



Flavonoid compounds as reversing agents of the P-glycoprotein-mediated multidrug resistance: An *in vitro* evaluation with focus on antiepileptic drugs



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ABSTRACT

The pharmacoresistance to antiepileptic drugs (AEDs) remains a major unsolved therapeutic need. The overexpression of multidrug transporters, as the P-glycoprotein (P-gp), at the level of the blood-brain barrier of epileptic patients has been suggested as a key mechanism underlying the refractory epilepsy. Thus, efforts have been made to search for therapeutically useful P-gp inhibitors. Herein, the strategy of flavonoid/AED combined therapy was exploited as a possible approach to overcome the P-gp-mediated pharmacoresistance. For this purpose, several *in vitro* studies were performed using Madin-Darby canine kidney II (MDCK II) cells and those transfected with the human multidrug resistance-1 (*MDR1*) gene, overexpressing the P-gp (MDCK-MDR1). Overall, the results showed that baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin, at 200 μM, produced a marked increase on the intracellular accumulation of rhodamine 123 in MDCK-MDR1 cells, potentially through inhibiting the P-gp activity. In addition, with the exception of lamotrigine, all other AEDs tested (phenytoin, carbamazepine and oxcarbazepine) and their active metabolites (carbamazepine-10,11-epoxide and licarbazepine) demonstrated to be P-gp substrates. Furthermore, the most promising flavonoids as P-gp inhibitors promoted a significant increase on the intracellular accumulation of the AEDs (excluding lamotrigine) and their active metabolites in MDCK-MDR1 cells, evidencing to be important drug candidates to reverse the AED-resistance. Thus, the co-administration of AEDs with baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin should continue to be explored as adjuvant therapy for refractory epilepsy.

List of chemical compounds studied in this article:

Baicalein (PubChem CID: 5,281,605); Carbamazepine (PubChem CID: 2554); Carbamazepine 10,11-epoxide (PubChem CID: 2555); (–)-Epigallocatechin gallate (PubChem CID: 65064); Kaempferol (PubChem CID: 5280863); Lamotrigine (PubChem CID: 3878); Licarbazepine (PubChem CID: 114709); Oxcarbazepine (PubChem CID: 34312); Phenytoin (PubChem CID: 1775); Silymarin (PubChem CID: 7073228); Quercetin (PubChem CID: 5280343); Verapamil (PubChem CID: 2520).

1. Introduction

Despite the clinical availability of more than twenty antiepileptic drugs (AEDs) with different pharmacokinetic profiles, mechanisms of action and potential for drug interactions, the development of drug-resistant epilepsy remains as a major unresolved problem, affecting 30–40% of patients (Baulac et al., 2015; Franco, French, & Perucca, 2016; Ventola, 2014). Although several pathomechanisms have been advocated to explain the drug resistance to AEDs, two major hypotheses have gained emphasis: the target hypothesis and the multidrug transporter hypothesis (Löscher, Klitgaard, Twyman, & Schmidt, 2013;

Rogawski, 2013; Wang, Wang, Liu, & Ma, 2016). The former postulates that AEDs lose efficacy due to changes in the structure/functionality of their target ion channels and neurotransmitter receptors; while the multidrug transporter hypothesis suggests an overexpression of multidrug efflux transporters such as P-glycoprotein (P-gp) in brain capillary endothelial cells, restricting AEDs penetration into the brain tissue of non-responsive epileptic patients (Ferreira, Pousinho, Fortuna, Falcão, & Alves, 2015; Gidal, 2014; Xiong, Mao, & Liu, 2015). This hypothesis has been supported by important clinical findings that demonstrated a greater expression of P-gp in patients with recurrent seizures (drug-resistant patients) than in those who have been seizure-free

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(Kwan et al., 2010b; Dombrowski et al., 2001; Józwiak, 2007; Marchi et al., 2004). Similar evidence has also been obtained from several animal models (Bartmann et al., 2010; Bauer et al., 2008; Liu, Yang, Yang, & Yang, 2007; Marchi et al., 2006; Nishimura, Honda, Sugioka, Takada, & Shibata, 2008). Furthermore, there are case reports of patients with drug-resistant epilepsy in which verapamil, a classic P-gp inhibitor, was successfully used as an add-on agent to conventional AED therapy, suggesting that P-gp inhibition could be one of the reasons underlying the improved seizure control (Iannetti, Spalice, & Parisi, 2005; Summers, Moore, & McAuley, 2004).

Thus, the multidrug transporter hypothesis has become particularly plausible in the context of drug-resistant epilepsy given that, similarly to other drug classes, several AEDs have been found to be substrates of the P-gp efflux pump (Luna-Tortós, Fedrowitz, & Löscher, 2008; Stouch & Gudmundsson, 2002; Weiss, Kerpen, Lindenmaier, Dormann, & Haefeli, 2003). Additionally, the exposure to certain AEDs seems to cause the P-gp upregulation; actually, some AEDs have been identified as P-gp inducers in both *in vitro* and *in vivo* conditions (Zhang, Kwan, Zuo, & Baum, 2012).

Given the relevance of the aforementioned P-gp-mediated multidrug resistance, efforts have been made to search for therapeutically useful P-gp inhibitors in order to overcome this functional barrier and reach higher drug concentrations into the target tissue (Chen et al., 2016). Nowadays, it is widely recognised the limited therapeutic success of first- and second-generation P-gp inhibitors, particularly due to their low potency, affinity and selectivity, requiring so high concentrations to inhibit P-gp that severe toxic effects are developed, compromising their clinical use (Bansal, Jaggi, Khar, & Talegaonkar, 2009; Palmeira, Sousa, Vasconcelos, & Pinto, 2012). In turn, the search for non-toxic third-generation P-gp inhibitors, which include herbal constituents like flavonoids, has gained a great attention (Bansal et al., 2009; Bansal, Awasthi, Jaggi, Khar, & Talegaonkar, 2008; Ferreira et al., 2015). As several flavonoids share some of the properties of an ideal P-gp inhibitor, their interest as P-gp modulators has increased in the last decade (Abdallah, Al-Abd, El-Dine, & El-Halawany, 2015; Ferreira et al., 2015; Mohana et al., 2016). As a result, compounds of this large group of polyphenolic constituents have exhibited P-gp inhibitory effects comparable to those of the classic P-gp inhibitors (verapamil and cyclosporine A) (Bansal et al., 2009; Ferreira et al., 2015; Schinkel & Jonker, 2012). In fact, some evidence of the potential of flavonoid compounds as P-gp inhibitors is supported by *in vitro* and *in vivo* studies as reviewed by Ferreira et al. (2015).

Hence, aware that the P-gp overexpression can be induced by seizures activity and chronic AED therapy, together with the fact that several AEDs are P-gp substrates, is of paramount importance the evaluation of the effect of multiple flavonoids on the activity of P-gp efflux pump and their influence on AEDs transport. In fact, the number of studies focusing on the effect of flavonoids in the disposition of central nervous system-acting drugs, including AEDs, is scarce; nevertheless, the inhibition of P-gp has previously demonstrated to restore the AEDs anticonvulsant activity in several animal models of refractory epilepsy (Brandt, Bethmann, Gastens, & Löscher, 2006; Clinckers, Smolders, Meurs, Ebinger, & Michotte, 2005; Höcht et al., 2007). Therefore, for this purpose, Madin-Darby canine kidney cell line transfected with the human multidrug resistance-1 (*MDR1*; *ABCB1*) gene encoding P-gp (MDCK-MDR1) and its respective wild-type Madin-Darby canine kidney II (MDCK II) cells were used to identify P-gp inhibitors among several flavonoids and evaluate their effects on the cell uptake of AEDs and their active metabolites. In parallel, P-gp substrates were identified among these drugs as well as their ability to interfere with the P-gp activity. The compounds under investigation included flavonoids as apigenin, baicalein, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin gallate, fisetin, hesperetin, kaempferol, naringin, quercetin and silymarin, and some of the most commonly prescribed AEDs [carbamazepine (CBZ), phenytoin (PHT), oxcarbazepine (OXC) and lamotrigine (LTG)] and their pharmacologically active metabolites [carbamazepine-10,11-epoxide (CBZ-E) and licarbazepine (LIC)].

2. Material and methods

2.1. Compounds and reagents

CBZ, CBZ-E, PHT, OXC, baicalein, (+)-catechin hydrate, fisetin, quercetin, silymarin, verapamil (a reference P-gp inhibitor used as positive control), rhodamine 123 (Rh123; a P-gp fluorescent probe substrate) and primidone (used as internal standard in chromatographic analysis) were all purchased from Sigma–Aldrich (St Louis, MO, USA). Silymarin is composed of silybinin, silydianin and silychristin, and the molar concentration was calculated based on the molecular weight of silybinin because this is the main flavonoid component present (Zhang & Morris, 2003a, 2003b). Apigenin, (–)-epigallocatechin gallate, kaempferol, hesperetin and naringin were obtained from Santa Cruz Biotechnology (Texas, USA) and (–)-epicatechin from Fluka (St Louis, MO, USA). LIC was supplied by Tocris Bioscience (Bristol, UK) and LTG was gently provided by Bluepharma (Coimbra, Portugal). All cell culture reagents including Dulbecco's Modified Eagle's Medium - high glucose (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), trypsin-EDTA (0.5 g/L porcine trypsin, 0.2 g/L EDTA) and phosphate-buffered saline (PBS) were acquired from Sigma-Aldrich (St Louis, MO, USA). Methanol and acetonitrile, both of high-performance liquid chromatography (HPLC) gradient grade, and ethyl acetate were 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).

