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Research paper

Rat intestinal precision-cut slices as an *in vitro* model to study xenobiotic interaction with transporters

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ABSTRACT

ATP-binding cassette (ABC) proteins play key role in tissue defence by transporting metabolic waste and toxic chemicals out of the cells. Consequently, intact cell systems are required to study xenobiotic interactions with ATP-dependent transporters. The aim of the present study was to set up an intestinal precision-cut slice technique to study the interactions of ABC transporters with xenobiotics. Rat intestinal slices were incubated with verapamil, indomethacin and glibenclamide, and the ability of the above-mentioned drugs to inhibit the multidrug resistance glycoprotein (MDR) and/or multidrug-resistance-associated protein (MRP) was assessed by measuring the intracellular conversion of calcein-AM to fluorescent calcein. The ABC transporters' inhibitors caused a time-dependent florescence increase which reached the maximum value at 30 min. Verapamil and glibenclamide promoted a concentration-dependent intracellular accumulation of calcein (IC_{50} 8.1×10^{-6} M, 1.9×10^{-4} M, respectively). The effect of glibenclamide was fully reversed by washing the slices, suggesting the reversible nature of calcein accumulation. These data suggest that the precision-cut intestinal slices are a reliable, simple and fast system to evaluate xenobiotic interactions with ABC transporters in rat and, hopefully, in human intestine.

1. Introduction

The intestine is a crucial organ with respect to toxicity of ingested compounds, because of its extensive exposed surface area as well as its absorptive and metabolic properties. There have been many studies of this organ regarding the investigation of the mechanism involved in detoxification or toxic activation of xenobiotics. Phase I and/or phase II of xenobiotic metabolism as well as active extrusion pumps (phase III) has been reported as a major physiological mechanism to protect intestine and, consequently, the whole organism from toxic compounds. Intestinal epithelial cells express a broad spectrum of proteins that interact with xenobiotics promoting their efflux into the intestinal lumen or into the circulation [1,2]. These processes make fundamental contribution to the bioavailability of xenobiotics and/or their metabolites by transporting them out of the cells [3].

ATP-binding cassette (ABC) family proteins includes the multidrug resistance 1/P-glycoprotein (MDR1/Pgp), which belongs to the ABCB1 subfamily, and the multidrug resistance–associated proteins (MRPs) that belong to the ABCC subfamily. Pgp, which transports amphipathic cations from cells [4], is encoded in human by *MDR1* gene, whereas it is encoded by *Mdr1a* and *Mdr1b* gene in the rat [5]. Pgp is predominantly located in the apical membranes of the epithelia on the luminal surface of small intestine and colon. Members of the MRPs family (MRP1–9) of xenobiotic transporters are important in the ATP-dependent transport of many organic anions including many phase II metabolites [6–13]. MRP1, 2 and 3 are export pumps responsible for the resistance of cells to cytotoxic drugs [14]. Because of the role of ABC transporters in the regulation of the xenobiotic bioavailability, it is important to set up a reliable *in vitro* model that mimics the interactions of drugs with transport proteins as it occurs in gut and other tissues *in vivo*. Several human cell lines, such as Caco-2 and LS180, have proven to be useful to study ATP-dependent transporters [15]. However, it is commonly known that these cell lines present incomplete enzyme- and transporter systems [16,17]. The Caco-2 cell has shown inter-laboratory variability and defective oxidative metabolism. Furthermore, it is deficient in the pregnane X receptor (PXR) and the constitutive receptor (CAR) [18], responsible for the regulation of phase I, II and III enzymes [19]. These problems make this cell line less suitable to study the regulation of xenobiotic disposable [15]. On the contrary, LS180 cells are a good model to study MDR1 and CYP3A4

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induction, but for drug transport studies, Caco-2 would be preferred as the more physiological model [20].

The use of intact tissue preserves the existence of different cell types, maintains cell–cell contacts, and the presence of enzyme, co-factors and transporters in a relative physiological context, permitting the study of the phase I, II and III reactions involved in intestinal detoxification.

Recently, van de Kerkhof et al. [19] have validated the precision-cut slice technique with rat intestinal tissue for metabolism and drug induction. This suggests that this model might be used to study several aspects of intestinal metabolism *in vitro*.

