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Ciências

In vitro assessment of cytochrome P450 2C9 inhibition: tolbutamide as probe substrate and HepaRG cells as human hepatic model

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Dedication

I would like to dedicate this work to the best parents in the world, my mother Paula and my father Luis, for all their unconditional love that had always given me strength even in the more difficult times. A thank you is not enough for you.

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A very special thanks to Filipa for the best supervision, guidance, companionship, friendship and professionalism. She was always available to help me, she was always ready to teach me and she always had the best advices. Thank you for all the support.

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Abstract

Pharmacokinetics information, especially regarding the drug metabolic profile, is a requirement during the preclinical and clinical development. *In vitro* methodologies represent more and more useful tools to predict drug safety and toxicity. The cytochrome P450 (CYP450) enzymes are the main responsible for the variability in pharmacokinetics and drug response, being the cytochrome (CYP) 2C9 isoform representative of about 20% of total hepatic CYP450 content, and one of the most important drug-metabolizing isoenzymes. Consequently, the prediction of drug interactions mediated by the inhibition of the CYP2C9 isoenzyme may be of great relevance in the development of new drugs. Due to the fact that HepaRG, a new human cell line derived from a hepatocellular carcinoma, is being considered a promising model to evaluate the *in vitro* metabolism of drugs, it was herein used for the development of an *in vitro* methodology for the investigation of CYP2C9 inhibition-based metabolic drug-drug interactions. The high-performance liquid chromatography–diode-array detection (HPLC-DAD) assay developed, enabled the simultaneously quantification of tolbutamide (TOL) and its metabolite 4-hydroxytolbutamide (4-OH-TOL), in HepaRG cell culture medium samples, supporting the subsequent *in vitro* studies. Chromatographic separation of the analytes (4-OH-TOL and TOL) and the internal standard (IS), carbamazepine (CBZ), was achieved in less than 20 minutes by a gradient elution on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm; 3 µm particle size), at 35 °C, using a mobile phase composed of phosphate buffer (10 mM) with 0.1% triethylamine (pH 3)/acetonitrile pumped at 0.6 mL/min. The analytes and IS were detected at 230 nm. The method proved to be selective, accurate, precise and linear ($r^2 \geq 0.9901$) over the concentration ranges of 0.25-200 µM for TOL and 0.25-20 µM for 4-OH-TOL. Furthermore, the absolute recovery of the analytes ranged from 73.2 to 84.8% and their stability was demonstrated in the studied conditions. HepaRG cells were used in the metabolic inhibition studies, in which the reference drugs used as CYP2C9 inhibitors (amiodarone, fluoxetine, ketoconazole, omeprazole and ticlopidine) or flavonoids [baicalein, (-)-epigallocatechin gallate, kaempferol and quercetin] were pre-incubated for 30 minutes, and then TOL (200 µM), a known CYP2C9 probe drug, was co-incubated with the CYP2C9 inhibitors or flavonoids (24 hours). The suitability of the *in vitro* technique and the experimental analytical procedures developed and validated in the evaluation of potential metabolic inhibition interactions involving the CYP2C9 was demonstrated by the inhibitory effect observed with almost all the concentrations of CYP2C9 inhibitors. Moreover, all the flavonoids also demonstrated to inhibit the CYP2C9 isoenzyme, with exception of baicalein at 50 µM, validating the methodology applicability. This work demonstrated the usefulness of the *in vitro* assay developed and validated in the HepaRG cell line as a useful *in vitro* approach to foresee metabolic interactions involving the CYP2C9 isoenzyme inhibition.

Keywords: CYP2C9 inhibition, Drug interactions, Flavonoids, HepaRG cells, High-performance liquid chromatography, *In vitro* studies, Tolbutamide

Resumo Alargado

Informação farmacocinética, principalmente em relação ao perfil metabólico dos fármacos, é uma exigência durante o desenvolvimento pré-clínico e clínico. Metodologias *in vitro* representam cada vez mais ferramentas úteis para prever a segurança e a toxicidade dos fármacos. As enzimas do sistema citocromo P450 (CYP450) são as principais responsáveis pela variabilidade na farmacocinética e na resposta aos fármacos, representando o citocromo (CYP) 2C9 cerca de 20% do conteúdo total de CYP450 hepáticos, sendo uma das isoenzimas mais relevantes na etapa de metabolização. Problemas relacionados com as suas características farmacocinéticas estão entre as principais razões para o fracasso no desenvolvimento de fármacos, estando estes problemas muitas vezes associados a interações farmacológicas. Por conseguinte, a previsão das interações ao nível do metabolismo mediadas pela inibição do CYP2C9 pode ser de grande relevância no desenvolvimento de novos fármacos.

Durante os últimos anos foram desenvolvidos vários modelos *in vitro* e *ex vivo* para o estudo do metabolismo hepático de fármacos, pois o fígado é o órgão principal responsável pelo metabolismo. As células HepaRG, uma nova linha celular derivada de um carcinoma hepatocelular humano, são consideradas cada vez mais um modelo promissor na avaliação do metabolismo *in vitro*. Assim, este foi o modelo escolhido para o desenvolvimento e validação de uma metodologia *in vitro* para a investigação de interações metabólicas mediadas pela inibição do CYP2C9.

O método de cromatografia líquida de alta resolução acoplado a um detetor de fotodíodos (HPLC-DAD) desenvolvido, permitiu a quantificação simultânea da tolbutamida (TOL) e do seu metabolito 4-hidroxitolbutamida (4-OH-TOL), em amostras de meio de cultura de células HepaRG, suportando assim os estudos *in vitro* subsequentes. A separação cromatográfica dos analitos (4-OH-TOL e TOL) e do padrão interno (PI), carbamazepina (CBZ), foi conseguida em menos de 20 min, com o uso de uma eluição por gradiente e de uma coluna cromatográfica LiChroCART® Purospher Star de fase reversa (C_{18}, 55 mm × 4 mm; 3 µm tamanho da partícula), a 35 °C, utilizando uma fase móvel composta por tampão fosfato (10 mM) com 0,1% de trietilamina (pH 3)/acetonitrilo a um fluxo de 0,6 mL/min. Os analitos e o PI foram detetados a um comprimento de onda de 230 nm. O método descrito provou ser seletivo, exato, preciso, e linear ($r^2 \geq 0,9901$) numa gama de concentrações de 0,25-200 µM para a TOL, e de 0,25-20 µM para a 4-OH-TOL. Além disso, a recuperação absoluta dos analitos variou entre 73,2 e 84,8%, e a estabilidade das amostras foi comprovada nas diferentes condições utilizadas, simulando o manuseamento e armazenamento das amostras.

A citotoxicidade dos inibidores de referência do CYP2C9 (amiodarona, fluoxetina, cetoconazol, omeprazol, ticlopidina) e dos compostos flavonoides [baicaleina, (-)-epigalocatequina galato, kaempferol, e quercetina] foi também avaliada, permitindo obter uma maior segurança na posterior interpretação dos resultados referentes aos estudos metabólicos.

As células HepaRG foram utilizadas nos estudos metabólicos de inibição, tendo sido os inibidores de referência do CYP2C9 e os flavonoides pré-incubados durante 30 minutos. De seguida a TOL (200 μM), uma sonda conhecida do CYP2C9, foi co-incubada com os inibidores de referência do CYP2C9 ou flavonoides (24 horas). A adequação da técnica *in vitro* e dos procedimentos analíticos e experimentais, desenvolvidos e validados na avaliação de potenciais interações que envolvem a inibição metabólica do CYP2C9, foi demonstrada através do efeito inibitório observado mediado pelos inibidores de referência do CYP2C9 a quase todas as concentrações testadas. Além disso, os flavonoides demonstraram também um efeito inibitório sobre a isoenzima CYP2C9, com exceção da baicaleina a 50 μM, validando assim a aplicabilidade da metodologia. Este trabalho demonstrou a utilidade do modelo *in vitro* desenvolvido e validado com a linha celular HepaRG, na previsão de interações metabólicas envolvendo a inibição da isoenzima CYP2C9.

Palavras-Chave: Células HepaRG, Cromatografia Líquida de Alta Resolução, Estudos *in vitro*, Flavonoides, Inibição do CYP2C9, Interações farmacológicas, Tolbutamida

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Abbreviations

ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
AUC	Area Under the Concentration-Time Curve
CBZ	Carbamazepine
CV	Coefficient of Variation
CYP	Cytochrome
CYP450	Cytochrome P450
DAD	Diode array detector
DMSO	Dimethyl Sulfoxide
EMA	European Medicines Agency
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
HPLC	High-performance liquid chromatography
IC_{50}	Half Maximal Inhibitory Concentration
IS	Internal standard
K_i	Inhibitory Constant
LLOQ	Lower Limit of Quantification
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NCE	New Chemical Entity
4-OH-TOL	4-hydroxytolbutamide
PBS	Phosphate-buffered saline
TOL	Tolbutamide
QC	Quality Control Samples

1. Introduction

1.1. Drug Discovery and Development

Drug discovery and development is a complex process by which a new chemical entity (NCE) may become a drug through a very strict, time-consuming and costly process (Kapetanovic 2008; Bunnage 2011). Actually, discovering and bringing one new drug to the market typically takes an average of 14 years of research, and clinical development efforts and cost around two billions U.S. dollars. Of ten thousand or more compounds tested in early discovery, only one may eventually lead to a drug that reaches the market (Adams & Van Brantner 2006; Paul et al. 2010).

The modern drug discovery follows a sequence of carefully planned events, starting with the identification of screening hits and following with the subsequent lead optimization in order to enhance the selectivity, efficacy, potency, metabolic stability, bioavailability and affinity (Van Dongen et al. 2002; Lombardino & Lowe III 2004). Most of these events are achieved through computer-aided or *in silico* drug design. This resourceful tool can help to reduce the million possibilities of NCEs, optimizing every steps of the process and even advise for making better and cheaper decisions in the arena of drug lead identification and optimization, **Figure 1** (Brown 1998; Lombardino and Lowe III 2004; Kapetanovic 2008; Paul et al. 2010; Hoelder et al. 2012).

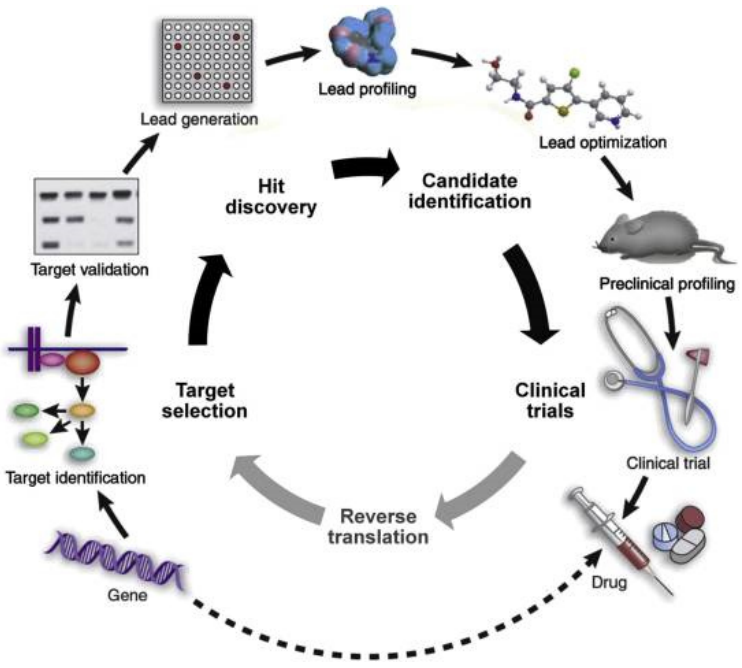

Figure 1 - Drug discovery and development: From gene to drug (Hoelder et al. 2012).

The first step in drug development is to discovery the best targets for treating or preventing a disease. Usually this step involves high-throughput screening methods that can evaluate libraries of chemical compounds, and give us the information about their ability to alter the target, and even reveal how selective the compounds are for the chosen target (Van Dongen et al. 2002; Lombardino and Lowe III 2004; Hoelder et al. 2012). The ideal compound should only interfere with the chosen target and no other. Regarding this, the cross-screening becomes important to know if the molecules discovered meddle other related targets. Actually, if this happens it is more likely that off-target toxicity will occur once the compound gets the clinic phase (Hoelder et al. 2012). In this early phase, the perspective to find the "perfect" compound is rare. Indeed, most of the times there is a need to improve the chemical features of the compound using quantitative structure-activity relationships (QSAR) and then create a lead compound (Hoelder et al. 2012). This process can also upgrade the drug likeness or ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of the compound, enhance the activity against the chosen target, or diminish the activity against unintended targets (Van Dongen et al. 2002; Lombardino and Lowe III 2004; Hoelder et al. 2012).

Once the properties of the NCE are improved, it will go forward to *in vitro* and *in vivo* tests. The *in vitro* tests will evaluated the physicochemical properties associated with drug absorption, including permeability, protein binding, ionization (pKa) and solubility; the *in vivo* tests will assess its pharmacokinetics and pharmacodynamics properties (Lombardino and Lowe III 2004; Paul et al. 2010).

Animals play a critical role in the drug discovery process as well. Actually, although much research can be done using various *in vitro* experiments or using computers, complex disease mechanisms can often be only simulated and understood in whole animal models. Indeed, the governments and regulatory authorities usually demand that drugs be tested in animals before they are tested in humans (Festing and Wilkinson 2007; FDA 2015a). Nevertheless, the use of animals should be kept to the minimum necessary, and always ensuring that the animal research is scientifically acceptable and performed in accordance with the current standards regulations. Hence, although the use of animal is still mandatory, it should be done committed to the policy of the 3R's - refinement, reduction and replacement, and always considering the highest standards in animal welfare (EMA 2010; FDA 2015a).

In a late pre-clinical stage further experiments are conducted on a drug candidate to ensure if it is safe enough for first-in-human studies. These first studies in man are required to explore the safety and pharmacokinetic properties of the drug candidate, like appropriated absorption and metabolism by the human body. These clinical trials are conducted with extraordinary diligences in order to minimized any risks to the human subjects involved (Ciociola et al. 2014). Clinical trial programs consist of several phases, each of them having specific aims but the focus is always on the assessment of drug safety and efficacy. Information about ongoing clinical trials can be found in *www.clinicaltrialsregister.eu*. In phase I clinical trials, the drug is usually tested in healthy volunteers to determine its safety and pharmacokinetics; in phase II clinical trials, the drug is giving to a group of about one hundred to two hundred and fifty patients with

the disease in order to explore its efficacy and to determine the potential optimal dose (Michael 2010; FDA 2015a). In addition, the safety of a drug and its side effects are evaluated, because these could be different in patients compared to the healthy volunteers included in Phase I clinical trials (Michael 2010; FDA 2015a). The data from Phase I and Phase II clinical trials provide the scientific confidence necessary to continue the development of the compound in a larger number of patients (Michael 2010). In Phase III clinical trials, one thousand to three thousand, or even more patients, are recruited; phase III clinical trials are undertaken to confirm the effectiveness of the new drug and to establish its positioning in relation to reference treatments already implemented in clinical practice (Michael 2010). Overall, the process of drug discovery and development is often graphically represented by a flowchart similar to that shown in **Figure 2**.

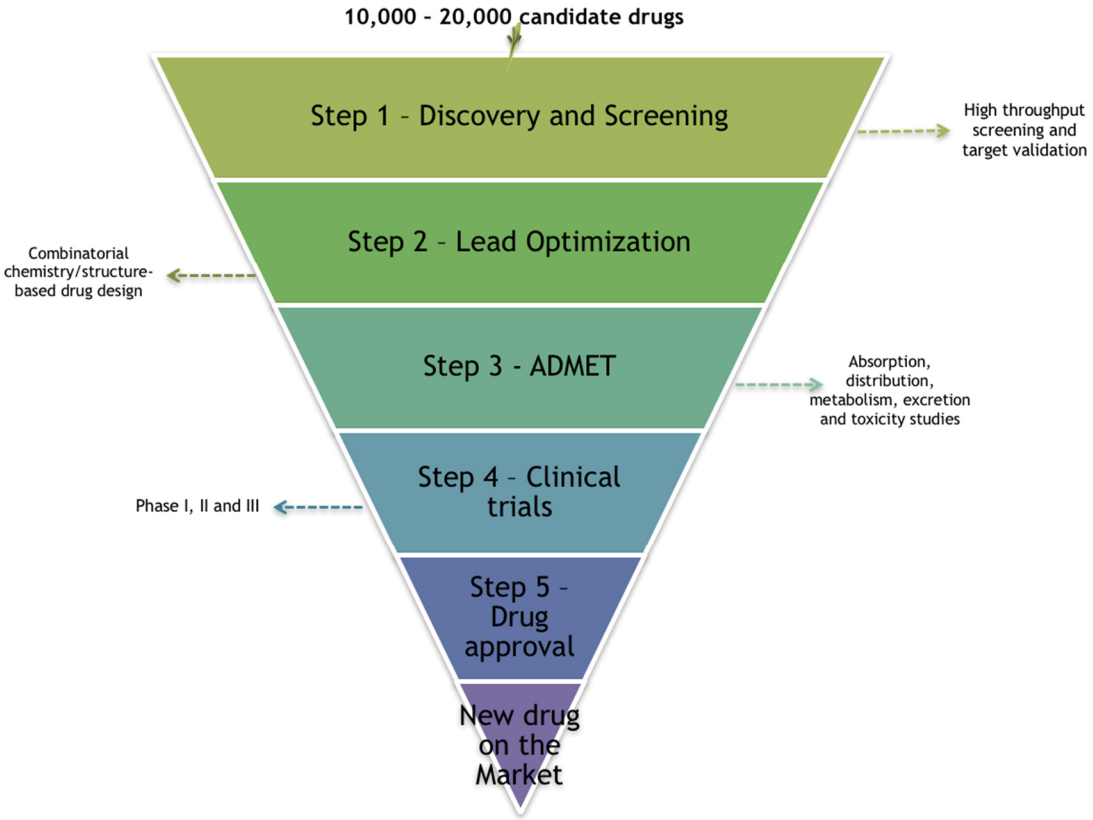

Figure 2 - General view of the process of drug discovery and development. Adapted from Kramer et al. (2007). ADMET, Absorption, Distribution, Metabolism, Excretion and Toxicity.

1.2. Drug Metabolism

The pharmacokinetics gives knowledge about the time course of a drug within the body and include the processes of ADMET (van de Waterbeemd and Gifford 2003). The contribution of metabolism in overall drug disposition is essential and it can have pharmacological and toxicological implications in the use of therapeutic drugs (Zhang et al. 2012a). Drug metabolism and other pharmacokinetic aspects are incorporated in early drug discovery, which allows to have a better understanding of the impact of drug metabolic enzymes and transporters (Zhang et al. 2012a).

Xenobiotics like drugs, toxins and other chemicals can access to the body through several routes (e.g. oral, rectal, sublingual, topical, inhalation, parenteral) that may determine differences in drug metabolism (Schonborn 2010). For instance, in the enteral routes of administration (e.g. oral, rectal, sublingual) in which any part of the gastrointestinal system is involved, the gut wall be decisive in the metabolism of many drugs. Nevertheless, the liver is considered to be the main metabolically active tissue (Schonborn 2010). As a result of its large size, and due to the presence of drug-metabolizing enzymes at very high levels in the hepatocytes (the functional unit of a liver), and the fact that it is continuously perfused by the bloodstream, the liver is undoubtedly accountable for the metabolism and/or excretion of the majority of xenobiotics that enters the body (van de Waterbeemd and Gifford 2003; Schonborn 2010).

The principal goal of the biotransformation of xenobiotics is to develop a more water soluble compound to facilitate the excretion of the drug in body fluids such as urine and bile, the primary routes of drug excretion (Michael 2010). The problem lies on the fact that most drugs and toxins are lipid-soluble, and therefore, their direct excretion is often hindered (FDA 1997; Michael 2010).

