity (MTT)	Correlation		55,57

Adapted from de Groot [34] and Ekins.[80,251].

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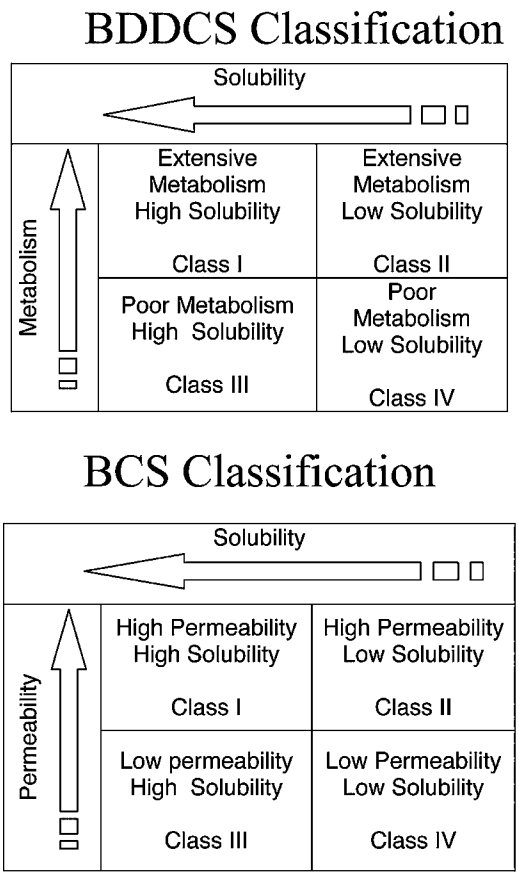

Figure 3. Biopharmaceutics classification system and biopharmaceutics drug disposition classification system.

under *in vitro* screening. Examples of their application have been tabulated in Table 2.

*In silic*o models are extensively applied to absorption descriptor's properties and bioavailability predictions. For instance, the quantitative structure–bioavailability relationship of 232 structurally diverse drugs was studied to evaluate the feasibility of constructing a predictive model for the human oral bioavailability of prospective new medicinal agents by Yoshida et al.[29] where they found lipophilicity to be a significant factor influencing oral bioavailability (see Tab. 1).

However, for many compounds, oral bioavailability may be limited by extensive metabolism rather than poor absorption. Metabolism can be defined as the chemical changes (biotransformation) that take place in a given chemical substance within an organism. The biotransformation that normally takes place within the body leads towards more hydrophilic or water-soluble moieties than the parent compound which facilitates/accelerates the excretion from the body. Thus, the potential of a compound containing a chemical moiety known to be extensively metabolized may be predicted with reasonable accuracy on the basis of abundant historical data.[30] Several *in vitro* methods are routinely used to establish the metabolic profile of a moiety[31,32] such as microsomes, supersomes, cytosol, S-9 fraction, cell-based models, primary hepatocytes, liver slices, and perfused liver. Because these assays are based on the native metabolic enzyme they are an excellent source for the experimental generation of metabolite profiles, which will help influence the selection of the appropriate pre-clinical safety species. Several *in vitro* experimental techniques have been briefly described later in this review (see Tab. 3).

At the level of Metabolism, there are also some predictive models. Bursi et al.[33] have derived a structure–pharmacokinetic relationship for a data set of 32 in-house steroidal androgens. The same group developed an electronic model for hydrogen abstraction in steroidal androgens in which the activation energies of steroid radical systems could be used to predict relative rates of metabolism to guide the design and redesign process of metabolically more stable steroidal androgens. Many 3D ligand-based and structure-based computational approaches have been used to predict the metabolism catalyzed by the enzymes of the cytochrome P450 superfamily (P450s) responsible for 70% of the metabolism of all drugs. Computational methodologies have focused on a few P450s that are directly involved in drug metabolism. Models derived for P450s help to explain and predict the involvement of P450s in the metabolism of specific compounds and guide the drug-design process.[34] See Table 1 for additional computational models for human P450s.

Drug administration has the main goal of achieving therapeutically effective drug concentrations at the site of their clinical activity while minimizing adverse effects (usually linked to non-target site drug concentrations).[35] However, the ability of drugs to reach the site of action depends on many pharmacokinetic aspects such us drug availability and drug metabolism, both already mentioned, but also binding to plasma proteins. Drug tissue distribution is often correlated with total plasma/serum concentrations (bound and unbound fractions), which for drugs with a high percentage of protein binding that act in peripheral tissues becomes a questionable assumption. Several techniques have been developed to study

Table 2. *In Vitro* Systems for Permeability and Protein Binding Studies

System	Origin	Comments	Reference
Dissolution studies	Simulated intestinal fluids	Establish the dissolution profile	253–255
CaCo-2	Human colorectal adenocarcinoma	Most well established cell model that expresses relevant carrier-mediated systems	256,257,258
MDCK	Canine kidney	Ideal for transfections. Low instrinsic expression of ABC family transporters	152,153
LLC-PK1	Pig kidney	Ideal for transfections. Low instrinsic expression of ABC family cassette transporters	154,259
2/4/A1	Rat fetal intestinal	Ideal for studying paracellular route of absorption	260,261,262
TC-7	CaCo-2 clone	Same than CaCo-2	263,264
HT-29	Human colorectal adenocarcinoma	Contains mucus-producing globet cells	265,266
IEC-18	Rat Ileum	Permeability and para-cellular transport	267
PAMPA	Phospholipid-coated filter	Permeability for passive absorption	20,125,268
IAM	cell membrane phospholipids immobilized to solid surfaces	Permeability for passive absorption	156,158
HDMs	Artificial Hexadecane Membranes	Passive transcellular permeability	262,141,142
BBMV	Brush border membrane vesicles	Passive transcellular permeability and active carrier-mediated systems	131–133
In situ intestinal perfusion	Rat small intesine	Passive transcellular permeability and active carrier-mediated systems	23,138,162
Liposomes	Bilayer vesicles	Passive absorption	129,269
Partition coefficient	N octanol/water	Lipophilicity parameter	124,270
Ultracentrifugation	Partitioning	Unbound drug fraction in plasma	170,171
Microdyalisis	Microdialysis probes	Unbound drug fraction in the interstitial fluid	271,272

Adapted from Balimane 2005[252].

