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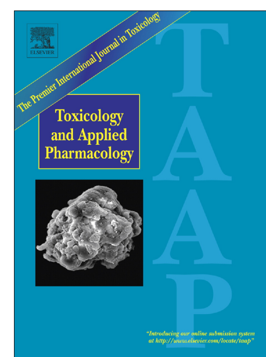

P-glycoprotein activation by 1-(propan-2-ylamino)-4-propoxy-9H-thioxanthen-9-one (TX5) in rat distal ileum: Ex vivo and in vivo studies

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P-GLYCOPROTEIN ACTIVATION BY 1-(PROPAN-2-YLAMINO)-4-PROPOXY-9*H*-THIOXANTHEN-9-ONE (TX5) IN RAT DISTAL ILEUM: *EX VIVO* AND *IN VIVO* STUDIES

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Abbreviations: ABC, ATP-binding cassette; AUC, area under the curve; BBM, brush border membranes; b.w., body weight; ip, intraperitoneal; MDR-1, multidrug resistance protein-1; P-gp, P-glycoprotein; RHO 123, rhodamine 123; TXs, thioxanthones; TX5, 1-(propan-2-ylamino)-4-propoxy-9*H*-tioxanthen-9-one.

ABSTRACT

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In vitro studies showed that 1-(propan-2-ylamino)-4-propoxy-9*H*-thioxanthen-9-one (TX5) increases P-glycoprotein (P-gp) expression and activity in Caco-2 cells, preventing xenobiotic toxicity. The present study aimed at investigating TX5 effects on P-gp expression/activity using Wistar Han rats: a) *in vivo*, evaluating intestinal P-gp activity; b) *ex vivo*, evaluating P-gp expression in ileum brush border membranes (BBM) and P-gp activity in everted intestinal sacs; c) *ex vivo,* evaluating P-gp activity in everted intestinal sacs of the distal and proximal ileum. TX5 (30 mg/kg, b.w.), gavage, activated P-gp *in vivo*, given the significant decrease in the AUC of digoxin (0.25 mg/kg, b.w.). The efflux of rhodamine 123 (200 μM), a P-gp fluorescent substrate, significantly increased in TX5-treated everted sacs from the distal portion of the rat ileum, when P-gp activity was evaluated in the presence of TX5 (20 μM), an effect abolished by the P-gp inhibitor verapamil (100 μM). No increases on P-gp expression or activity were found in TX5-treated BBM of the distal ileum and everted distal sacs, respectively, 24 hours after TX5 (10 mg/kg, b.w.) administration. *In vivo*, no differences were found on digoxin portal concentration between control (digoxin 0.025 mg/kg, b.w., intraduodenal) and TX5-treated (digoxin+TX5 20 μM, intraduodenal) rats. The observed discrepancies in digoxin results can be related to differences in TX5 dose administered and used methodologies. Thus, the results show that TX5 activates P-gp at the distal portion of the rat ileum, and, at the higher dose tested (30 mg/kg, b.w.), seems to modulate *in vivo* the AUC of P-gp substrates.

Keywords: P-glycoprotein; inducers; activators; thioxanthones; *in vivo*; *ex vivo*.

INTRODUCTION

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Permeability glycoprotein (P-glycoprotein; P-gp), encoded by the multidrug resistant gene 1 (*MDR1 or ABCB1*), is a carrier protein that belongs to the ATP-binding cassette (ABC) superfamily of membrane transporters and which activity, against a concentration gradient, depends on ATP hydrolysis (Gameiro et al., 2017). P-gp was originally identified in cancer cells resistant to chemotherapeutic drugs, reason by which it is also known as multidrug resistance protein-1 (MDR-1) (Juliano and Ling, 1976). P-gp promotes the efflux of both endo- and xenobiotics, thus protecting cells against their biological effects, namely toxicity (Silva et al., 2015b). In fact, P-gp has a broad substrate specificity, being able to bind structural and pharmacologically unrelated hydrophobic drugs, such as cardiac glycosides, calcium channel blockers, anticancer and immunosuppressant agents (Gottesman et al., 2002; Silva et al., 2015a).

P-gp is a transmembrane glycoprotein, showing a polarized expression in a variety of tissues (including the intestine, liver, lungs, kidneys) and body barriers (placenta, blood-brain and blood-testis barriers), both in physiological and pathological conditions. Particularly, P-gp is overexpressed in the blood-brain barrier, gastrointestinal tract, kidneys, liver, pancreas and cancerous cells (Gupta et al., 2015). P-gp activity can be modulated by inhibitors, inducers and/or activators, in order to increase or decrease, respectively, drugs intracellular accumulation (Gameiro et al., 2017).

Under the toxicological point of view, P-gp induction and/or activation could constitute an effective therapeutic strategy to detoxify the body from harmful compounds. In fact, Dinis-Oliveira and co-authors proposed P-gp induction in pneumocytes as a valuable therapeutic approach to increase the efflux of paraquat, a well-known toxic P-gp substrate (Dinis-Oliveira et al., 2006a; Dinis-Oliveira et al., 2006b). Additionally, several studies using *in vitro* models were performed showing a protective effect resulting from the increase of the *de novo* synthesis of P-gp, but also from the increase of its activity, which reduced the cytotoxicity elicited by toxic substrates (Silva et al., 2011a; Silva et al., 2013; Silva et al., 2015a; Silva et al.,