The drug metabolism is divided in two phases: phase I and phase II. The metabolic reactions of phase I (oxidation, reduction and hydrolysis) usually introduce some chemical modifications to make a compound more hydrophilic so that it can be easily excreted. Cytochrome P450 (CYP450) enzymes are the main responsible for the oxidative reactions occurring during this phase (Kwon 2014). On the other hand, phase II metabolic reactions, also known conjugation reactions, usually involve the addition of a larger polar group to further increment the compound's solubility. The enzymes responsible for phase II metabolic reactions are transferase enzymes such as uridine diphosphoglucuronosyl transferase (UGT), *N*-acetyl transferase (NAT), glutathione *S*-transferase (GST), and sulphotransferase (ST), **Figure 3** (Guengerich 2006; Kwon 2014).

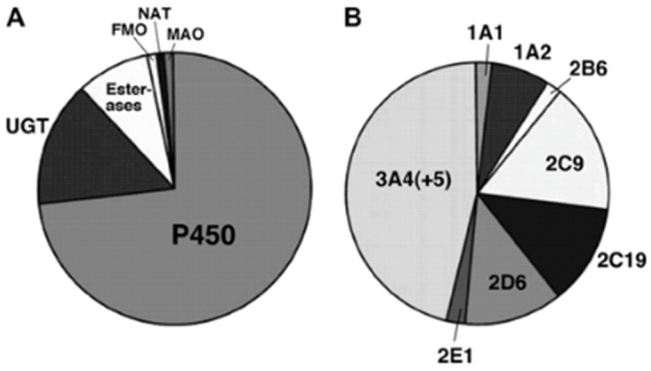

Figure 3 - (A) Contribution of individual enzyme systems to metabolism of marketed drugs; (B) Contribution of individual cytochrome P450s isoenzymes in metabolism of drugs (Guengerich 2006). UGT, uridine diphosphoglucuronosyl transferase; FMO, flavin monooxygenase; NAT, N-acetyl transferase; MAO, monoamine oxidase; P450, cytochrome P450.

In phase I metabolism in mammals, CYP450s are the most important enzymes and are primarily responsible for degradation and elimination of xenobiotics agents. These enzymes are located within the microsomes in the smooth endoplasmic reticulum of the hepatocyte (Michael 2010; Schonborn 2010; Zanger and Schwab 2013).

Presystemic and systemic metabolism may substantially affect the bioavailability of a drug (Heaney 2001). The presystemic metabolism or first pass metabolism occurs when an absorbed drug passes directly through the liver before reaching the systemic circulation, for instance, after oral administration. Other types of metabolism are the hydrolysis of the drug on the stomach fluids, the intestinal metabolism where the drug is metabolized in the intestine itself or during the passage through the intestinal wall. In addition, drug transporters, such as P-glycoprotein, may also influence the bioavailability of a drug (Michael 2010).

1.2.1. Cytochrome P450

CYP450 system is a superfamily of mono-oxygenases that include a heme-bound iron at the active site accountable for binding with and metabolizing the drug, connected to a protein chain (Zanger and Schwab 2013). There are more than 50 human CYP450's identified and classified according to their number of shared amino acid sequences into families and subfamilies (Lewis 2004; Zanger and Schwab 2013). The nomenclature for the families is given by cytochrome (CYP) 1 or CYP2, for example, and then cloven in subfamilies like CYP1A or CYP1B. Furthermore, these subfamilies are constituted by different isoforms such as CYP1A1 or CYP1A2 (Lewis 2004; Zanger and Schwab 2013).

There are about 57 functional CYP450 genes and 58 pseudogenes encoded by human genome, but only twelve of them belong to the three most important families of CYP450s (CYP1, CYP2, CYP3); these three families are responsible for the biotransformation of almost 70-80% of all drugs in clinical use (Zhou et al. 2009b; Ogu & Maxa 2000; Lewis 2004). Among all these

enzymes, the most expressed isoforms in the liver are CYP1A2, 2C8, 2C9, 2E1, and 3A4. CYP2A6, 2B6, 2C19, 2D6 and 3A5 are also expressed, however, these five are less abundant (Zhou et al. 2009b). In humans, almost 50% of the drugs are metabolized by CYP450's, but most of the times these drugs are metabolized for more than one CYP isoform, **Figure 4** (Zhou et al. 2009b; Zanger and Schwab 2013).

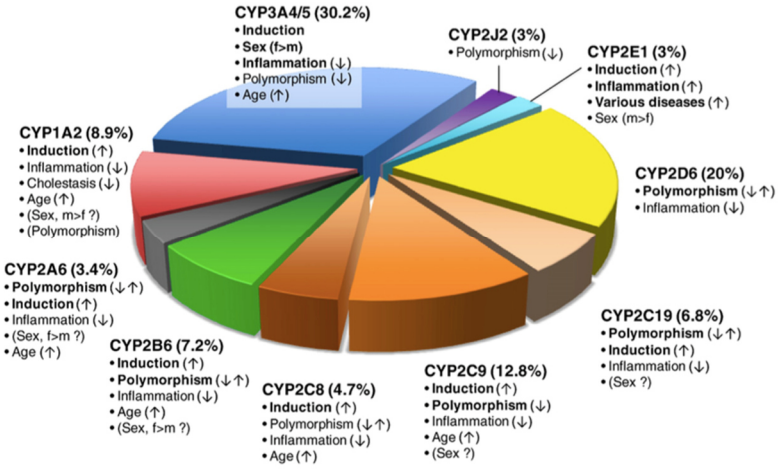

Figure 4 - Fraction of clinically used drugs metabolized by cytochrome (CYP) P450 isoforms and factors influencing variability (Zanger and Schwab 2013).

The variability in CYP450 content and their activities can have a huge impact on the response to medication. Actually, CYPs are often the main responsible for variability in pharmacokinetics and drug response (Zhou et al. 2009b; Ogu & Maxa 2000; Lewis 2004).

1.2.1.1. CYP2C9

The CYP2C9 isoform belongs to the CYP2C subfamily (CYP2C8, 2C9, 2C18 and 2C19), being CYP2C isoenzymes responsible for the metabolism of approximately 30% of clinical drugs (Zhou et al. 2009a). Investigation on the CYP450 system led to finding the importance of CYP2C9 isoenzyme; this CYP contributes about 20% of total hepatic CYP450 content and it is the second most prevalent CYP isoenzyme in the small intestine of humans (He et al. 2002; Zhou et al. 2009a). Consequently, CYP2C9 is one of the most important drug-metabolizing CYP isoenzymes, which is involved in the metabolism of drugs such as *S*-warfarin, glyburide, diclofenac, celecoxib, torasemide, acenocoumarol, losartan, tolbutamide (TOL), phenytoin, and plenty of non-steroidal anti-inflammatory drugs (He et al. 2002; Horn and Hansten 2007; Zhou et al. 2009a). A CYP2C9 substrate is a drug metabolized by this CYP, but most of the times such substrates are simultaneously metabolized by more than one CYP isoenzyme. Consequently, to conduct studies directed to examine the drug metabolism by specific CYP isoforms, it is essential to have a specific, or at least, selective CYP2C9 probe substrate. One of the most

characterized CYP2C9 probes and commonly used by pharmaceutical industry researchers is the TOL, **Figure 5** (Yuan et al. 2002; Zhou et al. 2009a; Horn & Hansten 2007).

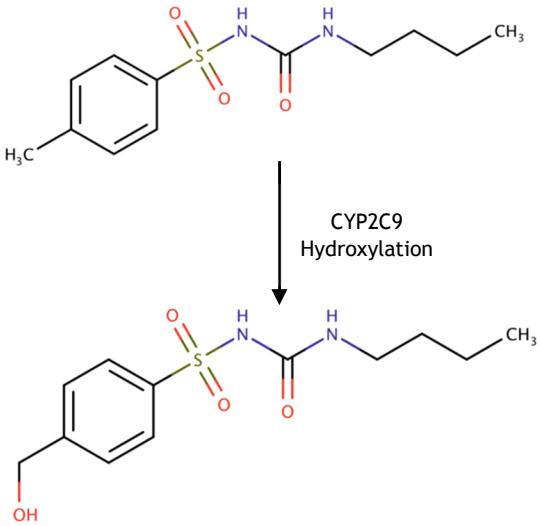

Figure 5 - Metabolic pathway of tolbutamide to its active metabolite 4-hydroxytolbutamide (Zhou et al. 2009a).

TOL (*N*-[(butylamino)carbonyl]-4-methylbenzenesulfonamide) is an oral antidiabetic drug, belonging to class of sulfonylureas, and in humans is essentially metabolized along a single CYP pathway (Miners and Birkett 1998; Sigma-Aldrich 2001). Evidence shows that CYP2C9 is responsible for TOL methyl-hydroxylation that forms its major metabolite called 4-hydroxytolbutamide (4-OH-TOL), and so TOL has been accepted as a prototypic substrate for the evaluation of hepatic CYP2C9 activity, both *in vitro* and *in vivo* (Miners and Birkett 1998).

1.2.1.1.1. CYP2C9 Inhibition and Induction

As it is well-known, many drug interactions are result of changes in CYP450 metabolism, which can induce numerous drug-related problems (Ogu and Maxa 2000; Roe et al. 2016). These CYP isoenzymes can suffer for induction or inhibition by xenobiotics, regulation by cytokines, and even genetic mutations, which causes a substantial variation in the enzyme activity. Actually, enzyme induction and inhibition are two important mechanisms responsible for CYP450-mediated drug interactions (Flockhart and Tanus-Santos 2002).

Induction denotes an increase in the enzyme activity, increasing the drug clearance, on the contrary the inhibition involves a decreases in the enzyme activity (Tanaka 1998; Flockhart and Tanus-Santos 2002). The time-course of enzyme induction is difficult to be predicted due to certain factors, which include the enzyme turnover and half-life that can rule the time-course of induction process. Actually, the induction phenomenon is time-dependent (Tanaka 1998; Roe

et al. 2016). Regarding the inhibition, the metabolic activity of one or more CYP isoenzymes may be almost immediately reduced in the presence of an inhibitor, i.e. the enzyme inhibition is not usually time-dependent (Ogu and Maxa 2000; Lynch et al. 2007). Consequently, inhibition of enzyme activity becomes more predictable than induction (Flockhart and Tanus-Santos 2002).

The extent to which an inhibitor affects the metabolism of a drug depends on multiple factors, such as the dose and ability of the inhibitor to bind to the enzyme (Lynch et al. 2007). Additionally, a drug can be both metabolized by and inhibit the same enzyme, or it can be metabolized by one enzyme and inhibit another enzyme. Indeed, sometimes drugs may be intentionally combined to take advantage of the CYP inhibition (Lynch et al. 2007). The process of inhibition can be either enzyme inactivation or mutual competition of substrates for a catalytic site. In any of these circumstances the net effect is a decrease in the rate of drug metabolism, and therefore, the drug (or active metabolite) half-life increases, and the therapeutic or toxic effects may be intensified (Flockhart and Tanus-Santos 2002). When a drug potently inhibits a drug-metabolizing enzyme, some clinically significant drug-drug interactions can occur (Roe et al. 2016).

Mechanistically, the inhibition of CYP isoenzymes can be either reversible or irreversible (Tanaka 1998; Flockhart and Tanus-Santos 2002). The mechanism responsible for documented drug interactions involves frequently the reversible inhibition, being the competitive inhibition the most important type, but it is unlikely to be the most relevant clinically (Flockhart & Tanus-Santos 2002; Scheller et al. 2014; Martikainen 2012). Besides the competitive inhibition, reversible inhibition is divided in two more types: uncompetitive and non-competitive mixed-type inhibition (Scheller et al. 2014). Competitive inhibition occurs as a result of competition between the inhibitor and substrate for the active site of an enzyme (Martikainen 2012; Wu 2014). In non-competitive inhibition, both active binding sites of the substrate and the inhibitor are different because the inhibitor binds to another site on the enzyme instead of its active site. Uncompetitive inhibition results from the binding of the inhibitor to the enzyme-substrate complex and not to the free enzyme. The mixed-type inhibition shows features of both competitive and non-competitive inhibition (Martikainen 2012). Mechanism-based irreversible inhibition results in the permanent inactivation of the enzyme. This inhibition can occur with the formation of metabolite intermediate complexes, or with the strong covalent binding of reactive intermediates to the protein or heme of the CYP (Martikainen 2012; Scheller et al. 2014; Lynch et al. 2007).

Some drugs can inhibit and induce CYP2C9, the most recognized were gathered in **Table 1** and **Table 2**, respectively. Inhibitors of CYP2C9 activity will boost the plasma concentrations of some drugs, therefore adverse effects may occur (Horn and Hansten 2007; Martikainen 2012; Roe et al. 2016). In the case of other drugs that induce CYP2C9, they could reduce the efficacy of CYP2C9 substrates, resulting in interactions deceitful, which may result in lack of efficacy rather than explicit adverse effects (Horn and Hansten 2007; Martikainen 2012; Roe et al. 2016).

Table 1 - List of the reference inhibitors of CYP2C9 based on Back et al. (1988); Baldwin et al. (1995); Schmider et al. (1997); Miners and Birkett (1998); Hemeryck et al. (1999); Ko et al. (2000); Wen et al. (2001); He et al. (2002); Kuroha et al. (2002); von Moltke et al. (2004); Greenblatt et al. (2005); Kumar et al. (2006a); Kumar et al. (2006b); Horn and Hansten (2007); Paris et al. (2009); Turpeinen et al. (2009); Zhou et al. (2009a); Zhang et al. (2012a); Sousa-Ferreira et al. (2014); Prasad et al. (2016).

7-Hydroxyisoflavone	Imatinib	Quercetin
Amiodarone	Imipramine	Quinoline-4-Carboxiamide
Baicalein	Ipriflavone	S-Warfarin
Benzbromarone	Isoniazid	Seratrodast
Capecitabine	Ketoconazole	Sertraline
Clotrimazole	Lansoprazole	Sulfamethoxazole
Clozapine	Linoleic acid	Sulfonamides
Cotrimoxazole	Lovastatin	Sulfaphenazole
Delavirdine	Luteolin	Sulfinpyrazone
Desmethylsertraline	Metronidazole	Suprofen
Efavirenz	Miconazole	Teniposide
Etravirine	Noscapine	Ticlopidine
Fenofibrate	Omeprazole	Tienilic Acid
Fluconazole	Oxandrolone	Tigecycline
Fluoxetine	Paroxetine	Valproato
Fluvastatin	Piroxicam	Voriconazole
Fluvoxamine	Probenecid	Zafirlukast
Glimepiride	Phenylbutazone	

Table 2 - The reference inducers of CYP2C9 based on Back et al. (1988); Baldwin et al. (1995); Schmider et al. (1997); Miners and Birkett (1998); Hemeryck et al. (1999); Ko et al. (2000); Wen et al. (2001); He et al. (2002); Kuroha et al. (2002); von Moltke et al. (2004); Greenblatt et al. (2005); Kumar et al. (2006a); Kumar et al. (2006b); Horn and Hansten (2007); Paris et al. (2009); Turpeinen et al. (2009); Zhou et al. (2009a); Zhang et al. (2012a); Sousa-Ferreira et al. (2014); Prasad et al. (2016).

Aprepitant	Nelfinavir
Avasimibe	Nevirapine
Bosentan	Phenobarbital
Carbamazepine	Rifampicin
Cyclophosphamide	Ritonavir
Dexamethasone	Secobarbital
Enzalutamide	St. John's Wort
Hyperforin	Tipranavir
Lopinavir	

Inducers and inhibitors can be classified as strong, moderate or weak, depending on the extension they can affect the optimal level of metabolism of the substrate. In case of the inhibition, a compound is considered a strong inhibitor for a specific CYP if it causes more than

a 5-fold increase in the plasma area under the concentration-time curve (AUC) values of a substrate, or more than a 80% decrease in clearance; a moderate inhibitor for a specific CYP originates a less than 5-fold increase, but equal to or more than 2-fold increase in the plasma AUC values of a sensitive substrate; and a weak inhibitor is defined as an inhibitor that induces a less than 2-fold increase in plasma AUC values of a sensitive substrate (FDA 2015b).
On the other hand, we are in the presence of a strong inducer when the decrease of AUC values is more than 80%; a moderate inducer when the decrease of AUC values is between 50 to 80%; and a weak inducer when the decrease of the AUC values is between 20 to 50% (FDA 2015b). Nevertheless, there is not enough information about the potency of all the reference CYP2C9 inhibitors and inducers. **Table 3** indicates the known qualitative potency of some inhibitors and inducers of CYP2C9.

Table 3 - Inhibitors and Inducers of cytochrome 2C9 (CYP2C9) classified by their potency. Adapted from FDA (2015a).

Inhibitors of CYP2C9	Potency of the inhibitor	Inducers of CYP2C9	Potency of the inducer
Amiodarone	Moderate	Carbamazepine	Moderate
Fluconazole	Moderate	Rifampicin	Moderate
Miconazole	Moderate	Phenobarbital	Weak
Oxandrolone	Moderate	St. John's Wort	Weak
Capecitabine	Weak		
Cotrimoxazole	Weak		
Etravirine	Weak		
Fluvastatin	Weak		
Fluvoxamine	Weak		
Metronidazole	Weak		
Sulfinpyrazone	Weak		
Tigecycline	Weak		
Voriconazole	Weak		
Zafirlukast	Weak		

1.2.1.2. Cytochrome P450 Probes

In vitro drug metabolism studies can pursue different types of methods and have distinct aims. Nevertheless, one crucial step of these experiments in the pharmaceutical industry is to analyse the potential of a new drug candidate to change CYP activities (Ogu and Maxa 2000). This is only possible to determine through something called probe substrates. The reactions of these probes can represent the activity of specific CYP isoenzymes under specific experimental conditions (Yuan et al. 2002). The applicability of probe substrates, specific for a certain CYP isoenzyme, can be either alone or in combination (cocktail approach), which enable to assess the metabolism of a particular or several substrates, and subsequently, the functionality of CYP

isoenzymes (Yuan et al. 2002; Turpeinen et al. 2006; Kumar et al. 2006b; Darnell et al. 2011). The Center for Drug Evaluation and Research (CDER) showed that the most common *in vitro* probes reactions used by pharmaceutical industry researchers are: phenacetin *O*-deethylation for CYP1A2, coumarin 7-hydroxylation for CYP2A6, 7-ethoxy-4-trifluoromethyl coumarin *O*-dealkylation for CYP2B6, TOL 4-hydroxylation for CYP2C9, *S*-mephenytoin 4-hydroxylation for CYP2C19, bufuralol 1-hydroxylation for CYP2D6, chlorzoxazone 6-hydroxylation for CYP2E1, and testosterone 6-hydroxylation for CYP3A4 (Yuan et al. 2002). This concept can grant the predicting potential for a new pharmaceutical compound to modify the metabolism of other drugs. However, the *in vitro* results depends on the probe chosen (Yuan et al. 2002). For example, when a specific inhibitor is analyse using different probe assays for the same CYP450 enzyme activity, the result of the drug interactions can be divergent (Yuan et al. 2002; Turpeinen et al. 2006; Kumar et al. 2006b; Darnell et al. 2011). Consequently, during the drug-candidate screening and development process, researchers need to recognize all the probe substrates, their inhibitors and inducers, the several solvents used, and every experimental condition.

1.3. *In vitro* metabolism studies

Many compounds fail during the drug discovery and development process due to many aspects such as poor pharmacokinetics, lack of efficacy, animal toxicity, adverse effects in man, miscellaneous, and commercial reasons, **Figure 6** (van de Waterbeemd and Gifford 2003).

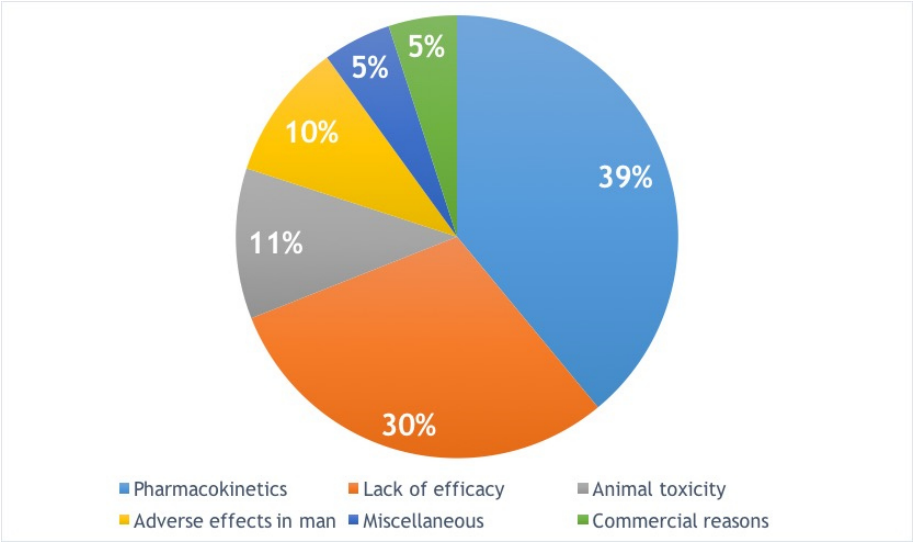

Figure 6 - An analysis of the main reasons for attrition in drug development. Adapted from van de Waterbeemd and Gifford (2003).