2.2. Cell lines and culture conditions

MDCK II (passages 8–10) and MDCK-MDR1 (passages 18–30) cells, originally obtained from The Netherlands Cancer Institute (NKI-AVL; Amsterdam, Netherlands), were cultured in 75-cm² culture flasks in DMEM supplemented with 10% FBS, 100 IU/mL of penicillin and 100 µg/mL of streptomycin. The cell lines were maintained at 37 °C in a humidified air incubator with 5% CO₂ and the medium was renewed every 2–3 days. Cells were seeded (density of 7.9 × 10⁴ cells/cm²) in 96-well plates for cytotoxicity and Rh123 accumulation assays and in 24-well plates for the AEDs/metabolites accumulation assays. In both cases the cells were maintained in culture for 4 days at 37 °C in an atmosphere of 5% CO₂ before being subjected to the experiments.

2.3. Cytotoxicity assays

The *in vitro* cell viability was determined by the MTT assay according to the procedure described by Freshney, 2010. Combination of each flavonoid (50–200 µM), verapamil (50 µM) or AED/metabolite [CBZ (20–50 µM), CBZ-E (2–16 µM), PHT (40–75 µM), OXC (2–20 µM), LIC (16–140 µM), LTG (12–55 µM)] with Rh123 (5 µM) were evaluated as well as each AED/metabolite alone (at the highest concentration level of the previously mentioned ranges) and their combination with baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin at 200 µM or verapamil (50 µM, positive control). The range of AEDs/metabolites concentrations tested correspond to the values of their therapeutic ranges (Hoyland, Hoy, Austin, & Wildman, 2013; Patsalos et al., 2008).

Briefly, cells were incubated for 4 h with the compounds of interest at the designated concentration levels prepared in FBS-free DMEM medium (1% DMSO, v/v). In the untreated control cells FBS-free DMEM medium with the same final concentration of DMSO (1%, v/v) was used. After incubation the well-content was removed and 100 µL of FBS- and antibiotic-free DMEM medium containing MTT (1 mg/mL) was loaded to each well, followed by a new incubation step for 3 h under the same conditions. Thereafter, the MTT-containing medium was removed and replaced with DMSO to dissolve the formazan

crystals. Afterwards, the content of the wells was transferred to a reading 96-well plate and the absorbance was measured at 570 nm using a microplate spectrophotometer xMark™ (Bio-Rad). Cell viability was expressed as a percentage relatively to the absorbance determined in the untreated control cells.

2.4. Intracellular rhodamine 123 accumulation assays

To identify the flavonoid compounds that are P-gp inhibitors and the AEDs/metabolites that are inducers of the P-gp activity, intracellular accumulation assays of Rh123 were performed according to Barthelemy et al. (2005) with some modifications. At confluence, MDCK-MDR1 cells were washed once with PBS (pH 7.4) at 37 °C and pre-incubated for 30 min with FBS-free DMEM medium containing the test compounds as described in the following Sections 2.4.1 and 2.4.2. Subsequently, 5 µM Rh123, prepared in FBS-free DMEM medium (1% DMSO, v/v), was added to each well and the cells were maintained at 37 °C/5% CO₂ for 2 h. These conditions were common through all studies. Finally, cells were washed with cold PBS in order to stop the accumulation of Rh123, and then lysed with 100 µL 0.1% Triton X-100 for 30 min at room temperature, protected from light. The fluorescence of cell lysates was measured with a SpectraMax Gemini spectrofluorometer (Molecular Devices) at excitation/emission wavelengths of 485 nm/538 nm. Untreated control cells, exposed only to the drugs vehicle [FBS-free DMEM medium containing DMSO (1%, v/v)], were also incubated with 5 µM Rh123 in order to estimate the basal levels of intracellular accumulation of Rh123. A Rh123 standard curve (0.003125–5 µM) was generated to quantify the total amount of Rh123 accumulated in each sample.

2.4.1. Identification of flavonoid compounds as P-glycoprotein inhibitors

MDCK-MDR1 cells were pre-incubated for 30 min with FBS-free DMEM medium containing apigenin, baicalein, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin gallate, fisetin, hesperetin, kaempferol, naringin, quercetin or silymarin. The positive control consisted of incubating the cells with verapamil instead of flavonoid. Flavonoid compounds were tested at 50, 100 and 200 µM, and verapamil was incubated at 50 µM, and all were prepared in FBS-free DMEM medium (1% DMSO, v/v).

2.4.2. Identification of antiepileptic drugs/metabolites as inducers of P-glycoprotein activity

MDCK-MDR1 cells were pre-incubated for 30 min with FBS-free DMEM medium containing each AED or active metabolite. These compounds were tested at three concentrations corresponding to the low, medium and high level of their therapeutic ranges: 20, 30 and 50 µM for CBZ; 2, 8 and 16 µM for CBZ-E; 12, 65 and 140 µM for LIC; 12, 35 and 55 µM for LTG; 2, 10 and 20 µM for OXC; and 40, 55 and 75 µM for PHT.

2.5. Intracellular antiepileptic drugs accumulation assays

2.5.1. Identification of antiepileptic drugs/metabolites as P-glycoprotein substrates

Confluent and washed MDCK II and MDCK-MDR1 cells were incubated for 2 h with FBS-free DMEM medium containing each AED or active metabolite. The AEDs and active metabolites were tested at therapeutic concentrations (50 µM for CBZ, 16 µM for CBZ-E, 140 µM for LIC, 55 µM for LTG, 20 µM for OXC and 75 µM for PHT) and prepared in FBS-free DMEM (1% DMSO, v/v). After incubation, cells were washed and lysed as described above in Section 2.4, and the content of each well was individually collected and frozen at –20 °C until HPLC analysis.

2.5.2. Effects of flavonoid compounds on the intracellular accumulation of antiepileptic drugs/metabolites

The flavonoids identified as P-gp inhibitors [baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin] were selected to evaluate their effects on the intracellular accumulation of each AED or active metabolite. For that, confluent and washed MDCK-MDR1 cells were pre-incubated for 30 min with FBS-free DMEM medium, in the presence of flavonoids or the positive control (verapamil). Flavonoids were tested at 200 µM and verapamil at 50 µM, and all were prepared in FBS-free DMEM (1% DMSO, v/v). Untreated control cells were exposed only to FBS-free DMEM with the same final concentration of DMSO (1%, v/v) in order to estimate the basal intracellular accumulation of AED/metabolite. Then, untreated and P-gp inhibitor treated cells were incubated with the AEDs or metabolites at the highest tested concentration (CBZ 50 µM, CBZ-E 16 µM, LIC 140 µM, LTG 55 µM, OXC 20 µM and PHT 75 µM) for 2 h. Afterwards, cells were washed and lysed as described above in Section 2.4, and the content of each well was separately collected and frozen at –20 °C until HPLC analysis.

2.6. HPLC analysis

The quantitative HPLC analysis of AEDs and their active metabolites was performed based on the methods previously reported by Ferreira, Rodrigues, Falcão, & Alves, 2016a, 2016b. For the determination of CBZ, CBZ-E, LIC, LTG and PHT in MDCK II or MDCK-MDR1 cell lysates, two HPLC methods were used. Briefly, to each aliquot (200 µL) of 0.1% Triton X-100 samples were added 20 µL of the internal standard working solution, 300 µL of acetonitrile and 1 mL of ethyl acetate. The mixture was vortex-mixed for 30 s and centrifuged at 13500 rpm (3 min). Then, the sample was re-extracted twice more with ethyl acetate (1 mL each time) using the same conditions. The whole organic extract was evaporated to dryness under a nitrogen stream at 45 °C and then reconstituted with 100 µL of mobile phase. An aliquot (20 µL) of this final sample was injected into the HPLC system.

Chromatographic analysis was carried out using an HPLC system (Shimadzu LC-2010A HT Liquid Chromatography) coupled with a diode array detector (Shimadzu SPD-M20A). All instrumental parts were automatically controlled by LabSolutions software (Shimadzu, Kyoto, Japan). The chromatographic separation was carried out at 35 °C on a reversed-phase LiChroCART® Purospher Star column (C₁₈, 55 mm × 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany). For the determination of CBZ, CBZ-E, OXC and LIC an isocratic elution was applied at a flow rate of 1.0 mL/min with a mobile phase composed of water/methanol/acetonitrile (69:25:6, v/v/v), and primidone was used as internal standard (working solution at 500 µg/mL). The wavelength of 215 nm was selected for detection of all compounds. On the other hand, the chromatographic analysis of LTG and PHT was carried out using an isocratic elution with acetonitrile (6%), methanol (25%) and a mixture (69%) of water–triethylamine (99.7:0.3, v/v; pH 6.0), pumped at 1 mL/min; in this case, CBZ was used as internal standard (working solution at 200 µg/mL). The compounds were detected at 215 nm (LTG and CBZ) and 235 nm (PHT). The mobile phases were filtered through a 0.2 µm filter and degassed ultrasonically for 15 min before use and the injected sample volume was always 20 µL.

