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Study of the metabolic stability profiles of perampanel, rufinamide and stiripentol and prediction of drug interactions using HepaRG cells as an *in vitro* human model

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ABSTRACT

New-generation antiepileptic drugs as perampanel, rufinamide and stiripentol emerged as alternatives in chronic epilepsy polytherapy. Hence, their metabolic stability and potential involvement in relevant drug-drug interactions (DDI) are of great clinical interest, being HepaRG cells herein used as an *in vitro* human model.

To characterize their metabolic stability profiles, HepaRG cells were incubated with perampanel (1 μM), rufinamide (100 μM) or stiripentol (5 μM) for 12-h. HepaRG cells, pretreated with known CYP450 isoenzymes inducers (rifampicin, phenytoin, phenobarbital, omeprazole and carbamazepine), were also incubated with perampanel, rufinamide or stiripentol to assess possible DDI mediated by CYP450 induction.

Results suggest a considerable decrease in perampanel and stiripentol concentrations over 12-h; contrary, rufinamide concentrations did not variated. Cells pretreatment with all inducers significantly decreased stiripentol concentrations (between 20.3% and 31.9%), suggesting a considerable potential for DDI. Rufinamide concentrations only decreased when preincubated with rifampicin and with the highest tested concentrations of the remaining inducers. Perampanel levels decreased with rifampicin, carbamazepine and phenobarbital, supporting the involvement of CYP3A4-mediated metabolism.

Besides relevant information concerning the metabolic stability profile and potential DDIs of the new antiepileptics here studied, it was also reinforced the HepaRG cells suitability as a reliable *in vitro* model to foresee *in vivo* metabolism in humans.

1. Introduction

Drug discovery and development is a costly and time-consuming process. In fact, the extensive characterization of the drug fate after administration is one of the major challenges to reduce late-stage attrition rates and improve clinical safety (Yildirim et al., 2016). Therefore, absorption, distribution, metabolism, and excretion (ADME) of drugs should be deeply studied, with drug metabolism playing an important role in optimizing pharmacokinetics, pharmacodynamics and safety profile (Zhang and Tang, 2018). Hence, the availability of *in silico*, *in vitro* and *in vivo* tools to understand multiple aspects on drug-related metabolism (*e.g.* metabolic stability, elimination routes, drug

Abbreviations: ADME, absorption, distribution, metabolism and excretion; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CE, carboxylesterase; CYP450, cytochrome P450; DAD, diode-array detection; DDI, drug-drug interactions; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FDA, US Food and Drug Administration; GST, glutathione *S*-transferase; HPLC, high-performance liquid chromatography; Km, Michaelis-Menten constant; $MgSO_4$, magnesium sulphate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PER, perampanel; PXR, pregnane X receptor; RFM, rufinamide; SEM, standard error of the mean; STP, stiripentol; UGT, uridine 5′-diphospho-glucuronosyltransferase..

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interactions, possible toxic metabolites) are of the utmost importance (Issa et al., 2017).

Three examples of new antiepileptic drugs, particularly licensed as adjunctive therapy in different epileptic seizure types, are perampanel (PER), rufinamide (RFM) and stiripentol (STP). After oral administration, their bioavailability is very high (≥85%), with PER presenting a bioavailability near 100% (Chung et al., 2011; Patsalos et al., 2016; Perucca et al., 2008; Verrotti et al., 2016). Based on clinical trials and animal studies, it is already known that hepatic metabolism, mostly mediated by phase I cytochrome P450 (CYP450) isoenzymes, is significantly responsible for their elimination. It is described that PER is considerably metabolized (98%) by CYP3A4 and CYP3A5, being these CYP450 isoenzymes mainly responsible for its oxidation (De Biase et al., 2018; Meirinho et al., 2021; Patsalos, 2013a; Patsalos et al., 2016; Rogawski and Hanada, 2013; Schulze-Bonhage, 2015). STP is also extensively metabolized in liver (73%) *via* CYP1A2, CYP2C19 and CYP3A4, forming approximately 13 different metabolites. It mostly involves four different metabolic pathways: glucuronidation, oxidation of the methylenedioxy ring system, hydroxylation of t-butyl group, and conversion of the allylic alcohol chain to an isomeric 3-pentanone structure (Buck and Goodkin, 2019; European Medicines Agency, 2021; Landmark and Patsalos, 2010; Meirinho et al., 2021; Nickels and Wirrell, 2017). On the other hand, RFM is known to be substantially deactivated in liver (96%) through hydrolysis mediated by carboxylesterases (CE) 1 and 2, with no involvement of CYP450 isoenzymes (European Medicines Agency, 2021; Landmark and Patsalos, 2010; Meirinho et al., 2021; Perucca et al., 2008). The knowledge of these metabolic pathways is of great interest since PER, RFM and STP are only prescribed in polytherapy regimens and there is a scarcity of information on their involvement either as victims or perpetrators in drug-drug interactions (DDI). Indeed, they are introduced as add-on therapy to other antiepileptic drugs and non-antiepileptic drugs, often involved in clinically relevant DDI (Landmark and Patsalos, 2010; Patsalos, 2013a). Therefore, metabolism induction by other drugs leads to a decrease in antiepileptic concentrations and, consequently, to a lack of therapeutic efficacy (Landmark and Patsalos, 2010; Patsalos, 2013a).

Concerning PER, RFM and STP, some DDI mediated by enzymatic induction are already identified. In this context, there are clinical reports describing that STP and RFM plasma concentrations can be decreased if they are co-prescribed with enzyme inducing antiepileptic drugs such as carbamazepine, phenytoin, phenobarbital or primidone. Additionally, RFM plasma concentrations decrease when co-administrated with vigabatrin, oxcarbazepine and methsuximide (Landmark and Patsalos, 2010; Patsalos, 2013a; Patsalos et al., 2018). Regarding PER, reports state that its plasma concentrations decrease when carbamazepine, phenytoin, oxcarbazepine or topiramate are co-prescribed (Patsalos, 2013a; Patsalos et al., 2018). However, up to date, to the best of our knowledge, there are no reports suggesting the induction of PER, RFM and STP metabolism by non-antiepileptic drugs, becoming a field worth of being explored (Patsalos, 2013b).