The major failure in drug development appears to be related with the pharmacokinetics. *In vitro* metabolism studies can provide important information about biotransformation of drugs, their transformation into soluble metabolites or toxic metabolites, and an early read on the metabolic profiles likely to be observed in humans (Schonborn 2010). The knowledge of this information can prevent the development of a drug candidate that would produce an inadmissible level of toxicity. Thus, it makes the *in vitro* screening of potential drug candidates in the early drug discovery phase an valuable stage to examine whether a drug is eliminated primarily by the excretion of unchanged drug, or by one or more routes of metabolism (FDA 1997; EMA 2012). To prevent those costly failures is crucial to perform *in vitro* studies as a more cost-effective approach to identify compounds that have unfavourable ADMET characteristics. Drug metabolism is the major determinant of the drug clearance and interindividual variability in pharmacokinetics, and it is an indirect determinant of the clinical efficacy and drug toxicity (Michael 2010).

The determination of the metabolic properties of a NCE is one of the most important steps during the drug discovery and development process, becoming imperative to determine the metabolic stability of the NCE, and evaluate its risk to be involved in drug-drug interactions related to inhibition and induction of drug-metabolizing enzymes (Ogu and Maxa 2000).

Since all these aspects have a significant impact on the drug development process, U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) recognized the value of data from *in vitro* drug metabolism studies and they have developed guidance's documents for the pharmaceutical industry;, these documents are called "*Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies in Vitro*" for FDA, and "*Guidance on the investigation of drug interactions" for EMA,* and they encourage routine, thorough evaluation of metabolism and interactions in *in vitro* studies whenever feasible and appropriate (FDA 1997; EMA 2012).

Generally, the use of *in vitro* methodologies to characterize drug metabolism is supported by some factors that include (1) efficient and relatively inexpensive nature of the methods; (2) allow early testing in the drug discovery and development process, providing important data on the chemical characteristics of molecules; (3) ready availability of human and animal tissues and enzyme systems; and (4) human cells or cell constituents can be used, increasing the relevance to man (Zhang et al. 2007). Ideally, from a metabolism perspective, the "perfect" drug candidate should be relatively stable, i.e. have a low first-pass effect and maintain an effective concentration in blood for a reasonable period of time; be metabolized by multiple CYP isoenzymes, and not largely dependent on CYPs that are polymorphically expressed, such as CYP2C9, CYP2C19, and CYP2D6; and lead to no pharmacologically-active (unless starting as a prodrug) or chemically-reactive metabolites (Lipinski et al. 1997; Lombardino and Lowe III 2004; Zanger and Schwab 2013).

In metabolic studies the ultimate goal is to ascertain (1) how the molecule is biotransformed, and whether the produced metabolites contribute to the pharmacological action of the compound (metabolic profile); (2) which enzymes are involved in its biotransformation and

whether this could be influenced by the concomitant use of other drugs; (3) whether the drug itself influences the expression or the activity of such enzymes which, in turn, could influence its metabolism and also the metabolism of other drugs; and (4) the drug toxicity associated with its metabolism (Donato et al. 2008).

All these factors and limitations make the choice of an *in vitro* metabolism system a planned decision. Researchers need to choose the system with less confounding factors, with easier application and interpretation and without complicated extrapolation to *in vivo*.

Typically, the *in vitro* studies performed on this area are inhibition or induction assays, metabolite profiling studies with liver microsomes, S-9 fraction or hepatocytes to perceive the metabolic profile across different toxicology species; and detailed reaction phenotyping studies to identify the enzymes responsible for the metabolism of the drug (Zhang et al. 2007). Three of these types of studies are focused on identifying the CYP isoenzymes involved, since practically all the drugs in the market are metabolized by CYP isoenzymes (Zhang et al. 2007). Inhibition and induction studies are conducted during drug discovery and development aiming to understand the potential of a drug candidate to inhibit or induce several CYP isoenzymes. Indeed, a powerful inhibition of CYPs may interrupt further development, especially if they inhibit key drug-metabolizing enzymes such as CYP1A2, CYP2A6, CYP3A4, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6 (Lynch et al. 2007; Zhang et al. 2007; Donato et al. 2008).

In vitro models are great systems to assess the degree of drug-drug interaction and the effects of NCEs on the activity of CYP isoenzymes (Donato et al. 2008). Acknowledging that clinically significant drug-drug interactions can occur, it is important to identify drug candidates that act as enzyme substrates, inhibitors or inducers (Ogu and Maxa 2000).

1.3.1. *In vitro* models to study drug metabolism

Several *in vitro* and *ex vivo* models have been developed for the study of drug hepatic metabolism (Anthérieu et al. 2010). In that way, the *in vitro* methods of drug metabolism can be divided into two classes: enzyme systems, which include subcellular fractions (microsomal or cytosolic) and isolated enzyme preparations (purified or cDNA expressed), and cellular systems, which include primary hepatocytes in suspension or monolayer culture, liver slices and hepatocyte-derived cell lines (Gunaratna 2000; Schoenwald 2013). *In vitro models* most used are: recombinant expressed enzymes, liver microsomes, fresh and cryopreserved hepatocytes, and tissue slices (Baranczewski et al. 2006). Unfortunately, all these models have some disadvantages like differences in the activity of metabolic enzymes per unit of microsomal protein, limited incubation time, limited availability, and not well-established for the prediction of metabolic clearance and drug-drug interactions, respectively (Baranczewski et al. 2006). Besides all these disadvantages, the only models that can retain the ability to express the complete metabolic pathways similarly to what occurs in man are those involving human liver cells (Gómez-Lechón et al. 2006; Guillouzo and Guguen-Guillouzo 2008; Ferreira et al. 2014b).

Primary hepatocytes derived from human liver tissue emerge as a unique model that permit to evaluate human-specific drug properties such as metabolic fate, drug-drug interactions and drug toxicity, and are widely recognized as being the "gold standard" for predicting CYP450 induction in both animals and humans (McGinnity et al. 2006; Li 2007; Andersson 2010; Lübberstedt et al. 2011). The liver consists of many different types of cells, being the hepatocytes those playing an important role in detoxifying xenobiotics, including drugs (Andersson 2010). For that reason, hepatocyte function strongly impacts on the pharmacokinetics, side effects and toxicity of drugs (Andersson 2010). As it is well recognized, isolated hepatocytes retain most of the key liver properties. Nevertheless, the value of the data obtained with primary human hepatocytes appears to be limited due to multiple reasons, including scarce and erratic availability, poor stability of their functions in culture, limited growth activity and life span (Li 2007; Ferreira et al. 2014b), making them not the best choice for high-throughput screenings (Ferreira et al. 2014b). Thus, new and improved alternatives to primary human hepatocytes have emerged.

The immortalized human hepatic tumour cell lines (HepG2 and Huh-7), proposed as alternatives to primary human hepatocytes, especially the HepG2 cells, have shown to be able to carry out biotransformation of xenobiotic compounds, being capable of activating mutagens and carcinogens, and carry no p53 mutations, which enables the cells to activate DNA damage response, induce growth arrest, and initiate apoptosis, becoming frequently applied in toxicogenomics studies (Jennen et al. 2010). However, both these cell lines express low to undetected levels of a number of human liver-specific CYP isoenzymes, limiting them as representative models of the primary human hepatocytes (Aninat et al. 2006; Lübberstedt et al. 2011; Ferreira et al. 2014b).

1.3.2. HepaRG cell line

Recently, a new human cell line derived from a hepatocellular carcinoma, HepaRG, has shown to be a potential model to evaluate the hepatic drug metabolism in *in vitro* conditions (Guillouzo and Guguen-Guillouzo 2008; Lübberstedt et al. 2011; Zanelli et al. 2012). This cell line is originated from a single donor, and they are a product of *in vivo* transformation (Anthérieu et al., 2010).

This promising cell line exhibits the ability to differentiate into both the biliary and hepatocyte lineage (Gripon et al. 2002; Lübberstedt et al. 2011), without losing the indefinite growth capacity of the transformed cells (Guguen-Guillouzo et al. 2010). Contrary to what happens at high density, when these cells are seeded at low densities, HepaRG cells revert to an undifferentiated morphology (Aninat et al. 2006; Guillouzo et al. 2007; Anthérieu et al. 2010). After reaching differentiation, they form hepatocyte-like colonies surrounded by epithelial biliary-like cells (Aninat et al. 2006; Guillouzo et al. 2007; Anthérieu et al. 2010). When the high-density is reached, the hepatocyte-type agglomerates typically retain their distinctive

morphology (Aninat et al., 2006). Thus, HepaRG cells represent one example of complete differentiation of liver progenitor cells *in vitro* while maintaining their proliferative capacity (Guguen-Guillouzo et al. 2010). According to Andersson (2010), in the differentiate stage, the cell line has a stable mRNA expression for the key proteins involved in the metabolism of specific drugs and in the liver functions for over 6 months.

HepaRG differentiated cells express the major CYPs such as CYP1A2, 2B6, 2C9, 2E, 2C19, and 3A4 (Guillouzo et al. 2007; Jossé et al. 2008; Kanebratt and Andersson 2008a; Hart et al. 2010; Jennen et al. 2010; Lübberstedt et al. 2011); enzymes of phase II metabolism (Guillouzo et al. 2007; Jossé et al. 2008; Anthérieu et al. 2010; Guguen-Guillouzo et al. 2010; Hart et al. 2010; Jennen et al. 2010; Darnell et al. 2011; Guguen-Guillouzo and Guillouzo 2011; Lübberstedt et al. 2011); influx and efflux transporters (Guillouzo et al. 2007; Jossé et al. 2008; Kanebratt and Andersson 2008a; Anthérieu et al. 2010; Hart et al. 2010; Jennen et al. 2010; Darnell et al. 2011; Guguen-Guillouzo and Guillouzo 2011) as well as apical and canalicular ATP-binding cassette (ABC) transporters (Guillouzo et al. 2007); and all nuclear receptors at levels comparable to those existing in primary hepatocytes (Anthérieu et al. 2010; Darnell et al. 2011; Guguen-Guillouzo and Guillouzo 2011). Guillouzo & Guguen-Guillouzo (2008) demonstrated a stable gene expression in HepaRG cells over up to 30 days.

For most metabolizing genes, the expression levels are associated with the presence dimethyl sulfoxide (DMSO) (Hoekstra et al. 2011). The expression of CYP isozymes decreases with the DMSO removal of the medium, but both carriers and liver specific factors remain unchanged (Kanebratt and Andersson 2008a). Thus, it is suggested a double effect of DMSO on the cells: DMSO affects not only their differentiation but also the gene expression in differentiated cells (Kanebratt and Andersson 2008a). As mentioned above, in HepG2 cells, mRNA has not been detected for several drug-metabolizing enzymes, having a quite different pattern in comparison to that observed for HepaRG (Kanebratt and Andersson 2008a).

About 85% of constitutively expressed genes in primary human hepatocytes are also expressed in HepaRG cells (Guguen-Guillouzo et al. 2010). Thus, when these cells reach a differentiated hepatocyte-like morphology, i.e., as seeded at high density, retain a single set of metabolizing enzymes at levels comparable to normal human hepatocytes in primary culture (Aninat *et al.*, 2006), **Table 4**. The HepaRG feature a large metabolizing capacity, including a comprehensive response to chemical modulators, which reflects the observed in populations of human hepatocytes in primary cultures (Anthérieu et al. 2010). The main difference between HepaRG cells and primary hepatocytes is the continuous expression of CYP1A1 in formation, and this activity is mostly related to its transformed state and not with the presence of bile duct cells (Anthérieu et al. 2010).

Table 4 - Comparative expression of cytochromes (CYPs) P450 mRNA levels in HepaRG cells, freshly isolated human hepatocytes, and HepG2 cells. Adapted from Aninat et al. (2006).

	CYP1A1	*CYP2B6*	*CYP2C9*	*CYP2D6*	*CYP2E1*	*CYP3A4*
FIH	100	100	**100**	100	100	100
HepG2 cells	0.3	0.5	**0**	2.3	0	0
HepaRG cells seeded at low density (days)						
5	0.1	0	**0**	1	0	0
8	0.1	0.1	**0.1**	1.2	0	0
15	0.3	3.2	**3.3**	0.9	0.1	4.5
30	2.3	3.5	**9.5**	1.2	0.2	8.1
30 (DMSO)	9.1	34.6	**34.7**	0.8	3.5	176
35 (-DMSO 24 h)	5.5	3.5	**26.5**	1	0.8	1.6
37 (-DMSO 72 h)	3.9	1.6	**16.7**	0.9	0.8	2.3
HepaRG cells seeded at high density after differentiation						
+ DMSO 24 h	17.8	72.4	**38.1**	1.1	13.3	243
- DMSO 24 h	7.5	4.9	**22.1**	0.8	0.7	6.8
+ DMSO 72 h	3.7	73.1	**43.8**	1	7	255.6
- DMSO 72 h	5.4	11.9	**22**	0.8	1	2.8

The HepaRG cells exhibits unique advantages, such as: the data are reproducible over several passages (Anthérieu et al. 2010; Pernelle et al. 2011); the functional activities are maintained relatively stable for 1-2 weeks after several weeks of confluence (Guillouzo et al. 2007; Jossé et al. 2008; Kanebratt and Andersson 2008a; Anthérieu et al. 2010; Guguen-Guillouzo et al. 2010; Hart et al. 2010; Lübberstedt et al. 2011); its enzymatic activity can be modulated allowing closer to inter-individual variations in metabolizing enzymes normally found when studied different populations of hepatocytes (Guguen-Guillouzo et al. 2010); the activity levels may be modulated by selection of appropriate culture conditions, such as composition of the culture medium (Anthérieu et al. 2010); both differentiated cells and non-differentiated derived from the same cell line can be used and compared, and they can be easily cryopreserved without marked cell loss (Guguen-Guillouzo et al. 2010).

In summary, the HepaRG cell line possesses the metabolic capacity characteristic of primary human hepatocytes and the indefinite proliferation property of hepatoma cell lines, making them perhaps the most relevant model for *in vitro* studies on drug metabolism, studying hepatic kinetics of drugs, drug-drug interactions and toxicity (Gripon et al. 2002; Anthérieu et al. 2010; Kotani et al. 2012; Zanelli et al. 2012; Ferreira et al. 2014b).

2. Aims

Drug metabolism can be affected by drug interactions involving the CYP450 system. Such drug interactions can result in reduced pharmacological effects, adverse drug reactions and drug toxicity. The application of high throughput screening methodologies with the aim of helping to recognize whether drugs or compounds in study act as enzyme substrates, inducers, or inhibitors may be useful to prevent clinically significant interactions. Given the requirement for new simple and fast methodologies, the development and implementation of an experimental *in vitro* assay able to be used to investigate drug–drug interactions mediated by CYP2C9 isoenzyme, which could be applied in high throughput screening during the development of new drugs, emerged as the global aim of this work.

More specifically, the partial aims of this work involved:

- The development and validation of a new high-performance liquid chromatography–diode-array detection (HPLC-DAD) assay for the quantification of TOL and its main metabolite 4-OH-TOL in HepaRG cell culture samples;
- The accomplishment of several metabolic inhibition studies, using a set of model compounds widely described in the literature as CYP2C9 inhibitors, being TOL employed as a selective probe drug for CYP2C9 isoenzyme, in order to demonstrate the interest of this *in vitro* methodology for the evaluation of potential metabolic inhibition interactions involving CYP2C9;
- The application of the *in vitro* methodology developed and validated to investigate the interference of flavonoid compounds with the CYP2C9-mediated metabolism.

3. Materials and Methods

3.1. Reagents and chemicals

Amiodarone hydrochloride (minimum 98%), baicalein, omeprazole, quercetin and carbamazepine (CBZ), used as internal standard (IS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). (-)-Epigallocatechin gallate and kaempferol were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Fluoxetine hydrochloride and ticlopidine hydrochloride were kindly provided by Bluepharma (Coimbra, Portugal). Ketoconazole was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). TOL and 4-OH-TOL were acquired from Sigma-Aldrich (St. Louis, MO, USA).

HepaRG cells (lot no. #48588) were obtained from Life Technologies – Invitrogen™ (through Alfagene, Portugal). All cell culture reagents including Williams' E medium, fetal bovine serum (FBS), hydrocortisone hemisuccinate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, trypsin–EDTA, phosphate-buffered saline (PBS), and sodium hydrogen carbonate were purchased from Sigma–Aldrich (St Louis, MO, USA). Acetonitrile HPLC grade was purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultra-pure water (HPLC grade, >18 MΩ cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). All other reagents were of analytical grade: sodium phosphate monobasic purris p.a. (Sigma–Aldrich GmbH; Seelze, Germany) and triethylamine (Fisher Scientific, Lisbon, Portugal).

3.2. HPLC-DAD method for the quantification of tolbutamide and 4-hydroxytolbutamide

3.2.1. Stock solutions, calibration standards and quality control samples

Stock solutions of TOL (20 mM) and 4-OH-TOL (2 mM) were individually prepared by dissolving appropriate amounts of each compound in methanol. Stock solutions of TOL and 4-OH-TOL were diluted with methanol to obtain the corresponding working solutions. Stock and working solutions of TOL and 4-OH-TOL were then properly mixed to afford seven combined spiking solutions with final concentrations of 2.5, 5, 10, 50, 250, 1250 and 2000 μM for TOL, and of 2.5, 5, 10, 20, 50, 125 and 200 μM for 4-OH-TOL. Each one of these combined solutions was daily used for spiking aliquots of blank supplemented Williams' E medium in order to prepare seven calibration standards with the concentrations of 0.25, 0.5, 1, 5, 25, 125 and 200 μM for TOL, and 0.25, 0.5, 1, 2, 5, 12.5 and 20 μM for 4-OH-TOL. A stock solution of CBZ (IS; 10 mM) was also prepared in methanol. An aliquot of the IS stock was diluted daily with water/methanol

(75:25, v/v) to obtain the working solution at 200 µM. All solutions were protected from light and stored at about 4 °C, with the exception of IS working solution that was prepared daily. Before were used, the spiking solutions were degassed ultrasonically for 5 minutes.
Quality control samples (QC) were also independently prepared in the supplemented Williams' E medium at three concentration levels representative of the low (QC_1), medium (QC_2), and high (QC_3) ranges of the calibration curve. Aliquots of blank supplemented Williams' E medium were appropriately spiked to achieve the final concentrations of 0.75 µM in QC_1; 100 µM in QC_2 and 180 µM in QC_3 for TOL, and 0.75 µM in QC_1; 10 µM in QC_2 and 18 µM in QC_3 for 4-OH-TOL. An additional QC at the concentration of the lower limit of quantification (QC_{LLOQ}) was also prepared.

3.2.2. Sample preparation

The sample preparation procedure has been previously optimized and the final conditions are as follows. To each aliquot (200 µL) of supplemented Williams' E culture medium were added 20 µL of IS working solution (200 µM), 40 µL of 20% trichloroacetic acid (precipitating agent) and 1 mL of ethyl acetate (used as liquid-liquid extraction solvent). The mixture was vortex-mixed for 1 minute and centrifuged at 13,500 rpm for 3 minutes at room temperature. Posteriorly, approximately 1 mL of the upper organic layer was transferred to a glass tube; the sample was re-extracted twice more with ethyl acetate (1 mL each) under the conditions mentioned above. The total organic extract (3 mL) was evaporated to dryness under a nitrogen stream at 45 °C, and the residue was reconstituted in 100 µL of ultra-pure water and vortex-mixed for approximately 1 minute. At the end, an aliquot (20 µL) of the final sample was injected into the HPLC system for analysis of TOL and 4-OH-TOL.