Table 3. *In Vitro* Systems for Metabolism and Toxicity Studies

System	Origin	Comments	Reference
Liver microsomes	Liver tissue	Phase I metabolism	173–175,183
Intestinal microsomes	Intestinal tissue	Phase I metabolism	176,177
Cytosolic fractions	Liver tissue	Phase II metabolism	178,179
Supersomes	Liver tissue (Baculovirus-insect-cell expressed)	Specific CYP/UGT mediated metabolism	32,183
S9 fractions	Liver tissue	Phase I and II metabolism	180,181
		Detecting DNA damage	182
Isolated perfused liver	Liver	Hepatotoxicity	204–206
		Metabolism	207,208
Liver Slices	Liver	Hepatotoxicity	184,185
		Metabolism	184,186
Hepatocytes	Liver	Hepatotoxicity	199–201,234–236
		Metabolism	203
HepG2	Human hepatoma cell line	Hepatotoxicity	188–190
		Metabolism	187,191
HLE	Human lens epithelial cell line	Hepatotoxicity	193,194
		Metabolism	192,193
BC2	Human hepatoma cell line	Hepatotoxicity	196,197
		Metabolism	195,198
DNA microarray	Rat liver	Phase I metabolism	211
	Rat liver	Phase II metabolism	212
Genotoxicity	COMET assay	Detecting DNA damage	44,45
	Ames test		41,42
Renal toxiciy	DNA microarray	Changes in gene expression	273,274
Hepatotoxicity			275,276
Cytotoxicity	MTT assay	Cell proliferation	48,49,54,57
	SRB assay		50,51
	Clonogenic assay		52,53
hERG potassium channels	I_{Kr} assay	Electrophysiology study	237,238

drug protein binding in plasma, with the two predominant methods being ultrafiltration (UF) and equilibrium dialysis (ED).[36,37]

There is a clear need for companies to find ways to evaluate safety of drug candidates earlier in the development process. Animal toxicology studies are the foundation of an IND. The principal safety concerns are usually in the area of genetic toxicology, target organ toxicity and cardiosafety. The identification of HTS assays that can accelerate the advance of the drug candidate into more relevant *in vivo* testing as soon as possible is the main goal in the discovery stages. There are several toxicity studies that are routinely performed depending on the nature of the drug. Genotoxicity, cytotoxicity, and target organ toxicity may be, at least in a first instance, evaluated through *in vitro* screening. The use of positive (known toxic reagents) and negative controls (non toxic reagents) is necessary to assure that the *in vitro* systems respond as the *in vivo* tissue would. *In vitro* studies should be conducted with concentrations and exposure times similar to the *in vivo* conditions. The *in vitro* models may allow high throughput screening, decreasing the number of chemicals tested in whole animals.

Mutagenicity screening is a regulatory requirement for drug approval since they imply a toxic risk in humans.[38] The International Agency for Research on Cancer (IARC) discussed in a consensus report[39] the term "genotoxicity," considering that this term includes both direct and indirect effects in DNA. Direct effects are considered inductions of mutations (gene, chromosomal, genomial, and recombinational) that at the molecular level are similar to events known to be involved in carcinogenesis. Indirect DNA effects involve surrogate events associated with mutagenesis (e.g., unscheduled DNA synthesis (UDS) and sister chromatid exchange (SCE), or DNA

Table 4. Online Information and Software Resources for Absorption as Well as Other LADME Aspects

URL	Description	Name
http://www.accelrys.com/products/cerius2/cerius2products/c2adme.html	This is a package of six predictive ADME/Tox models—human intestinal absorption, aqueous solubility, human CYP 2d6, blood–brain barrier penetration, serum protein binding, and human hepatotoxicity.	
http://www.compudrug.com/	Since the early 1960s, lipophilicity has proven to be very important molecular description, often well-correlated with the bioactivity of chemical entities. Lipophilicity and hydrophobicity are measured by lipophilic and hydrophobic indices, such as the logarithm of a partition coefficient, which reflects the equilibrium partitioning of a molecule between a nonpolar and polar (aqueous) phase. A new artificial neural network using atomic fragmental descriptors has been developed to predict the octanol-water partition coefficient (logP). The fragmental descriptors were obtained from the Atomic2005 linear logP calculation method implemented in Pallas PrologP program. Using a numerically optimized weighted average of the older methods and the current one, the result is significantly more accurate than the previous method, and provides an exceedingly accurate prediction. The new logP prediction method was implemented into the Pallas 3.4 version.	Pallas
http://www.simulations-plus.com/products.html	In an effort to help pharmaceutical companies bring new drugs to market faster and for less cost, Simulations Plus has developed GastroPlus^{TM}, a unique software program that simulates the dissolution and absorption of a drug in the human gastrointestinal tract.	Gastro-Plus
http://www.simulations-plus.com/products.html	ADMET Predictor is an advanced computer program that enables pharmaceutical researchers to estimate ADME properties (such as permeability, solubility, lipophilicity, diffusivity, etc.) of new chemical entities (NCE's) from their molecular structure.	ADMET Predictor
http://www.simulations-plus.com/products.html	(Dose Disintegration and Dissolution) is a new tool for forulation scientists that simulates the *in vitro* disintegration and dissolution of dosage forms in USP Paddle, Basket, and Flow-Through experiments.	DDDPlus
http://www.pasteur.fr/recherche/unites/pmtg/abc/database.iphtml	Database describing the properties and the protein composition of experimentally investigated ATP-binding cassette (ABC) systems. In addition, we report complete inventories of the predicted ABC systems for organisms whose complete genome sequence is known. In the latter case, ABC proteins and their interacting partners were identified by comparing the proteomes of these organisms to the full content of ABCISSE. Systems were reconstructed and sorted in families and subfamilies according to the phylogenetic and functional classification, which was described in our publications. We predict the polarity of transport, the substrate specificity, and the biological role of these systems.	ABCISSE
http://133.9.194.61/tp-search/index.html	TP-Search is a comprehensive database on drug transporters, which are thought to play an important role in the pharmacokinetics of drugs. All the infomation is extracted from a large number of published papers. With this database, users can obtain various kinds of basic information on drug transporters.	TP-Search
http://nutrigene.4t.com/humanabc.htm	Human ATP-binding cassette transporters	

(*Continued*)

Table 4. (*Continued*)

URL	Description	Name
http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html	ABC-transporter genes	
http://lab.digibench.net/transporter/	Human membrane transporter database	
http://xin.cz3.nus.edu.sg/group/admeap/admeap.asp	A database for facilitating the search for drug Absorption, Distribution, Metabolism, Excretion associated proteins. It contains information about known drug ADME associated proteins, functions, similarities, substrates/ligands, tissue distributions, and other properties of the targets. Associated references are also included.	
http://www.mhc.com/PGP/index.html	P-glycoprotein drug interactions	

damage (e.g., the formation of adducts) being the most relevant ones, which may eventually lead to mutations. Mutations are permanent hereditary changes in the cell lines. Genotoxicity thus preceeds mutagenicity although most of genotoxicity is repaired and is never expressed as mutations.[40] The Ames test and Comet assay (Single-Cell Gel, SCG) are *in vitro* cell-based assays that are routinely used to assess genotoxicity induced by xenobiotics[41–47] and they have been briefly described later in this review.