The aim of the present study was to set up an experimental model, based on rat intestine precision-cut slice technique, to assay the interactions of xenobiotics with ATP-dependent transporters. In particular, the effects of glibenclamide, indomethacin (specific MRP inhibitors) [21] and verapamil (Pgp inhibitor) [22] on ABC transporters were studied by following the intracellular de-esterification of calcein-AM to the fluorescent compound calcein.

2. Materials and methods

2.1. Materials

RPMI 1640 culture medium and foetal bovine serum were purchased from BioWhittaker, Cambrex, (Belgium); D-glucose, insulin, hydrocortisone-21 hemisuccinate, amphotericin B, L-methionine, gentamycin, NADH, sodium pyruvate, low gelling temperature agarose (type VII-A), calcein acetoxymethyl ester (calcein-AM), verapamil, indomethacin, glibenclamide and MK571 were purchased from Sigma Aldrich (Milan, Italy). MC176 was synthesized as described elsewhere [23]. All other chemicals and solvents were of the highest grade available and obtained from common commercial sources.

2.2. Animals

All experiments were performed in strict compliance with the recommendation of the EEC (86/609/CEE) for the care and use of laboratory animals, and the protocols were approved by the Animal Care and Ethics Committee of the University of Siena, Italy. Male Wistar albino rats (250 g body weight, Charles River Italia, Calco, Italy) were kept in large cages under a 12:12 h day–night cycle at 20 °C (ambient temperature). Drinking water and conventional laboratory rat food were available *ad libitum*. Rats were fasted overnight prior to the sacrifice and anaesthetized by intraperitoneal injection of xylazine chloride (10 mg kg^{-1}, Rompun® Vet., Bayer AG, Germany) and ketamine hydrochloride (35 mg kg^{-1}, Ketavet®, Parke Davis/Warner-Lambert, USA).

2.3. Preparation of precision-cut tissue slices

Intestine precision-cut slices were prepared as described by de Kanter et al. [24]. Briefly, the small intestine was rapidly excised and immerged in ice-cold, oxygenated Krebs–Henseleit buffer with the following composition (mM): NaCl 116, KCl 4.6, $NaHCO_3$ 25.4, $MgSO_4$ and KH_2PO_4 1.16, $CaCl_2$ 2.5, Hepes 20 and D-glucose 25, final pH 7.4. The jejunum was rinsed with cold buffer, cut in a 12 cm segment length and tightly closed at one side, filled with 3% (w/v) low-melting agarose solution dissolved in 37 °C-heated 0.9% NaCl solution and cooled in ice-cold Krebs–Henseleit buffer allowing the agarose solution to gel. The filled segment was embedded in 37 °C agarose and allowed to gel at 4 °C. Afterwards, 400 μm thickness precision-cut slices were prepared (Krumdieck tissue slicer, Alabama Research and Development, Munford, USA) and maintained in RPMI 1640 culture medium for 30 min to allow maximal recovery from slicing trauma [24,25].

Slices were then individually placed in a 12-well culture plate and 0.5 ml of RPMI 1640, supplemented with 5% foetal bovine serum, 0.5 mM L-methionine, 1 μM insulin, 50 μg/ml gentamycin, 0.1 mM hydrocortisone 21-hemisuccinate and 2.5 μg/ml, final concentration, amphotericin B [26]. Culture plates were incubated at 37 °C in a humidified 5% CO_2-containing atmosphere under shaking (50 times/min).

2.4. Histomorphology of slices

Slices viability were investigated after 120 min of incubation with RPMI. At the end of incubation time, samples were rinsed thrice for 5 min with fresh medium and then fixed with Karnovsky's fixative in 0.1 M cacodylate buffer (pH 7.35) for 1 h and post-fixed for 1 h with 1% osmium tetroxide dissolved in 0.1 M cacodylate buffer. Finally, samples were dehydrated and embedded in Epon 812 according to standard procedures.