2.7. Statistical analysis

Data were reported as mean ± standard error of the mean. The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Whenever a simpler statistical analysis was needed to evaluate the effect of each treatment versus the control

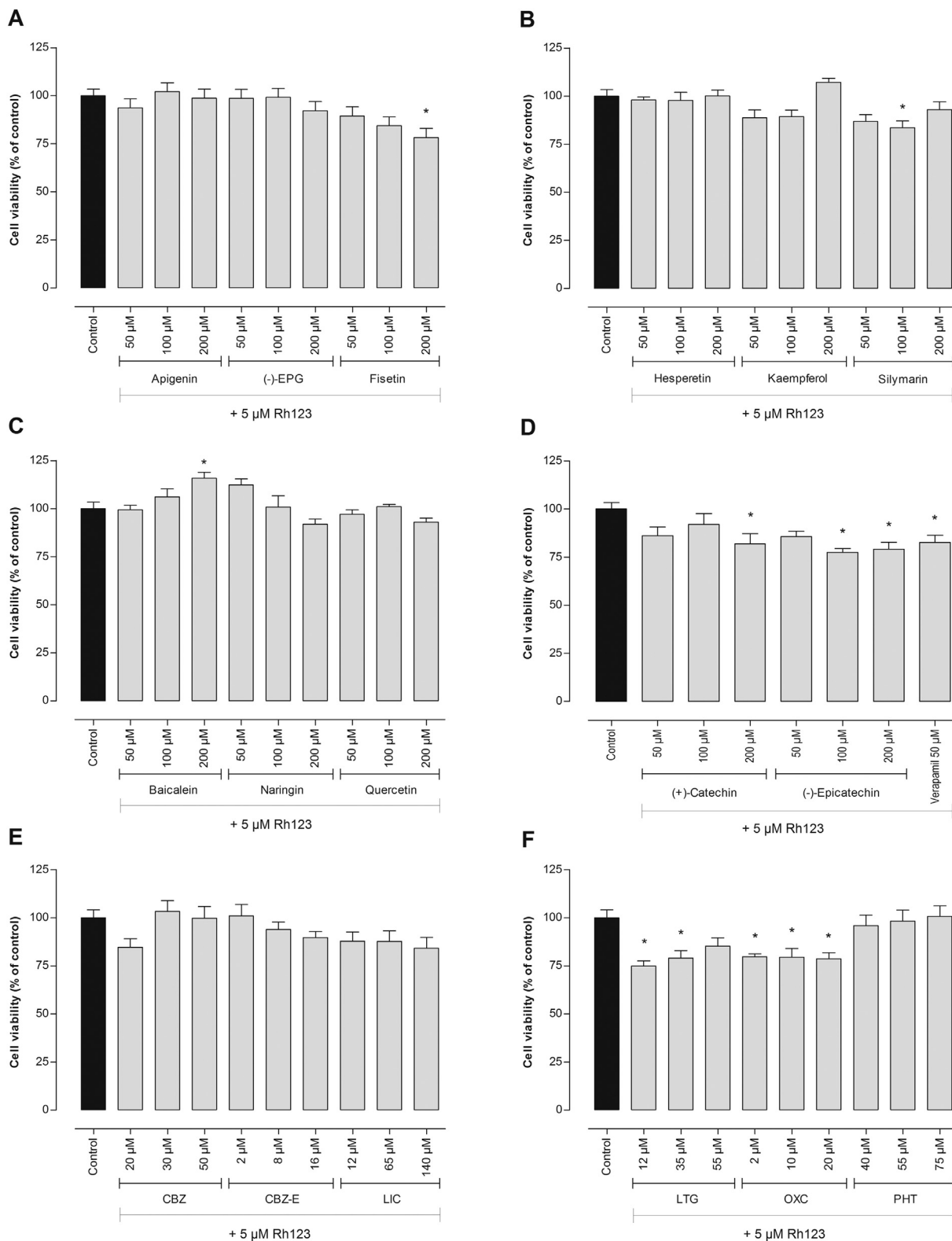


Fig. 1. Cell viability data for flavonoids (A–D), verapamil (D) and antiepileptic drugs or metabolites (E–F) together with 5 μM rhodamine123 (Rh123) after 4 h of incubation in MDCK-MDR1 cells. Data are expressed as the mean ± standard error of the mean (n = 6). Multiple comparisons between treated cells vs. untreated control cells as well as among the different concentrations of flavonoids or antiepileptics/metabolites were performed by one-way ANOVA with the *post hoc* Tukey's test (*p < 0.05). CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; EPG, (-)-epigallocatechin gallate; LIC, licarbazepine; PHT, phenytoin.

group, a one-way ANOVA with the *post hoc* Dunnett's test was conducted. To check for statistically significant differences not only with the control group but also for comparing multiple treatments a one-way ANOVA with the *post hoc* Tukey's test for multiple comparisons was

performed. In some specific cases, a Student's *t*-test was applied to compare two groups. A difference was considered to be statistically significant for a *p*-value lower than 0.05 (p < 0.05). The statistical tests used were appropriately identified in the figure legends.

3. Results

3.1. Cytotoxicity assays

The effects of the different combinations of each flavonoid (or verapamil) with Rh123 in cell viability was investigated during 4 h. As shown in Fig. 1A–D, only a few concentrations of flavonoids induced a statistically significant reduction of cell viability ($p < 0.05$) when compared to untreated control cells [fisetin, (+)-catechin and (–)-epicatechin at 200 μM ; and silymarin and (–)-epicatechin at 100 μM], but without a very marked effect. It is worthy of note that the cytotoxicity of verapamil at 50 μM (82.6%) was similar or higher than that observed with the highest tested concentration of flavonoids (200 μM). In turn, baicalein did not reduce the cell viability, instead it appeared to increase the cell proliferation at 200 μM (Fig. 1C).

Regarding the combination of AEDs or metabolites and Rh123 at 5 μM (Fig. 1E–F), statistically significant differences in reduction of cell viability were observed for OXC over the tested concentration range (2–20 μM ; $p < 0.05$) and also for LTG at the lower concentrations assayed (12 and 35 μM ; $p < 0.05$); however, cytotoxicity for LTG at 55 μM was not significant. In addition, the effects on the cell viability produced by the different concentrations of LTG and OXC were tested by the one-way ANOVA with the *post hoc* Tukey's test and no statistically significant differences were found ($p > 0.05$), supporting the use of the highest concentration levels of both AEDs in the following intracellular Rh123 accumulation assays.

Fig. 2 represents the data of cell viability obtained for each AED (or metabolite) alone and in combination with each one of the selected flavonoid compounds or verapamil. Overall, the AED (or metabolite)/flavonoid combinations did not appear to produce important cytotoxic

effects, which supports the use of these concentrations in the subsequent studies. Once again, in general, no marked loss of cell viability was induced by combinations of verapamil (50 μM) and AEDs or metabolites.

3.2. Identification of flavonoid compounds as P-glycoprotein inhibitors

According to the results depicted in Fig. 3, five [baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin] of the eleven flavonoids tested induced a marked increase on the intracellular accumulation of Rh123 in MDCK-MDR1 cells. The results obtained showed statistically significant differences between flavonoid treated cells and untreated control cells ($*p < 0.05$) in almost all tested concentrations, the last representing the basal intracellular accumulation of Rh123 (negative control). Moreover, as it can be seen in Fig. 3, the effects of baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin on the intracellular accumulation of Rh123 occurred in a concentration-dependent manner ($^{\#}p < 0.05$). Actually, the intracellular accumulation of Rh123 increases as the concentration of these five flavonoids increases (a predictive marker of P-gp inhibition). Specifically, (–)-epigallocatechin gallate at 200 μM produced an increase in the intracellular accumulation of Rh123 similar to that exhibited by verapamil (positive control as P-gp inhibitor). On the contrary, apigenin, (+)-catechin, (–)-epicatechin and fisetin decreased the intracellular accumulation of Rh123, suggesting to be inducer agents of the P-gp activity, whereas none effect was found for hesperetin and naringin.

Taking into account these results, baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin at 200 μM were selected to be evaluated in the subsequent experiments involving the combination