Cytotoxicity can be defined as the quality of a xenobiotic to confer toxicity to cells. Cytotoxicity in different cell lines will provide useful information about potential target organs toxicities. MTT, SRB (Sulforhodamine B), and clonogenic assays are used for measuring drug-induced cytotoxicity by measuring cellular proliferation in mammalian cell lines.[48–53] Moreover, a routine screening for a wide range of pharmaceutical products is cutaneous and ocular toxicity, where epidermal keratocyte and corneal epithelial cultures have become relevant models that can provide valuable information about the mechanisms of cutaneous and ocular toxicities of test compounds.[54–57]

Hepatotoxicity is one of the major reasons for withdrawal of marketed drugs over the past two decades.[58,59] The pharmaceutical industry has dedicated much emphasis on developing *in vitro* screening systems to detect hepatotoxicity (isolated perfused liver, liver slices, primary hepatocyte cultures, human liver-derived cell lines, etc).Toxins can be classified as hepatotoxins, which cause liver damage in the majority of the population with or without metabolic activation. Further in this nomenclature, hepatotoxins can be subdivided into cytotoxics if they injure the hepatocytes and cholestatics if they interfere with the bile flow.[60]

The International Conference of Harmonisation (ICH) guideline S7B describes a nonclinical testing strategy for assessing the potential of a test substance to delay ventricular repolarization (VR). VR is determined by the duration of the cardiac action potential as a result of the activities of many membrane ion channels and transporters. In this guideline are descriptions of *in vitro* (patch clamp) and *in vivo* electrophysiology studies for competition binding protocols in which test substances are studied for their ability to displace a hERG channel blocker (human Ether-a-go-go Related Gene).[61,62] The hERG gene encodes a potassium ion channel responsible for the repolarizing I_{Kr} current in the cardiac action

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Table 5. Online Information and Software Resources Mainly Focused in Metabolism

URL	Description	Name
http://drnelson.utmem.edu/CytochromeP450.html	Cytochrome P450 Homepage	
http://www.cypalleles.ki.se/	Home Page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee	
http://www.icgeb.org/~p450srv/	The goal of this www Directory is to facilitate access to electronic resources world-wide for all researchers working in the field of P450 proteins and P450-containing systems.	
http://medicine.iupui.edu/flockhart/	Cytochrome P450 drug interactions table P450 inhibiting drugs	
http://www.accelrys.com/products/cerius2/cerius2products/c2adme.html	This is a package of six predictive ADME/Tox models—human intestinal absorption, aqueous solubility, human CYP 2D6, blood–brain-barrier penetration, serum protein binding, and human hepatotoxicity	C2 adme
http://www.accelrys.com/products/chem_databases/databases/metabolism.html	This database has been compiled from and based on two noteworthy journals of the Royal Society of Chemistry (RSC): *'Biotransformations'* and *'Metabolic Pathways of Agrochemicals'*. It covers the metabolic pathways of drugs, agrochemicals and industrial chemicals in various species. On this strong foundation, new entries are added based on the expert abstraction of pertinent and relevant literature references	Metabolism
http://www.compudrug.com/	The knowledge base of the MetabolExpert module of Pallas has been extended with new metabolic reactions. The extension includes a set of special metabolic reactions collected from the scientific literature focusing on the metabolisms of toxic and drug-like organic compounds. Thanks to the inserted reactions, the new version of MetabolExpert will manage a series of special metabolisms, including ring opening and closing reactions	MetabolExpert
http://www.lhasalimited.org/index.php?cat=2& sub_cat=68	Meteor is a computer program that helps scientists who need information about the metabolic fate of chemicals and want to be more efficient, more effective and make better decisions. The program uses expert knowledge rules in metabolism to predict the metabolic fate of chemicals and the predictions are presented in metabolic trees. The only information needed by the program to make its prediction is the molecular structure of the chemical	METEOR
http://multicase.com/products/products.htm	An expert system capable, when coupled with appropriate dictionaries, to predict the metabolic transformations that may be produced when the molecules are ingested or dumped in the environment. The program is totally interfaced with MCASE and permits a complete evaluation of the potential toxicological effect of a molecule and its metabolites	META
http://mhc.com/Cytochromes/	P450, UGT, and P-gp drug interactions	

Table 6. Online Resources in Toxicology.

URL	Description	Name
http://www.accelrys.com/products/cerius2/cerius2products/c2adme.html	This is a package of six predictive ADME/Tox models—human intestinal absorption, aqueous solubility, human CYP 2D6, blood–brain-barrier penetration, serum protein binding, and human hepatotoxicity	
http://www.compudrug.com/	Elimination of cytotoxic compounds in the early phases of drug discovery can save substantial amounts of research and development costs. A new artificial neural network based approach using atomic fragmental descriptors has been developed to categorize compounds according to their *in vitro* human cytotoxicity. Fragmental descriptions were obtained from the linear logP calculation method implemented in Pallas PrologP program	Pallas proLogP
http://multicase.com/products/products.htm	Knowledge-based systems are computer programs aimed at organizing relevant experimental data for the purpose of helping a user make a decision about real world problems. Our group, over the years, has developed two such programs, CASE (Computer Automated Structure Evaluation) and its successor, MCASE/MC4PC, designed for the specific purpose of organizing biological/toxicological data obtained from the evaluation of diverse chemicals. These programs can automatically identify molecular substructures that have a high probability of being relevant or responsible for the observed biological activity of a learning set comprised of a mix of active and inactive molecules of diverse composition. New, untested molecules can then be submitted to the program, and an expert prediction of the potential activity of the new molecule is obtained. MCASE differs from CASE in a great many ways, but the major algorithmic difference is the use of hierarchy in the selection of descriptors, leading to the concept of biophores and modulators	CASE/MCASE

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Table 7. *In Silico* Online information and Databases

URL	Description	Name
http://iwrwww1.fzk.de/biostruct/RLD/mtx.htm	Application of STUN to *in silico* database screening	
http://redpoll.pharmacy.ualberta.ca/drugbank/	Bioinformatics and cheminformatics resource that combines detailed drug (i.e., chemical, pharmacological and pharmaceutical) data with comprehensive drug target (i.e., sequence, structure, and pathway) information	DRUGBANK
http://www.psc.edu/general/software/packages/genbank/genbank.html	GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences. A new release is made every two months. GenBank is part of the International Nucleotide Sequence Database Collaboration, which is comprised of the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at the National Center for Biotechnology Information.Each GenBank entry includes a concise description of the sequence, the scientific name and taxonomy of the source organism, and a table of features that identifies coding regions and other sites of biological significance, such as transcription units, sites of mutations or modifications, and repeats. Protein translations for coding regions are included in the feature table	GENBANK
http://iwrwww1.fzk.de/biostruct/RLD/flexscreen.htm	Flexscreen performs fully automated in-silico screening of a large 3D database of ligands against a structurally resolved protein receptor. Each ligand of the database is docked against the receptor with the stochastic tunneling method using an all-atom representation of both ligand and receptor. Both ligand and receptor can change their conformation in the docking process. Using an all-atom scoring function based on physical principles, the affinity of the each ligand to the receptor is evaluated. The ligands with the best affinity are selected as lead candidates for drug development. Using our secure, high-performace computational platform, we presently screen the open ligand database of the National Cancer Institute (USA) which contains about 250000 ligands, but other databases can also be used	
http://www.accelrys.com/products/mstudio/modeling/visualizationandstatistics/qsarplus.html	QSAR Plus builds on the base tools available in QSAR and extends them to include a neural networks model building method and accurate quantum mechanical descriptors. QSAR Plus enables you to identify compounds with optimal physicochemical properties	QSAR PLUS

DOI 10.1002/jps

potential. Abnormalities in this channel may lead to either Long QT syndrome (LQT2) (with loss of function mutations) or Short QT syndrome (with gain of function mutations). Both are potentially fatal cardiac arrhythmia due to repolarization disturbances of the cardiac action potential. Erroneous drug binding to this channel may lead to acquired Long QT Syndrome.[63]