As far as we know, all the aforementioned reports are based on data from clinical trials and not from *in vitro* approaches, contrary to what would be expected given the regulations and the standard policies that guide the development of new drugs (De Biase et al., 2018; Buck and Goodkin, 2019; Patsalos, 2013a; Patsalos et al., 2018; Perucca et al., 2008; Vlach et al., 2019). Actually, *in vitro* assays using primary human hepatocytes in culture are considered the gold standard approach to accurately predict metabolism and hepatotoxicity of different drug candidates during the preclinical stage of drug development (Gomez-Lechon et al., 2004; Lübberstedt et al., 2011). However, primary human hepatocytes present limited growth and life-span, large inter-donor functional variability and early phenotypic changes when placed in culture, greatly limiting their use (Anthérieu et al., 2012; Guillouzo et al., 2007; Vlach et al., 2019). A viable alternative to primary human hepatocytes is the human hepatoma cell line called as HepaRG (Anthérieu et al., 2010; Ashraf et al., 2019). Once differentiated, these cells resemble typical mature hepatocyte-like cells surrounded by biliary-like cells (Fig. 1) (Andersson et al., 2012; Anthérieu et al., 2010; Guillouzo et al., 2007; Kanebratt and Andersson, 2008; Lübberstedt et al., 2011).

In the differentiated state, HepaRG cells express most of the CYP450 isoenzymes in levels comparable to those found in primary human hepatocytes, (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4), some phase II metabolic enzymes [as uridine 5′-diphosphoglucuronosyltransferase (UGT) and glutathione *S*-transferase (GST)], and several drug transporters (Ashraf et al., 2019; Guillouzo et al., 2007; Kanebratt and Andersson, 2008; Lübberstedt et al., 2011). To prove it, many studies have used cocktails of CYP450 substrates to demonstrate the expression of CYP450 isoenzymes by HepaRG cells and to determine their basal and induced functional activities (Table S1, see Supplementary material). This cell line has also proven to be an excellent *in vitro* model to study metabolic induction, once it expresses the main nuclear transcription factors responsible for most of the CYP450 induction, as pregnane X receptor (PXR), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR) (Kanebratt and Andersson, 2007; Ogasawara et al., 2018). Considering this information, DDI mediated by enzyme induction can be studied using both primary hepatocytes or hepatic cell lines such as HepaRG (Anthérieu et al., 2010; Gerets et al., 2012; Ogasawara et al., 2018; Sugiyama et al., 2016). Therefore, bearing in mind the aforementioned advantages of using HepaRG cells, and being well established in literature their ability to highly express the majority of CYP450 isoenzymes and their inducibility capacity (Table S1, see Supplementary material), the present work aimed to assess the metabolic stability profiles of PER, RFM and STP in this cell line. Moreover, using HepaRG cells as an *in vitro* human model, we also

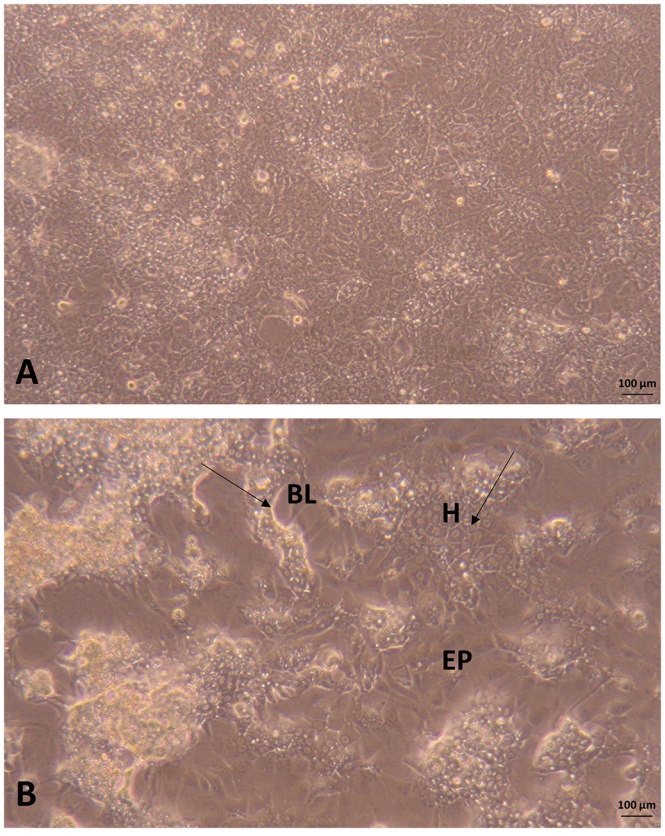

Fig. 1. Phase-contrast micrographs of HepaRG cells at 10× amplification. (A) Cells cultured in basal growth medium for 15 days; (B) differentiated HepaRG cells after 30 days of seeding and exposed to 2% DMSO for 15 days, showing epithelial-like cells (EP), hepatocyte-like cells (H) and formed bile canaliculus (BL).

proposed to foresee the potential for PER, RFM and STP to be targets of DDI involving enzyme induction, particularly when co-incubated with non-antiepileptic drugs.