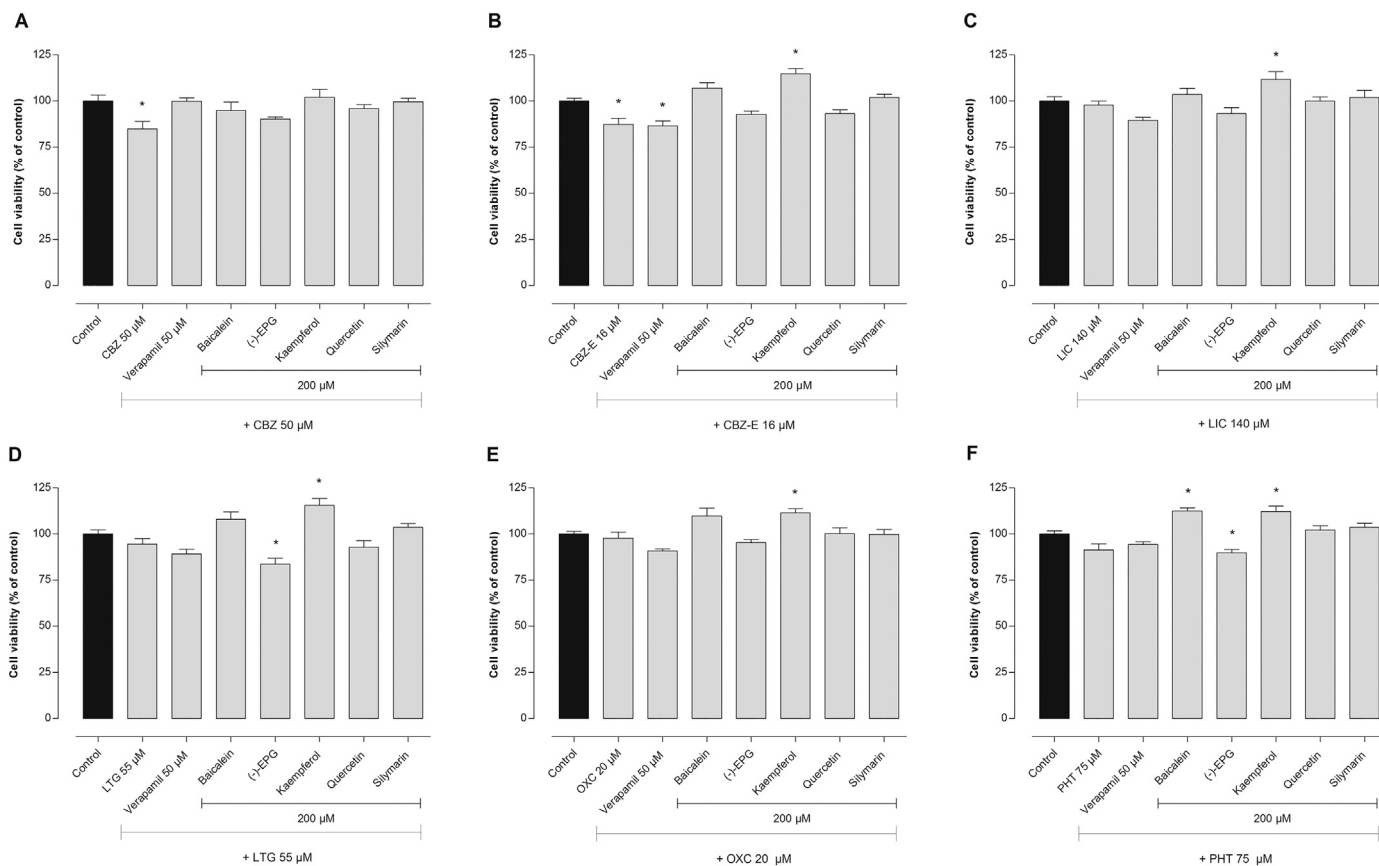


Fig. 2. Cell viability data for (A) carbamazepine (CBZ), (B) carbamazepine-10,11-epoxide (CBZ-E), (C) licarbazepine (LIC), (D) lamotrigine (LTG), (E) oxcarbazepine (OXC) and (F) phenytoin (PHT) alone and together with verapamil or flavonoids after 4 h of incubation in MDCK-MDR1 cells. Data are expressed as the mean values \pm standard error of the mean ($n = 6$). Comparisons between treated cells vs. untreated control cells were performed by one-way ANOVA with the *post hoc* Dunnett's test ($p < 0.05$). EPG, (–)-epigallocatechin gallate.

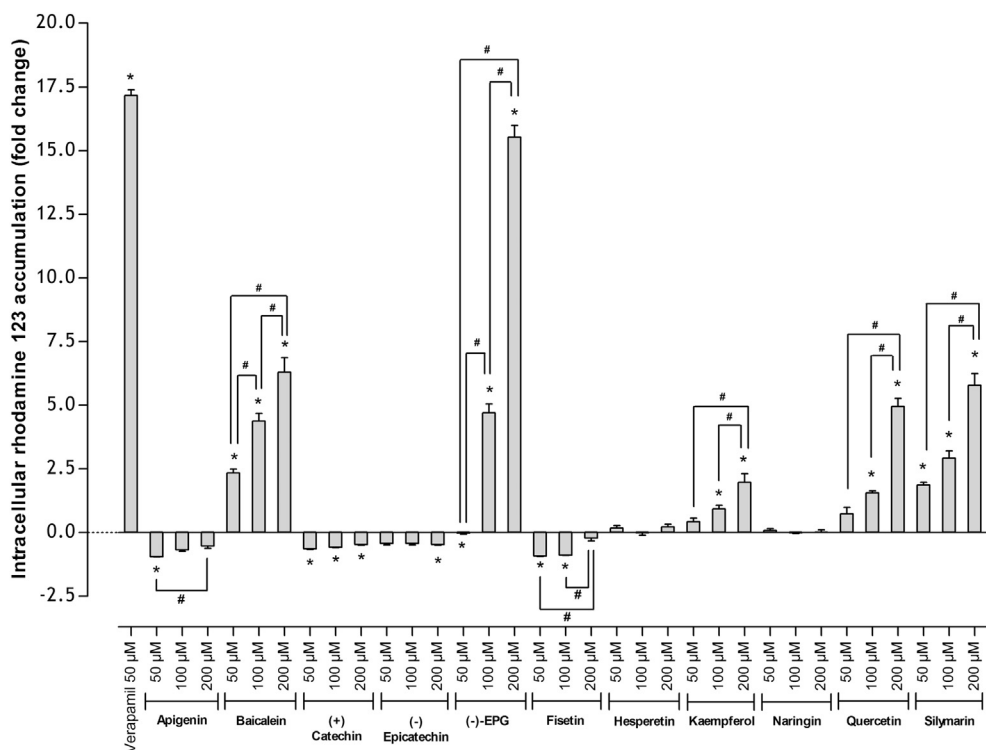


Fig. 3. Effect of flavonoids on the intracellular accumulation of rhodamine 123 (Rh123) in MDCK-MDR1 cells. The results were obtained by comparing the flavonoid (or verapamil) pre-treated cells vs. untreated cells (control). Data are expressed as the mean values \pm standard error of the mean ($n = 6$). The basal intracellular accumulation of Rh123 in the control group was $0.0097 \pm 0.0026 \mu\text{M}$. Multiple comparisons between flavonoids/verapamil group vs. control group ($*p < 0.05$) as well as among the different concentrations of flavonoids ($\#p < 0.05$) were performed by one-way ANOVA with the *post hoc* Tukey's test. (-)-EPG, (-)-epigallocatechin gallate.

with AEDs or their active metabolites.

3.3. Identification of antiepileptic drugs/metabolites as inducers of P-glycoprotein activity

Although with different magnitudes and with the exception for CBZ at 50 μM , CBZ-E (2, 8 and 16 μM) and OXC at 2 μM , a statistically significant decrease in the accumulation of Rh123 into MDCK-MDR1

cells was found for the AEDs or metabolites, appearing to exhibit an inducer effect on the P-gp activity (Fig. 4). Overall, it is noteworthy the concentration-dependent effect found for OXC on the intracellular accumulation of Rh123, which significantly decreases as the concentration of OXC increases ($\#p < 0.05$). In contrast to OXC, no statistically significant difference was detected among the different concentrations of the other AEDs or metabolites tested.

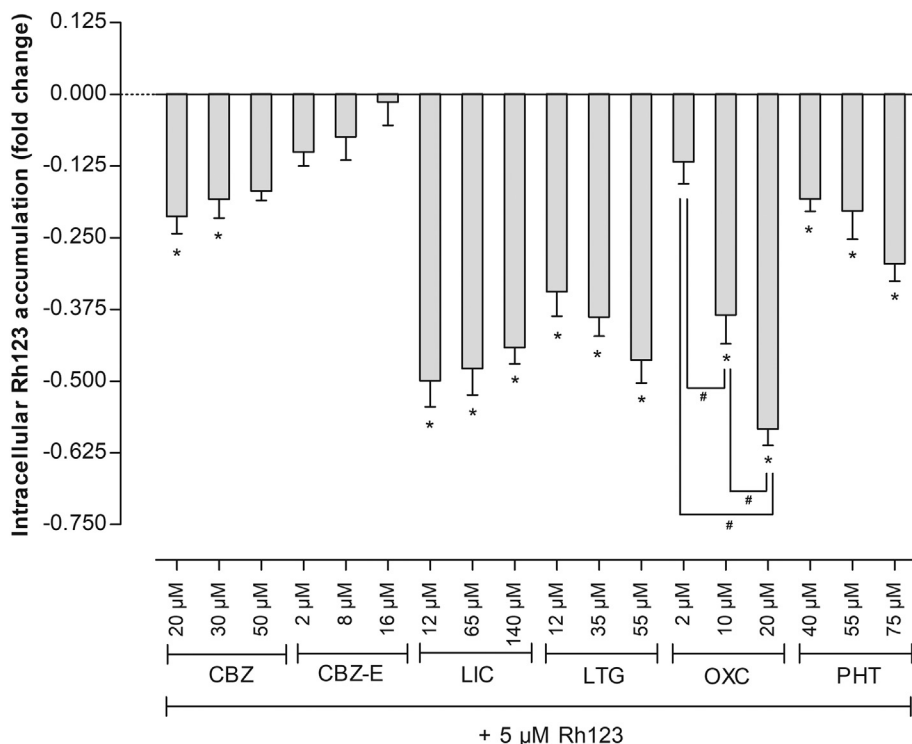


Fig. 4. Identification of P-glycoprotein inducers among the antiepileptics/metabolites through rhodamine 123 (Rh123) accumulation assays performed in MDCK-MDR1 cells. The results were obtained by comparing the intracellular accumulation of Rh123 in antiepileptics or metabolites pre-treated cells vs. untreated cells (control). Data are expressed as the mean values \pm standard error of the mean ($n = 6$). The basal intracellular accumulation of Rh123 in the control group was $0.0091 \pm 0.0016 \mu\text{M}$. Multiple comparisons between antiepileptics/metabolite group vs. control group ($*p < 0.05$) as well as among the different concentrations of antiepileptics/metabolites ($\#p < 0.05$) were performed by one-way ANOVA with the *post hoc* Tukey's test. CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; EPG, (-)-epigallocatechin gallate; LIC, licarbazepine; LTG, lamotrigine; OXC, oxcarbazepine; PHT, phenytoin.