In silico models, described below, are also being used for predicting activity as well as toxicity[15]. Klopman et al.[25] have developed a model for MDR reversal agents (to overcome Multi-Drug Resistance) to estimate the MDR reversal activity of compounds. The same author discussed the importance of lipophilicity values (represented as the logarithm of the n-octanol/water partition coefficient) and its correlation with their pharmacological and toxic activities.[29] Kazius et al.[43] were able to perform mutagenicity predictions of an independent validation set of 535 compounds with an error percentage of 15%. The authors concluded that toxic properties can often be related to substructures, which are generally identified as toxicophores, and that these toxicophores can be applied to risk assessment processes and can guide the design of chemical libraries for hit and lead optimization. Yoshida et al. have used some physicochemical descriptors (n-octanol/water partition coefficient, topological polar surface area, diameter, summed surface area of atoms with partial charges) to carry out 2D-quantitative structure–activity relationship (2D-QSAR) studies on 104 hERG channel blockers with diverse structures collected from the literature, thus formulating interpretable models to guide chemical-modification studies and virtual screening.[64] Combination of predictive models has also been performed with great success. O'Brian et al. combined hERG channel blocking and CYP450 2D6 inhibition computational models with better predicted values than their individual predictions.[17]

IN SILICO LADMET

The line between *in silico* LADME-T and *in vitro-in silico* LADME-T is difficult to define since the majority of the *in silico* models use not only physicochemical parameters but also some *in vitro* data (see Tab. 1).

In recent years, the number of computational models for the different LADMET processes has considerably increased with the aim of promising leads and the elimination of unsuitable ones. Although these models can never be accurate enough to replace real circumstances, many of them can be extremely useful if they are built under the correct assumptions and right set of data. When the *in silico* models have been carefully developed and rigorously validated the information they provide can be valuable in early Drug Discovery. It is, therefore, not surprising that there is considerable interest in developing mathematical models capable of accurately predicting some LADME-T key information for new drug candidates. However, the misleading use and interpretation of *in silico* LADMET is often the reason why these models have been extensively criticized by a large part of the scientific community.[65] These models are usually based in *in vitro* data and/or physico-chemical properties. Two different types of computational models are being used currently: molecular and data modeling. A brief description of the fundaments of the most used computational models is presented below and listed in Table 1.

Molecular Modeling

The main objective of molecular modeling is to assess the potential interaction between the studied drug and proteins involved in LADMET processes (e.g., carrier-mediated systems such us p-gp, enzymes responsible of either phase I (e.g., CYPs) or II biotransformation(e.g., Glutathione tranferases). Under molecular modelling, we can distinguish: Ligand-based models, Structure-based models, and Homology Models.

Ligand-Based Models

These attempts to link chemical structures with observed activities based on information about active site, shape, electronic properties and conformation of substrates, inhibitors or metabolic products. The simplest one is QSAR: Quantitative Structure–Activity relationship. Three-dimensional quantitative Structure–Activity relationship (3D-QSAR) refers to the analysis of the quantitative relationship between the biological activity of a set of compounds and their spatial properties using statistical methods.[66–69] 3D-QSAR are often based on Molecular Field Analysis, MFA. MFA employs a combination of reasonable molecular description, statistical analysis, and graphical display of results.[67,70,71] Molecular structures are described with molecular interaction energies as steric and

electrostatic fields surrounding the molecules; the statistics is computed by partial least square (PLS) regression analysis and the output is displayed as contours superimposed on the molecules. The comparative MFA, (CoMFA) methodology assumes that a suitable sampling of steric and electrostatic fields around a set of aligned molecules provides all the information necessary for understanding their biological properties.[72] If no structural information is available, an alternative means to assess potential interactions is to use pharmacophore models. These are ligand-based models where different structures of ligands or their properties overlay in 3D space in an attempt to describe the physical, spatial, and chemical properties of the active site.[10,73–82]

Structure-Based Models

Included in this category are X-ray crystallography,[83] nuclear magnetic resonance (NMR),[84] spectroscopy and electron microscopy. These models determine the 3D structure of proteins through a variety of means. However, it is often very difficult to use these techniques due to the nature of the proteins (e.g., difficult to crystallize, poor solubility, large molecular size).

Homology Models

As a result of the difficulties mentioned above when trying to elucidate the 3D structure of proteins, these models were developed. Homology models are an alternative method to elucidate the 3D structure of proteins. These models are based on the fact that the 3D structure of a protein is related to its amino acid sequence since proteins with similar amino acid sequence tend to adopt similar 3D structure.[85–90]

Data Modeling

Data modeling uses statistical tools to search for correlations between a given property and a set of physicochemical descriptors. Quantitative structure–activity relationship (QSAR),[28,29,64,91–95] quantitative structure–property relationship (QSPR),[96–99] population Analysis by topology-based QSAR (PATQSAR)[100] and Artificial Neural Networks (ANN) are some examples of data modeling. ANN is an adaptive system that changes its structure based on external or internal information that flows through the network. ANN may include molecular modeling and data modeling.[101–103]

Li et al.[104] have done excellent work describing the most recent explored statistical learning approaches such as neural networks (NN), support vector machines (SVM), etc. The authors concluded that both classification-based and regression-based statistical learning methods have consistently shown promising capability for predicting chemical agents of diverse ranges of structures and of a wide variety of LADMET poperties.

Table 1 contains a list of references of published *in silico* work in LADME-T.

In Vitro In Vivo Correlations (IVIVC)

Bioequivalent products are those whose rate and extent of absorption do not show significant differences when administered at the same dose.[21] The bioequivalence of two drug products is usually evaluated through *in vivo* assays in human volunteers. However, under some particular conditions, it should not be necessary to carry out an *in vivo* pharmacokinetic study to assure bioequivalence; a well validated *in vitro* study should be able to assess that. From an ethical point of view, if the assay with human volunteers is not essential to demonstrate the equivalence between two formulations, the assay should not be performed, but, on the other hand, we need to assure that the *in vitro* surrogate is reliable. The factors that we have to analyze to establish the theoretical basis for correlating *in vitro* dissolution and *in vivo* absorption are the parameters that control rate and extent of absorption. The basic concept is if two drug products containing the same drug have the same concentration time profiles at the intestinal membrane surface, then they will have the same rate and extent of absorption. Two conditions are necessary for this statement to be true: the two drug products have the same *in vivo* dissolution profile under all luminal conditions and none of the formulation components affects the membrane permeability or intestinal transit time.

IVIVC has been defined by the Food and Drug Administration (FDA) as a "predictive mathematical model describing the relationship between an *in vitro* property of a dosage form and an *in vivo* response".[21,27] Generally, the *in vitro* property is the rate or extent of drug dissolution or release while the *in vivo* response is the plasma

drug concentration or amount of drug absorbed. The main objective of developing and evaluating an IVIVC is to establish the dissolution test as a surrogate for human bioequivalence studies, which may reduce the number of bioequivalence studies performed during the initial approval process as well as with certain scale-up and post approval changes.[105,106] Two step or one step methods can be applied to obtain these correlations. Using the two-step method, by deconvolution or by a mass balance method, the *in vivo* function is computed and from dissolution assays, the *in vitro* variable is calculated, then, in a second step, plasma concentration are predicted by convolution based on the *in vitro* data. A one step method involves a convolution step where plasma concentrations predicted from the model and those observed are directly compared. Published work in this matter has been referenced in Table 1.