3.2.3. Apparatus and chromatographic conditions

The chromatographic analysis was performed using an HPLC system Shimadzu LC-2010A HT Liquid Chromatography coupled with DAD (Shimadzu SPD-M20A). All instrumental parts were automatically controlled by Lab-Solutions software (Shimadzu, Kyoto, Japan). Chromatographic separation of TOL, 4-OH-TOL and IS was achieved in less than 20 minutes by a gradient elution on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany), protected by a LiChroCART® 4-4 (C_{18}, 5 µm) precolumn, and thermostatically controlled at 35 ºC. A gradient elution program was employed at a flow rate of 0.6 mL/min with a mobile phase of phosphate buffer (10 mM) with 0.1% triethylamine (pH 3)/acetonitrile. During the first 4 minutes of the run, a linear gradient was applied from 22% to 30% of acetonitrile and it was kept during 15 minutes; subsequently, the percentage of acetonitrile was restored to 22% within 1 minute and maintained until the end of the run. The aqueous fraction of the mobile phase was filtered through a 0.45 mm filter.

The injection volume was 20 µL and 230 nm was the selected wavelength for the detection of all compounds (TOL, 4-OH-TOL and IS).

3.2.4. Method validation

The validation of a bioanalytical method comprises all procedures to demonstrate that a particular method used for quantifying an analyte in a given array is reliable and reproducible for the intended use (FDA 2013; EMA 2011). The described method was validated according to the general recommendations published in the last few years concerning bioanalytical method validation and the validation parameters investigated were: selectivity, linearity, precision, accuracy, lower limit of quantification (LLOQ), recovery and stability (FDA 2013; EMA 2011).

Selectivity

Aiming at testing the chromatographic similarity between the supplemented Williams' E medium collected after the culture of HepaRG cells, in order to reproduce what happens in real metabolic studies, and the simple supplemented Williams' E medium, a set of samples from these two related matrices were analysed and compared. To obtain aliquots of medium from cultured HepaRG cells the following procedures were conducted. HepaRG cells were maintained in the Williams' E medium supplemented with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin and $5x10^{-5}$ M hydrocortisone hemisuccinate during 15 days. HepaRG cells were maintained in culture at 37 °C in a humidified air incubator with 5% CO_2 and the medium was renewed each 3 days. Then, the HepaRG cells were seeded at a high density ($4.5x10^5$ cells/cm^2) in 24-well plates and maintained during 24 hours. After that, the medium was removed, the cells were washed with 200 µL of PBS, and then incubated with 200 µL of supplemented Williams' E medium (simulating the drug incubation assay). After 24 hours (simulating the time of drug incubation assay), the medium was collected to an Eppendorf tube and the sample analysed by the HPLC-DAD method.

Calibration Curve

The linearity of the analytical method was assessed in the range 0.25-200 µM for TOL and 0.25-20 µM for 4-OH-TOL. For that, calibration curves were prepared on three different days (n = 3) using calibration standards prepared in supplemented Williams' E medium at seven different concentration levels. Calibration curves were constructed by plotting the peak-area ratios (analyte/IS) as a function of the respective nominal concentrations. The data were subjected to a weighted linear regression analysis using $1/x^2$ as the weighting factor for both analytes (TOL and 4-OH-TOL). This weighting factor was selected because it yielded the best fit of peak-area ratios v*ersus* concentration (Almeida et al. 2002).

Lower Limit of Quantification

The LLOQ for TOL and 4-OH-TOL, defined as the lowest concentration on the calibration curve that can be measured with acceptable intraday and interday precision and accuracy, was established by analysing spiked samples of supplemented Williams' E medium in replicate. The precision was expressed by the percentage of coefficient of variation (CV) not exceeding 20%, and the accuracy by the percentage of deviation from nominal value (% *bias*), which should be within ±20% (EMA 2011; FDA 2013).

Precision and Accuracy

Intraday and interday precision and accuracy were assessed by using QC analysed in replicate (n = 3) at four concentration levels (QC_{LOQ}, QC_1, QC_2 and QC_3), representative of the calibration range. The concentrations tested were 0.25, 0.75, 100 and 180 µM for TOL, and 0.25, 0.75, 10 and 18 µM for 4-OH-TOL. The acceptance criterion for intraday and interday precision (expressed as % CV) was a CV not exceeding 15% (or 20% in the LLOQ), and the criterion for accuracy (expressed as % *bias*) was a *bias* value within ±15% (or ±20% in the LLOQ) (EMA 2011; FDA 2013).

Recovery

The recovery of the analytes (TOL and 4-OH-TOL) from supplemented Williams' E medium samples, submitted to the sample preparation procedures described above in *section 3.2.2*, was determined using QC samples at three concentration levels (QC_1, QC_2 and QC_3) and analysed in replicate (n = 5). The recovery of the analytes was calculated by comparing the analyte peak area of extracted QC supplemented Williams' E medium samples against those obtained after direct injection of non-extracted solutions at the same nominal concentrations. The recovery of the IS was also evaluated at the concentration used in the analysis of samples, calculating the ratio of its peak areas in extracted samples and non-extracted solutions.

Stability

The stability of TOL and 4-OH-TOL was assessed in supplemented Williams' E medium at low (QC_1) and high (QC_3) concentration levels. The stability was evaluated during 4 hours at room temperature and 8 days at –20 ºC, in order to simulate sample handling and storage time in the freezer before analysis (n = 5). The stability of the analytes (TOL and 4-OH-TOL) was also studied at 12 hours at room temperature in the processed samples, in order to reproduce the time that samples can spend in the autosampler before analysis. Stability was assessed by comparing the data of QC samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). A stability/reference samples ratio of 85–115% was accepted as stability criterion (n = 5) (EMA 2011).

3.3. Metabolic studies

3.3.1. Cell culture

HepaRG cells were seeded in Williams' E medium supplemented with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin and $5x10^{-5}$ M hydrocortisone hemisuccinate, and maintained at 37 °C in a humidified air incubator with 5% CO_2. The medium was renewed every 2–3 days until cells reach confluence. Then, the cells were expanded by gentle trypsinization and maintained in culture by serial passages and used when appropriate for the specific studies.

3.3.2. Cell viability analysis (MTT assay)

The *in vitro* cytotoxicity was studied by the MTT assay according to the procedure described by Freshney (2012). For this purpose, HepaRG cells were seeded in 96-well plates ($1x10^4$ cells/well) in supplemented Williams' E medium. After 24 hours of adherence, the cells were treated with three different concentrations of each known CYP2C9 inhibitor or flavonoid compounds to be further tested (**Table** 5), plus TOL at 200 µM, and incubated for 24 hours at 37 °C in an atmosphere of 5% CO_2. Untreated cells were used as controls (DMSO, 2%). After the incubation period (24 hours) the medium was removed, 200 µL of PBS were used to wash the cells, and 100 µL of MTT solution (5 mg/mL) prepared in William's E medium (without FBS, antibiotics, insulin and hemisuccinate hydrocortisone) was added to each well, followed by an incubation for 4 hours under the conditions mentioned above. Then, the MTT containing medium was removed and the formazan crystals were dissolved with 200 µL of DMSO. The contents were transferred to a reading plate and the absorbance was measured at 570 nm using a microplate spectrophotometer (BIORAD XMark, USA). Cell viability values were expressed in percentage relatively to the absorbance determined in the cells used as controls.

Table 5 - Studied concentrations of the known cytochrome (CYP) 2C9 inhibitors and flavonoid compounds. K_i, Inhibitory Constant.

Substance	Studied concentrations (μM)	K_i (μM)	Reference
CYP2C9 inhibitors			
Amiodarone	0.4 / 0.8 / 1.6	0.81	Kumar et al. (2006a)
Fluoxetine (RS-fluoxetine)	10 / 20 / 38	19	Schmider et al. (1997)
Ketoconazole	0.7 / 1.5 / 3	1.46	Kumar et al. (2006b)
Omeprazole	0.9 / 1.8 / 4	1.79	Kumar et al. (2006b)
Ticlopidine	38 / 76 / 152	76	Ko et al. (2000)
Studied flavonoids			
Baicalein	2.5 / 15 / 50	-	Si et al. (2009)
(-)-Epigallocatechin gallate	2.5 / 15 / 50	-	Si et al. (2009)
Quercetin	2.5 / 15 / 50	-	Si et al. (2009)
Kaempferol	2.5 / 15 / 50	-	Si et al. (2009)

3.3.3. Metabolic inhibition assays

HepaRG cells were seeded in 150 cm^2 flasks in supplemented Williams' E medium and maintained at 37 °C in an atmosphere of 5% CO_2 for about 15 days. The medium was renewed each 3 days. The HepaRG cells in confluence were then used in the metabolic inhibition studies. The culture medium of HepaRG cells was removed and the cells were washed immediately with 30 mL of PBS. Thereafter, 10 mL of trypsin was added into the flask and it was maintained for approximately 10 minutes at 37 °C in a humidified air incubator with 5% CO_2. After this time, 10 mL of the culture medium was added into the flask in order to stop the trypsin action. The whole volume was collected into a Falcon tube and centrifuged at 1414 rpm for 2 minute to obtain the cell pellet. The supernatant was discarded and replaced by William's medium E supplemented with 5 μg/mL insulin, $5x10^{-5}$ M hydrocortisone hemissucinate and 100 IU/mL penicillin and 100 μg/mL streptomycin. Then, the HepaRG cells were seeded at high density ($4.5x10^5$ cells/cm^2) in 24-well plates and allowed to adhere to the plates over a period of 24 hours. At the end of 24 hours, the medium was removed, the cells were washed with 200 μL of PBS, and then pre-incubated for 30 minutes with 200 μL of supplemented Williams' E medium, without FBS, fortified with the CYP2C9 inhibitors or flavonoids at three different concentrations (as shown in **Table 5**). In the controls the percentage of the drug vehicle (DMSO, 1%) was maintained. After 30 minutes, the fortified medium was removed, and the cells were incubated with the CYP2C9 inhibitors or flavonoids, plus the CYP2C9 probe drug TOL at 200 μM for 24 hours. The controls were incubated also with TOL (200 μM) and the percentage of the CYP2C9 inhibitors or flavonoids vehicle (DMSO, 1%). After the incubation period, the total volume of

each well was separately collected and frozen at -20 °C until HPLC analysis.

3.3.4. Statistical analysis

Data were reported as the mean ± standard error of the mean. Comparisons between two groups (experimental group *vs*. control group) were performed by student's *t* test. A difference was considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

4. Results

The results obtained for the development and validation of the HPLC-DAD method for quantifying TOL and 4-OH-TOL in HepaRG cells, the cell viability analysis and metabolic inhibition assays are presented below.

4.1. HPLC-DAD method for the quantification of tolbutamide and 4-hydroxytolbutamide

4.1.1. Method validation

The chromatographic separation of IS and the analytes of interest (TOL and 4-OH-TOL) in spiked supplemented Williams' E medium culture samples was successfully achieved by using the analytical conditions previously described. Under these conditions, the last-eluting analyte was the TOL, being its retention time of approximately 15 minutes. The elution order was as follows: 4-OH-TOL, IS and TOL (**Figure 7**). **Figure 7** shows typical chromatograms of samples of blank and spiked supplemented Williams' E culture medium.

Selectivity

The chromatograms obtained by the analysis of blank samples of supplemented Williams' E medium collected after the culture of HepaRG cells (to reproduce the metabolic studies) and those generated by the analysis of blank samples of simple supplemented Williams' E medium were broadly comparable. As result, since there is no difference between them, the most convenient blank matrix, this is, the simple supplemented Williams' E medium, was chosen for the development and validation of this HPLC–DAD assay. The analytical method must be able to differentiate and quantify analyte(s) of interest and IS in the presence of endogenous matrix components or other constituents of the sample (FDA 2013; EMA 2011). The absence of response in samples of blank supplemented Williams' E culture medium in the retention times of the analytes or IS was demonstrated at 230 nm.

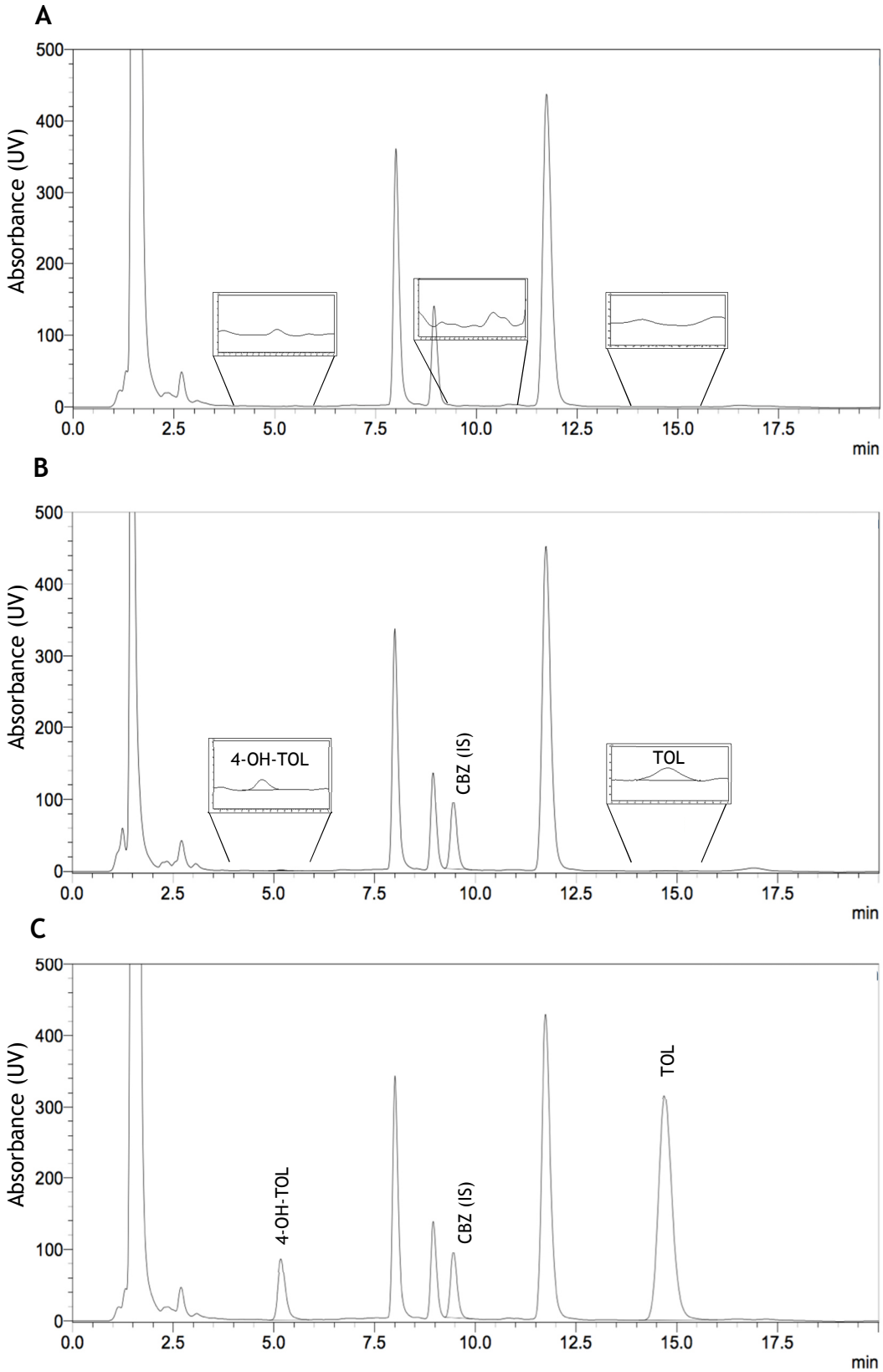

Figure 7 - Typical chromatograms of extracted supplemented Williams' E medium samples generated by the HPLC-DAD assay developed: blank supplemented Williams' E medium (**A**); supplemented Williams' E medium spiked with internal standard (IS) and the analytes at concentrations of the lower limit of quantification (**B**); and supplemented Williams' E medium spiked with IS and the analytes at the maximum concentration of the calibration ranges (**C**). CBZ, carbamazepine; 4-OH-TOL, 4-hydroxytolbutamide; TOL, tolbutamide; UV, ultraviolet.

Calibration Curves

The calibration curves obtained from blank supplemented Williams' E culture medium samples over 3 days (n = 3) showed linearity in the concentration range of 0.25-200 µM for TOL, and 0.25-20 µM for 4-OH-TOL. The equations of the calibration curves are shown in **Table 6**. The obtained results showed good linearity ($r^2 \geq 0.9901$) for the ratios of the peak areas (analyte/IS) *versus* nominal concentration. Due to the wide calibration range and to compensate the heteroscedasticity detected, the calibration curves for all the analytes were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor. This factor was chosen taking into account the plots and the sums of absolute percentage of relative error as statistical criteria. The regression equations of the calibration curves and the corresponding regression coefficients (r^2) achieved for each analyte are summarized in **Table 6**.

Table 6 - Mean calibration parameters obtained for tolbutamide (TOL) and 4-hydroxytolbutamide (4-OH-TOL) in supplemented Williams' E culture medium (n = 3).

Analyte	Calibration parameters		
	Concentration range (µM)	Equation[a]	r^2
TOL	0.25-200	y=0.0422x-0.0014	0.9901
4-OH-TOL	0.25-20	y=0.0642x-0.0015	0.9924

[a] Equation for the calibration curve: y = bx + a, where x is the analyte concentration, expressed in µM, and y is the ratio of peak areas (analyte/IS) expressed in arbitrary units of area; r^2, regression coefficient.

Lower Limit of Quantification

The LLOQ of the method was established at 0.25 µM for TOL and 4-OH-TOL, as this was the lowest concentration considered in the calibration range, quantified with acceptable intraday and interday precision and accuracy (n = 3) (**Table 7**).

Precision and Accuracy

The precision of an analytical method describes the closeness of repeated individual measures of analyte, while accuracy describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte (FDA 2013; EMA 2011). The data for intraday and interday precision and accuracy obtained from QC's supplemented Williams' E medium samples at the four different concentration levels (QC_{LLOQ}, QC_1, QC_2 and QC_3) are shown in **Table 7**). All the data fulfilled the acceptance criteria internationally established.

Table 7 - Interday and intraday precision (% CV) and accuracy (% bias) values obtained for tolbutamide (TOL) and 4-hydroxytolbutamide (4-OH-TOL) in supplemented Williams' E culture medium samples at the concentrations of the lower limit of quantification (QC_{LLOQ}) and at low (QC_1), medium (QC_2) and high (QC_3) concentrations representative of the calibration ranges.

Analyte	Nominal concentration (μM)	Interday		Intraday	
		Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)
TOL	0.25	16.1	1.2	16.8	3.3
	0.75	13.6	11.1	3.5	12.3
	100	14.1	-1.2	6.5	-11.4
	180	14.8	-6.7	5.3	-11.6
4-OH-TOL	0.25	19.0	-11.3	10.6	8.4
	0.75	6.2	3.2	9.6	4.6
	10	6.2	7.6	4.8	1.5
	18	7.1	-2.3	5.6	-9.8

CV, coefficient of variation; *bias*, deviation from nominal concentration value.

Accordingly, the overall interday and intraday CV values did not exceed 14.8% (or 19.0% in the QC_{LLOQ}), and the overall interday and intraday *bias* values varied between −11.6 and 12.3% (or −11.3 and 8.4% in the QC_{LLOQ}), which supports that the HPLC–DAD method herein described is precise and accurate.

Recovery

The absolute recovery of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte (FDA 2013; EMA 2011). The overall absolute recovery of TOL and 4-OH-TOL from supplemented Williams' E culture medium samples was determined at three concentration levels (QC_1, QC_2 and QC_3) by repeated analysis (n = 5). These data are shown in **Table 8**. The mean absolute recoveries ranged from 73.2 to 84.8% and showed CV values lower than 14% for all analytes (TOL and 4-OH-TOL). The recovery of the IS was also evaluated, being its absolute recovery of 81.9 ± 8.0% with a CV value of 9.8%. These data undoubtedly support a consistent recovery over the evaluated concentration range using the sample preparation procedure implemented.