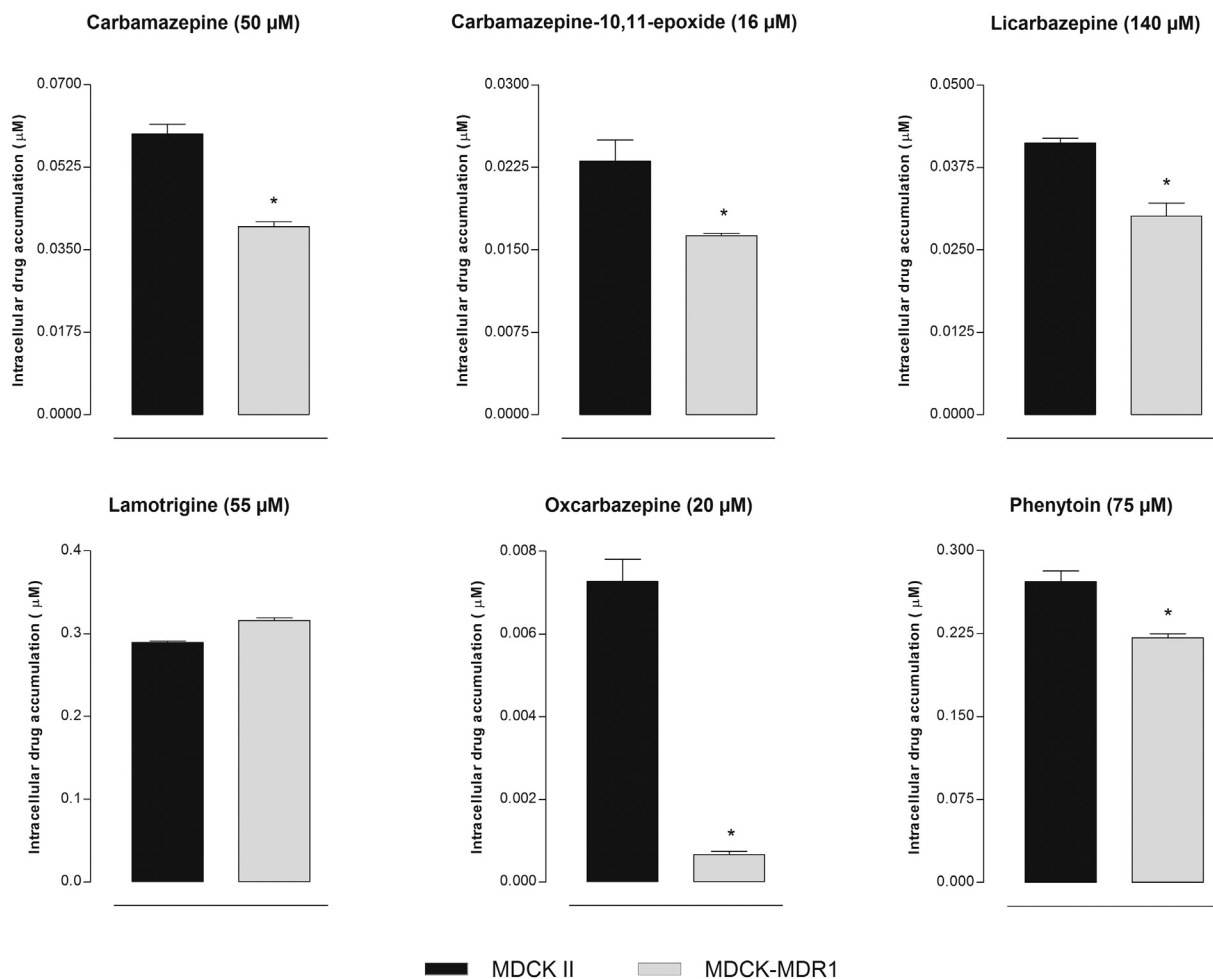


Fig. 5. Comparison of the intracellular accumulation of antiepileptic drugs/metabolites between MDCK II and MDCK-MDR1 cells. Data are expressed as the mean values \pm standard error of the mean ($n = 3$). Comparisons between MDCK II vs. MDCK-MDR1 group were performed by Student's *t*-test. * $p < 0.05$, compared to control group (MDCK II cell line).

3.4. Identification of antiepileptic drugs/metabolites as P-glycoprotein substrates

The intracellular accumulation of the AEDs or metabolites was evaluated and compared in both MDCK II cells and MDCK-MDR1 cells. Taking into account the results presented in Fig. 5, the intracellular accumulation of CBZ ($p = 0.0011$), CBZ-E ($p = 0.0254$), LIC ($p = 0.0060$) and PHT ($p = 0.0071$) was 1.2 to 1.5 times higher in MDCK II than in MDCK-MDR1 cells. Nevertheless, the degree of intracellular accumulation of OXC was very much steeper in MDCK II (a 16-fold increase) than in MDCK-MDR1 cells ($p = 0.0003$). On the contrary, no relevant differences were observed in the intracellular accumulation of LTG between the two cell lines. Contrarily to the LTG, the lower intracellular accumulation of CBZ, CBZ-E, LIC, OXC and PHT in the MDCK-MDR1 cells than in MDCK II cells indicates that these AEDs and metabolites are P-gp substrates in this cell model.

3.5. Effects of flavonoid compounds on the intracellular accumulation of antiepileptic drugs/metabolites

According to the results shown in Fig. 6, the majority of flavonoids significantly increased the intracellular accumulation of the AEDs and their active metabolites in MDCK-MDR1 cells comparatively to the untreated control cells ($p < 0.05$). LTG was the unique AED whose intracellular accumulation was not affected by any of the tested flavonoids (Fig. 6D). Overall, silymarin was the flavonoid compound that most increased the AED or metabolite accumulation, suggesting to be

the most promising flavonoid as P-gp inhibitor. Actually, in some of the cases, the effect of silymarin on the intracellular accumulation of AEDs/metabolites was very close to that produced by verapamil. In opposition, kaempferol was the flavonoid that less increased the intracellular accumulation of AEDs or metabolites.

4. Discussion

The search for new therapeutic options continues to be pursued in order to overpass the pharmacoresistance in epilepsy. The recognition of P-gp-mediated drug efflux as a major factor responsible for drug resistance that may restrict the penetration or accumulation of AEDs into the epileptic brain tissue has increased the demand for P-gp inhibitors that could enhance the AEDs concentration in the site of action (Löscher et al., 2013; Robey, Lazarowski, & Bates, 2008; Zhang et al., 2012). These efforts are particularly justified by the fact that this glycoprotein is overexpressed in patients with the phenotype of drug-resistant epilepsy, who are resistant to several, if not all, AEDs despite their different mechanisms of action. Moreover, evidence has been reported supporting the inducer effects of AEDs on the P-gp activity. All these findings support the transporter hypothesis of AED-resistant epilepsy and emphasize the potential of P-gp inhibitors in an attempt to reverse this drug resistance phenomenon (Gidal, 2014; Luna-Tortós et al., 2008; Zhang et al., 2012).

Bearing in mind this possible therapeutic approach, the current study had as a starting point the evaluation of the potential of a series of flavonoids to inhibit the P-gp. The selection of these flavonoid

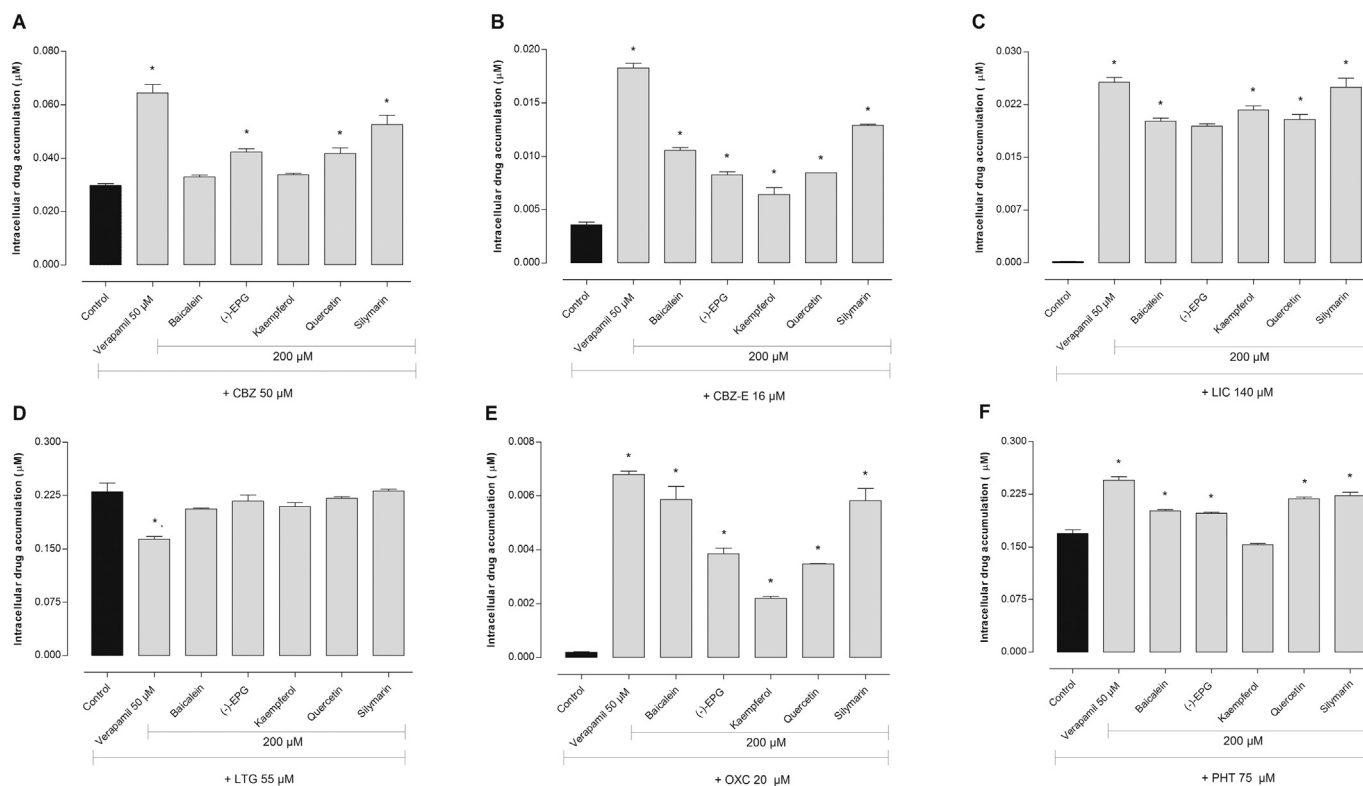


Fig. 6. Effect of selected flavonoid compounds or verapamil in the intracellular accumulation of (A) carbamazepine (CBZ), (B) carbamazepine-10,11-epoxide (CBZ-E), (C) licarbazepine (LIC), (D) lamotrigine (LTG), (E) oxcarbazepine (OXC) and (F) phenytoin (PHT) in MDCK-MDR1 cells. Data are expressed as the mean values \pm standard error of the mean ($n = 3$). Comparisons between flavonoids and verapamil groups vs. control (basal drug accumulation) group were performed by one-way ANOVA with the *post hoc* Dunnett's test. $^*p < 0.05$, compared to control group. EPG, (–)-epigallocatechin gallate.