Genomics, Proteomics, and Metabonomics

Lately, these terms have been incorporated into many scientists vocabulary with not always a clear idea of the intended meaning.

In addition to the traditional information about the disease state, we now posses a set of new descriptors obtained by molecular profiling. In other words, we have access to large scale of systematic readouts at various levels such as DNA content (genomics), protein expression (proteomics), and measurements of metabolites (metabonomics). Hopefully, the combination of these different readouts will provide us a combination of potential biomarkers as well as better predictors of a disease state.

Genomics

Genomics is the study of an organism's genome and the identification of the genes involved in biological processes. Genomics has the potential of offering new therapeutic methods for the treatment of some diseases, as well as new diagnostic methods. As a consequence of the identification of genome sequences, it is possible to engineer DNA microarrays, which can measure gene expression of thousands of genes simultaneously.[60,107,108]

New applications in the field of genomics are emerging in two major areas.[109] The first one focuses on understanding the mechanism of action involved in disease states or compound-induced phenotypic changes. The second one involves specific biomarker identification for various purposes ranging from disease monitoring to disease progression and prognosis.

Proteomics

Proteomics is a high throughput study of proteins, particularly their structures and functions. Proteomics is much more complicated than genomics. Most importantly, while the genome is a rather constant entity, the proteome differs from cell to cell and is constantly changing through its biochemical interactions with the genome and the environment. The entirety of proteins in existence in an organism throughout its life cycle, or on a smaller scale the entirety of proteins found in a particular cell type under a particular type of stimulation, are referred to as the *proteome* of the organism or cell type respectively.[110–112] Since proteins play a central role in the life of an organism, proteomics is instrumental in the discovery of biomarkers, such as markers that indicate a particular disease.[113–117]

Metabonomics

Metabonomics has been defined as the quantitative measurement and identification of the biochemicals contained in a biological sample such as the metabolic response of living systems to pathophysiological stimuli or genetic modification. This approach has been used in toxicology, disease diagnosis, and a number of other fields. This technology has been used to identify biomarkers for disease as well as to identify off-target side effects in marketed drugs and new chemical entities in development.[15,60,118–120]

A similar and related concept that is worth defining here is Metabolomics, which refers to the study of the chemical fingerprints that specific cellular processes may produce. The study of metabolite profiles will fall into this category. Although there is still no absolute agreement, there is a growing consensus that the difference between the two terms resides in the fact that "metabolomics" places a greater emphasis on comprehensive metabolic profiling, while "metabonomics" is used to describe multiple (but not necessarily comprehensive) metabolic changes caused by a biological perturbation.

The integration of genomic, proteomic and metabonomic data constitutes a very powerful data source for *in silico* analysis. Although we do not fully understand the complexity of the

biological systems and pathologies, by gathering the various sources of information and analyzing the data together, we will certainly see increased contributions toward the establishment of fingerprints for toxicity, metabolism and other LADMET-related processes.

IN VITRO LADMET

The following *in vitro* assays are probably the most common screening tools for the PK aspects already commented on throughout this review. Tables 2 and 3 list these *in vitro* assays pointing out the parameter assayed and some relevant published work in that matter.

Dissolution Studies

Dissolution studies are routinely performed as a part of the quality protocol of solid dosage forms, because these studies help to ensure that the manufacturing process has not deviated significantly from the established standards. The use of dissolution assays as a quality control index requires a simple dissolution media with simple dissolution conditions in order to minimize practical problems, such as analytical complications, and to keep the cost of the test at the minimum value.[121–123] However, if a test is required that provides more information about what will happen *in vivo*, the BCS can simplify the requirements of the test and provide guidance regarding the inferences that we can obtain from in vitro assays.[23] As mentioned previously, BCS is a framework for classifying drug substances based on their aqueous solubility and permeability and provides the basis for establishing *in vitro–in vivo* correlations (IVIVC) and for justifying "biowaivers," or in other words, permission to use dissolution test data as a surrogate for pharmacokinetic data. Hence, a dissolution test can be used as an *in vitro* bioequivalence study instead of an *in vivo* bioequivalence study.

Absorption

N-Octanol/Water Partition Coefficient

This parameter is often expressed by the −Log(P) value. The −Log(P) is basically a parameter of lipophilicity where the distribution between a solute dissolved in an aqueous buffer (aqueous phase) and n-octanol, as organic phase, is measured.[124–126] The measure of disappearance of the solute from the aqueous phase indicates how much solute travelled to the organic phase. Both phases, aqueous and organic, should be saturated in each other to avoid changes in volume when they contact as that will introduce considerable error in the determination of this parameter. The partition coefficient is defined as the concentration ratio between the organic and aqueous phase as follows:

$$P = \frac{C_o}{C_a} = \frac{(Q_{ai} - Q_{af})/V_o}{Q_{af}/V_a}$$

Where Q_{ai} and Q_{af} represent the amount of solute in the aqueous phase before and after being in contact for a specific period of time with the organic phase in continuous agitation. The pH value of the aqueous phase along with the temperature used for the partitioning are the variables that determine the value of this parameter.

Liposomes

Liposomes are lipid bilayer vesicles used as models for biological lipid bilayer membranes for the study of drug partitioning from aqueous phase into the liposome.[127] Several authors have suggested that this parameter correlates better with human drug absorption than n-octanol-water partition coefficient.[128,129]

Membrane Vesicles

The most commonly used are Brush Border Membrane Vesicles (BBMV) and Basolateral membrane vesicles (BLMV), both subcellular fractions. Its preparation involves tissue homogenation and differential sedimentation, fractionation, and differential precipitation. For BLMV there is an additional subfractionation step. Basically, these systems are used for transcellular absorption studies[130] as well as active and facilitated transport mechanisms.[131–133] The tissues may be of human origin but most frequently are derived from different animal species such us rabbits, pigs, and rats. BBM contain a variety of hydrolytic enzymes, which are valuable tools in studying drug stability. The distribution of these enzymes is well known[134–136] enabling rational approaches to assessing protection of the drug by formulation or synthetic techniques. BBM matrix is especially useful in studying the specificity of targeted prodrug reconversion at the intestinal wall.[137]

Everted Intestinal Rings

Everted Intestinal rings provide a relatively quick technique for measuring uptake of drug into tissue.[138,139] The viability of the tissue over the time course of the experiment may be compromised.[140]

Filter-Immobilized Hexadecane Membrane (HDM)

This technique consists of a hexadecane liquid layer immobilized between two aqueous compartments.[141,142] An HTS technique used to study passive transport pathways.

Parallel Artificial Membrane Permeability Assay (PAMPA)

PAMPA is a phospholipid-based parallel artificial membrane that lacks active transport and also paracellular pathways.[20,125,142–144]

Cell Culture-Based Permeability Models

There exists a significant variety of cell lines now routinely cultivated as a monolayer on permeable filters. Caco-2 cell monolayers (human epithelial colon adenocarcinoma cell line) are extensively used because it is an excellent system for the study of transcellular transport and also active transport. Dipeptide carrier[145,146] and P-glycoprotein[147,148] (P-gp) are some of the transporters expressed in Caco-2 cells. Other cell lines are extensively used due to their low intrinsic expression of ATP-binding cassette transporters superfamily[149–151] (ABC) such us Madin–Darby Canine Kidney (MDCK) and LLC-PK1 which make them ideal for transfections.[72,152–154] Another cell line that is being routinely used is 2/4/A1 which lacks functional expression of several important active drug transporters and does not present tight junctions, rendering this system ideal for studying paracellular permeability.[142]

Immobilized Artificial Membranes (IAM)

The IAM system was developed by Pidgeon et al.[155] and described in several papers.[156–159] In essence, IAMs are a chromatographic model of lipidic membranes for studying passive absorption.