2. Material and methods

2.1. Reagents and cells

PER (99.9% purity) and RFM (100.63% purity) were kindly supplied by MSN Laboratories Ltd. (Hyderabad, India), and STP (≥ 98% purity) was purchased from Tokyo Chemical Industry (Tokyo, Japan). For CYP450 induction studies, phenobarbital was kindly given by BIAL-Portela & Ca, S.A. (S. Mamede do Coronado, Portugal) and carbamazepine, omeprazole, phenytoin and rifampicin were acquired from Sigma-Aldrich (St. Louis, MO, USA). HepaRG cells (lot no. #48588; passages 6–13) were obtained from Life Technologies – Invitrogen™ (through Alfagene, Portugal). All cell culture reagents including Williams' E medium, fetal bovine serum (FBS), insulin, hydrocortisone hemisuccinate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and trypsin-EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). For samples preparation procedures, several chemical reagents as acetonitrile (Fisher Scientific; Leicestershire, UK), dichloromethane (Fisher Scientific; Leicestershire, UK), magnesium sulphate ($MgSO_4$) (Panreac Quimica) and isopropanol (Honeywell Riedel-de Haën™; Seelze, Germany) were used. Terbinafine hydrochloride (99.5% purity), used as internal standard for PER and STP quantification, was kindly supplied by Bluepharma (Coimbra, Portugal), whereas chloramphenicol (> 99% purity), used as internal standard for RFM quantification, was purchased from Sigma-Aldrich (St. Louis, MO, USA). For bioanalytical purposes, acetonitrile (HPLC grade) was purchased from Fisher Scientific (Leicestershire, UK), ultra-pure water (HPLC grade, > 18 MΩ.cm) was prepared by a Milli-Q water apparatus from Millipore (Milford, MA, USA) and 85% *ortho*-phosphoric acid was obtained from Fisher Scientific (Leicestershire, UK).

2.2. HepaRG cells culture

All incubations of HepaRG cells were carried out at 37 °C with 95% humidity in an atmosphere of 5% CO_2. Following the manufactures' instructions, after properly defrost, HepaRG cells were seeded on 75 cm^2 T-flasks using Williams' E culture medium supplemented with 10% FBS, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 5 μg mL^{-1} insulin, 2 mM glutamine and 5 × 10^{-5} M hydrocortisone hemisuccinate (basal growth medium). Every 2–3 days, medium was renewed and, when necessary, cells were expanded by trypsinization with trypsin-EDTA and maintained in basal growth medium until use for assays.

2.3. Cell viability studies

HepaRG cell viability was assessed after 12 h exposure to PER (0.01–500 μM), RFM (0.1–800 μM) and STP (0.01–800 μM). After trypsinization and counting, HepaRG cells were plated in 96-wells plates (1 × 10^4 cells/well) and left to adhere for 48 h. After that period, cells were incubated for 12 h with different solutions of culture medium fortified with the different concentrations of PER, RFM and STP under study. Control experiments were performed by incubating the cells with drug vehicle (0.2% DMSO). After that period, the medium was removed and cells were washed with 100 μL of phosphate buffer saline. Then, 100 μL of the MTT solution (5 mg mL^{-1}), prepared in serum-free medium, was added to each well, followed by incubation of approximately 4 h at 37 °C. Next, the MTT containing medium was removed and the formazan crystals were dissolved in DMSO. The final absorbances were read at 560 nm using a microplate reader Anthos 2020 (Alfagene). Cell viability was expressed as a percentage relatively to the absorbance of control wells, and cell viability is considered not be compromised when it was maintained above 85% comparatively with controls (Bicker et al., 2017; Li et al., 2014).

2.4. Metabolic stability assays

To characterize the metabolic stability profiles of PER, RFM and STP and for the metabolism interaction studies, HepaRG cells were seeded at low density (2.6 × 10^4 cells cm^{-2}) in 24-well plates and grew to confluence for 15 days in basal growth medium (Fig. 1A). Thereafter, basal culture medium was supplemented with 2% DMSO (differentiation medium) and cells were kept under this condition for an additional 15-day period until complete differentiation was achieved (Fig. 1B). To characterize the metabolic stability profiles of PER, RFM and STP, the differentiation medium was replaced by 400 μL of fresh FBS-free basal growth medium (0.2% DMSO) fortified with PER (1 μM), RFM (100 μM) or STP (5 μM). The concentrations to be tested of the different antiepileptic drugs were selected based on the results of the MTT assay and having as requirement a cell viability above 85% (Section 2.3.) and, if possible, being within the therapeutic range of each drug (Reimers and Berg, 2018). Based on methodologies already described (Ferreira et al., 2016, 2014; Lübberstedt et al., 2011), immediately after incubation with each antiepileptic drug (0 h) and after 1, 2, 4, 8 and 12 h of incubation, the metabolism was stopped by adding to each well 400 μL of ice-cold acetonitrile (in cells incubated with RFM) or ice-cold isopropanol (in cells incubated with PER or STP). Cells were scrapped and the total content of each well was transferred to a 2 mL centrifuge tube and stored at −20 °C until analysis.

To induce CYP450 isoenzymes, differentiated HepaRG cells were incubated with serum-free basal growth medium containing rifampicin (4, 8 and 16 μM), phenytoin (40, 80 and 160 μM), omeprazole (30, 50 and 100 μM), phenobarbital (50, 150 and 500 μM), carbamazepine (50, 100 and 150 μM) or vehicle (0.2% DMSO). The induction process took place over 72 h, with the renewal of the medium every 24 h. Then, cells were incubated with basal growth medium (free of inducer drug) spiked with PER (1 μM), RFM (100 μM) or STP (5 μM) for 12 h and the metabolism was stopped as previously described, with total content of each well collected and stored at −20 °C until analysis. All inducers were selected considering the proper US Food and Drug Administration (FDA) guidelines for *in vitro* metabolism and DDI studies (Food and Drug Administration, 2020; Food And Drug Administration, 2017) and the gathered information about the most commonly used specific CYP450 isoenzymes inducers (Table S1, see Supplementary material).