Table 8 - Absolute recovery of tolbutamide (TOL) and 4-hydroxytolbutamide (4-OH-TOL) in supplemented Williams' E culture medium.

Analyte	Nominal concentration (µM)	Absolute recovery (%)[a]	Precision (% CV)
TOL	0.75	73.2 ± 10.3	14.0
	100	77.0 ± 9.1	11.8
	180	78.5 ± 8.5	10.8
4-OH-TOL	0.75	74.9 ± 5.3	7.1
	10	84.8 ± 7.6	8.9
	18	79.7 ± 7.1	8.9

[a] Mean ± standard deviation, *n* = 5.

Stability

The stability of the TOL and 4-OH-TOL in supplemented Williams' E culture medium was evaluated under the different conditions previously described, which simulate the handling and sample storage conditions to be likely required during the analytical process. The analytes demonstrated to be stable in unprocessed samples at room temperature for 4 hours and at -20 °C for 8 days, and also in processed samples in the autosampler of the HPLC during 12 hours. The stability data are shown in **Table 9.**

Table 9 - Stability (values in mean percentage) of tolbutamide (TOL) and 4-hydroxytolbutamide (4-OH-TOL) in unprocessed and processed samples of supplemented Williams' E culture medium under the expected sample handling and storage conditions (*n* = 5).

Analyte	Nominal concentration (µM)	Supplemented Williams' E culture medium		
		Unprocessed sample		Processed sample
		Room temperature (4 hours)	−20 °C (8 days)	Room temperature (12 hours)
TOL	0.75	112.2	101.9	109.8
	18	104.8	90.5	85.9
4-OH-TOL	0.75	104.0	112.8	104.8
	18	97.6	109.9	95.9

4.2. Metabolic studies

4.2.1. Cell viability analysis (MTT assay)

The study of the cytotoxicity potentially induced by the CYP2C9 inhibitors (amiodarone, fluoxetine, ketoconazole, omeprazole and ticlopidine) and by the flavonoid compounds [baicalein, (-)-epigallocatechin gallate, kaempferol and quercetin] in HepaRG cells had, as main purpose, to understand the concentrations of these inhibitors and flavonoids that could be used in the following metabolic studies, without significant loss of cell viability. In this context, **Figure 8** shows the results of the cytotoxicity assays performed after the incubation with 200 µM TOL and CYP2C9 inhibitors, at three different concentrations, in HepaRG cells for 24 hours.

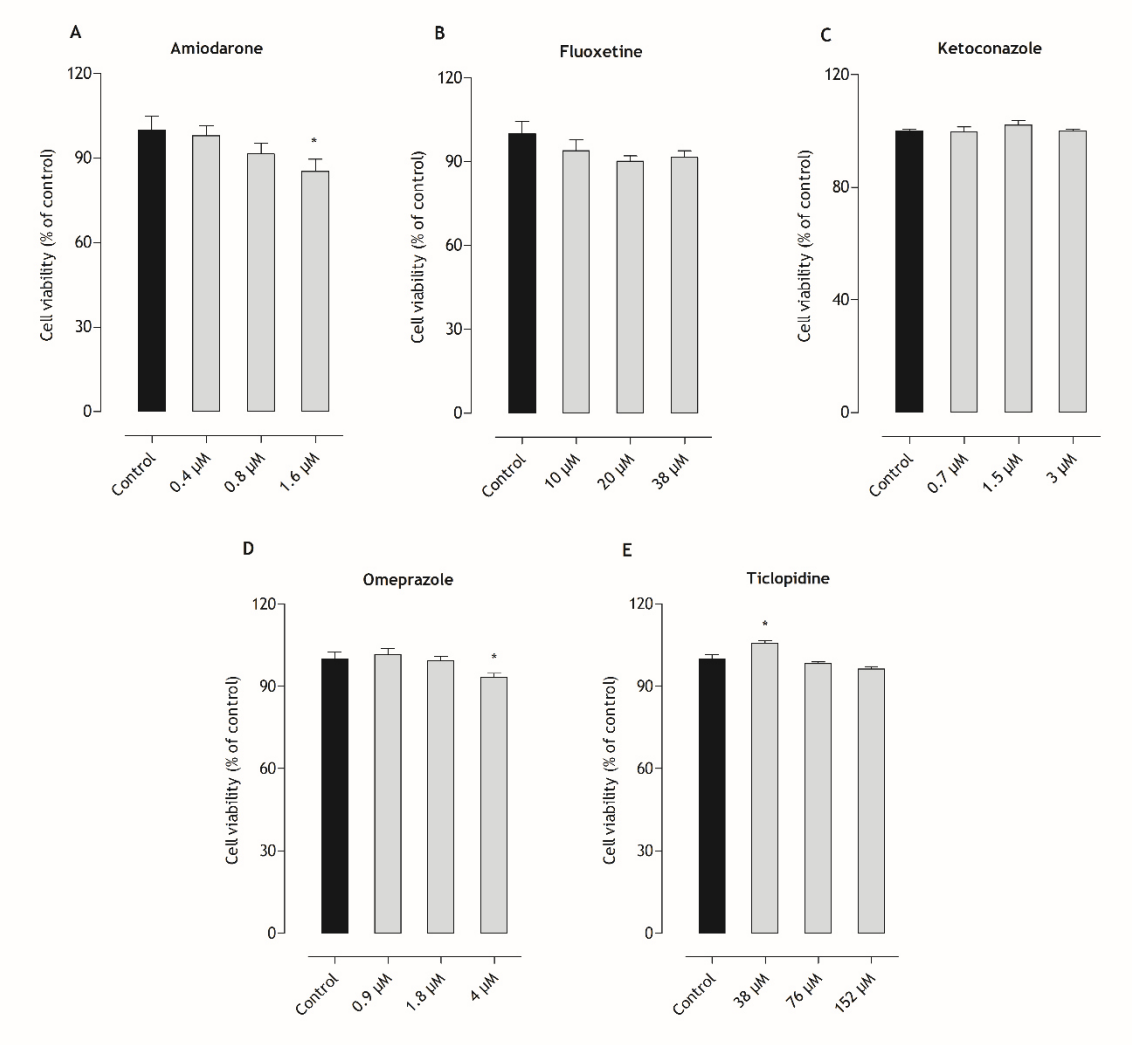

Figure 8 - Cytotoxicity assay of amiodarone (A), fluoxetine (B), ketoconazole (C), omeprazole (D) and ticlopidine (E) after 24 hours of incubation with 200 µM tolbutamide in HepaRG cells. Data are expressed as the mean values ± standard error of the mean (*n* = 6).

The obtained results depicted in **Figure 8** shown that none of the CYP2C9 inhibitors caused significant loss of cell viability in HepaRG cells. As expected, in general, there was an increased loss of the cell viability with the highest concentrations of the CYP2C9 inhibitors tested.
In **Figure 9** are shown the results of the cytotoxicity assays performed after the incubation with 200 µM TOL and the different flavonoid compounds in HepaRG cells for 24 hours.

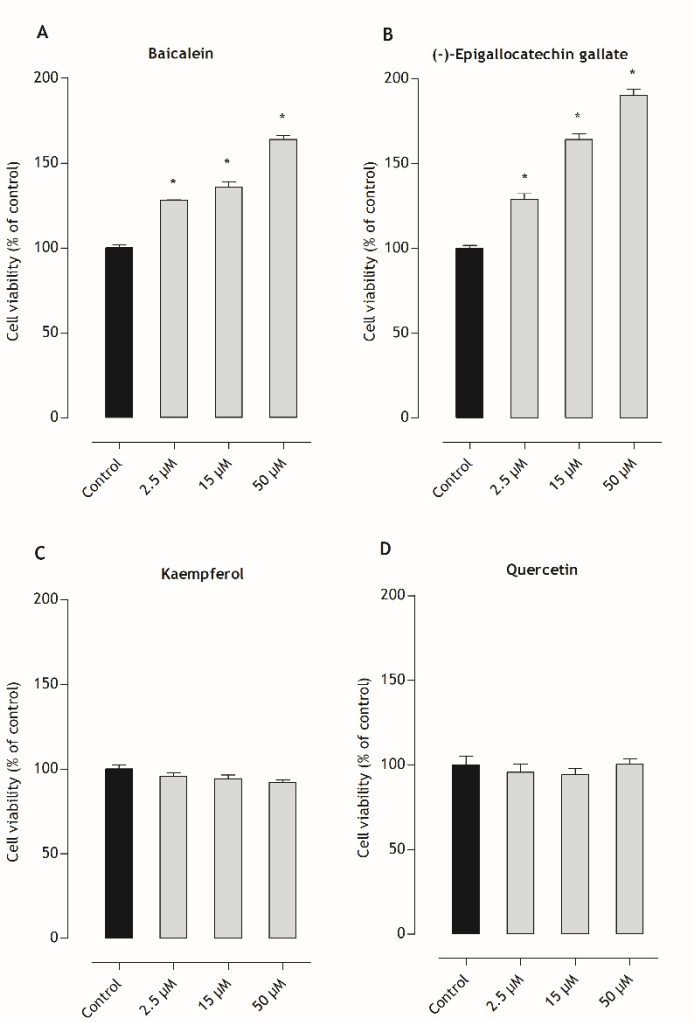

Figure 9 - Cytotoxicity assay of baicalein (**A**), (-)-epigallocatechin gallate (**B**), kaempferol (**C**) and quercetin (**D**) after 24 hours of incubation of flavonoids with 200 µM tolbutamide in HepaRG cells. Data are expressed as the mean values ± standard error of the mean (n = 6). *p < 0.05, compared to control group.

The obtained results depicted in **Figure 9** demonstrated that none of the flavonoids caused significant loss of cell viability in HepaRG cells. Nevertheless, baicalein and (-)-epigallocatechin gallate demonstrated to increase the HepaRG cell proliferation, being these effects concentration-dependent and more markedly as the concentrations of these flavonoids increase.

4.2.2. Metabolic inhibition assays

HepaRG cell line exhibits different morphology when starts to differentiate. After reaching confluence, these cells form hepatocyte-like colonies surrounded by epithelial biliary-like cells (Gripon et al. 2002; Aninat et al. 2006; Guillouzo et al. 2007; Anthérieu et al. 2010). In **Figure 10**, it can be observed HepaRG cells seeded at high density before (A) and after (B) two weeks of confluence.

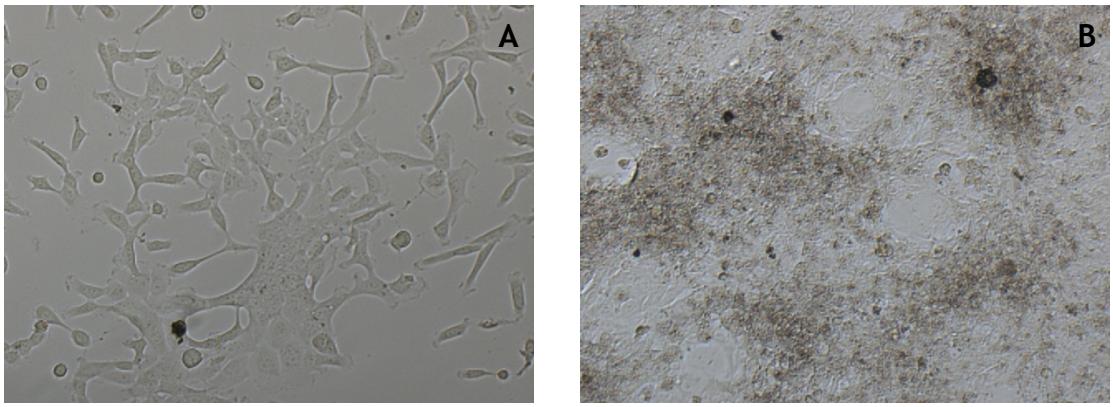

Figure 10 - HepaRG cells seeded at high density before (A) and after (B) two weeks of confluence. (100x magnification, Olympus IX51, Japan, OCTAX Eyeware v.1.5 Build 406, Germany).

In order to evaluate the suitability of the developed methodology for the screening of CYP2C9 drug interactions in HepaRG cells, a set of metabolic inhibition assays were performed using recognized CYP2C9 inhibitors and TOL as a selective CYP2C9 probe drug. For this purpose, the CYP2C9 inhibitors amiodarone, fluoxetine, ketoconazole, omeprazole and ticlopidine were used. The results of these studies are presented in the **Figure 11**. It can be observed that the presence of CYP2C9 inhibitors usually decreased the formation of the metabolite 4-OH-TOL, when compared to the control, and it occurred at almost all the tested concentrations. Nevertheless, the concentration of 0.9 µM omeprazole appeared to significantly induce the formation of 4-OH-TOL, unlike the other two concentrations. Apart from fluoxetine and amiodarone, the inhibitory effect originated by the others CYP2C9 inhibitors increased as the inhibitor concentration increased. In the case of amiodarone, its inhibitory effect appeared also to be concentration-dependent, but in this case this effect decreased as the concentrations of the drug increased. In the case of fluoxetine, the highest inhibitor effect was achieved at 20 µM.

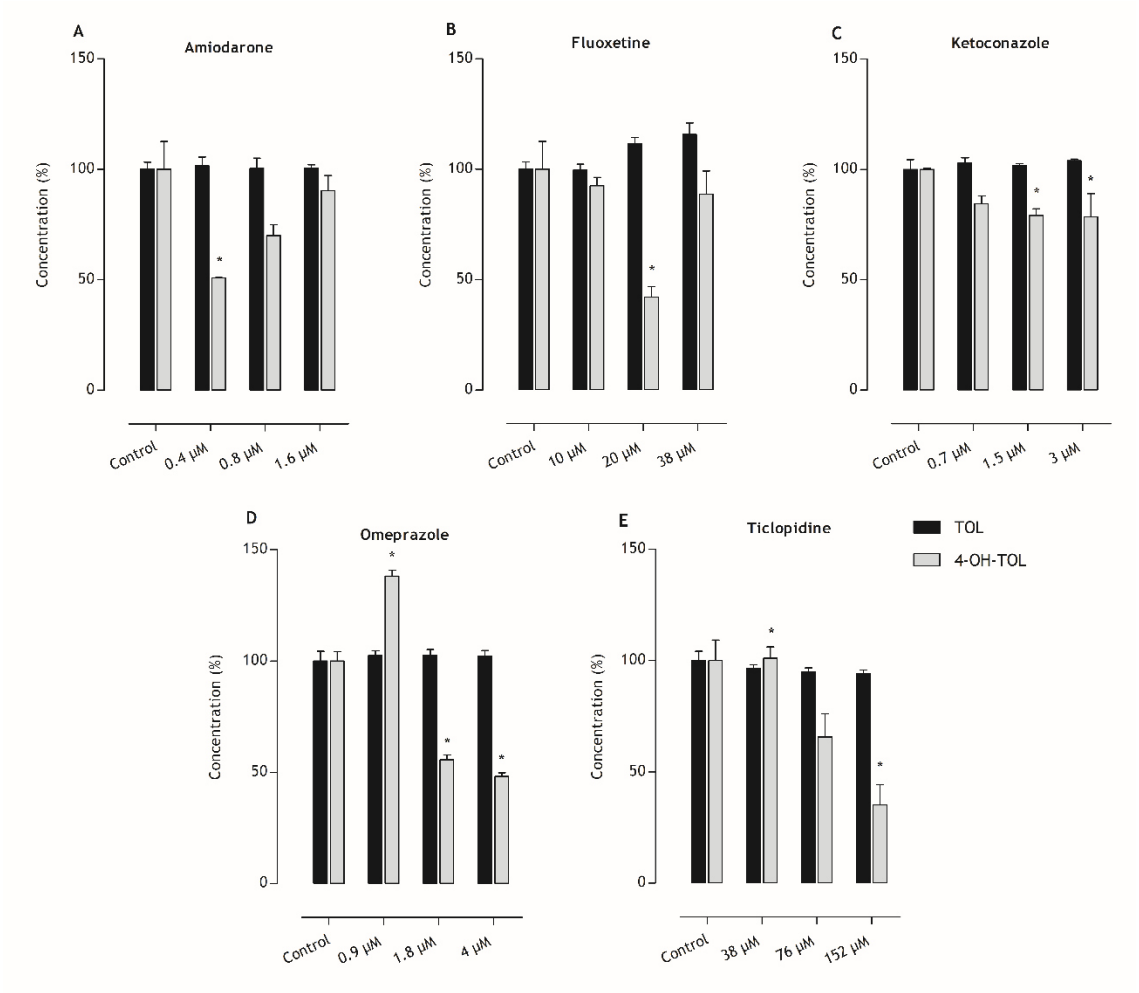

Figure 11 - Concentrations (%) of tolbutamide (TOL) and 4-hydroxytolbutamide (4-OH-TOL) after a pre-incubation of 30 minutes with the CYP2C9 inhibitors at the different concentrations tested and 24 hours of incubation of 200 µM TOL in HepaRG cells with: 0.4, 0.8 and 1.6 µM of amiodarone (**A**); 10, 20 and 38 µM of fluoxetine (**B**); 0.7, 1.5 and 3 µM of ketoconazole (**C**); 0.9, 1.8 and 4 µM of omeprazole (**D**); and 38, 76 and 152 µM of ticlopidine (**E**). Data are expressed as the mean values ± standard error of the mean (n = 3). $^{*}p < 0.05$ compared to control group.

In the **Figure 12** could be observed the effects induced by the flavonoid compounds [baicalein, (-)-epigallocatechin gallate, kaempferol and quercetin] in the metabolism of the TOL (selective CYP2C9 probe drug).

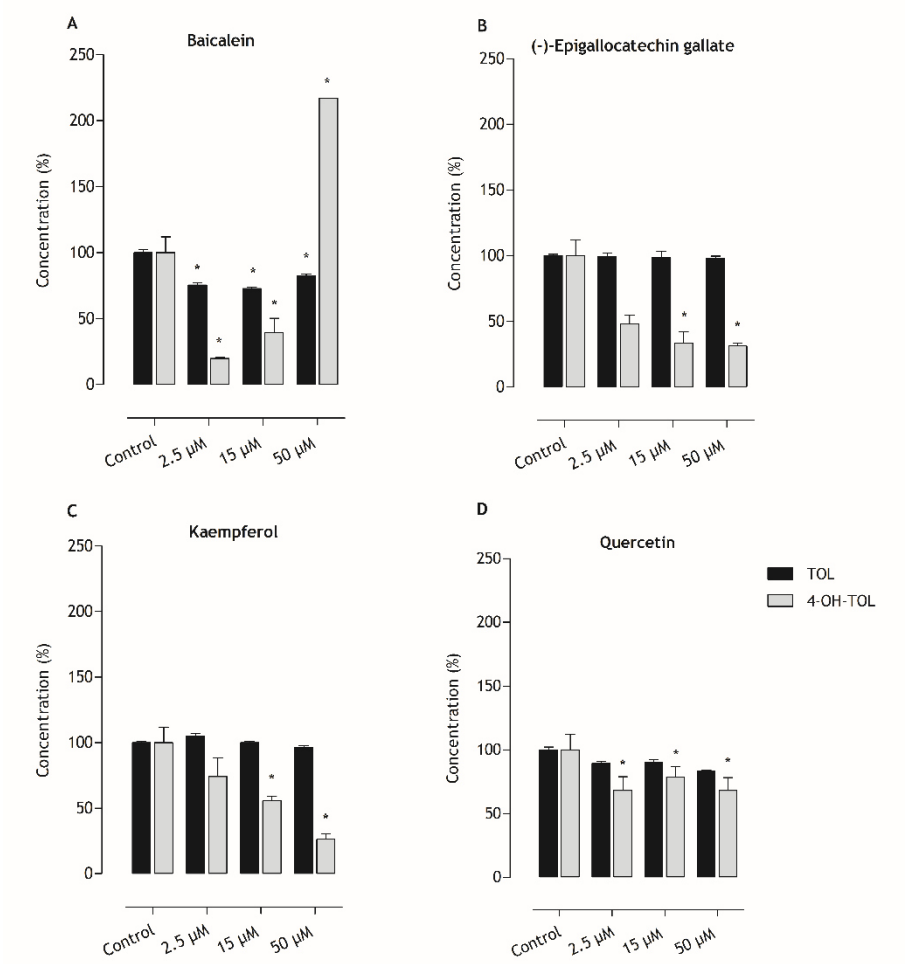

Figure 12 - Concentrations (%) of tolbutamide (TOL) and 4-hydroxytolbutamide (4-OH-TOL) after a pre-incubation of 30 minutes with the flavonoids at the different concentrations tested and 24 hours of incubation of 200 µM TOL in HepaRG cells with: 2.5, 15 and 50 µM of baicalein (**A**), (-)-epigallocatechin gallate (**B**), kaempferol (**C**), and quercetin (**D**). Data are expressed as the mean values ± standard error of the mean (n = 3). $^{*}p < 0.05$ compared to control group.