In Situ Intestinal Perfusion

The *in situ* perfusion technique provides enhanced tissue viability as well as several sampling sites.[160,161] The technique typically involves isolation of an intestinal segment or the whole small intestine, which remains *in situ* and is perfused (close or open loop) with a solution containing a known concentration of the test moiety. Samples of the intestinal perfusate are taken at specific time measuring the disappearance of the studied molecule.[126,160–163]

Isolated Sections of Intestinal Tissue

In this setting, sections of intestinal tissue are mounted in a chamber as the barrier between two compartments.[164] Both the serosal and mucosal surfaces are bathed with oxygenated buffer solution and the passage of the compound in solution across the tissue is measured by standard analytical techniques. The integrity of the membrane is monitored by measurement of transepithelial electrical resistance across the tissue.[126,164] Using chambers can be also utilized having as a barrier cultured cell monolayer as oppose to epithelial tissue.[165]

Protein Binding

Equilibrium Dialysis (ED)

Dialysis works on the principle of the diffusion of solutes along a concentration gradient across a semipermeable membrane. Usually, the device contains two chambers divided by a semipermeable membrane with a specific molecular weight cut-off, which means that only molecules with a molecular weight smaller than the cut-off size will permeate through the membrane. In a typical experiment, plasma containing the test article is on one side of the membrane and a buffer is placed in the other dialysis chamber. A variant of ED is comparative equilibrium dialysis (CED), where plasma is placed on either side of the dialysis membrane.[36] Microdialysis, as a specific type of dialysis system, allow you to continuously sample the unbound fraction on the interstitial space fluid(ISF), which may be the actual target compartment for many drugs. Microdialysis is an *in vivo* probe-based sampling method linked to an analytical device for measurement of drug concentration profiles. When a physiological salt solution is slowly pumped through the microdialysis probe, the solution equilibrates with the ISF which then contains a representative proportion of the tissue fluid's molecule.[35,166–168]

Ultrafiltration (UF)

It is the most common methodology for determination of unbound plasma concentrations.[169–171] Ultrafiltration is a variety of membrane filtration in which hydrostatic pressure forces a liquid against a semipermeable membrane. Partitioning occurs without dilution, change in physiologic pH, ion composition, or unbound microsolute concentration. Protein leakages and adsorption to the UF device are the major drawbacks of this technique.[172]

Metabolism

Liver and Intestinal Microsomes

Microsomes are small vesicles from fragmented hepatocyte or enterocytes endoplasmic reticulum prepared by differential centrifugation. Microsomes contain phase I enzymes. The basic processes included in phase I reactions are oxidation, reduction, and/or hydrolysis mainly catalyzed by the CYP system requiring NADPH as cofactor.[173–177]

Liver Cytosol Fraction

The cytosolic fraction of the liver contains soluble phase II enzymes. It is obtained by differential centrifugation of whole-liver homogenate. Addition of co-factor is necessary.[178,179]

Liver S9 Fraction

S9 fraction contains both microsomal and cytosolic fractions. Co-factors are required.[180,181] Liver fractions can be used in combination with the Ames test (see below) for predicting potential mutagenicity of a compound.[182]

Human Recombinant Enzymes

Human Recombinant Enzymes are basically microsomes prepared from baculovirus-infected insect cells that express specific human CYPs and Uridine Diphosphoglucuronosyl Transferase (UGTs). This system is useful for isozyme-specific drug biotransformation and drug–drug interaction studies.[32,183]

Liver Slices

These pieces of liver tissue possess active phase I and II xenobiotic metabolism. Toxicity can be measured by MTT, changes in ATP levels, Lactate dehydrogenase release, protein synthesis, K content, glutathione, glutathione S-tranferases, DNA adduct formation, etc.[60,184,185] Liver slices are a valuable model for studying the regulation of a larger number of enzymes by single compounds.[184,186]

Cell-Based Cultures

Liver cell lines do not present all families of metabolic enzymes. Cultures of human cell lines are preferred versus animal liver cell lines. When used for cell-based hepatotoxicity, these cultures are relevant for elucidating the mechanism of action of the studied toxin. The most commonly used human liver cell lines are HepG2,[187–191] HLE,[192–194] and BC2.[195–198] (see Tab. 3)

Hepatocytes

Primary hepatocytes are a well-controlled, relatively easy to handle *in vitro* system that is well-accepted for investigating xenobiotic biotransformation, enzyme induction, and inhibition, and (biotransformation-mediated) hepatotoxicity.[199–201] However, it has been observed that a number of liver-specific functions are progressively lost with time when hepatocytes are isolated and cultivated.[202] Hepatocytes are the closest *in vitro* model to the human liver, and they are the only model which can produce a metabolic profile of a drug which is very similar to that found *in vivo*. Hepatocytes contain phase I and II metabolic enzymes as opposed to microsomes that lack phase II metabolism. Phase II reactions generally inactivate the drug if it is not already inactive following phase I metabolism and makes the drug more polar or water soluble facilitating its elimination.[203]

Isolated Perfused Liver

Pefusion of a freshly isolated liver is considered the closest system to *in vivo* experiments. It maintains the three dimensional architecture, the bile flow and the liver's hemodynamics.[60,204–208] Livers are connected to a temperature-controlled perfusion system (open or closed system) and the perfusion medium is continuously gassed with a mixture of CO_2 and O_2. The test article is added to the circulated perfusate. Several parameters are monitored during the length of the experiment to determine the impact of the test article in the functionality of the liver (liver enzymes, oxygen

consumption, bile content, ATP, perfusion flow rate,...etc).

DNA Microarray Assay (DNA chips)

DNA Microarray assays consist of a collection of microscopic DNA fragments attached to a support material forming an array for the purpose of expression profiling, monitoring expression levels for thousands of genes simultaneously. It allows the evaluation of expression of the mRNA transcripts for a large number of genes by a single experiment (HTS).[209,210]

Measuring gene expression using DNA microarrays is relevant in metabolism studies when we are investigating whether the gene expression of drug enzymes involved either in phase I[211] or Phase II[212] metabolism is affected by a potential drug. The measurement of gene expression levels upon exposure to a xenobiotic may provide information about its mechanism of action/elimination forming a sort of genetic signature.