2.5. Measurement of perampanel, rufinamide and stiripentol levels

Samples obtained from each independent assay described in Section 2.4. were analyzed based on HPLC methods already full validated (Meirinho et al., 2019, 2020). Since the type of biological matrix changed from mice matrices to culture medium containing HepaRG cells, a partial validation of both HPLC techniques was herein accomplished following the FDA and European Medicines Agency guidelines for the bioanalytical methods validation (European Medicines Agency, 2011; Food and Drug Administration, 2018). Selectivity, lower limit of quantification, calibration curve (linearity), as well as intra and interday precision and accuracy, and recovery were assessed (Table S2, see Supplementary material).

For the analysis of samples containing RFM, 20 μL of chloramphenicol (100 μg mL^{-1}) were added as internal standard to 200 μL of each cell culture sample. Then, 300 μL of ice-cold acetonitrile and 1.5 mL of dichloromethane were added, vortex-mixed for 1 min and then centrifuged at 12300*g* for 3 min. The obtained organic layer was collected into a glass tube, evaporated to dryness under a gentle nitrogen stream at 45 °C and then reconstituted using 100 μL of the mobile phase used for analysis [water/acetonitrile (82:18, *v/v*)]. The instrumental analysis was performed using an HPLC system (Shimadzu LC-2010A HT Liquid Chromatography) coupled to a diode-array detector (DAD) set at 210 nm

(Meirinho et al., 2019). On the other hand, for the analysis of samples containing PER or STP, to 200 µL of cell culture samples were added 20 µL of terbinafine (100 µg mL^{-1}) as internal standard followed by the addition of 100 µL of $MgSO_4$ and 100 µL of isopropanol. Then, samples were vortex-mixed for 1 min followed by a centrifugation step at 12300*g* for 3 min, which leads to a salting-out effect that allowed the recovery of the organic phase. The extract recovered was evaporated until dryness using a gentle nitrogen stream at 45 °C and then reconstituted with 200 µL of the mobile phase used for PER and STP analysis in HPLC [1% TEA in water (pH 2.5 adjusted with 85% *ortho*-phosphoric acid)/acetonitrile (53:47, v/v)] (Meirinho et al., 2020). For PER and STP quantification, an HPLC system (Shimadzu Corporation, Japan) equipped with a fluorescence detector was used, setting an excitation/emission wavelength pair of 275/430 nm for PER detection and a pair of 254/372 nm for both terbinafine and STP detection. In both methods, mobile phases were isocratically pumped at a flow rate of 1 mL min^{-1} and a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm; 3 µm particle size), protected by a LiChroCART® Purospher Star pre-column (C_{18}, 4 mm × 4 mm; 5 µm), both purchased from Merck KGaA (Darmstadt, Germany), were used as stationary phase in thermostatized conditions (35 °C).

2.6. Statistical analysis

Data were reported as the mean ± standard error of the mean (SEM) ($n = 4$) and statistical comparisons between the experimental groups *vs.* control group were performed using one-way ANOVA with *post-hoc* Dunnett's test. The determination of IC_{50} was performed by sigmoidal dose-response fitting analysis [log (inhibitor) *vs.* response – Variable slope]. Considering a confidence interval of 95%, differences were statistically significant if the obtained *p*-value was lower than 0.05 ($p < 0.05$). All statistical data analysis and graphical representation were performed with GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Cell viability studies

Aiming to properly evaluate the metabolic stability profiles of PER, RFM and STP over a 12 h period, the cell viability of HepaRG cells was firstly assessed in the presence of each antiepileptic drug to select the concentrations that would not lead to less than 85% of cell viability (Bicker et al., 2017; Li et al., 2014). Fig. 2 shows the viability percentages of HepaRG cells obtained in three independent experiments in which cells were treated with PER (0.01–500 µM), RFM (0.1–800 µM) or STP (0.01–800 µM) for 12 h.

Accordingly, the concentrations selected for the following studies were 1 µM for PER, 100 µM for RFM and 5 µM for STP. All these values are significantly lower than the calculated IC_{50} of each antiepileptic after 12 h incubation (363.8 µM for PER, 362.6 µM for RFM and 215.4 µM for STP), thus ensuring the absence of antiproliferative activity of the selected drugs concentrations against HepaRG cells. Although the selected PER, RFM and STP concentrations have ensured a cell viability greater than 85%, only in the case of PER and RFM those concentrations are within their corresponding therapeutic ranges (Reimers and Berg, 2018). On the other hand, STP induced an important cytotoxic effect when HepaRG cells were incubated with the higher concentrations tested (≥ 100 µM); thus, in this case, the selected concentration was below the lower limit of the therapeutic range but still kept under its Michaelis-Menten constant (Km) value of 2.2 ± 1.2 µg mL^{-1} (equivalent to 9.4 ± 0.5 µM) (Levy et al., 1984). This is particularly important since STP is the only antiepileptic drug here studied that presents a non-linear pharmacokinetics. So, at the selected concentration of 5 µM, it is anticipated that STP follows a linear, non-saturable metabolism, being this an important aspect to avoid difficulties in the interpretation of STP metabolism data (Baranczewski et al., 2006).