All the flavonoids studied demonstrated to inhibit the formation of 4-OH-TOL at almost all the concentrations tested. Baicalein exhibited the highest inhibitory effect at the lowest concentration used (2.5 µM). On the contrary, with this flavonoid, it was observed an extremely high formation of the metabolite 4-OH-TOL at the highest concentration tested (50 µM). Relatively to quercetin, this flavonoid showed a similar inhibition degree on the 4-OH-TOL formation at all the concentrations assayed. Kaempferol and (-)-epigallocatechin gallate demonstrated to originate a concentration-dependent inhibitory effect regarding the formation 4-OH-TOL metabolite, with their highest concentration tested (50 µM) causing the highest inhibitory effects.

An example of the chromatograms obtained during the HPLC-DAD analysis of the real samples resulting from the metabolic inhibition studies are presented in the **Figure 13**. This figure shows chromatograms obtained from the analysis of a real sample collected in the *in vitro* metabolic studies in which none compound was tested (A), and where the effect of the concentration of 2.5 µM baicalein on the formation of 4-OH-TOL was assessed (B).

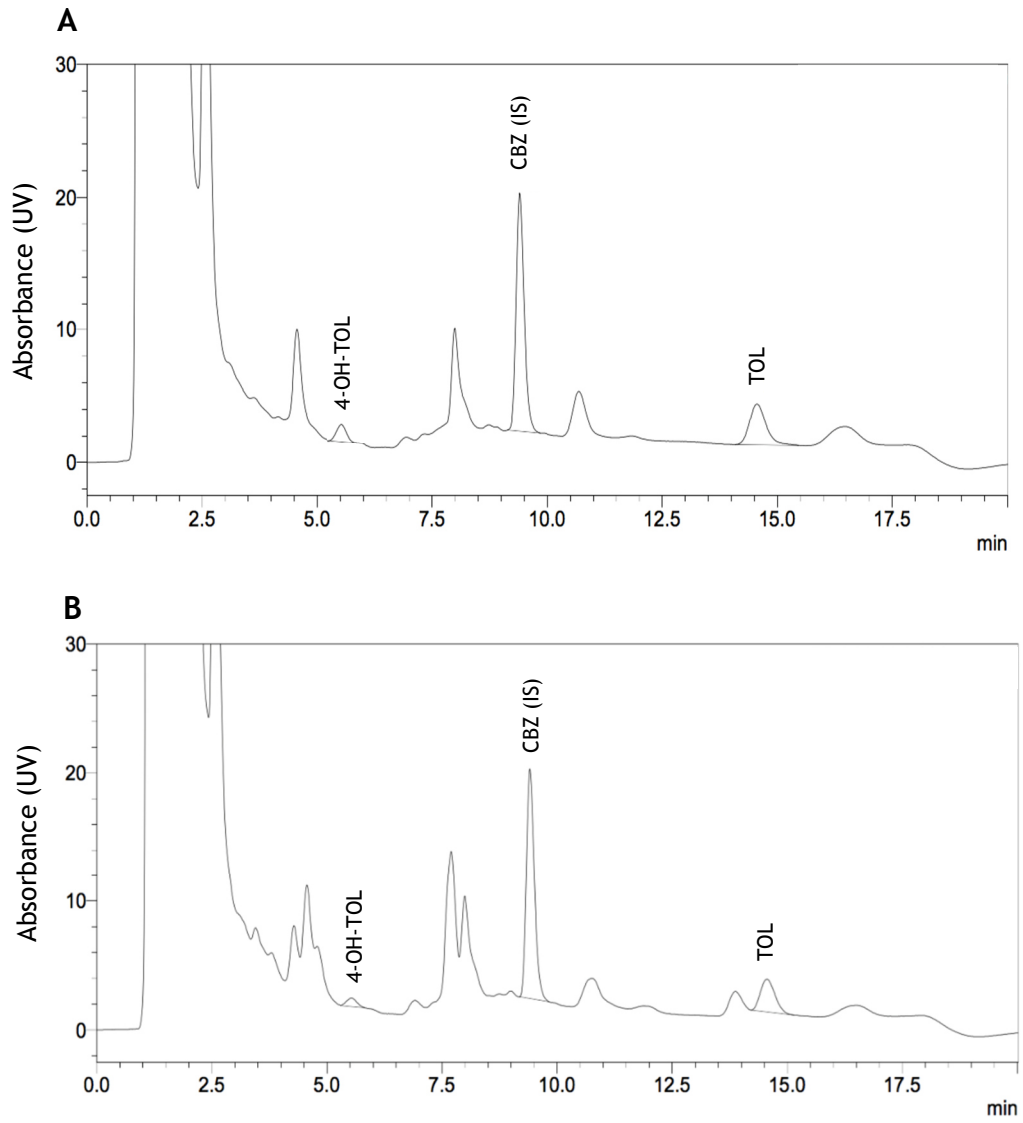

Figure 13 - Representative chromatograms of the analysis of real samples obtained from the metabolic inhibition studies. The first chromatogram concerns to a real sample where a pre-incubation of 30 minutes of the supplemented William's E medium, without FBS, with 1% DMSO, and an incubation of 200 µM TOL with 1% DMSO during 24 hours were performed (A). The second chromatogram depicts a real sample were HepaRG cells were pre-incubated with 2.5 µM baicalein for 30 minutes, and incubated with 200 µM TOL and 2.5 µM baicalein for 24 hours (B). CBZ (IS), carbamazepine (internal standard); 4-OH-TOL, 4-hydroxytolbutamide; TOL, tolbutamide; UV, ultraviolet.

5. Discussion

During the process of drug discovery and development, the bioanalysis was recognized to be a critical tool, essential for pharmacokinetics and pharmacodynamics characterization of a drug (Ferreira et al. 2016a). Indeed, there is a continuous need of the development of analytical methodologies, which permit not only the quantification of the drug but also its metabolites in several biological samples, supporting the various stages of the drug discovery and development process (Korfmacher 2011; Ferreira et al. 2016b).

Taking into account the experimental advantages of HepaRG cells, the availability of rapid, sensitive and reliable bioanalytical methods emerges as a critical need to support subsequent metabolic drug interaction studies in this kind of biological samples. Most of the methodologies described in the literature for the simultaneous determination of TOL and/or 4-OH-TOL are HPLC assays using ultraviolet detection (Hemeryck et al. 1999; Lin et al. 2012). The use of an ultraviolet detector gives response only at one fixed wavelength at the same time, while a DAD detector measures a wide range of wavelengths at one time. In that way, apart from ultraviolet, previous reports presented several different HPLC assays for the quantification of TOL and/or 4-OH-TOL using other detection methods, namely: fluorescence (Ko et al. 2000), mass spectrometry (Walsky and Obach 2004; Zanelli et al. 2012; Zhang et al. 2012b), and DAD (Medvedovici et al. 2000; Zhai and Lu 2013). However, none of the HPLC-DAD methods described in the literature was used to the quantification of TOL and/or 4-OH-TOL in HepaRG cells culture medium. Actually, the quantification of TOL and/or 4-OH-TOL by the available methodologies were performed in the matrices of human plasma (Medvedovici et al. 2000; Lin et al. 2012), rat microsomal medium (Zhai and Lu 2013), rat plasma (Zhang et al. 2012b), and human liver microsomes (Back et al. 1988; Hemeryck et al. 1999; Ko et al. 2000; He et al. 2002; Walsky and Obach 2004). Aninat et al. (2006) referred that TOL 4-hydroxylation samples resulting from the *in vitro* studies performed in the HepaRG cell line were analysed by HPLC. However, none information regarding the chromatographic or extraction conditions was indicated, as well as regarding the robustness and reliability of the methodology applied. The extraction procedures that were applied to the preparation of the samples in these methodologies were diversified and included not only the liquid-liquid extraction, which was also applied in our assay (Hemeryck et al. 1999; Ko et al. 2000; Medvedovici et al. 2000; Lin et al. 2012; Zhang et al. 2012b), but also the solid-phase extraction (Zhai & Lu 2013), and protein precipitation (Medvedovici et al. 2000). Over the last years, sample preparation techniques have evolved, enabling a more efficient sample preparation, which allow the matrix-analyte recovery and eliminating endogenous substances that potentially may interfere with the analysis. Sample preparation has mostly been performed through classic sample extraction procedures such as liquid–liquid extraction, solid-phase extraction, solid-phase microextraction, supercritical fluid extraction, membrane extraction and the simple protein precipitation (Orlando et al. 2009). All these sample extraction procedures have advantages

and disadvantages, making the choice of the most suitable procedure dependent on the characteristics of the analytes and the purpose of the method in developing. The sample preparation procedure considered in this assay involves the use of liquid-liquid extraction, which is the most easy-to-use approach in this case due to the necessity of a less expensive, faster and simpler analytical methodologies to support drug interaction screening studies.

This work describes a HPLC-DAD method for the quantification of TOL and its metabolite 4-OH-TOL in HepaRG cell culture samples. The analytical method was fully validated in HepaRG cell culture medium samples, enabling the fast chromatographic analysis of TOL and 4-OH-TOL in HepaRG cell culture samples, in approximately 20 minutes, using simple instrumentation and uncomplicated chromatographic conditions. These aspects suggest that the method is appropriate for implementation in almost all laboratories, because fast bioanalytical assays are essential whenever a large number of samples have to be analysed. Moreover, the method was successfully applied to the evaluation of potential metabolic inhibition interactions involving the CYP2C9.

The degree of development and quality of the bioanalytical assays used tend to increase as the lead drug candidates progress to more advanced stages. In that way, the levels of analytical acceptance criteria become stricter in the later stages, being the results confirmed by appropriate validation assays, which attest its reliability, robustness and accuracy. Actually, the various validation criteria defined by the international guidelines are not usually applied in the early stages of drug discovery and development, being used only minimal standards in the higher throughput assays performed in these steps (Srinivas 2006; Pandey et al. 2010; Korfmacher 2011). This aspect could potentially explain the scarcity of HPLC methodologies fully validated in the literature to support *in vitro* studies. Notwithstanding, the HPLC-DAD assay herein reported was extensively validated taking into account the international criteria of FDA and EMA guidelines. Indeed, to the reliable application of this method to pharmacokinetic assays, the validation parameters selectivity, intraday and interday precision and accuracy, and stability, were extensively evaluated in HepaRG cell culture medium samples, to ensure confidence in the obtained data.

Whenever possible, drug interactions should be prevented and early identified (Ferreira et al. 2016b). Indeed, drug–drug interactions are an essential aspect to be considered in the process of drug development, representing a major concern for pharmaceutical industry and regulatory agencies, and at clinical level for healthcare professionals and their patients. Therefore, *in vitro* models are being increasingly used during preclinical drug development, arising as screening tools to predict drug–drug interactions and providing useful data to extrapolate to man (Lin and Lu 1997; Ferreira et al. 2016b). In fact, there is increasing evidence that appropriate *in vitro* drug interaction studies can accurately reflect what happens in *in vivo* conditions (Lin and Lu 1997; Costa et al. 2014).

When *in vitro* models are used, it is important to first make sure that the results are not compromised by cytotoxicity of the substance to be studied. Accordingly, it may be prudent the previous evaluation of cytotoxicity for concentration-selection purposes (Maurel 2010). In

this work, the MTT assay was used to evaluate the cell cytotoxicity of the studied compounds. The reduction of MTT is a rapid colorimetric method, often used to assess cellular proliferation and cytotoxicity in *in vitro* conditions (Mosmann 1983). This assay depends on the number of viable cells present as well as of the mitochondrial cell activity (van Meerloo et al. 2011). In this assay, MTT is accumulated by the cells via endocytosis. The reduction of the tetrazolium ring of the salt results in the formation of formazan crystals (exhibit blue color), which accumulate in endosomal and/or lysosomal compartments and are then transported out of cells by exocytosis (van Meerloo et al. 2011). This reduction occurs by the mitochondrial succinate dehydrogenase enzyme existing within the viable cells (van Meerloo et al. 2011). Consequently, the amount of formazan produced is proportional to the number of viable cells present, and which have an active cellular respiration. That is, the higher the absorbance detected in the spectrophotometer, the greater the number of live cells (Mosmann 1983; Liu et al. 1997; van Meerloo et al. 2011). Due to the fact that endocytosis is a fundamental mechanism of living cells, the MTT assay has been often used as a cell viability assay (Mosmann 1983; Liu et al. 1997; van Meerloo et al. 2011). The combined cytotoxicity of TOL (200 μM) with the CYP2C9 inhibitors drugs or flavonoids, at three different concentrations, was evaluated in HepaRG cells during 24 hours. The main purpose of this assay was to realize the concentrations that could be used in the metabolic studies, without induction of important loss of cell viability. In the literature it has been reported the frequent use of CYP2C9 inhibitors that were studied in this work (Zhou et al. 2009a). Nevertheless, few of these studies involved the use of *in vitro* methodologies. Amiodarone revealed toxicity, in a MTT assay, only at a concentration superior to 50 μM after 24 hours of incubation in HepaRG cells (Aninat et al. 2006). These results are concordant with our data, since the highest concentration used in our MTT assay was 1.6 μM and also did not exhibit cytotoxicity in HepaRG cells. Gerets et al. (2012), showed that ketoconazole did not cause hepatotoxicity after 24 hour of incubation in HepaRG cells and revealed an LC_{50} (lethal concentration, 50%) higher than 50 μM in this cell line. Nevertheless, in this case, a xCELLigence System from Roche Diagnostics was the chosen assay to evaluate ketoconazole cytotoxicity (Gerets et al. 2012). In case of fluoxetine, omeprazole and ticlopidine, to the best of our knowledge, the cytotoxicity of these inhibitors was not previously studied in HepaRG cells, therefore, the results obtained in this study were new in literature. As expected, an increased loss of the cell viability was achieved at the highest concentrations of the CYP2C9 inhibitors tested; it should be note that the highest concentrations tested are twice their inhibitory constant (K_i) values. Nevertheless, none of CYP2C9 inhibitors induced a cytotoxicity that could be alarming in terms of HepaRG cell viability, which could compromise the reliability of the metabolic inhibition assays to be performed later.

Few studies have reported cytotoxicity assays with flavonoid compounds (Peng et al. 2005; Yadegarynia et al. 2012). Yadegarynia et al. (2012) showed that several flavonoids, including kaempferol and quercetin, were cytotoxic at 50 μM in breast cancer cell lines and they assessed a possible structure-function relationship for cellular cytotoxicity based on the various chemical structures of flavonoids. However, in our work, using the MTT colorimetric assay, we

demonstrated that the flavonoids baicalein, (-)-epigallocatechin gallate, kaempferol, and quercetin did not cause any significant loss of cell viability in HepaRG cells. Nevertheless, baicalein and (-)-epigallocatechin gallate demonstrated to increase the HepaRG cell proliferation, being these effects concentration-dependent and they are more marked as flavonoid concentrations increase. The conduction of these cell viability assays allowed to achieve additional security in the interpretation of the results of metabolic studies. In the literature there are no other studies with flavonoids in cancer cell lines evidencing the cell proliferation demonstrated in the HepaRG cell line. Nevertheless, several *in vivo* studies have highlighted the fact that the biologic activities of flavonoids may play a dual role in mutagenesis and carcinogenesis; indeed, depending on the levels consumed, as well as the physiological conditions in the individual, these compounds can act as antimutagens or promutagens (Kato et al. 1984; Plakas et al. 1985; Dunnick and Hailey 1992; Formica and Regelson 1995; Skibola and Smith 2000; Nijveldt et al. 2001; Galati and O'Brien 2004; Kyselova 2012).

In the decision making process during the development of new drug candidates, the evaluation of induction and inhibition of CYP450 isoenzymes is one of the major points of concern (McGinnity et al. 2006; Ferreira et al. 2016b). Therefore, the main aim of this work was to develop and validate an *in vitro* assay to the identification and evaluation of drug interactions involving the CYP2C9 inhibition, using a very recent cellular model, the HepaRG cells. Some methods have been reported in literature for determination of metabolic profiles using *in vitro* models (Hemeryck et al. 1999; He et al. 2002; Yuan et al. 2002; Aninat et al. 2006; Kumar et al. 2006a). Some of them also used TOL (Hemeryck et al. 1999; He et al. 2002; Yuan et al. 2002). Actually, the probe drug TOL has also been previously used in HepaRG cell, however, in this case, the main purpose was the caracterization of the enzymatic profile of this cell line (Aninat et al. 2006).

As previously mentioned, HepaRG cell line has similarities to human hepatocytes and brings advantages over the other known cell lines, like HepG2 or Huh-7, in particular the possibility of its use in inhibition and induction studies (Kanebratt and Andersson 2008b; Turpeinen et al. 2009; Ferreira et al. 2014b). Indeed, many non-clinical research studies have been developed targeting to identify drug interactions involving the CYP2C9 using human liver microsomes (Back et al. 1988; Baldwin et al. 1995; Hemeryck et al. 1999; Ko et al. 2000; He et al. 2002; von Moltke et al. 2004; Greenblatt et al. 2005; Baranczewski et al. 2006; Paris et al. 2009; Greenblatt et al. 2011), but there are few studies of this nature carried out in the new promising cell line HepaRG (Turpeinen et al. 2009; Ferreira et al. 2014b). The HepaRG cells have several advantages over other metabolic models, one of them is the stable expression of CYP450 enzymes, phase II enzymes, transporters, and nuclear transcription factors over a time period of 6 weeks in culture (Kanebratt and Andersson 2008a). These are ideal characteristics for conducting metabolic studies. Taking into account the fact that enzymatic induction process is time-dependent and its adverse effects on humans are mainly observed with continuous treatment regimens, the occurrence of drug interactions based on enzymatic inhibition is

usually more frequent than induction (Flockhart and Tanus-Santos 2002), thus justifying our focus on metabolic inhibition studies.

HepaRG cells have the ability to differentiate into hepatocyte-like colonies surrounded by epithelial biliary-like cells (Guguen-Guillouzo et al. 2010). If we intend to assess more information and expression of genes or CYP isoenzymes, these cells can be treated with DMSO (Gripon et al. 2002; Lübberstedt et al. 2011), which can helps them to differentiate and to increase their genetic expression (Aninat et al. 2006). Nevertheless, because we intended to develop a rapid assay for screening purposes, and due to the fact that HepaRG cells after 2 weeks of confluency have shown accepable metabolic ability that enabled enough analytical sensitivity, the cells were used in these conditions.

Taking into account the CYP2C9 inhibitors described in the literature, and those available in our lab, we have chosen seven CYP2C9 inhibitors to attest the validity of our developed *in vitro* assay. The TOL concentration (200 µM) used in these metabolic studies was chosen based on Aninat et al. (2006), who have also studied drug-metabolizing enzyme activities and metabolic profiles in HepaRG cells. Regarding the CYP2C9 inhibitors concentrations to be studied, as few data are available in literature the concentration ranges to be used in *in vitro* models, the range of concentrations tested in our assays were chosen based on their corresponding K_i values. The K_i values for the CYP2C9 inhibitors obtained from literature were gathered in **Table 5** (Schmider et al. 1997; Ko et al. 2000; Kumar et al. 2006a). The range of values was also compared with the few concentration values found in the literature (Hemeryck et al. 1999; Wen et al. 2001; Micuda et al. 2004; Roymans et al. 2004; Kumar et al. 2006a; Paris et al. 2009; Si et al. 2009; Toda et al. 2009; Turpeinen et al. 2009; Greenblatt et al. 2011; Nayadu et al. 2013; Sousa-Ferreira et al. 2014; Prasad et al. 2016).