Microarray applications include the identification of disease-associated genes,[213–215] drug target validation,[216] biological pathways dissection,[217–219] discovery of gene functions,[220] experimental annotation of the human genome,[221] compound toxicity and safety assessment studies,[222,223] tumor classifications,[224–226] diagnostic and prognostic predictions,[227–230] and other biomarker identification.[231–233]

Toxicity

Ames Test

In the Ames test, several strains of *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis are used. The bacteria require histidine for growth. The variable being tested is the mutagen's ability to cause a reversion to growth on a histidine-free medium. The tester strains are specially constructed to have both frame shift and point mutations in the genes required to synthesize histidine, which allows for the detection of mutagens acting via different mechanisms. Some compounds are quite specific, causing reversions in just one or two strains. The tester strains also carry mutations in the genes responsible for lipopolysaccharide synthesis, making the cell wall of the bacteria more permeable, and in the excision repair system to make the test more sensitive. Rat liver extract (e.g., rat liver –S9 mix) is added to simulate the effect of metabolism as some compounds, like benzopyrene, are not mutagenic themselves but their metabolic products are. The bacteria are spread on a histidine-free agar plate in the middle of which the mutagen to be tested is added. The plates are then incubated for 48 h. The mutagenicity of a substance is proportional to the number of colonies observed.

COMET Assay

Basically, a cell is embedded in agar and exposed to a DNA-damaging agent such as UV radiation or a chemical mutagen. The cell is then permeabilized by adding detergent and an electric field applied. If the cell's genomic DNA has been broken into small fragments then these fragments move out of the cell by electrophoresis and form a streak or "tail" leading away of the cell. This looks a bit like a comet, hence the name of the assay.

Hepatotoxicity

Hepatotoxicity can be studied through some of the *in vitro* metabolism assays described above, such as: liver slices, cell-based cultures, hepatocytes, and perfused liver. Mitochondrial dysfunction is often detected in liver cultures[234–236] since the liver is the main organ in contact with xenobiotics due to its detoxifying body function and the fact that is a very well-perfused tissue. Table 3 lists recent published *in vitro* toxicity work.

MTT Assay

A colorimetric assay based on the tetrazolium salt MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) that measures only living cells and can be read on a scanning multi-well spectrophotometer.

SRB Assay

The goal of using Sulforhodamine B is to measure drug-induced cytotoxicity and cell proliferation for large-scale drug-screening applications. Its principle is based on the ability of the protein dye Sulforhodamine B to bind electrostatically in a pH-dependent manner to proteins and basic amino acid residues of trichloroacetic acid-fixed cells. Under mildly acidic conditions, it binds and can be extracted from cells and solubilized for measurement.

Clonogenic Assay

This is a microbiology technique for studying the effect of specific agents on the proliferation of cells. The term "clonogenic" refers to the fact that these cells are clones of one another.

Ventricular Repolarization Assays: hERG Assay

The hERG assay is used to characterize the potential of a test substance to delay ventricular repolarization. In these studies, test substances are studied for their ability to displace a hERG channel blocker (human Ether-a-go-go Related Gene). We can differentiate *in vitro* (patch clamp) and *in vivo* (electrophysiology) studies. For *in vitro* studies I_{Kr} assay, or patch clamp, is commonly used. This technique traditionally uses a glass pipette with a very small and smooth rounded surface tip diameter (1 micrometer). This type of electrode is known as a "patch clamp electrode." The interior of the pipette is filled with a solution that approximates the intracellular fluid. A metal electrode in contact with this solution conducts the electrical changes to a voltage clamp amplifier. During the experiment, the researcher can manipulate the contents of this solution or add drugs to study the ion channels under different conditions. The patch clamp electrode is pressed against a cell membrane and suction is applied to the inside of the electrode to pull the cell's membrane inside the tip of the electrode. The suction causes the cell to form a tight seal with the electrode (also-called "gigaohm seal," since the electrical resistance of that seal is in excess of a gigaohm). The patch clamp recording uses a single electrode to voltage clamp a cell. This allows a researcher to keep the voltage constant while observing changes in current. Alternately, the cell can be current clamped, keeping current constant while observing changes in membrane voltage.[237,238] For the *in vivo* test, The QT interval of the electrocardiogram (ECG) is the most common endpoint. Additionally, blood pressure, heart rate, PR interval, QRS duration, and arrhythmias can be simultaneously monitored.

DNA Microarray Assay

DNA Microarray technology is becoming very popular in the toxicological field. Information on the global gene expression profile may provide clues to understanding biological actions of toxic substances at the molecular, cellular, tissue, and individual animal levels.[239] Gene expression profiling is now being used in screening for toxicity of new and existing chemical structures.[240] The Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI) has coordinated an international study involving several pharmaceutical companies and governmental and academic institutions to evaluate the harmonization of gene expression data an analyses showing that (1) patterns of gene expression relating to biological pathways are robust enough to allow insight into mechanisms of toxicity, (2) gene expression data can provide meaningful information on the physical location of the toxicity, (3) dose-dependent changes can be observed, and (4) concerns about oversensitivity of the technology may be unfounded.[240] See Table 3 for some examples of gene markers of toxicity.

PHARMACOKINETICS: DIFFERENT APPROACHES, DIFFERENT GOALS

Pharmacokinetics is a discipline that uses mathematical models to describe and predict the time-course of drug concentrations in body fluids. In this respect, different methodologies or mathematical approaches have been described. Basically, we can classify PK strategies under two categories: Non-compartmental analysis (NCA) and Modeling.[241] Under this last category, we have the classical compartmental analysis, Physiologically-Based PK analysis (PBPK), and Hybrid models where the classical compartmental analysis and physiological based models are mixed.

Non-Compartmental Analysis

The NCA method uses algebraic equations and provides a descriptive knowledge of the test compound. Very minimal assumptions are made (the principal one regarding first order exponential terminal phase), which minimizes bias by eliminating the assumption required in any modeling. It is the method of choice for Bioequivalence studies and the only analysis required by the FDA for a new drug application. However, no simulations can be performed using this analysis.

Modeling

Modeling uses differential equations. The model can be totally empirical where the compartments are black boxes (the drug scheme is commonly generalized into one- two- or three-compartment structures) or have some physiological meaning assigned to the compartments defined in the model (PBPK).[242] The selection of the best fit model is done by using some sort of statistical criterion along with diagnostic plots. However, in pharmacokinetics there is no such thing as "the model." A good pharmacokinetic analysis will provide a mathematical model that will be able to fit the data, simulate, and predict various case scenarios with a certain degree of comfort. This will not necessarily be the best model; it should be the simplest model that successfully describes the dataset.

The main purpose of a pharmacokinetic analysis is to obtain a set of parameters that describe the kinetic behavior of the drug in the body upon administration. Thus, with the PK analysis, we tend to extrapolate the results obtained in one study to the whole population: the potential target for the test article in study. According to the FDA, the term population pharmacokinetics (popPK) is "*the study of the sources and correlates of variability in drug concentrations among individuals who are the target patient population receiving clinically relevant doses of a drug of interest.*"[243,244] However, this definition is very vague; popPK involves the analysis of data from a group (population) of individuals, with all their data analyzed simultaneously to provide information about the variability of the model's parameters. The richness of this information will depend on the mathematical approach performed and the dataset available. Although the phrase "population analysis" is being used to refer to one-stage analysis, it is equally applicable to naïve pooled data (NPD), naïve averaged data (NAD), standard two stage approach (S2S), Three stage analysis (3S), Bayesian Estimation, and one-stage analysis (linear and nonlinear mixed effects modeling).

Table 8 summarizes some of the most relevant internet resources for Pharmacokinetics/Pharmacodynamics, including online PK courses, definitions, and examples.