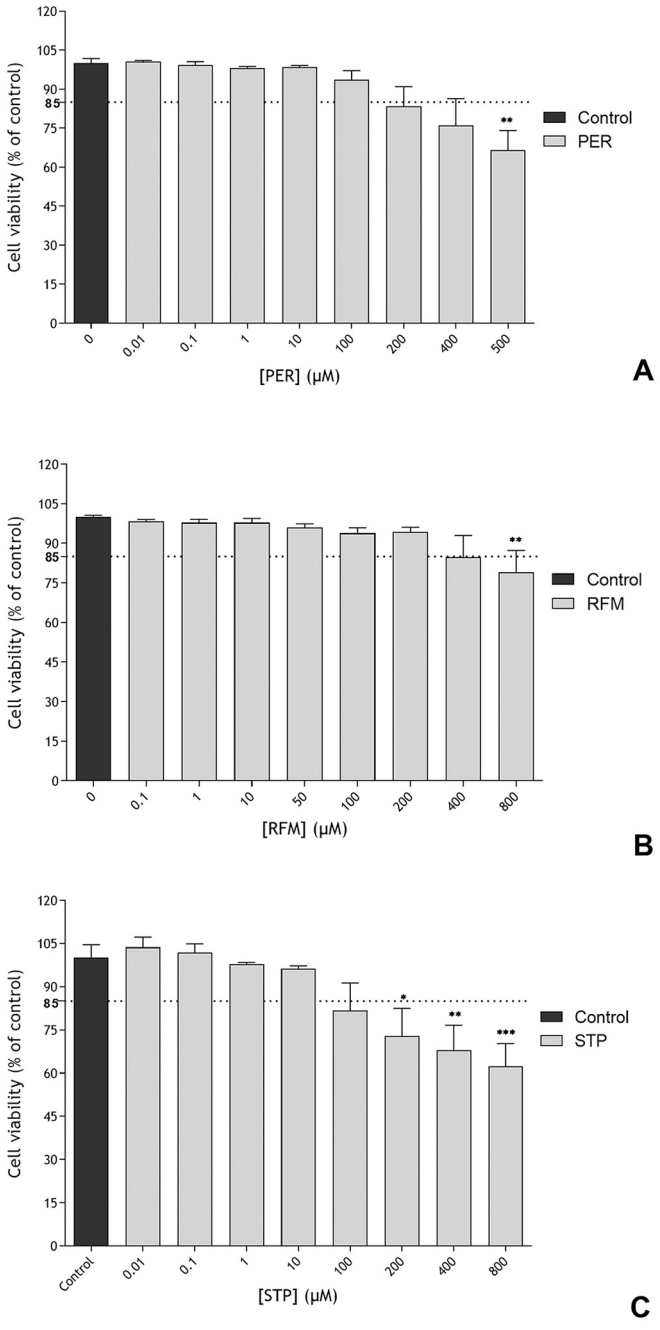

Fig. 2. Cell viability of perampanel (PER) (A), rufinamide (RFM) (B) and stiripentol (STP) (C) after 12 h of incubation in HepaRG cells using a standardized MTT test. Data expressed as mean ± SEM ($n = 3$, three independent replicates). *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$, compared with control group.

3.2. Metabolic stability profiles of perampanel, rufinamide and stiripentol

Metabolic stability profiles for each antiepileptic drug were plotted over a period of 12 h after incubation with PER (1 µM), RFM (100 µM) or STP (5 µM) in differentiated HepaRG cells. The obtained profiles are depicted in Fig. 3, which roughly represents the rate of metabolism of PER (A), RFM (B) and STP (C) in differentiated HepaRG cells.

A decrease of PER and STP concentrations within the study period was observed. Even though in absolute values this decrease is not very high (*i.e.*, 11.2% for PER and 20.3% for stiripentol), it was found

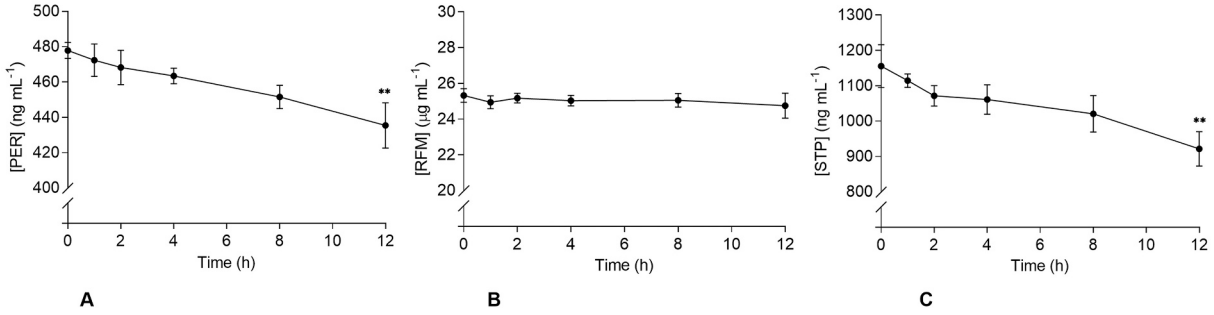

Fig. 3. Metabolic stability profiles for metabolism of (A) perampanel (PER), (B) rufinamide (RFM) and (C) stiripentol (STP) incubated at 1 µM, 100 µM and 5 µM, respectively, in differentiated HepaRG cells. Data expressed as mean ± SEM ($n = 4$). $^{**}p < 0.01$, compared with control group.

statistically significant ($p < 0.05$) at 12 h post-incubation compared with time zero. This suggests that differentiated HepaRG cells express the main CYP450 isoenzymes already recognized as being involved in the metabolism of these antiepileptic drugs, making it a reliable *in vitro* model to study the metabolism of PER and STP. This decrease of PER and STP concentrations over time is in accordance with the available clinical data. In fact, it is settled that PER is primarily metabolized by CYP3A4 and CYP3A5 (De Biase et al., 2018; Meirinho et al., 2021; Patsalos, 2013a; Patsalos et al., 2016; Rogawski and Hanada, 2013; Schulze-Bonhage, 2015), and STP is extensively metabolized by CYP1A2, CYP2C19 and CYP3A4 (Buck and Goodkin, 2019; European Medicines Agency, 2021; Meirinho et al., 2021; Nickels and Wirrell, 2017). These are all phase I drug-metabolizing enzymes by now proved to be highly expressed in differentiated HepaRG cells (Andersson et al., 2012; Guillouzo et al., 2007; Kanebratt and Andersson, 2008; Vlach et al., 2019). On the contrary, by analyzing Fig. 3B, RFM concentrations remain almost constant over the 12 h of incubation. According to the current clinical evidence, after oral administration, RFM is almost completely deactivated by hydrolysis in liver through CE1 and CE2 enzymes (European Medicines Agency, 2021; Perucca et al., 2008), which was not demonstrated from our results. Hence, even though HepaRG cells are able to express most phase I and phase II drug-metabolizing enzymes present in primary human hepatocytes, a possible explanation for the lack of RFM metabolism in HepaRG cells may be the low expression levels of CE enzymes in these cells.