Amiodarone is the most commonly used antiarrhythmic agent, which can control a wide spectrum of atrial and ventricular antiarrhythmic disorders (Micuda et al. 2004; Zhou et al. 2009a; Kumar et al. 2006a). The amiodarone showed to inhibit the formation of the metabolite 4-OH-TOL in our metabolic inhibition studies, being the highest inhibitory effect achieved at 0.4 µM. In this case, the inhibitory effect of amiodarone appeared to be concentration-dependent, decreasing as the drug concentrations increases. Nevertheless, previous reports about the effect of amiodarone on the CYP2C9 isoenzyme presented results that are contradictory to those obtained by us (Kumar et al. 2006a). Using naproxen as the probe drug for the CYP2C9 isoenzyme, Kumar et al. (2006a) demonstrated that at low concentrations (<1 µM) amiodarone produced a weak activation of naproxen demethylation, but when the drug concentration was increased above 1.25 µM this activation was reversed and, eventually, an inhibitory effect was noted (Kumar et al. 2006a). Fluoxetine is a potent and selective serotonin reuptake inhibitor in the central nervous system and it is extensively used to treat depression and obsessive-compulsive behaviour (Schmider et al. 1997; Hemeryck et al. 1999; Zhao-Qian et al. 2001; Turpeinen et al. 2009). This drug has showed to inhibit the CYP2C9 (Schmider et al. 1997; Hemeryck et al. 1999; Zhao-Qian et al. 2001; Turpeinen et al. 2006). Turpeinen et al. (2006) measured a half maximal inhibitory concentration (IC_{50} value = 48.6 µM) for fluoxetine

by the traditional single substrate assay in human liver microsomes, using TOL as the CYP2C9 substrate. We have tested three different concentrations of fluoxetine (10, 20 and 38 μM) and it was observed a discrepancy in the results, since 20 μM of fluoxetine exhibit a meaningful inhibition on the formation of the metabolite 4-OH-TOL, and at 38 μM this inhibition was, apparently, lost. Ketoconazole is an antifungal agent and it is reported to be a general inhibitor of CYP450 at millimolar concentrations in both animals and humans (Baldwin et al. 1995). Ketoconazole is frequently used as a CYP3A reference inhibitor. However, its effects in the CYP2C9 isoenzyme did not receive much attention (Baldwin et al. 1995; Kuroha et al. 2002; Toda et al. 2009; Greenblatt et al. 2011). Nevertheless, Baldwin et al. (1995) and Kumar et al. (2006b) reported some inhibition of the CYP2C9 by this drug. The later addressed the impact of the probe selection for assays with the CYP2C9. The K_i of 28 effector molecules was assessed with five commonly used CYP2C9 probes [diclofenac, (S)-flurbiprofen, (S)-warfarin, phenytoin and TOL]. Among the 28 chosen effector molecules, the ketoconazole was classified as moderate-strong inhibitor in both CYP2C9 isoforms (CYP2C9.1 and CYP2C9.3) using TOL as the probe drug (Kumar et al. 2006b). This report is consistent with our results. Ketoconazole at 3 μM showed an inhibition of 22%, on average, in the formation of the metabolite 4-OH-TOL. Omeprazole is a proton-pump inhibitor widely used for the treatment of gastric ulcers and it is converted to hydroxyomeprazole and omeprazole sulphone primarily by CYP2C19 and CYP3A4, respectively (Michael 2010). This drug was also studied by Kumar et al. (2006b), and it was also classified as moderate-strong inhibitor in both CYP2C9 isoforms using TOL as a probe drug. Nevertheless, omeprazole is commonly used as reference inducer to other CYP450, more specifically, CYP3A4 and CYP1A2 (Roymans et al. 2004; Kanebratt and Andersson 2008b; Paris et al. 2009; Kaneko et al. 2010). From the results obtained by us, omeprazole demonstrated to be a CYP2C9 inhibitor at 1.8 μM and 4 μM concentrations, inhibiting the formation of the metabolite 4-OH-TOL in 45% and 42%, on average, respectively. Notwithstanding, at 0.9 μM, omeprazole revealed to be an inducer of the CYP2C9, increasing in 37%, on average, the formation of the metabolite 4-OH-TOL. However, to the best of our knowledge, this inducer effect shown by the drug on this CYP is not previously described in the literature. Omeprazole was previously described as a competitive inhibitor of the CYP2C9-catalized conversion of TOL to 4-OH-TOL, when tested at the range of 5-50 μM (Ko et al. 1997). The prodrug ticlopidine, when metabolized to its active form, is a potent and long-acting inhibitor of platelet aggregation acting through inhibition of the P2RY12 receptor, and an effective drug in preventing atherothrombotic events in cardiovascular, cerebrovascular, and peripheral vascular diseases (Zhou et al. 2009a). In the literature, ticlopidine is reported as a potent mechanism-based inhibitor of CYP2B6 (IC_{50} value = 0.32 μM), but it is also an inhibitor of CYP2C19 and CYP2C9, impairing also the metabolic activities associated with CYP1A2 and CYP2D6 (Martikainen 2012). Ko et al. (2000) examined in *in vitro* conditions the potency of ticlopidine as CYP2C9 inhibitor using human liver microsomes and the TOL as probe substrate. The authors reported that ticlopidine (0-40 μM) was a moderate inhibitor of this CYP450. These results were consistent with the data obtained from our metabolic inhibition studies. The

inhibitory potency of ticlopidine in HepaRG cells revealed to be concentration-dependent, increasing as the drug concentrations increase. Ticlopidine originated a potent inhibition of the formation of the metabolite 4-OH-TOL at 152 μM (65%).

The consume of flavonoid compounds in the form of dietary supplements and plant extracts has been steadily increasing in order to improve health status of humans (Si et al. 2009; Bansal et al. 2009; Ferreira et al. 2014a). The flavonoids are among the most ubiquitous groups of polyphenolic compounds found in foods and beverages of herbal origin (Boumendjel et al. 2002; Kitagawa et al. 2005; Badhan and Penny 2006; Brand et al. 2006; Wesołowska et al. 2009; Chan et al. 2009). Flavonoids and their metabolites have generally a low bioavailability, and most of them are rapidly metabolized in the intestinal mucosa and liver (Si et al. 2009).

An extremely important aspect that should be thoroughly considered in relation to the consumption of flavonoid compounds, mainly taking into account their extensive use, is their potential for interactions with drugs (Si et al. 2009; Kyselova 2012). In fact, these type of compounds are not yet fully investigated through pharmacological studies, besides natural compounds they have been used for medical purposes (Kyselova 2012). Consequently, due to the widespread belief of its security provided by their natural origin, the risk of adverse effects due to pharmacological interactions between herbal medicinal products and conventional therapies are often underestimated (Rietjens et al. 2008; Kyselova 2012).

Actually, besides the modulation of the expression and activity of the P-glycoprotein efflux transporter (Ferreira et al. 2014a), flavonoids have been shown to modulate the CYP450 system, being well-recognized as inducers and inhibitors of CYP450 drug-metabolizing enzymes (Galati and O'Brien 2004). The interactions of flavonoids with human CYP450 can be related to either enzyme inhibition or induction depending upon their structures, concentrations, or experimental conditions (Galati and O'Brien 2004; Moon et al. 2006). Analysing the available data on CYP450-flavonoid drug interactions, it could be drawn that flavonoids possessing hydroxyl groups inhibit CYP450 activity, while those lacking hydroxyl groups may be inducers (Galati and O'Brien 2004; Moon et al. 2006). In fact, our results confirmed this idea, as baicalein, (-)-epigallocatechin gallate, kaempferol and quercetin have 3, 8, 4 and 5 hydroxyl groups respectively, and they demonstrated to be CYP2C9 inhibitors in general. Thus, the simultaneous intake of flavonoids and drugs could increase the risk of flavonoid-drug interactions through the flavonoids modulation of the pharmacokinetics of certain drugs, which may result in an increased toxicity or a decline of their therapeutic effect, depending on the flavonoid involved (Galati and O'Brien 2004; Moon et al. 2006).

All these facts justify the evaluation of the effect of the flavonoid compounds baicalein, (-)-epigallocatechin gallate, kaempferol and quercetin on the CYP2C9 isoenzyme, that was performed in this work. Relatively to flavonoids, the range of concentrations to be tested were chosen differently of those considered for CYP2C9 inhibitors. Actually, as the K_i values of all the flavonoid compounds are not available in the literature, we considered as reference the concentration range (0-40 μM) studied by Si et al. (2009), where the effects of some flavonoids on the CYP2C9 was also explored. Regarding this, three concentration values were tested (2.5,

15 and 50 μM) (Si et al. 2009). Actually, there are few studies in the literature exploring the interactions of flavonoids with CYP450. For example, α-naphthoflavone was recognised as an inhibitor of human CYP1A1 and CYP1A2 and as an inducer of CYP3A4 (Tassaneeyakul et al. 1993; Guengerich et al. 1994); quercetin is an inducer of CYP1A enzymes and CYP1A1 expression (Vrba et al. 2012); scutellarin is an inhibitor of CYP1A2 (Jian et al. 2012); and the isoflavone biochanin A appears to be an inducer of CYP3A4 (Singh et al. 2012).

In fact, to the best of our knowledge, there is only one study in the literature that assessed the effect of flavonoids on CYP2C9. Si et al. (2009) evaluated the *in vitro* inhibitory effect of several flavonoids on CYP2C9 mediated 4'-hydroxylation of diclofenac (another CYP2C9 probe drug), using the CYP2C9 RECO system (a purified, reconstituted enzyme system containing recombinant human CYP2C9, CYP450 reductase, CYP *b*5, and liposomes), human liver microsomes, and S9 fraction. In this report, the flavonoids baicalein, kaempferol and quercetin, tested at the concentration range of 0-40 μM, showed to markedly inhibit the CYP2C9 isoenzyme. These data support the validity of the results obtained in our work. Kinetic analysis of diclofenac 4-hydroxylation formation from computer docking studies revealed that baicalein, kaempferol, quercetin and other flavonoids were competitive inhibitors (Si et al. 2009), because no time-dependent inhibition was observed. Notwithstanding, although the flavonoids kaempferol, (-)-epigallocatechin gallate, and quercetin demonstrated to be inhibitors of CYP2C9 at all the tested concentrations, it was observed an extremely high formation of the metabolite 4-OH-TOL with the higher concentration of baicalein tested (50 μM). This event could be at least partially explained by the great increase of the cell proliferation observed with this concentration of baicalein in the MTT assay. However, although (-)-epigallocatechin gallate also demonstrated to increase the HepaRG proliferation, mainly at higher concentrations, this great increase in the formation of 4-OH-TOL was not observed. From this fact we can infer that (-)-epigallocatechin gallate is a powerful inhibitor of CYP2C9; indeed, despite its deeply increase in the HepaRG cells proliferation at the highest concentration tested, its inhibitory effect on the CYP2C9 isoenzyme was very marked at all the tested concentrations.

6. Conclusion

Over the last years, the strategies for drug discovery and development have changed considerably, and new approaches are being taken into account by the pharmaceutical industry in the making decision process. Pharmaceutical companies are currently under huge pressure to reduce costs. Consequently, in the early phase of drug discovery and development, *in vitro* high-throughput screening studies can be applied for the prediction of the possible involvement of CYP450 isoenzymes in the metabolism of drug candidates. These studies could potentially make the drug design more efficient, less costly, improve the drug safety, and even possibly replace some *in vivo* experiments.

In recent years, HepaRG cells emerged as a very promising model to evaluate the hepatic drug metabolism under *in vitro* conditions. The implementation of this experimental work enabled the development, validation and application of a bioanalytical HPLC technique capable of supporting many of the metabolic studies that could be applied to a large panoply of compounds, improving the early recognition of their properties regarding the CYP2C9 inhibition. Thus, the development of an analytical methodology to support the studies conducted with this cell model are of extreme relevancy. Actually, until now, to the best of our knowledge, it is not reported any methodology in the literature to the simultaneously quantification of the considered analytes in HepaRG cell culture medium samples. Actually, this is the first report describing the development and full validation of a reversed-phase HPLC-DAD method for the quantification of TOL and its metabolite 4-OH-TOL, to be applied to HepaRG cell culture medium samples. Furthermore, the high absolute recoveries obtained for all the compounds of interest (TOL, 4-OH-TOL and IS) demonstrated the suitability of the extraction sample preparation procedure described, involving liquid-liquid extraction. The LLOQs of the method achieved for both analytes (TOL and 4-OH-TOL) were considerably low, despite the nature of the procedures applied in sample preparation, and taking into account the use of a HPLC-DAD system, which is simpler and cheaper than other more sensitive chromatographic systems as liquid chromatography–mass spectrometry or liquid chromatography-tandem mass spectrometry. It was concluded that the bioanalytical assay developed for the quantification of TOL and 4-OH-TOL in HepaRG cell culture medium samples is selective, sensitive, accurate, reliable, and reproducible. Consequently, the reported HPLC–DAD method, validated according to the international requirements of EMA and FDA, will be suitable and essential to support not only the metabolic inhibition studies performed in this work, but also future *in vitro* metabolic, drug interaction, and pharmacokinetics-based screening studies involving TOL and 4-OH-TOL.

This work also permitted to provide some new knowledge about the cytotoxicity of the CYP2C9 inhibitors drugs and flavonoids studied in the HepaRG cell line. Actually, the reference drugs used as CYP2C9 inhibitors (amiodarone, fluoxetine, ketoconazole, omeprazole and ticlopidine), as well as the flavonoid compounds [baicalein, (-)-epigallocatechin gallate, kaempferol and

quercetin] selected for the metabolic *in vitro* studies, did not exhibit a marked cytotoxicity in the HepaRG cell line at the tested concentrations.

The suitability of the *in vitro* technique and of the experimental analytical procedures developed and validated was successfully demonstrated with the metabolic inhibition studies performed, using known CYP2C9 inhibitors described in the literature, and TOL as the probe drug. Effectively, notorious inhibitory effects in the formation of 4-OH-TOL determined by the CYP2C9 inhibitors were observed. Thus, in the near future, we intend to extend these studies to many other drugs and CYP450s, in order to increase the information available on drug interactions involving CYP450.

The applicability of the *in vitro* methodology developed and validated was confirmed evaluating the effect of four flavonoid compounds [baicalein, (-)-epigallocatechin gallate, kaempferol and quercetin] on the CYP2C9 isoenzyme. Effectively, it was observed an inhibitory effect in the 4-OH-TOL formation, with all the tested concentrations of flavonoids, with exception of baicalein at 50 μM, which originated an extremely high formation of the metabolite 4-OH-TOL.

The CYP2C9 drug interactions identified in the early screening phases could be subject to further investigation by additional pharmacokinetic studies, appropriately designed to confirm the *in vitro* results, and also to predict the clinical impact of such potential interactions.

In conclusion, this work demonstrated the applicability of this *in vitro* assay developed and validated in the HepaRG cell line as a useful *in vitro* approach to foresee metabolic interactions involving the CYP2C9 isoenzyme inhibition, becoming increasingly an alternative of great relevance to the primary human hepatocytes.

7. Bibliography

Almeida AM, Castel-Branco MM, Falcão AC (2002) Linear regression for calibration lines revisited: Weighting schemes for bioanalytical methods. *J Chromatogr B* 774:215–222.

Andersson TB (2010) The Application of HepaRG Cells in Evaluation of Cytochrome P450 Induction Properties of Drug Compounds. In: Maurel P (ed) *Hepatocytes Methods and Protocols*. Springer Science+Business Media, pp 375–387

Aninat C, Piton A, Glaise D, et al (2006) Expression of Cytochrome P450 Enzymes and Nuclear Receptors in Human Hepatoma HepaRG Cells. *Drug Metab Dispos* 34:75–83.

Anthérieu S, Chesne C, Li R, et al (2010) Stable Expression, Activity, and Inducibility of Cytochromes P450 in Differentiated HepaRG Cells. *Drug Metab Dispos* 38:516–525.

Back DJ, Tjia JF, Karbwang J, Colbert J (1988) In vitro inhibition studies of tolbutamide hydroxylase activity of human liver microsomes by azoles, sulphonamides and quinolines. *Br J Clin Pharmacol* 26:23–29.

Badhan R, Penny J (2006) In silico modelling of the interaction of flavonoids with human P-glycoprotein nucleotide-binding domain. *Eur J Med Chem* 41:285–295.

Baldwin SJ, Bloomer JC, Smith GJ, et al (1995) Ketoconazole and sulphaphenazole as the respective selective inhibitors of P4503A and 2C9. *Xenobiotica* 25:261–270.

Bansal T, Jaggi M, Khar RK, Talegaonkar S (2009) Emerging significance of flavonoids as P-glycoprotein inhibitors in cancer chemotherapy. *J Pharm Pharm Sci* 12:46–78.

Baranczewski P, Stanczak A, Kautiainen A, et al (2006) Introduction to early in vitro estimation of metabolic stability and drug interactions of new chemical entities in drug discovery and development. *Pharmacol Reports* 58:453–472.

Boumendjel A, Di Pietro A, Dumontet C, Barron D (2002) Recent advances in the discovery of flavonoids and analogs with high-affinity binding to P-glycoprotein responsible for cancer cell multidrug resistance. *Med Res Rev* 22:512–29.

Brand W, Schutte ME, Williamson G, et al (2006) Flavonoid-mediated inhibition of intestinal ABC transporters may affect the oral bioavailability of drugs, food-borne toxic compounds and bioactive ingredients. *Biomed Pharmacother* 60:508–519.

Brown FK (1998) Chemoinfomatics: What is it and How does it Impact Drug Discovery. *Annu Rep Med Chem* 33:375–384.

Bunnage ME (2011) Getting pharmaceutical R&D back on target. *Nat Chem Biol* 7:335–339.

Chan K-F, Zhao Y, Chow TWS, et al (2009) Flavonoid dimers as bivalent modulators for p-glycoprotein-based multidrug resistance: structure-activity relationships. *ChemMedChem*

4:594–614.

Ciociola AA, Cohen LB, Kulkarni P (2014) How Drugs are Developed and Approved by the FDA: Current Process and Future Directions. *Am J Gastroenterol* 109:620–623.

Costa A, Sarmento B, Seabra V (2014) An evaluation of the latest in vitro tools for drug metabolism studies. *Expert Opin Drug Metab Toxicol* 10:103–119.

Darnell M, Schreiter T, Zeilinger K, et al (2011) Cytochrome P450-Dependent Metabolism in HepaRG Cells Cultured in a Dynamic Three-Dimensional Bioreactor. *Drug Metab Dispos* 39:1131–1138.

Donato MT, Lahoz A, Castell J V., Gómez-Lechón MJ (2008) Cell lines: a tool for in vitro drug metabolism studies. *Curr Drug Metab* 9:1–11.

Dunnick JK, Hailey JR (1992) Toxicity and carcinogenicity studies of quercetin, a natural component of foods. *Fundam Appl Toxicol* 19:423–431.

EMA (2010) Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. http://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/directive/en.pdf. Accessed 16 May 2016

EMA (2012) Guideline on the investigation of drug interactions. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf. Accessed 16 May 2016

EMA (2011) Guideline on bioanalytical method validation. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf. Accessed 16 May 2016

FDA (2015a) Product Development Under the Animal Rule - Guidance for Industry. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/default.htm. Accessed 16 May 2016

FDA (1997) Guidance for industry: Drug metabolism/drug interaction studies in the drug development process: Studies in vitro. http://www.fda.gov/downloads/AboutFDA/CentersOffices/CDER/UCM142439.pdf. Accessed 16 May 2016

FDA (2015b) Drug Development and Drug Interactions: Table of Substrates, Inhibitors, and Inducers. http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm. Accessed 12 May 2016

FDA (2013) Guidance for industry: Bioanalytical method validation. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf. Accessed 16 May 2016

Ferreira A, Pousinho S, Fortuna A, et al (2014a) Flavonoid compounds as reversal agents of the P-glycoprotein-mediated multidrug resistance: biology, chemistry and pharmacology. *Phytochem Rev* 14:233–272.

Ferreira A, Rodrigues M, Falcão A, Alves G (2016a) A Rapid and Sensitive HPLC–DAD Assay to Quantify Lamotrigine, Phenytoin and Its Main Metabolite in Samples of Cultured HepaRG Cells. *J Chromatogr Sci* bmw088 [Epub ahead of print].