2.5. Assay of intracellular accumulation of calcein-AM/calcein

Calcein-AM is a nonfluorescent and lipophilic compound, which rapidly diffuses through the plasma membrane into the cells and is effluxed by Pgp; furthermore, it is metabolized by intracellular esterases to calcein, a hydrophilic and highly fluorescent compound, which is effluxed by MRP1 and MRP2 but not by Pgp [9,27–29]. Thus, drug-induced inhibition of Pgp and/or MRPs promotes an increase in the intracellular concentration of calcein, which can be measured spectrofluorimetrically (λ_{ex} 488 nm and λ_{em} 518 nm). DMSO or water stock solutions of glibenclamide, verapamil, MC176 and MK571 were prepared (200 mM) and diluted with incubation *medium* to the established final concentration immediately prior to use. The final concentration of DMSO in the incubation medium was equal or lower than 0.02% v/v.

Time-course studies of calcein accumulation were performed by incubating intestinal slices at different times (1–60 min) in the presence of 0.5 μM calcein-AM and vehicle (controls), 0.5 μM calcein-AM and 100 μM glibenclamide, or a mixture containing 50 μM of either glibenclamide, indomethacin or verapamil. The slices were then rinsed twice with 1 ml of phosphate buffer saline (PBS) in order to stop incubation and then homogenized in 0.1% Triton X-100. After centrifugation (5000 *g* for 6 min), the fluorescence of calcein was measured in the supernatant.

Concentration dependence studies were performed by incubating the intestinal slices for 30 min with 0.5 μM calcein-AM and increasing concentrations of drugs (i.e. 1×10^{-6}–5×10^{-4} M verapamil, 1×10^{-6}–1×10^{-4} M indomethacin, 1×10^{-6}–2.5×10^{-3} M glibenclamide, 1×10^{-10}–1×10^{-5} M MC176 and 2.5×10^{-5}–2.5×10^{-4} M MK571).

To assess whether drug-induced calcein accumulation was a reversible process, glibenclamide, the most potent inhibitor tested in previous experiments, was used; 100 μM glibenclamide-pretreated slices (30 min) were washed with PBS (three times for 10 min), and then 0.5 μM calcein-AM or 0.5 μM calcein-AM and 100 μM glibenclamide were added. Controls were performed by omitting glibenclamide in the first incubation step. For these experiments, the use of a single inhibitor was preferred to a mixture of inhibitors in order to avoid drug–drug interactions that could hamper the interpretation of the results.

2.6. Statistical analysis

All the experiments were performed by using intestinal slices derived from at least four different rats. Data are reported as mean ± SEM, and "*n*" is defined as the number of samples.

In concentration dependence studies, data were expressed as the percentage of calcein found in homogenates of drug-treated slices compared to that found in untreated slices. Sigmoidal concentration-dependent inhibition curves were calculated by best fitting data obtained by plotting the percentage of calcein *versus* log of inhibitor concentration, and the concentration that induced 50% of the maximum calcein accumulation was calculated by using GraphPadPrism5 program (GraphPad Inc., USA). Statistical analysis was performed by using Student's *t*-test or one-way ANOVA followed by *post hoc* Turkey test, as appropriate. $p < 0.05$ was considered significant.

3. Results

3.1. Slices viability

As shown in Fig. 1, the general morphology of the tissue is well preserved after 120 min of incubation. Intestinal *villi* are still completely covered by the epithelium and they appear shortened. Intestinal crypts are also well maintained. The viability of intestinal slices was maintained up to 24 h (data not shown).

3.2. Time-dependent accumulation of calcein-AM

Fig. 2 shows the time-course of calcein accumulation in the intestinal precision-cut slices incubated with 0.5 μM calcein-AM. Fluorescence did not change significantly under control conditions, whereas it peaked after 30 min, returning to control value at 60 min, in the slices treated with 100 μM glibenclamide. When the intestinal slices were incubated with the MRP- or Pgp inhibitors (50 μM verapamil + 50 μM glibenclamide + 50 μM indomethacin), the maximum of calcein accumulation occurred at 30 min, with a threefold increase in fluorescence, as compared to the control slices. These results show that the inhibitor mixture is more potent than glibenclamide alone in inhibiting the intestinal slice transporters. After 60 min incubation, calcein accumulation decreased, without reaching the control value. Thus, 30 min incubation period was selected as the *optimum* time to perform inhibition studies.