3.3. Metabolism induction studies

As far as we know, up to date, there are no published studies using an *in vitro* model that demonstrate the relationship between the induction of drug-metabolizing enzymes and the decrease in PER, RFM and STP concentrations. Therefore, the present study can be settled as an innovation in this scope. In fact, HepaRG cells have proven to be an excellent *in vitro* model for evaluation of CYP450 induction in humans (Table S1, see Supplementary material), as they respond to prototypical inducers as rifampicin, omeprazole, phenobarbital, phenytoin and carbamazepine (Ogasawara et al., 2018; Ramachandran et al., 2015; Wang et al., 2004). Indeed, important nuclear factors involved in CYP450 induction are

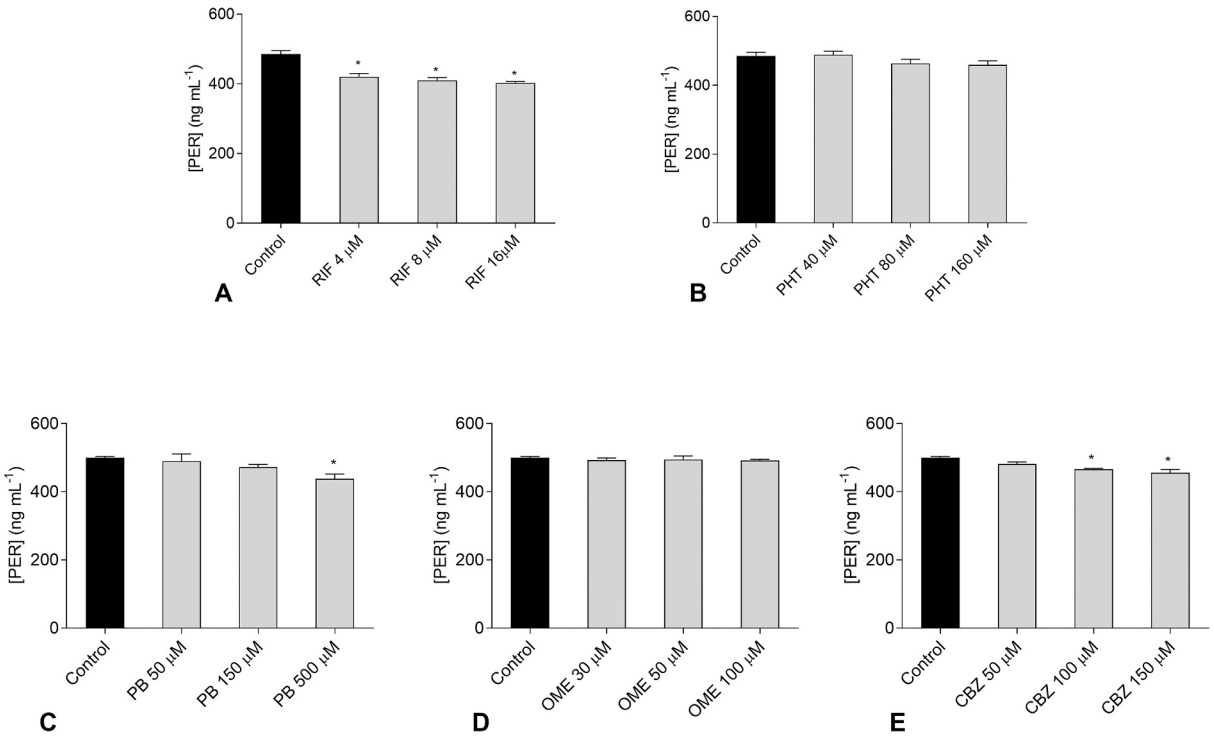

Fig. 4. Concentrations of perampanel (PER) after 12 h of incubation in differentiated HepaRG previously pre-treated during 72 h with different concentrations of the inducers (A) rifampicin (RIF), (B) phenytoin (PHT), (C) phenobarbital (PB), (D) omeprazole (OME) and (E) carbamazepine (CBZ). Data expressed as mean ± SEM ($n = 4$). $^{*}p < 0.05$, compared with control group.

expressed in HepaRG cells at similar levels to those found in human hepatocytes (Andersson et al., 2012; Anthérieu et al., 2010; Ashraf et al., 2019; Kanebratt and Andersson, 2007; Ogasawara et al., 2018). After analyzing the results presented in Figs. 4, 5 and 6, it can be observed that, when HepaRG cells were pre-treated with rifampicin, the concentrations of PER, RFM and STP were significantly lower ($p < 0.05$) in all experimental groups compared with controls. More specifically, PER, RFM and STP concentrations have a decrease of 15.3 ± 1.81%, 31.9 ± 4.81% and 32.4 ± 2.60%, respectively.

These results corroborate the information already published regarding the ability of rifampicin to induce CYP3A4, mostly by activation of PXR transcription factor (Andersson et al., 2012; Kanebratt and Andersson, 2007; Lübberstedt et al., 2011; Ogasawara et al., 2018). However, it must be considered that, in a lesser extent, rifampicin can likewise induce other CYP450 isoenzymes such as CYP2B6 and CYP2C19 (Andersson et al., 2012; Kanebratt and Andersson, 2007; Lübberstedt et al., 2011), which can also play a role in decreasing the concentration of each studied antiepileptic drug. Clinical data corroborate that PER, RFM and STP are affected by CYP450 inducers, with a consequent decrease of their plasma levels. This occurs particularly when PER, RFM and STP are co-prescribed with other antiepileptic drugs recognized as metabolic inducers, such as carbamazepine, phenytoin and phenobarbital (Landmark and Patsalos, 2010; Patsalos, 2013a; Patsalos et al., 2018; Perucca et al., 2008). However, until now, the available information is scarce regarding possible DDI involving PER, RFM and STP with other co-prescribed non-antiepileptic drugs. Hence, the current study comes to demonstrate that DDI mediated by CYP450 induction with non-antiepileptic drugs can actually occur, particularly when PER, RFM and STP are administrated with rifampicin and omeprazole. These findings were expectable in HepaRG cells since the inducible CYP3A4, CYP2B6 and CYP2C19 isoenzymes are also responsible for PER and STP metabolism (De Biase et al., 2018; Buck and Goodkin, 2019; European Medicines Agency, 2021; Meirinho et al., 2021; Nickels and Wirrell, 2017; Patsalos et al., 2016).