Ferreira A, Rodrigues M, Falcão A, Alves G (2016b) HPLC–DAD Method for the Quantification of Carbamazepine, Oxcarbazepine and their Active Metabolites in HepaRG Cell Culture Samples. *Chromatographia* 79:581–590.

Ferreira A, Rodrigues M, Silvestre S, et al (2014b) HepaRG cell line as an in vitro model for screening drug-drug interactions mediated by metabolic induction: Amiodarone used as a model substance. *Toxicol Vitr* 28:1507–1520.

Festing S, Wilkinson R (2007) The ethics of animal research. Talking Point on the use of animals in scientific research. *EMBO Rep* 8:526–530.

Flockhart DA, Tanus-Santos JE (2002) Implications of cytochrome P450 interactions when prescribing medication for hypertension. *Arch Intern Med* 162:405–412.

Formica J V, Regelson W (1995) Review of the biology of Quercetin and related bioflavonoids. *Food Chem Toxicol* 33:1061–1080.

Freshney RI (ed) (2012) *Culture of animal cells - A manual of basic technique*, 6th edn. Wiley-Blackwell

Galati G, O'Brien PJ (2004) Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. *Free Radic Biol Med* 37:287–303.

Gerets HHJ, Tilmant K, Gerin B, et al (2012) Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biol Toxicol* 28:69–87.

Gómez-Lechón MJ, Castell JV, Donato MT (2006) Hepatocytes-the choice to investigate drug metabolism and toxicity in man: In vitro variability as a reflection of in vivo. *Chem Biol Interact* 168:30–50.

Greenblatt DJ, von Moltke LL, Perloff ES, et al (2005) Effect of ginkgo on CYP2C9: in vitro and in vivo studies. *Clin Pharmacol Ther* 77:48.

Greenblatt DJ, Zhao Y, Venkatakrishnan K, et al (2011) Mechanism of cytochrome P450-3A inhibition by ketoconazole. *J Pharm Pharmacol* 63:214–221.

Gripon P, Rumin S, Urban S, et al (2002) Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci* 99:15655–15660.

Guengerich FP (2006) Cytochrome P450s and other enzymes in drug metabolism and toxicity. *AAPS J* 8:E101–E111.

Guengerich FP, Shimada T, Yun CH, et al (1994) Interactions of ingested food, beverage, and tobacco components involving human cytochrome P4501A2, 2A6, 2E1, and 3A4 enzymes. *Environ Heal Perspect* 102:49–53.

Guguen-Guillouzo C, Corlu A, Guillouzo A (2010) Stem cell-derived hepatocytes and their use in toxicology. *Toxicology* 270:3–9.

Guguen-Guillouzo C, Guillouzo A (2011) General review on in vitro hepatocyte models and their applications. *Methods Mol Biol* 610:1–40.

Guillouzo A, Corlu A, Aninat C, et al (2007) The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem Biol Interact* 168:66–73.

Guillouzo A, Guguen-Guillouzo C (2008) Evolving concepts in liver tissue modeling and implications for in vitro toxicology. *Expert Opin Drug Metab Toxicol* 4:1279–1294.

Gunaratna C (2000) Drug metabolism and pharmacokinetics in drug discovery: a primer for bioanalytical chemists, part I. *Curr Sep* 19:17–23.

Hart SN, Li Y, Nakamoto K, et al (2010) A Comparison of Whole Genome Gene Expression Profiles of HepaRG Cells and HepG2 Cells to Primary Human Hepatocytes and Human Liver Tissues. *Drug Metab Dispos* 38:988–994.

He N, Zhang WQ, Shockley D, Edeki T (2002) Inhibitory effects of H1-antihistamines on CYP2D6- and CYP2C9-mediated drug metabolic reactions in human liver microsomes. *Eur J Clin Pharmacol* 57:847–851.

Heaney RP (2001) Factors Influencing the Measurement of Bioavailability, Taking Calcium as a Model. *J Nutr* 131:1376–1382.

Hemeryck A, De Vriendt C, Belpaire FM (1999) Inhibition of CYP2C9 by selective serotonin reuptake inhibitors: in vitro studies with tolbutamide and (S)-warfarin using human liver microsomes. *Eur J Clin Pharmacol* 54:947–951.

Hoekstra R, Nibourg GAA, Van Der Hoeven T V., et al (2011) The HepaRG cell line is suitable for bioartificial liver application. *Int J Biochem Cell Biol* 43:1483–1489.

Hoelder S, Clarke PA, Workman P (2012) Discovery of small molecule cancer drugs: Successes, challenges and opportunities. *Mol Oncol* 6:155–176.

Horn JR, Hansten PD (2007) Get to Know an Enzyme: CYP2C9. *Pharm Times* 76.

Jennen DGJ, Magkoufopoulou C, Ketelslegers HB, et al (2010) Comparison of HepG2 and HepaRG by whole-genome gene expression analysis for the purpose of chemical hazard identification. *Toxicol Sci* 115:66–79.

Jian T-Y, He J-C, He G-H, et al (2012) Scutellarin inhibits cytochrome P450 isoenzyme 1A2 (CYP1A2) in rats. *Phyther Res* 26:1226–1230.

Jossé R, Aninat C, Glaise D, et al (2008) Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. *Drug Metab Dispos* 36:1111–1118.

Kanebratt KP, Andersson TB (2008a) Evaluation of HepaRG Cells as an in Vitro Model for Human Drug Metabolism Studies. *Drug Metab Dispos* 36:1444–1452.

Kanebratt KP, Andersson TB (2008b) HepaRG Cells as an in Vitro Model for Evaluation of Cytochrome P450 Induction in Humans. *Drug Metab Dispos* 36:137–145.

Kaneko A, Kato M, Endo C, et al (2010) Prediction of clinical CYP3A4 induction using cryopreserved human hepatocytes. *Xenobiotica* 40:791–799.

Kapetanovic IM (2008) Computer aided drug discovery and development (CADDD): in silico-chemico-biological approach. *Chem Biol Interact* 171:165–176.

Kato K, Mori H, Fujii M, et al (1984) Lack of promotive effect of quercetin on methylazoxymethanol acetate carcinogenesis in rats. *J Toxicol Sci* 9:319–25.

Kitagawa S, Nabekura T, Takahashi T, et al (2005) Structure-activity relationships of the inhibitory effects of flavonoids on P-glycoprotein-mediated transport in KB-C2 cells. *Biol Pharm Bull* 28:2274–2278.

Ko JW, Desta Z, Soukhova N V., et al (2000) In vitro inhibition of the cytochrome P450 (CYP450) system by the antiplatelet drug ticlopidine: Potent effect on CYP2C19 and CYP2D6. *Br J Clin Pharmacol* 49:343–351.

Ko JW, Sukhova N, Thacker D, et al (1997) Evaluation of omeprazole and lansoprazole as inhibitors of cytochrome P450 isoforms. *Drug Metab Dispos* 25:853–862.

Korfmacher WA (2011) Bioanalytical Support for Both In Vitro and In Vivo Assays Across Drug Discovery and Drug Development. *Encycl Drug Metab Interact* 1–18.

Kotani N, Maeda K, Debori Y, et al (2012) Expression and transport function of drug uptake transporters in differentiated HepaRG cells. *Mol Pharm* 9:3434–3441.

Kramer JA, Sagartz JE, Morris DL (2007) The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates. *Nat Rev Drug Discov* 6:636–649.

Kumar V, Locuson CW, Sham YY, Tracy TS (2006a) Amiodarone analog-dependent effects on CYP2C9-mediated metabolism and kinetic profiles. *Drug Metab Dispos* 34:1688–1696.

Kumar V, Wahlstrom JL, Rock DA, et al (2006b) CYP2C9 inhibition: Impact of probe selection and pharmacogenetics on in vitro inhibition profiles. *Drug Metab Dispos* 34:1966–1975.

Kuroha M, Kuze Y, Shimoda M, Kokue E (2002) In vitro characterization of the inhibitory effects of ketoconazole on metabolic activities of cytochrome P-450 in canine hepatic microsomes. *Am*

J Vet Res 63:900–905.

Kwon Y (2014) *Handbook of Essential Pharmacokinetics, Pharmacodynamics and Drug Metabolism for Industrial Scientists*. Kluwer Academic Publishers

Kyselova Z (2012) Toxicological aspects of the use of phenolic compounds in disease prevention. *Interdiscip Toxicol* 4:173–183.

Lewis DF V (2004) 57 varieties: the human cytochromes P450. *Pharmacogenomics* 5:305–318.

Li AP (2007) Human hepatocytes: Isolation, cryopreservation and applications in drug development. *Chem Biol Interact* 168:16–29.

Lin D, Wang Z, Pan P, et al (2012) Determination of Tolbutamide and its Metabolite in Human Plasma by High Performance Liquid Chromatography and its Application to Pharmacokinetics. *Lat Am J Pharm* 31:2383.

Lin JH, Lu AY (1997) Role of pharmacokinetics and metabolism in drug discovery and development. *Pharmacol Rev* 49:403–449.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and developmental settings. *Adv Drug Deliv Rev* 23:3–25.

Liu Y, Peterson DA, Kimura H, Schubert D (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J Neurochem* 69:581–593.

Lombardino JG, Lowe III JA (2004) The role of the medicinal chemist in drug discovery-then and now. *Nat Rev Drug Discov* 3:853–862.

Lübberstedt M, Müller-Vieira U, Mayer M, et al (2011) HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment in vitro. *J Pharmacol Toxicol Methods* 63:59–68.

Lynch T, Price A, Virginia E (2007) The Effect of Cytochrome P450 Metabolism on Drug Response,Interactions, and Adverse Effects. *Am Fam Physicians* 76:1–6.

Martikainen L (2012) *In Vitro and in Silico Methods to Predict Cytochrome P450 Enzyme Inhibition*. University of Eastern Finland

Maurel P (2010) *Hepatocytes Methods and Protocols*. Humana Press Inc.

McGinnity DF, Berry AJ, Kenny JR, et al (2006) Evaluation of Time-Dependent Cytochrome P450 Iinhibition Using Cultured Human Hepatocytes. *Drug Metab Dispos* 34:1291–1300.

Medvedovici A, David V, Miron D, Mircioiu C (2000) Comparison of Two Sample Preparation Methods for a HPLC-DAD Assay of Tolbutamide from Human Plasma. *Anal Lett* 33:2219–2230.

Michael DC (2010) *Human Drug Metabolism: An Introduction*. John Wiley & Sons Ltd

Micuda S, Mundlova L, Anzenbacherova E, et al (2004) Inhibitory effects of memantine on human

cytochrome P450 activities: Prediction of in vivo drug interactions. *Eur J Clin Pharmacol* 60:583–589.

Miners JO, Birkett DJ (1998) Cytochrome P4502C9: An enzyme of major importance in human drug metabolism. *Br J Clin Pharmacol* 45:525–538.

Moon YJ, Wang X, Morris ME (2006) Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. *Toxicol Vitr* 20:187–210.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63.

Nayadu S, Behera D, Sharma M, et al (2013) Fluorescent Probe Based Cyp Inhibition Assay: a High Throughput Tool for Early Drug Discovery Screening. *Int J Pharm Sci* 5:303–307.

Nijveldt RJ, van Nood E, van Hoorn DE, et al (2001) Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr* 74:418–425.

Ogu CC, Maxa JL (2000) Drug interactions due to cytochrome P450. *BUMC Proc* 13:421–423.

Orlando RM, Cordeiro DD, Elisa A, et al (2009) Pré-Tratamento de Amostras. *Vita Sanitas* 3:122–139.

Pandey S, Pandey P, Tiwari G, Tiwari R (2010) Bioanalysis in drug discovery and development. *Pharm Methods* 1:14–24.

Paris BL, Ogilvie BW, Scheinkoenig JA, et al (2009) In Vitro Inhibition and Induction of Human Liver Cytochrome P450 Enzymes by Milnacipran. *Pharmacology* 37:2045–2054.

Paul SM, Mytelka DS, Dunwiddie CT, et al (2010) How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat Rev Drug Discov* 9:203–214.

Pelkonen O, Turpeinen M, Hakkola J, et al (2008) Inhibition and induction of human cytochrome P450 enzymes: Current status. *Arch Toxicol* 82:667–715.

Peng L, Wang B, Ren P (2005) Reduction of MTT by flavonoids in the absence of cells. *Colloids Surfaces B Biointerfaces* 45:108–111.

Pernelle K, Le Guevel R, Glaise D, et al (2011) Automated detection of hepatotoxic compounds in human hepatocytes using HepaRG cells and image-based analysis of mitochondrial dysfunction with JC-1 dye. *Toxicol Appl Pharmacol* 254:256–266.

Plakas SM, Lee TC, Wolke RE (1985) Absence of overt toxicity from feeding the flavonol, quercetin, to rainbow trout (Salmo gairdneri). *Food Chem Toxicol* 23:1077–1080.

Prasad GS, Srisailam K, Sashidhar RB (2016) Metabolic inhibition of meloxicam by specific CYP2C9 inhibitors in Cunninghamella blakesleeana NCIM 687: in silico and in vitro studies. *Springerplus* 5:166.

Rietjens IMCM, Slob W, Galli C, Silano V (2008) Risk assessment of botanicals and botanical preparations intended for use in food and food supplements: emerging issues. *Toxicol Lett*

180:131–136.

Roe AL, Paine MF, Gurley BJ, et al (2016) Assessing Natural Product-Drug Interactions: An End-to-End Safety Framework. *Regul Toxicol Pharmacol* 76:1–6.

Roymans D, Van Looveren C, Leone A, et al (2004) Determination of cytochrome P450 1A2 and cytochrome P450 3A4 induction in cryopreserved human hepatocytes. *Biochem Pharmacol* 67:427–437.

Schmider J, Greenblatt DJ, von Moltke LL, et al (1997) Inhibition of CYP2C9 by selective serotonin reuptake inhibitors in vitro: studies of phenytoin p-hydroxylation. *Br J Clin Pharmacol* 44:495–498.

Schoenwald RD (ed) (2013) *Pharmacokinetics in Drug Discovery and Development*. CRC Press LLC

Schonborn JL (2010) The role of the liver in drug metabolism. *Anaesth Tutor Week* 1:1–6.

Shah VP, Midha KK, Findlay JW, et al (2000) Bioanalytical method validation-a revisit with a decade of progress. *Pharm Res* 17:1551–1557.

Si D, Wang Y, Zhou YH, et al (2009) Mechanism of CYP2C9 inhibition by flavones and flavonols. *Drug Metab Dispos* 37:629–634.

Sigma-Aldrich (2001) Tolbutamide - Product Information. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Fluka/Product_Information_Sheet/t0891pis.pdf. Accessed 10 May 2016

Singh SP, Wahajuddin, Raju KSR, et al (2012) Reduced bioavailability of tamoxifen and its metabolite 4-hydroxytamoxifen after oral administration with biochanin A (an isoflavone) in rats. *Phyther Res* 26:303–307.

Skibola CF, Smith MT (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med* 29:375–83.

Sousa-Ferreira L, Aveleira C, Botelho M, et al (2014) Fluoxetine induces proliferation and inhibits differentiation of hypothalamic neuroprogenitor cells in vitro. *PLoS One* 9:e88917.

Srinivas NR (2006) Applicability of bioanalysis of multiple analytes in drug discovery and development: review of select case studies including assay development considerations. *Biomed Chromatogr* 20:383–414.

Tanaka E (1998) Clinically important pharmacokinetic drug-drug interactions: role of cytochrome P450 enzymes. *J Clin Pharm Ther* 23:403–416.

Tassaneeyakul W, Birkett DJ, Veronese ME, et al (1993) Specificity of substrate and inhibitor probes for human cytochromes P450 1A1 and 1A2. *J Pharmacol Exp Ther* 265:401–7.

Toda T, Eliasson E, Ask B, et al (2009) Roles of different CYP enzymes in the formation of specific fluvastatin metabolites by human liver microsomes. *Basic Clin Pharmacol Toxicol*

105:327–332.

Turpeinen M, Korhonen LE, Tolonen A, et al (2006) Cytochrome P450 (CYP) inhibition screening: Comparison of three tests. *Eur J Pharm Sci* 29:130–138.

Turpeinen M, Tolonen A, Chesne C, et al (2009) Functional expression, inhibition and induction of CYP enzymes in HepaRG cells. *Toxicol Vitr* 23:748–753.

van de Waterbeemd H, Gifford E (2003) ADMET in silico modelling: towards prediction paradise? *Nat Rev Drug Discov* 2:192–204.

Van Dongen M, Weigelt J, Uppenberg J, et al (2002) Structure-based screening and design in drug discovery. *Drug Discov Today* 7:471–478.

van Meerloo J, Kaspers GJL, Cloos J (2011) *Cell Sensitivity Assays: The MTT Assay. In: Cancer Cell Culture: Methods and Protocols*, 2th edn. Cree, Ian A., pp 237–245

von Moltke LL, Weemhoff JL, Bedir E, et al (2004) Inhibition of human cytochromes P450 by components of Ginkgo biloba. *J Pharm Pharmacol* 56:1039–1044.

Vrba J, Kren V, Vacek J, et al (2012) Quercetin, quercetin glycosides and taxifolin differ in their ability to induce AhR activation and CYP1A1 expression in HepG2 cells. *Phyther Res* 26:1746–52.

Walsky RL, Obach RS (2004) Validated Assays for Human Cytochrome P450 Activities. *Drug Metab Dispos* 32:647–660.

Wen X, Wang JS, Kivisto KT, et al (2001) In vitro evaluation of valproic acid as an inhibitor of human cytochrome P450 isoforms: preferential inhibition of cytochrome P450 2C9 (CYP2C9). *Br J Clin Pharmacol* 52:547–553.

Wesołowska O, Hendrich AB, Łaniapietrzak B, et al (2009) Perturbation of the lipid phase of a membrane is not involved in the modulation of MRP1 transport activity by flavonoids. *Cell Mol Biol Lett* 14:199–221.

Wu J (ed) (2014) *Cytochrome P450 Enzymes: Biochemistry, Pharmacology and Health Implications*. Nova Science Publishers, Inc.

Yadegarynia S, Pham A, Ng A, et al (2012) Profiling Flavonoid Cytotoxicity in Human Breast Cancer Cell Lines: Determination of Structure-Function Relationships. *Nat Prod Commun* 7:1295–304.

Yuan R, Madani S, Wei X-X, et al (2002) Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab Dispos* 30:1311–1319.

Zanelli U, Caradonna NP, Hallifax D, et al (2012) Comparison of cryopreserved HepaRG cells with cryopreserved human hepatocytes for prediction of clearance for 26 drugs. *Drug Metab Dispos* 40:104–110.

Zanger UM, Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* 138:103–141.

Zhai X, Lu Y (2013) Development and validation of a simple LC method for the determination of phenacetin, coumarin, tolbutamide, chlorzoxazone, testosterone and their metabolites as markers of cytochromes 1A2, 2A6, 2C11, 2E1 and 3A2 in rat microsomal medium. *Pharmazie* 68:19–26.

Zhang D, Luo G, Ding X, Lu C (2012a) Preclinical experimental models of drug metabolism and disposition in drug discovery and development. *Acta Pharm Sin B* 2:549–561.

Zhang D, Zhu M, Humphreys WG (eds) (2007) *Drug Metabolism in Drug Design and Development: Basic Concepts and Practice*. John Wiley & Sons, Inc.

Zhang X, Ma J, Hu L, Zhang Q (2012b) Simultaneous Determination of Tolbutamide and Its Metabolite Hydroxytolbutamide in Rat Plasma By LC-MS. *Liq Chromatogr Relat Technol* 35:1627–1637.

Zhao-Qian L, Yan S, Song-Lin H, et al (2001) Effects of CYP2C19 genotype and CYP2C9 on fluoxetine N-demethylation in human liver microsomes. *Acta Pharm Sin B* 22:85–90.

Zhou S-FF, Zhou Z-WW, Yang L-PP, Cai J-PP (2009a) Substrates, inducers, inhibitors and structure-activity relationships of human cytochrome P450 2C9 and implications in drug development. *Curr Med Chem* 16:3480–3675.

Zhou SF, Liu JP, Chowbay B (2009b) Polymorphism of human cytochrome P450 enzymes and its clinical impact. *Drug Metab Rev* 41:89–295.