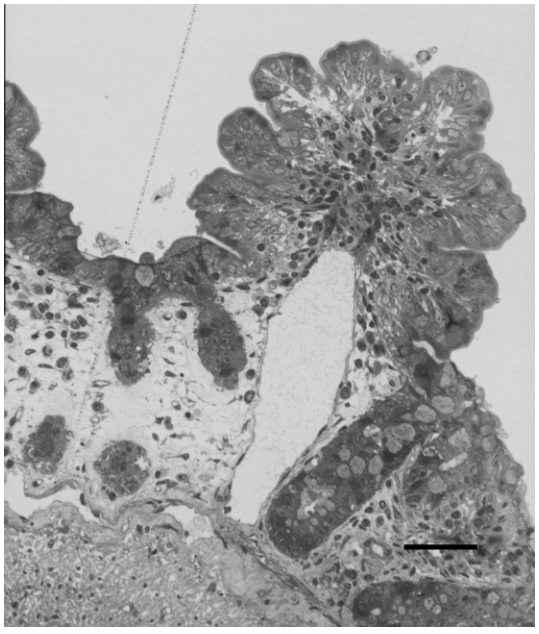

Fig. 1. Semi-thin section of an intestinal slice incubated for 30 min. The general morphology of the tissue is well preserved. Intestinal *villi* are still completely covered by the epithelium and they appear shortened. Intestinal crypts are also well maintained. In the longitudinal axis of the *villus*, it is evident as a large lymphatic vessel. Staining was done with toluidine blue. For further details, see Section 2. Magnification bar = 80 μm.

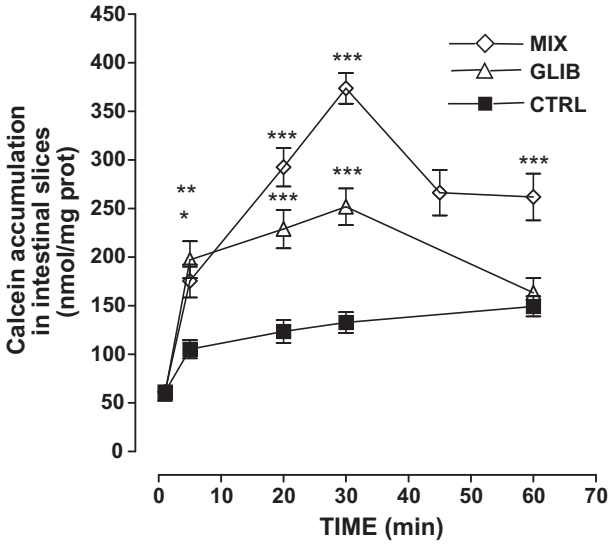

Fig. 2. Time-course of calcein accumulation in the intestinal precision-cut slices in the presence (△) and absence of (■) 100 μM glibenclamide or with a mixture (◇) containing 50 μM either verapamil, glibenclamide or indomethacin. Results are expressed as means ± SEM of fluorescent value recorded in the homogenate of intestinal slices from four different rats. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.z

3.3. Inhibition studies

Concentration dependence studies showed that verapamil induced intracellular calcein accumulation to about 150% at the highest concentration used (5×10^{-4} M) (Fig. 3). Indomethacin, which is a fairly specific MRP inhibitor, increased calcein accumulation to 165% at the highest concentration tested (1×10^{-4} M) (Fig. 4). It was not possible to test higher concentrations due to its very low water solubility. Glibenclamide, MRP and Pgp inhibitor [30] increased calcein accumulation to 409% at the highest concentration tested (2.5×10^{-3} M) with 1.9×10^{-4} M IC_{50} value (Fig. 5). Verapamil was shown to have a low efficacy, when compared to glibenclamide, since florescence values of intestinal slices reached a plateau value of 252%.

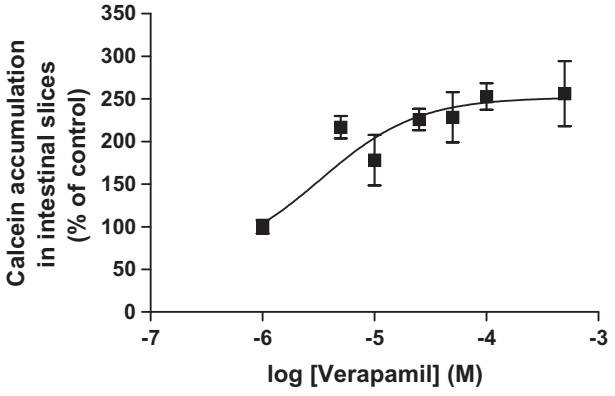

Fig. 3. Concentration-dependent increase in intracellular fluorescence in rat precision-cut intestine slices caused by increasing concentration of verapamil (1×10^{-6}–5×10^{-4} M). Each point represents the mean ± SEM of results recorded in the homogenate of intestinal slices from four different rats; 100% corresponded to the formation of 41 nmol/mg protein of calcein.