Surprisingly, RFM concentration decreased when incubated in HepaRG cells pretreated with different CYP450 inducers, even though its main metabolic route is the hydrolysis mediated by CE1 and CE2, with no evidence of CYP450 involvement (Perucca et al., 2008). Actually, considering the RFM metabolic stability profile obtained in HepaRG cells over a 12 h period (Fig. 3B), the lack of CEs expression in HepaRG cells is a strong possibility. However, there was a significant decrease ($p < 0.05$) in RFM concentrations in HepaRG cells pretreated with rifampicin (31.9 ± 4.81%) and with the highest concentration tested of phenobarbital (17.7 ± 6.45%), carbamazepine (14.1 ± 6.13%) and omeprazole (19.7 ± 2.58%) (Fig. 5). This corroborates the scientific evidence that CEs are also susceptible to induction mediated by CYP450 inducers such as phenobarbital (Perucca et al., 2008; Satoh and Hosokawa, 2006); even though it appears that, when not subject to inducers, HepaRG cells do not express CE enzymes as demonstrated by the metabolic stability profile obtained for RFM after 12 h incubation (Section 3.2.).

Considering the HepaRG cells pre-treated with phenytoin, only STP showed a significant decrease (31.3 ± 1.68%) in its concentration when compared with control group, even though literature describes that both PER, RFM and STP are subject to metabolism induction when administrated with phenytoin (Landmark and Patsalos, 2010; Patsalos, 2013a). This decrease in STP concentrations might be explained by the fact that both phenytoin and carbamazepine are inducers of CYP2B6 and CYP3A4. However, in HepaRG cells, the induction mechanism of these CYP450 enzymes is mediated by selective activation of CAR that, in this *in vitro* model, only cause a moderate upregulation of CYP2B6 and CYP3A4 mRNA (Kanebratt and Andersson, 2007; Sugiyama et al., 2016; Wang et al., 2004). So, this moderate upregulation might not be enough to explain the induction metabolism of PER and RFM in HepaRG cells, since they are not so extensively metabolized in liver as STP (Buck and Goodkin, 2019; Nickels and Wirrell, 2017). In opposition to phenytoin and carbamazepine, a strong upregulation of CYP1A2, CYP2B6 and CYP3A4 activity after HepaRG pretreatment with omeprazole, phenobarbital and rifampicin inducers was also expected (Kanebratt and Andersson, 2007). This can be another additional explanation to the

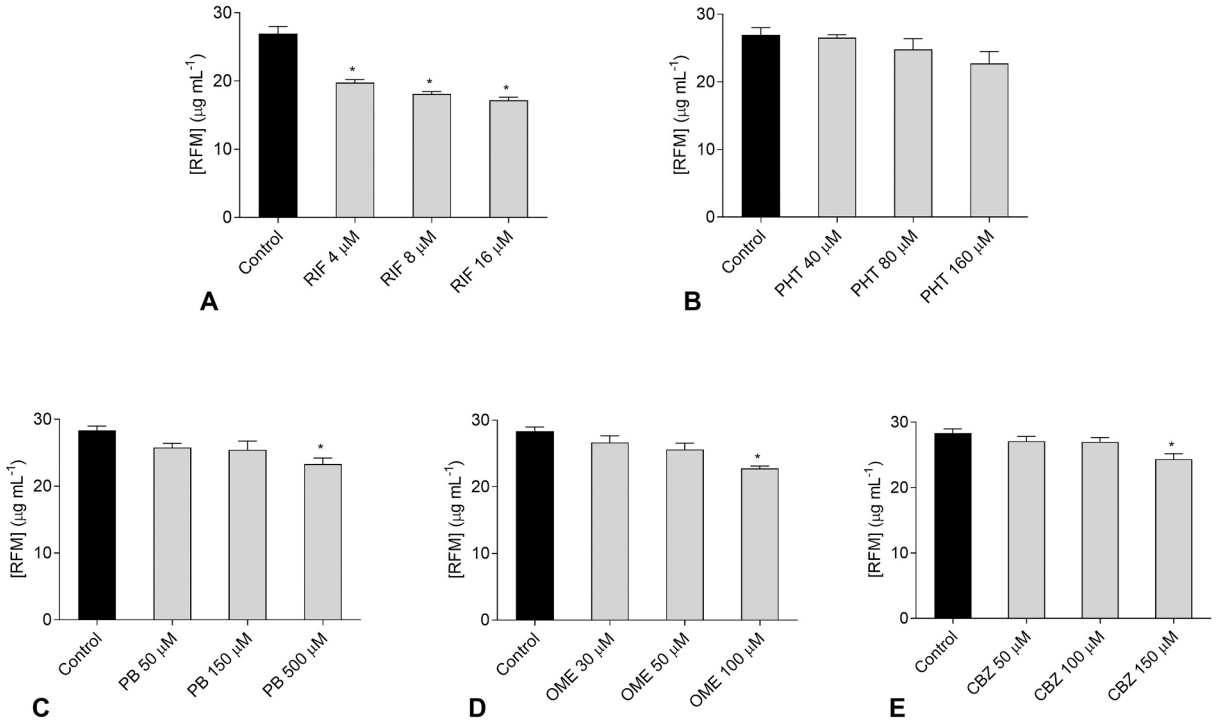

Fig. 5. Concentrations of rufinamide (RFM) after 12 h of incubation in differentiated HepaRG previously pre-treated during 72 h with different concentrations of the inducers (A) rifampicin (RIF), (B) phenytoin (PHT), (C) phenobarbital (PB), (D) omeprazole (OME) and (E) carbamazepine (CBZ). Data expressed as mean ± SEM ($n = 4$). *$p < 0.05$, compared with control group.