The intent of this review is not to conclude that one approach is better than another. The goal is to understand when to use one or another based on what assumptions we can make, what is/are the answer(s) that we are looking for and what is the set of data that we have to work with. Depending on what PK analysis we perform, we will be able to answer different questions which will drive the conclusions that will be extracted out of that analysis. In summary, we need to understand the different PK mathematical approaches to make a smart choice of which methodology to use, and this depends upon what information is required from the study. In general, for early discovery up to lead optimization a NCA as a "high throughput analysis" would be sufficient in the majority of the cases. Even for filing an NDA, NCA is all that is required. Supporting an NDA with modeling would be recommendable in certain cases where nonlinearities due to metabolism or carried-mediated systems are significant in the dose range that is considered clinically relevant.

SUMMARY

The analysis of the factors that impact drug discovery and development is extremely relevant for the success of new chemical entities. More than half of the compounds that reached the "first in humans" stage failed due to toxicity and safety concerns (see Fig. 1). Therefore, it is imperative that the pharmaceutical industry remains vigilant for faster, more efficient methods of screening in order to survive in the highly competitive pharmaceutical marketplace. Expediting the discovery phase has proven to be a crucial element of success in Drug Development.

In summary, there exists an extensive variety of tools which LADMET scientists should take advantage of in Drug Discovery. Good understanding of these systems allows us to wisely apply them in an integrated fashion, always keeping in mind their caveats and limitations, to extract more meaning from the data. We believe this global overview and collection of methodologies will benefit Drug Discovery research in taking the right approach to meet each specific goal that arises. Using the tools presented here is one step towards saving time while making smart choices in the design and direction of the research, through out the Drug Discovery/Development continuum.

Table 8. Online Information and Software Resources in Pharmacokinetics/Pharmacodynamics

URL	Description	Name
http://www.globomax.net/products/nonmem.cfm	NONMEM: Globomax	NONMEM
ftp://ftp.globomaxnm.com/Public/nonmem/	Shared resource for files and information for the NONMEM community	
http://www.pharsight.com/main.php	Pharsight facilitates strategic decision making in drug development. Pharsight software improves the value and availability of preclinical and clinical program data for PK/PD modeling, analysis, drug attribute visualization, reporting and trial simulation	WINONLIN/WINONMIX
http://xpose.sourceforge.net/	Xpose is an R-based model building aid for population analysis using NONMEM. It facilitates data set checkout, exploration and visualization, model diagnostics, candidate covariate identification and model comparison	XPOSE
http://www.mrc-bsu.cam.ac.uk/bugs/	The BUGS (Bayesian inference Using Gibbs Sampling) project is concerned with flexible software for the Bayesian analysis of complex statistical models using Markov chain Monte Carlo (MCMC) methods	BUGS
http://www.exprimo.com/	Exprimo is a European consulting company with the emphasis of its activities focused towards the application of quantitative, model-based approaches at all stages of pharmaceutical development	Exprimo NV
http://www.emf-consulting.com/	EMF Consulting is a group of pharmaceutical consultants who provide drug development services, especially related to study design, analysis and development planning	EMF Consulting
http://www.lapp.nl/	LAP&P provides interdisciplinary support on Pharmacokinetic and Pharmacodynamic aspects during preclinical and clinical Drug Development	LAP&P Consultants
http://www.boomer.org/pkin/	The purpose of this page is to provide links to information about the discipline of Pharmacokinetics and Pharmacodynamics	
http://depts.washington.edu/rfpk/	The Resource Facility for Population Kinetics is a computer resource facility sponsored by the National Institute of Biomedical Imaging and Bioengineering at the National Institutes of Health	
http://anesthesia.stanford.edu/pkpd/	On this server you will find PK/PD software, computer controlled drug administration programs, and simulation programs. This server is maintained by Steve Shafer of the Department of Anesthesia at Stanford	
http://www.lapk.org/	The Laboratory of Applied Pharmacokinetics (LAPK) of the School of Medicine at the University of Southern California is a resource for optimal study and control of pharmacokinetic systems and for individualized drug therapy supported in part by the National Library of Medicine (NLM) and National Center for Research Resources	LAPK
http://www.drudevo.com/	More than 20+ years experience in modeling & simulation with a special focus on optimizing drug development and software development	DruDev0
http://members.aol.com/rdppweb/index.htm	Research and development for population pharmacokinetics	RDPP

(*Continued*)

Table 8. (*Continued*)

URL	Description	Name
http://www.aster-cephac.com/index.asp	Leading European contract research organization (CRO) providing clinical and bioanalytical services to pharmaceutical companies	ASTER.CEPHAC
http://www.metrumrg.com/	It is a research-based organization focused on the investigation and application of innovative mathematical modeling and simulation methods in clinical pharmacology and related biomedical sciences. MetrumRG provides quality research and consulting services to the pharma/biotech industries and non-profit research centers	Metrum Research Group LLC
http://www.nextlevelsolns.com/	Next level solutions offers both traditional and rapid analysis solutions to population pharmacokinetics/pharmacodynamics models	Next Level Solutions
http://accp1.org/pharmacometrics/index.html	The objective of this free for all site is to aid in pharmacometrics training	American College of Clinical Pharmacology
http://www.icp.org.nz/	By Dr Matt Doogue, Christchurch Hospital	ONLINE text books or courses
http://www.curvefit.com/index.htm	By Harvey Motulsky, GraphPad Software, Inc.	
http://web.vet.cornell.edu/public/pharmacokinetics/pharmacokinetics.html	By The College of Veterinary Medicine at Cornell University	
http://www.4um.com/tutorial/science/pharmak.htm	By Pat Neligan	
http://www.pharmacy.ualberta.ca/pharm415/	By Dr. F. Jamali, Univ. of Alberta	
http://www.boomer.org/c/p4/index.php?Loc=Advance	By David W.A. Bourne	

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GLOSSARY

3-D-QSAR	three dimensional quantitative structure-affinity relationship analysis
3S	three stage analysis
ANN	artificial neural network
BBMV	brush border membrane vesicles
BCS	biopharmaceutics classification system
BDDCS	biopharmaceutics drug disposition classification system
BLMV	basolateral membrane vesicles
CED	comparative equilibrium dialysis
CoMFA	comparative molecular field analysis
CYP	cytochrome P 450
ECG	electrocardiogram
ED	equilibrium dialysis
FDA	Food and Drug Administration
HDM	hexadecane membrane
Herg	human Ether-go-go-related gene
IAM	immobilized artificial membrane
ICH	international conference of harmonization
IVIVC	*in vitro-in vivo* correlations
NAD	naïve averaged data
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of NADP
NCA	non-compartmental analysis
NDA	new drug application
NPD	naïve pooled data
ORMUCS	ordered multicategorical classification method using the Simplex
PAMPA	parallel artificial membranes permeability assay
PATQSAR	population analysis by topology-based QSAR
PBPK	physiologically-based pharmacokinetics
PK	pharmacokinetics
popPK	population pharmacokinetics
QSAR	quantitative structure-affinity relationship
QSPR	quantitative structure-property relationship technique
S2S	standard two stage approach
SVM	support vector machines
UDP	uridine diphosphate
UF	ultrafiltration

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