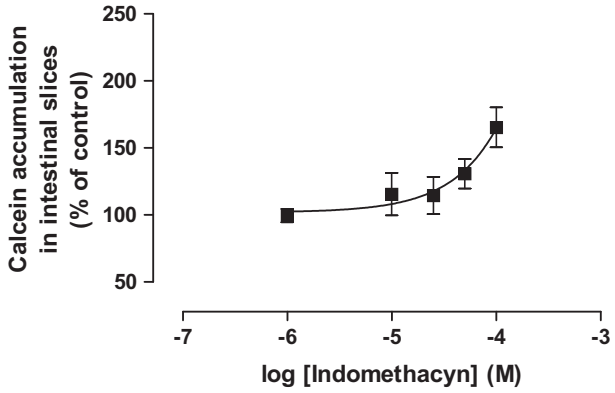

Fig. 4. Concentration-dependent increase in intracellular fluorescence in rat precision-cut intestine slices in the presence of increasing concentration of indomethacin (1×10^{-6}–1×10^{-4} M). Each point represents the mean ± SEM of results recorded in the homogenate of intestinal slices from four different rats; 100% corresponded to the formation of 43 nmol/mg protein of calcein.

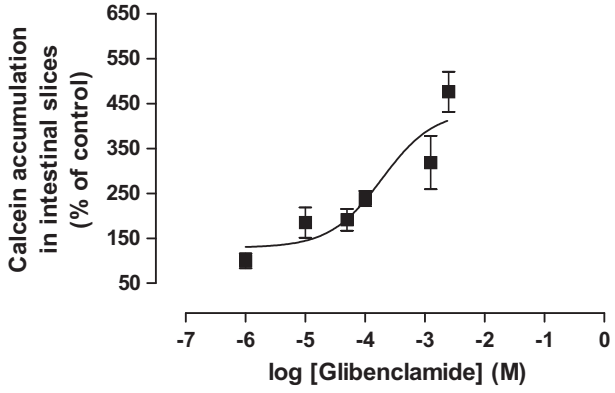

Fig. 5. Concentration-dependent increase in intracellular fluorescence in rat precision-cut intestine slices in the presence of increasing concentration of glibenclamide (1×10^{-6}–2.5×10^{-3} M). Each point represents the mean ± SEM of results recorded in the homogenate of intestinal slices from four different rats; 100% corresponded to the formation of 45 nmol/mg protein of calcein.

MC176, a *N,N*-bis(cyclohexanol)amine aryl ester, synthesized as novel Pgp reverter compound [23,31,32] was tested. As shown in Fig. 6, the compound caused a 360% *maximum* increase in calcein accumulation at the concentration of 1×10^{-5} M, with an IC_{50} of 1.6×10^{-8} M, i.e. four orders of magnitude more potent than glibenclamide.

Finally, MK571 was used as specific inhibitor for the MRPs. The compound caused an increase in calcein accumulation up to 160%, at the *maximum* concentration used (2.5×10^{-4} M) with an IC_{50} value of 8.6×10^{-5} M, a value twofold lower than IC_{50} of glibenclamide (Fig.7).

3.4. Reversibility of transporter inhibition

To confirm that calcein accumulation was solely related to ATP-dependent transporters and not dependent on nonspecific and/or irreversible processes, reversibility experiments were performed by determining the recovery of fluorescence upon dilution of the inhibitor/transporter mixture. As shown in Fig. 8, the inhibition of calcein accumulation by glibenclamide (GL) was reversed by washing (W − GL) and restored by the addition of fresh glibenclamide (W + GL), indicating that the inhibition exerted by this drug was fully reversible. Since the florescence of homogenates of W + GL slices was similar to that observed in GL sample, it can be concluded that calcein accumulation was strictly dependent on the presence of glibenclamide in the culture medium.