compounds was based on an extensive literature review within this scope conducted by Ferreira et al. (2015). Moreover, as our main focus was the improvement of the epilepsy treatment, whenever possible a particular attention was given to flavonoid compounds with intrinsic anticonvulsant properties, aiming to find promising compounds potentially with dual and complementary mechanisms of action. According to our results, five flavonoids [baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin] were identified as promising P-gp inhibitors due to their ability to increase the intracellular accumulation of Rh123 in MDCK-MDR1 cells. Although we expected to find some flavonoid compounds capable of inhibiting P-gp, the increase on the intracellular accumulation of Rh123 promoted by (–)-epigallocatechin gallate at 200 μM was truly remarkable (a 16-fold increase), being similar to that found with verapamil, a standard P-gp inhibitor (Fig. 3).

The exact mechanisms underlying flavonoid–P-gp interactions are not yet clear. Nevertheless, several hypotheses have been proposed: (1) flavonoids can directly bind to the C-terminal nucleotide-binding domain of P-gp (NBD2) and modulate P-gp by interacting in a bifunctional way with the vicinal ATP-binding site and the steroid binding site within a cytosolic domain of P-gp (chrysin, flavone, quercetin, rutin, apigenin, 3-hydroxyflavone, genistein, kaempferide, and kaempferol) (Conseil et al., 1998); (2) flavonoids can act as substrate and may directly interact with P-gp either by competitive binding to the substrate-binding site or by binding to other drug-binding sites and changing the P-gp conformation (genistein, epicatechin gallate, catechin gallate, epigallocatechin gallate and silymarin) (Castro & Altenberg, 1997; Jodoin, Demeule, & Beliveau, 2002; Shapiro & Ling, 1997; Zhang & Morris, 2003a); and (3) flavonoids may bind to an allosteric site (epicatechin) (Wang, Barecki-Roach, & Johnson, 2002). It is worthy to mention that in our studies the inhibitory activity of baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin appeared to be concentration-dependent, increasing with flavonoids

concentrations. These results are in accordance with the literature (Ferreira et al., 2015).

In addition, other pharmacological properties that have been exhibited by flavonoid compounds can also contribute to enhance the interest in these agents to reverse the AED-resistant epilepsy. Indeed, the potential of flavonoids as antiepileptic/anticonvulsant drugs has been widely debated. For instance, baicalein (Yoon et al., 2011) and quercetin (Baluchnejadmojarad, Roghani, & Homayounfar, 2010; Nassiri-Asl et al., 2013, 2014) demonstrated some anticonvulsant activity in rodent models of acute and chronic seizures. Furthermore, the therapeutic potential of flavonoid compounds for frequent epilepsy comorbidities, due to their activity on cognition and neurodegeneration, inflammation and depression, emphasizes, even more, the interest in these multi-target compounds for complex central nervous system (CNS) disorders such as epilepsy. Particularly, quercetin demonstrated to have neuroprotective effects on hippocampal injury post status epilepticus (Hu et al., 2011), whereas baicalein evidenced a protective role against global ischemia (Romano et al., 2013). Moreover, quercetin demonstrated to inhibit both cyclooxygenase and lipoxygenase activities, diminishing the formation of inflammatory mediators. Additionally, flavonoids appear to inhibit eicosanoid biosynthesis and neutrophil degranulation (Nijveldt et al., 2001). Some flavonoids like quercetin and kaempferol also revealed antidepressant activity through their effects by the stimulation of brain-derived neurotrophic factor and the reduction of amyloid-beta peptide toxicity (Hou et al., 2010).

Although there are studies reporting some AEDs as P-gp substrates, this point was also herein investigated using MDCK II and MDCK-MDR1 cells, because there is still no consensus on this subject (Zhang et al., 2012). Taking into account our results, only the LTG did not demonstrate to be a P-gp substrate in the MDCK-MDR1 cell model; in contrast, OXC appears to have been subjected to a strong efflux transport mediated by P-gp (Fig. 5). According to the results of other *in vitro* and *in vivo* studies, including clinical trials, AEDs as LTG, PHT and OXC are classified as

definitive P-gp substrates, while CBZ, CBZ-E and S-LIC are classified only as probable P-gp substrates as deeply reviewed by Zhang et al. (2012). The main divergence between our results and those of the literature involves the LTG, which has not yet been investigated in MDCK-MDR1 cells; however, LTG was found to be a P-gp substrate in LLC-PK1 cells transfected with human MDR1 and in OS2.4/Doxo cells, but not in the Caco-2 cell line (Zhang et al., 2012). According to an *in vitro* transportation study performed by Zhang, Zuo, Kwan, and Baum (2011), CBZ analogues (eslicarbazepine acetate, OXC, S-LIC and CBZ-E) were found to be P-gp substrates. Indeed, there are few *in vitro* studies in the literature evaluating OXC as P-gp substrate but, in this particular one, OXC exhibited a higher rate of P-gp-mediated efflux transport than its derivatives (except for the eslicarbazepine acetate) (Zhang et al., 2011). Despite the availability of some studies that evaluated the structure-activity relationship of multiple P-gp substrates (Ekins et al., 2002; Raub, 2006; Wang, Kuo, Lien, & Lien, 2003), the reports analysing the structure-activity relationship regarding the interactions between AEDs and P-gp are limited (Knight & Weaver, 1998). Nevertheless, CBZ and its analogues have the identical dibenzazepine (iminostilbene) nucleus, differing at the 10,11-position; this planar structure has been reported to be very important for the interaction with P-gp (Ferreira et al., 2015). Consequently, the ketone group exhibited by OXC at the 10,11-position can be responsible for the higher affinity for P-gp evidenced by OXC when compared to CBZ and its structural analogues (Fig. 5).

After identifying the most promising flavonoids as P-gp inhibitors, and classifying the AEDs and their active metabolites as P-gp substrates or not in MDCK-MDR1 cells, the potential of the promising flavonoids [baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and silymarin] to promote the intracellular accumulation of the AEDs was also assessed. Although with different degrees of potency, almost all the selected flavonoids demonstrated to enhance the intracellular accumulation of AEDs or their active metabolites into MDCK-MDR1 cells. As expected, only the intracellular accumulation of LTG was not changed in the presence of flavonoids once it did not appear to be a P-gp substrate in the MDCK-MDR1 cells (Fig. 6D). The remarkable effect produced by tested flavonoids and verapamil on the intracellular accumulation of OXC is in accordance with its profile as P-gp substrate. Moreover, we have recently demonstrated in an exploratory study conducted in Wistar rats that silymarin and (–)-epigallocatechin gallate increase the systemic and brain exposure to OXC and its active metabolite LIC. More specifically, the pre-treatment of rats with silymarin and (–)-epigallocatechin gallate combinations originated peak plasma concentrations of OXC and LIC similar to those achieved in the presence of verapamil (positive control); additionally, the effects promoted by silymarin/(–)-epigallocatechin gallate combinations on the magnitude of systemic drug exposure to OXC and LIC were also reflected in the corresponding drug levels attained in the brain (biophase) (Ferreira, Rodrigues, Marques, Falcão, & Alves, 2017). Actually, other studies have also demonstrated that the huge differences observed in the transportation of OXC in MDCK and LLC MDR1-transfected *versus* non-transfected cells were almost completely abolished by verapamil and tariquidar, both P-gp inhibitors (Zhang et al., 2011). Indeed, the positive effect of verapamil on the OXC bioavailability was even reported in healthy volunteers (Antunes et al., 2016).

Furthermore, the set of tested flavonoid compounds did not seem to importantly compromise the cell viability. This is important when remembering that the first- and second-generation P-gp inhibitors are associated with severe toxicity at the concentrations necessary to significantly inhibit the P-gp (Potschka, 2012). Moreover, several flavonoid preparations are already found on the market as herbal medicines, or dietary supplements, alleged without nontoxic effects. Indeed, a variety of flavonoid-containing dietary supplements and herbal products are nowadays marketed for their antioxidant, anticarcinogenic, anti-inflammatory, antiproliferative, antiangiogenic and antiestrogenic (or estrogenic) effects, with no evidence of toxicity (Bansal et al., 2009; Ferreira et al., 2015). Additionally, in some animal studies flavonoid

compounds have revealed a weak potential of toxicity. Notwithstanding, to document the clinical value of these compounds as P-gp inhibitors capable of reversing the drug-resistant epilepsy requires the conduction of well-designed clinical trials.