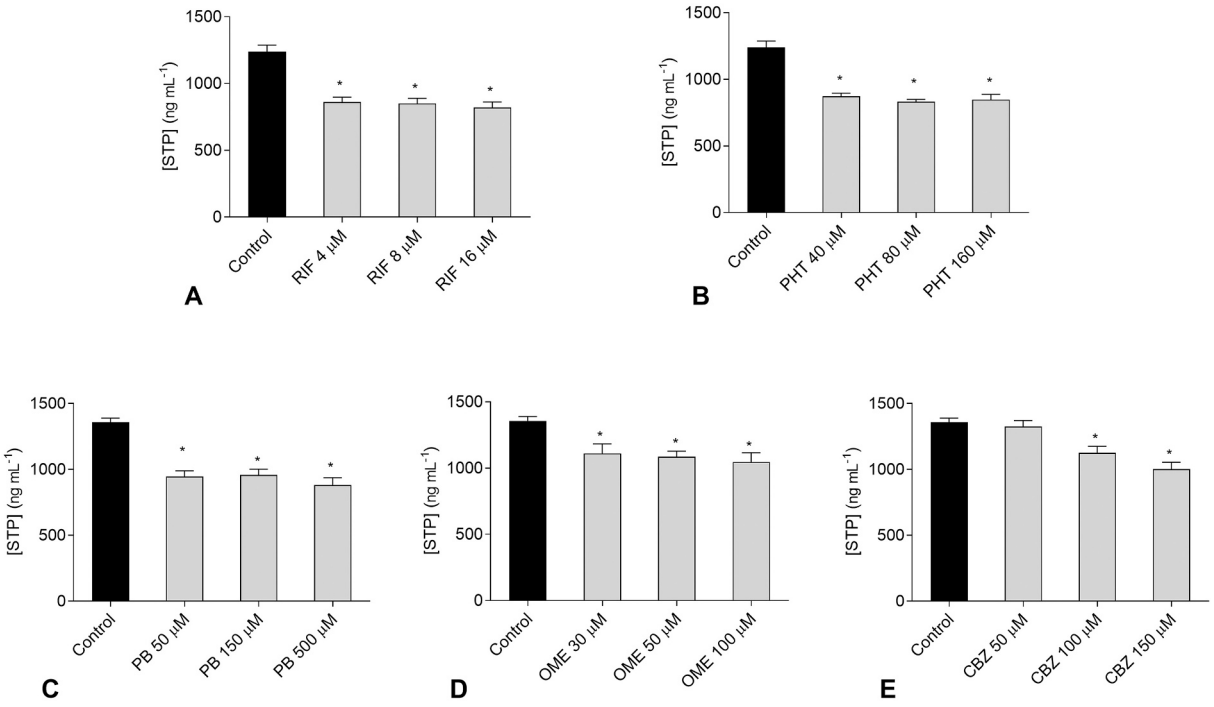

Fig. 6. Concentrations of stiripentol (STP) after 12 h of incubation in differentiated HepaRG previously pre-treated during 72 h with different concentrations of the inducers (A) rifampicin (RIF), (B) phenytoin (PHT), (C) phenobarbital (PB), (D) omeprazole (OME) and (E) carbamazepine (CBZ). Data expressed as mean ± SEM ($n = 4$). *$p < 0.05$, compared with control group.

decrease in STP concentrations when incubated in HepaRG cells pre-treated with all those inducers, with a significant decrease ($p < 0.05$) in STP concentration values when co-incubated with omeprazole (20.3 ± 2.40%), phenobarbital (31.6 ± 3.02%) and rifampicin (31.9 ± 1.66%) (Fig. 6).

Hence, the results obtained after treating differentiated HepaRG cells with different concentrations of each inducer (rifampicin, omeprazole, phenobarbital, phenytoin and carbamazepine) clearly suggest that this cell line represents a valid *in vitro* model to predict possible pharmacokinetic-based drug interactions mediated by induction involving the new antiepileptics PER, RFM and STP.

4. Conclusion

The use of HepaRG cell line as an *in vitro* model of human metabolism shows to be suitable for prediction of drug metabolism and DDI, particularly mediated by induction of CYP450 isoenzymes. In fact, the obtained results using the new generation antiepileptics PER, RFM and STP as drug models are in accordance with the information already reported in clinical trials regarding the metabolism of these drugs. Besides, the HepaRG cell line also demonstrated to be highly suitable to be used in more studies implying PER, RFM and STP, since only higher concentrations of these drugs produced cytotoxicity and antiproliferative effects. The metabolic stability profiles of PER and STP, in which was observed a decrease of each concentration along the time of incubation, seems to be related to the metabolism mediated by CYP450 isoenzymes. On the contrary, the lack of CEs expression was evident by the obtained metabolic stability profile of RFM, which is in accordance with the literature data. Moreover, by using HepaRG cells, it was demonstrated that PER, RFM and STP can be victim drugs in DDI when administrated with other antiepileptic drugs recognized as metabolic inducers, but also with other non-antiepileptic drugs recognized as CYP450 inducers. Indeed, the ability of the non-antiepileptic drugs rifampicin and omeprazole to induce the metabolism of STP, RFM and PER was herein demonstrated, which, to the best of our knowledge, was not previously described. Hence, despite the inherent limitations of an *in vitro* system, HepaRG cells might be a promising model to resemble the *in vivo* metabolism of antiepileptics and other new drugs in humans.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2022.105389.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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