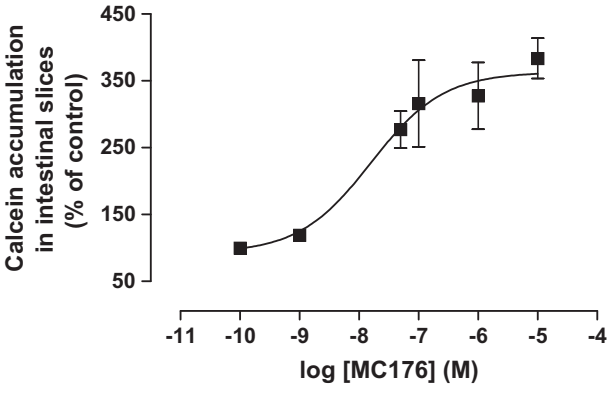

Fig. 6. Concentration-dependent increase in intracellular fluorescence in rat precision-cut intestine slices in the presence of increasing concentration of MC176 (1×10^{-10}–1×10^{-5} M). Each point represents the mean ± SEM of results recorded in the homogenate of intestinal slices from four different rats; 100% corresponded to the formation of 38 nmol/mg protein of calcein.

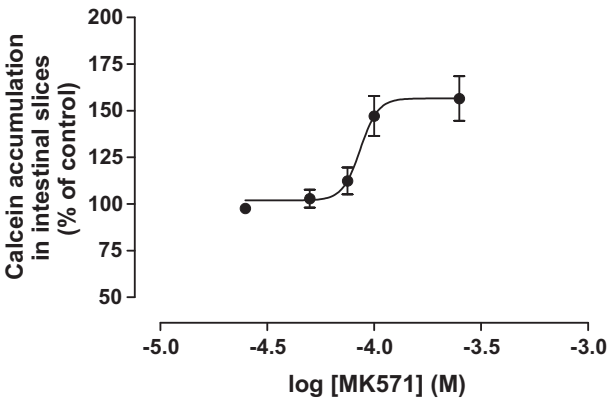

Fig. 7. Concentration-dependent increase in intracellular fluorescence in rat precision-cut intestine slices in the presence of increasing concentration of MK571 (2.5×10^{-5}–2.5×10^{-4} M). Each point represents the mean ± SEM of results recorded in the homogenate of intestinal slices from four different rats; 100% corresponded to the formation of 41 nmol/mg protein of calcein.

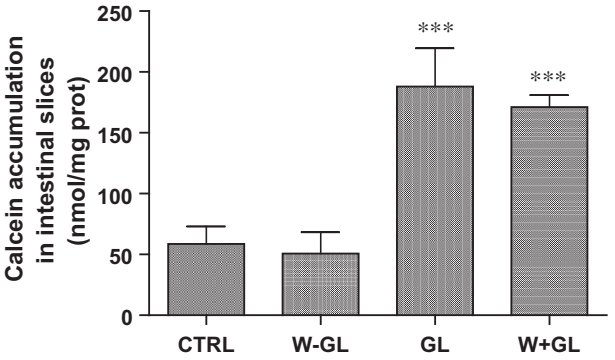

Fig. 8. Reversibility of ABC inhibition. Studies were performed by incubating slices for 30 min in the presence of 100 μM glibenclamide (GL) and, subsequently, slices were washed three times with PBS and incubated in the presence (W + GL) or in the absence (W − GL) of the same concentration of glibenclamide. Controls were performed by omitting glibenclamide in the first incubation step (CTRL). GL represented the result obtained in inhibition experiments without washing. Each point represents the mean ± SEM of results obtained with slices from three different rats ($n = 3$). ***$p < 0.001$.

4. Discussion

ABC transporters, as well as phase I and phase II metabolism reactions, represent the major mechanism underlying xenobiotic detoxification at the intestinal level. Studies of the interaction of exogenous compounds with ABC transporters are important for evaluating intestinal ability to eliminate toxic compounds or to limit their bioavailability as well as to predict drug–drug interaction [33,34]. Consequently, it is important to establish a reliable physiological experimental model to evaluate the interactions of chemicals with ABC transporters, which might also be used to investigate the other mechanisms involved in xenobiotic detoxification, such as phase I and phase II metabolic reactions. Previous studies have demonstrated that intestinal precision-cut slices are suitable for investigating *in vitro* quantitative drug metabolism, since many key enzymes, including those involved in AhR-, PXR- and CAR pathways, are active in this experimental system [19]. Furthermore, intestinal slices provide a reliable, simple and rapid system to study xenobiotics interaction with ABC transporters, because the physiological architecture of the organ is conserved.