In addition to the identification of AEDs or metabolites as P-gp substrates, the recognition of AEDs as P-gp inducers also highlights the importance of this strategy to reverse the drug resistance mediated by P-gp. In fact, the investigated AEDs and their active metabolites induced the P-gp activity (herein assessed by the decrease in intracellular accumulation of Rh123; Fig. 4). Hence, besides our recognition that all the AEDs tested, with exception of CBZ-E, had an inducer effect on the P-gp activity, the existing literature is not extensive regarding these aspects. In fact, only PHT and CBZ are particularly recognised as P-gp inducers (Akamine, Miura, Yasui-Furukori, Kojima, & Uno, 2012; Chhun et al., 2009; Owen, Goldring, Morgan, Park, & Pirmohamed, 2006; U.S. Food and Drug Administration, 2011).

5. Conclusions

In conclusion, five out of eleven flavonoid compounds tested were found to increase the intracellular accumulation of Rh123 P-gp probe substrate in MDCK-MDR1 cells, as well as of AEDs (CBZ, OXC and PHT) and their active metabolites (CBZ-E and LIC) recognised as substrates of the P-gp. Hence, these results corroborate that the strategy of co-administration of AEDs with safe and potent P-gp inhibitors should continue to be exploited to reverse the pharmacoresistance in epilepsy, and flavonoids such as baicalein, (–)-epigallocatechin gallate, kaempferol, quercetin and particularly silymarin are promising drug candidates to be developed as P-gp inhibitors.

Abbreviations

AEDs	Antiepileptic drugs
CBZ	Carbamazepine
CBZ-E	Carbamazepine-10,11-epoxide
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium - high glucose
FBS	Fetal bovine serum
HPLC	High-performance liquid chromatography
LIC	Licarbazepine
LTG	Lamotrigine
MDCK II	Madin-Darby canine kidney cell line
MDCK-MDR1	Madin-Darby canine kidney cell line overexpressing the human multidrug resistance-1
MDR1	Human multidrug resistance-1
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
OXC	Oxcarbazepine
PBS	Phosphate-buffered saline
P-gp	P-glycoprotein
PHT	Phenytoin
Rh123	Rhodamine 123

Conflict of interest statement

The authors have declared no conflict of interest.

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References

- Abdallah, H. M., Al-Abd, A. M., El-Dine, R. S., & El-Halawany, A. M. (2015). P-glycoprotein inhibitors of natural origin as potential tumor chemo-sensitizers: A review. *Journal of Advanced Research*, 6(1), 45–62.
- Akamie, Y., Miura, M., Yasui-Furukori, N., Kojima, M., & Uno, T. (2012). Carbamazepine differentially affects the pharmacokinetics of fexofenadine enantiomers. *British Journal of Clinical Pharmacology*, 73(3), 478–481.
- Antunes, N., Wichert-Ana, L., Coelho, E. B., Della Pasqua, O., Alexandre Junior, V., Takayanagi, O. M., ... Lanchote, V. L. (2016). Influence of verapamil on the pharmacokinetics of oxcarbazepine and of the enantiomers of its 10-hydroxy metabolite in healthy volunteers. *European Journal of Clinical Pharmacology*, 72(2), 195–201.
- Baluchnejadmojarad, T., Roghani, M., & Homayounfar, H. (2010). Inhibitory effect of high dose of the flavonoid quercetin on amygdala electrical kindling in rats. *Basic Clinical Neurosciences*, 1(3), 57–61.
- Bansal, T., Awasthi, A., Jaggi, M., Khar, R. K., & Talegaonkar, S. (2008). Pre-clinical evidence for altered absorption and biliary excretion of irinotecan (CPT-11) in combination with quercetin: Possible contribution of P-glycoprotein. *Life Sciences*, 83(7–8), 250–259.
- Bansal, T., Jaggi, M., Khar, R. K., & Talegaonkar, S. (2009). Emerging significance of flavonoids as P-glycoprotein inhibitors in cancer chemotherapy. *Journal of Pharmacy & Pharmaceutical Sciences*, 12(1), 46–78.
- Barthoumeuf, C., Grassi, J., Demeule, M., Fournier, C., Boivin, D., & Béliveau, R. (2005). Inhibition of P-glycoprotein transport function and reversion of MDR1 multidrug resistance by cniadin. *Cancer Chemotherapy and Pharmacology*, 56(2), 173–181.
- Bartmann, H., Fuest, C., la Fougere, C., Xiong, G., Just, T., Schlichtiger, J., ... Potschka, H. (2010). Imaging of P-glycoprotein-mediated pharmacoresistance in the hippocampus: Proof-of-concept in a chronic rat model of temporal lobe epilepsy. *Epilepsia*, 51(9), 1780–1790.
- Bauer, B., Hartz, A. M. S., Pekcec, A., Toellner, K., Miller, D. S., & Potschka, H. (2008). Seizure-induced up-regulation of P-glycoprotein at the blood-brain barrier through glutamate and cyclooxygenase-2 signaling. *Molecular Pharmacology*, 73(5), 1444–1453.
- Baulac, M., de Boer, H., Elger, C., Glynn, M., Kälviäinen, R., Little, A., & Ryvlin, P. (2015). Epilepsy priorities in Europe: A report of the ILAE-IBE epilepsy advocacy Europe task force. *Epilepsia*, 56(11), 1687–1695.
- Brandt, C., Bethmann, K., Gastens, A. M., & Löscher, W. (2006). The multidrug transporter hypothesis of drug resistance in epilepsy: Proof-of-principle in a rat model of temporal lobe epilepsy. *Neurobiology of Disease*, 24(1), 202–211.
- Castro, A. F., & Altenberg, G. A. (1997). Inhibition of drug transport by genistein in multidrug-resistant cells expressing P-glycoprotein. *Biochemical Pharmacology*, 53(1), 89–93.
- Chen, Z., Shi, T., Zhang, L., Zhu, P., Deng, M., Huang, C., ... Li, J. (2016). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in multidrug resistance: A review of the past decade. *Cancer Letters*, 370(1), 153–164.
- Chhun, S., Verstuyft, C., Rizzo-Padoin, N., Simoneau, G., Becquemont, L., Peretti, I., & Mouly, S. (2009). Gefitinib-phenytoin interaction is not correlated with the C-erythromycin breath test in healthy male volunteers. *British Journal of Clinical Pharmacology*, 68(2), 226–237.
- Clinckers, R., Smolders, I., Meurs, A., Ebinger, G., & Michotte, Y. (2005). Quantitative in vivo microdialysis study on the influence of multidrug transporters on the blood-brain barrier passage of oxcarbazepine: Concomitant use of hippocampal monoamines as pharmacodynamic markers for the anticonvulsant activity. *The Journal of Pharmacology and Experimental Therapeutics*, 314(2), 725–731.
- Conseil, G., Baubichon-Cortay, H., Dayan, G., Jault, J. M., Barron, D., & Di Pietro, A. (1998). Flavonoids: A class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America*, 95(17), 9831–9836.
- Dombrowski, S. M., Desai, S. Y., Marroni, M., Cucullo, L., Goodrich, K., Bingaman, W., ... Janigro, D. (2001). Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. *Epilepsia*, 42(12), 1501–1506.
- Ekins, S., Kim, R. B., Leake, B. F., Dantzig, A. H., Schuetz, E. G., Lan, L.-B., & Wrighton, S. A. (2002). Application of three-dimensional quantitative structure-activity relationships of P-glycoprotein inhibitors and substrates. *Molecular Pharmacology*, 61(5), 974–981.
- Ferreira, A., Pousinho, S., Fortuna, A., Falcão, A., & Alves, G. (2015). Flavonoid compounds as reversal agents of the P-glycoprotein-mediated multidrug resistance: Biology, chemistry and pharmacology. *Phytochemistry Reviews*, 14(2), 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. *Journal of Chromatographic Science*, 54(8), 1352–1358.
- 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(9), 581–590.
- Ferreira, A., Rodrigues, M., Marques, A., Falcão, A., & Alves, G. (2017). Influence of the dual combination of silymarin and (–)-epigallocatechin gallate, natural dietary flavonoids, on the pharmacokinetics of oxcarbazepine in rats. *Food and Chemical Toxicology*, 106, 446–454.
- Franco, V., French, J. A., & Perucca, E. (2016). Challenges in the clinical development of new antiepileptic drugs. *Pharmacological Research*, 103, 95–104.
- Freshney, R. I. (2010). *Culture of animal cells - a manual of basic technique and specialized applications* (6th ed.). Hoboken, New Jersey: Wiley-Blackwell, Ed.
- Gidal, B. E. (2014). P-glycoprotein expression and pharmacoresistant epilepsy: Cause or consequence? *Epilepsy Current*, 14(3), 136–138.
- Höcht, C., Lazarowski, A., Gonzalez, N. N., Auzmendi, J., Opezzo, J. A. W., Bramuglia, G. F., ... Girardi, E. (2007). Nimodipine restores the altered hippocampal phenytoin pharmacokinetics in a refractory epileptic model. *Neuroscience Letters*, 413(2), 168–172.
- Hou, Y., Aboukhatwa, M. A., Lei, D.-L., Manaye, K., Khan, I., & Luo, Y. (2010). Anti-depressant natural flavonols modulate BDNF and beta amyloid in neurons and hippocampus of double TgAD mice. *Neuropharmacology*, 58(6), 911–920.
- Hoyland, K., Hoy, M., Austin, R., & Wildman, M. (2013). Successful use of haemodialysis to treat phenobarbital overdose. *BMJ Case Reports* (pii: bcr2013010011).
- Hu, K., Li, S.-Y., Xiao, B., Bi, F., Lu, X.-Q., & Wu, X.-M. (2011). Protective effects of quercetin against status epilepticus-induced hippocampal neuronal injury in rats: Involvement of X-linked inhibitor of apoptosis protein. *Acta Neurologica Belgica*, 111(3), 205–212.
- Iannetti, P., Spalice, A., & Parisi, P. (2005). Calcium-channel blocker verapamil administration in prolonged and refractory status epilepticus. *Epilepsia*, 46(6), 967–969.
- Jodoin, J., Demeule, M., & Béliveau, R. (2002). Inhibition of the multidrug resistance P-glycoprotein activity by green tea polyphenols. *Biochimica et Biophysica Acta*, 1542(1–3), 149–159.
- Jóźwiak, S. (2007). Contemporary opinions on classification, pathogenesis and treatment of drug-resistant epilepsy. *Wiadomości Lekarskie*, 60(5–6), 258–264.
- Knight, J. L., & Weaver, D. F. (1998). A computational quantitative structure-activity relationship study of carbamate anticonvulsants using quantum pharmacological methods. *Seizure*, 7(5), 347–354.
- Kwan, P., Li, H. M., Al-Jufairi, E., Abdulla, R., Gonzales, M., Kaye, A. H., & O'Brien, T. J. (2010). Association between temporal lobe P-glycoprotein expression and seizure recurrence after surgery for pharmacoresistant temporal lobe epilepsy. *Neurobiology of Disease*, 39(2), 192–197.
- Liu, X., Yang, Z., Yang, J., & Yang, H. (2007). Increased P-glycoprotein expression and decreased phenobarbital distribution in the brain of pentylentetrazole-kindled rats. *Neuropharmacology*, 53(5), 657–663.
- Löscher, W., Klitgaard, H., Twyman, R. E., & Schmidt, D. (2013). New avenues for anti-epileptic drug discovery and development. *Nature Reviews. Drug Discovery*, 12(10), 757–776.
- Luna-Tortós, C., Fedrowitz, M., & Löscher, W. (2008). Several major antiepileptic drugs are substrates for human P-glycoprotein. *Neuropharmacology*, 55(8), 1364–1375.
- Marchi, N., Guiso, G., Caccia, S., Rizzi, M., Gagliardi, B., Noé, F., ... Vezzani, A. (2006). Determinants of drug brain uptake in a rat model of seizure-associated malformations of cortical development. *Neurobiology of Disease*, 24(3), 429–442.
- Marchi, N., Hallene, K. L., Kight, K. M., Cucullo, L., Moddel, G., Bingaman, W., & Janigro, D. (2004). Significance of MDR1 and multiple drug resistance in refractory human epileptic brain. *BMC Medicine*, 2, 37.
- Mohana, S., Ganesan, M., Agilan, B., Karthikeyan, R., Srithar, G., Beulah Mary, R., ... Ambudkar, S. V. (2016). Screening dietary flavonoids for the reversal of P-glycoprotein-mediated multidrug resistance in cancer. *Molecular BioSystems*, 12(8), 2458–2470.
- Nassiri-Asl, M., Hajiali, F., Taghiloo, M., Abbasi, E., Mohseni, F., & Yousefi, F. (2014). Comparison between the effects of quercetin on seizure threshold in acute and chronic seizure models. *Toxicology and Industrial Health*, 32(5), 936–944.
- Nassiri-Asl, M., Moghbelinejad, S., Abbasi, E., Yonesi, F., Haghghi, M.-R., Lotfizadeh, M., & Bazahang, P. (2013). Effects of quercetin on oxidative stress and memory retrieval in kindled rats. *Epilepsy & Behavior*, 28(2), 151–155.
- Nijveldt, R. J., van Nood, E., van Hoorn, D. E., Boelens, P. G., van Norren, K., & van Leeuwen, P. A. (2001). Flavonoids: A review of probable mechanisms of action and potential applications. *The American Journal of Clinical Nutrition*, 74(4), 418–425.
- Nishimura, A., Honda, N., Sugioka, N., Takada, K., & Shibata, N. (2008). Evaluation of carbamazepine pharmacokinetic profiles in mice with kainic acid-induced acute seizures. *Biological & Pharmaceutical Bulletin*, 31(12), 2302–2308.
- Owen, A., Goldring, C., Morgan, P., Park, B. K., & Pirmohamed, M. (2006). Induction of P-glycoprotein in lymphocytes by carbamazepine and rifampicin: The role of nuclear hormone response elements. *British Journal of Clinical Pharmacology*, 62(2), 237–242.
- Palmeira, A., Sousa, E., Vasconcelos, M. H., & Pinto, M. M. (2012). Three decades of P-gp inhibitors: Skimming through several generations and scaffolds. *Current Medicinal Chemistry*, 19(13), 1946–2025.
- Patsalos, P. N., Berry, D. J., Bourgeois, B. F. D., Cloyd, J. C., Glauser, T. A., Johannessen, S. I., ... Perucca, E. (2008). Antiepileptic drugs-best practice guidelines for therapeutic drug monitoring: A position paper by the subcommission on therapeutic drug monitoring, ILAE commission on therapeutic strategies. *Epilepsia*, 49(7), 1239–1276.
- Potschka, H. (2012). Role of CNS efflux drug transporters in antiepileptic drug delivery: Overcoming CNS efflux drug transport. *Advanced Drug Delivery Reviews*, 64(10), 943–952.
- Raub, T. J. (2006). P-glycoprotein recognition of substrates and circumvention through rational drug design. *Molecular Pharmaceutics*, 3(1), 3–25.
- Robey, R., Lazarowski, A., & Bates, S. (2008). P-glycoprotein - a clinical target in drug-refractory epilepsy? *Molecular Pharmacology*, 73(5), 1343–1346.
- Rogawski, M. A. (2013). The intrinsic severity hypothesis of pharmacoresistance to antiepileptic drugs. *Epilepsia*, 54(Suppl. 2), 33–40.
- Romano, B., Pagano, E., Montanaro, V., Fortunato, A. L., Milic, N., & Borrelli, F. (2013). Novel insights into the pharmacology of flavonoids. *Phytotherapy Research*, 27(11), 1588–1596.
- Schinkel, A. H., & Jonker, J. W. (2012). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: An overview. *Advanced Drug Delivery Reviews*, 55(1), 3–29.
- Shapiro, A. B., & Ling, V. (1997). Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *European Journal of Biochemistry*, 250(1), 130–137.
- Stouch, T. R., & Gudmundsson, O. (2002). Progress in understanding the structure-

- activity relationships of P-glycoprotein. *Advanced Drug Delivery Reviews*, 54(3), 315–328.
- Summers, M. A., Moore, J. L., & McAuley, J. W. (2004). Use of verapamil as a potential P-glycoprotein inhibitor in a patient with refractory epilepsy. *The Annals of Pharmacotherapy*, 38(10), 1631–1634.
- U.S. Food and Drug Administration. *Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers*. (2011). Retrieved from <http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm> (Consulted in May 2017).
- Ventola, C. L. (2014). Epilepsy management: Newer agents, unmet needs, and future treatment strategies. *P T*, 39(11), 776–792.
- Wang, E., Barecki-Roach, M., & Johnson, W. W. (2002). Elevation of P-glycoprotein function by a catechin in green tea. *Biochemical and Biophysical Research Communications*, 297(2), 412–418.
- Wang, G.-X., Wang, D.-W., Liu, Y., & Ma, Y.-H. (2016). Intractable epilepsy and the P-glycoprotein hypothesis. *The International Journal of Neuroscience*, 126(5), 385–392.
- Wang, R. B., Kuo, C. L., Lien, L. L., & Lien, E. J. (2003). Structure-activity relationship: Analyses of p-glycoprotein substrates and inhibitors. *Journal of Clinical Pharmacy and Therapeutics*, 28(3), 203–228.
- Weiss, J., Kerpen, C. J., Lindenmaier, H., Dormann, S.-M. G., & Haefeli, W. E. (2003). Interaction of antiepileptic drugs with human P-glycoprotein in vitro. *The Journal of Pharmacology and Experimental Therapeutics*, 307(1), 262–267.
- Xiong, J., Mao, D.-A., & Liu, L.-Q. (2015). Research progress on the role of ABC transporters in the drug resistance mechanism of intractable epilepsy. *BioMed Research International*, 2015, 194541.
- Yoon, S. Y., dela Peña, I. C., Shin, C. Y., Son, K. H., Lee, Y. S., Ryu, J. H., ... Ko, K. H. (2011). Convulsion-related activities of Scutellaria flavones are related to the 5,7-dihydroxyl structures. *European Journal of Pharmacology*, 659(2–3), 155–160.
- Zhang, C., Kwan, P., Zuo, Z., & Baum, L. (2012). The transport of antiepileptic drugs by P-glycoprotein. *Advanced Drug Delivery Reviews*, 64(10), 930–942.
- Zhang, C., Zuo, Z., Kwan, P., & Baum, L. (2011). In vitro transport profile of carbamazepine, oxcarbazepine, eslicarbazepine acetate, and their active metabolites by human P-glycoprotein. *Epilepsia*, 52(10), 1894–1904.
- Zhang, S., & Morris, M. E. (2003a). Effect of the flavonoids biochanin A and silymarin on the P-glycoprotein-mediated transport of digoxin and vinblastine in human intestinal Caco-2 cells. *Pharmaceutical Research*, 20(8), 1184–1191.
- Zhang, S., & Morris, M. E. (2003b). Effects of the flavonoids biochanin A, morin, phloracetin, and silymarin on P-glycoprotein-mediated transport. *The Journal of Pharmacology and Experimental Therapeutics*, 304(3), 1258–1267.