In the present study, drug inhibition of ATP-dependent transporters was evaluated by measuring changes in the intracellular accumulation of the fluorescence dye calcein. Calcein-AM and its metabolic product calcein are substrates for different transporters, including Pgp and MRPs, and have been wildly used in cell culture models to study ABC transporter function [35]. The results demonstrated that calcein accumulation by intestinal slices was dependent on the concentration of the drug as well as the incubation time up to 30 min. The observation that there was a decrease in fluorescence after 60 min incubation might be ascribed to a transitory inhibition of the transporter proteins or by a time-dependent decay in drug concentration at the target site, caused by metabolism and/or a diffusion process involved in the regulation of inhibitor(s) concentration at ABC target site. The incubation of 0.5 μM calcein-AM with intestinal homogenate resulted in a de-esterification rate of calcein linear up to 120 min, which was similar in the presence or in the absence of the used drugs (data not shown), suggesting that the decrease in fluorescence could not be due to a time-dependent inhibition of calcein-AM de-esterification or by a quenching phenomenon. It should be noted that it was not possible to entirely block ATP-dependent transporters by using the irreversible inhibitor sodium orthovanadate, because this compound inhibited calcein-AM de-esterification. On the other hand, the use of calcein alone was not suitable since its high diffusion in paracellular compartments gave rise to a high fluorescence noise that hampered the detection of intracellular concentration of the fluorescence dye, while the fast intracellular esterase metabolism of calcein-AM enhances the sensitivity of the present method, as previously demonstrated in different tumour cell lines [28,29].

Verapamil, glibenclamide and indomethacin caused a concentration-dependent increase in intracellular calcein. The fact that both Pgp and MRP inhibitors were effective was consistent with different transporters being present in rat intestinal slices, in agreement with previous observations [19]. The concentrations of verapamil that induced 50% of maximum calcein accumulation observed in the present study were two orders of magnitude lower than that of glibenclamide and close to that reported in isolated porcine brain capillary endothelial cells [36]. Moreover, verapamil was found to have a lower efficacy than glibenclamide in agreement with results observed elsewhere [21]. These results confirm the high potency of the calcium antagonist to inhibit ABC transporter (in particular MDR1 [21]) together with an intermediate efficacy. MC176 and MK571 were tested as specific inhibitors of Pgp and MRP, respectively. MC176, which belongs to a new class of highly potent transporter-dependent MDR inhibitors, showed low nanomolar potency as Pgp inhibitor in MDR1 gene–transfected mouse T-lymphoma L5178 cells and was able to reverse Pgp-dependent resistance in anthracycline-resistant erythroleukaemia K562 cells [23,31,32].

It is interesting to outline that the potency of MC176 observed in the present study is in agreement with the above-mentioned observations as well as its efficacy comparable to glibenclamide.

On the contrary, MK571, despite an IC_{50} close to that reported in the literature [37], possesses a lower efficacy when compared to glibenclamide. Taking together, these results strongly suggest that MRP and MDR are both involved in the intracellular calcein accumulation, although in a different fashion. The present results indicate that intestinal precision-cut slices are suitable to study the effect of various compounds on different ABC transporters expressed in the healthy and intact tissue. However, it is to underline that in the present experimental model, it is not possible to discriminate a donor and a receiver compartment and consequently to use it to measure drug permeability as it happens in Caco-2 model [38]. On the contrary, it is possible to calculate the inhibition constant (*Ki*) towards calcein transport. The apparent affinity constant (*Km*) of ABC transporters should be determined by using a suitable analytical method for measuring the intracellular concentration of the drugs.

In conclusion, the present study indicates that rat intestinal precision-cut slices are suitable experimental models to assess xenobiotic interactions with intestinal ABC transporters under conditions comparable to those *in vivo*. As many drug interactions are highly species-specific, this model could be extended to humans.

Acknowledgements

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