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2014b; Silva et al., 2011b; Vilas-Boas et al., 2013). Some of these studies used thioxanthones (TXs), which are S-heterocycles with a dibenzo-γ-thiopyrone scaffold and constitute a class of compounds able to modulate P-gp activity (Lima et al., 2016; Paiva et al., 2013; Palmeira et al., 2012). In our previous *in vitro* study (Silva et al., 2015a), it was demonstrated that the 1-(propan-2-ylamino)-4-propoxy-9*H*-thioxanthen-9-one (TX5) can reduce paraquat-mediated toxicity in Caco-2 cells as a consequence of P-gp induction (increased expression levels) and activation (increased activity). Based on this promising result, we decided to continue the study of TX5 by evaluating its putative modulatory role over P-gp expression and activity, both *ex vivo* and *in vivo*, focusing at the intestinal barrier. For that, we selected a 30 mg/kg (b.w.) dose of the compound for a preliminary *in vivo* efficacy study, whose results are herein presented. Given the motivating results obtained, we proceeded to a safety assessment of the compound (Rocha-Pereira et al., 2019), which led us to reduce the dose to 10 mg/kg (b.w.) to be administered, by gavage, in the subsequent *ex vivo* functional assays described in the present work. Direct effects of TX5 (20 μM) on P-gp expressed in the intestine, in *ex vivo* and *in vivo* studies, were also assessed.

Our main goal is to find compounds capable of increasing P-gp activity and/or expression *in vivo*. By studing P-gp inducers/activators as TX5, these ones can be used as xenobiotic safety enhancers through their ability to decrease the absorption of potentially toxic compounds at the intestinal barrier.

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MATERIALS AND METHODS

Chemicals and drugs

Rhodamine 123 (RHO 123), digoxin, verapamil, sodium chloride, bovine serum albumin (BSA), Tris hydrochloride, Tris base, phenylmethylsulfonyl fluoride (PMSF) and mannitol were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). Meanwhile, cOmplete™ Protease Inhibitor Tablets were purchased from Roche (Amadora, Portugal). Ethanol was obtained from Aga (Prior Velho, Portugal) and Hepes from Fisher Scientific (Geel, Belgium). Ketamine and xylazine were obtained from Novavet (Bragança, Portugal). Meanwhile, isoflurane (Isoflo®) was purchased from Abbott (IL, USA) and lidocaine 25 mg/g + Prilocaine 25 mg/g (EMLA®) was acquired from AstraZeneca (London, UK). Heparin was acquired from Braun (Germany). Dimethyl sulfoxide (DMSO), potassium chloride, sodium hydrogenocarbonate, potassium dihydrogen phosphate, calcium chloride and glucose were purchased from Merck (Darmstadt, Germany). Magnesium sulfate heptahydrate was from Labkem (Barcelona, Spain). TX5 was synthesized by the Organic and Pharmaceutical Chemistry Laboratory of the Faculty of Pharmacy of University of Porto, according to described procedures (Palmeira et al., 2012). An ethyl ether solution was prepared with TX5 (purity > 95 %) and was cooled at - 4 °C. A solution of hydrogen chloride 2.0 M in diethyl ether solution (1 ml) was added. An orange precipitate was formed and it was placed at - 4 °C overnight. The solid thus obtained was filtered, washed with 100 ml of diethyl ether, and dried in a desiccator containing phosphorus pentoxide furnishing 1-(propan-2-ylamino)-4-propoxy-9*H*-thioxanthen-9-one hydrochloride (TX5.HCl). Stock solutions were made up in ultrapure water or DMSO and diluted in Krebs-Henseleit buffer or saline solution (0.9 % NaCl) immediately before use. DMSO was added to the Krebs-Henseleit solution in parallel control experiments. All the reagents used were of analytical grade or from the highest available grade.

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Animals

Adult male Wistar Han rats (12-16-week-old) were purchased from Charles River Laboratories (Barcelona, Spain). Animals were kept under standard laboratory conditions (12/12 h light/dark cycles, 22 ± 2 °C room temperature, 50 – 60 % humidity) for an acclimation period of at least 1 week before starting experiments. During this period, animals had free access to tap water and rat chow *ad libitum*. Animal experiments were approved by the *Organismo Responsável pelo Bem-Estar Animal* (ORBEA; protocol number 250/2018) from the Institute of Biomedical Sciences Abel Salazar of University of Porto (ICBAS-UP). Housing and experimental treatment of the animals were in accordance with the guidelines defined by the European Council Directive for animal experiments (2010/63/EU) and the current Portuguese Law (Decreto-Lei no. 113/2013, de 7 de Agosto).

Experimental protocol

Assessment of P-glycoprotein transport activity in vivo after administration of TX5 by gavage

To assess P-gp activity *in vivo*, we started by carrying out a first assay in which a 30 mg/kg (b.w.) dose of TX5 hydrochloride was tested. The selection of this dose was based on a previous *in vivo* study recently reported by Lima and colleagues (Lima et al., 2018) in which increasing doses of a TX5-like compound, TXA1, administered subcutaneously, three times per week, for two weeks, did not cause evident signs of rat discomfort until the dose of 50 mg/kg. Animals were kept in standard cages and groups allowing social interactions. Eighteen animals were fasted for 12 h prior administration, by gavage, of digoxin (0.25 mg/kg b.w.), a well-known P-gp substrate, or digoxin+TX5 hydrochloride (0.25 mg/kg b.w. + 30 mg/kg b.w.). Immediately before the administration of the compound(s) by gavage, each animal was subjected to a brief inhalational anesthesia with isoflurane to reduce the discomfort associated

to the administration (Murphy et al., 2001). Each animal was locally anesthetized with a mixture of lidocaine (2.5 %) and prilocaine (2.5 %) (EMLA® cream), applied in the rat tail 30 minutes prior blood collection (Flecknell et al., 1990). Blood was collected from caudal vein at different time points during seven hours and local anesthesia was reapplied between each sampling. Blood was then centrifuged (1670 x *g*, 10 min, room temperature) and the obtained plasma samples were stored at -20 °C until further analysis. Digoxin concentration in plasma samples was measured on an AutoAnalyzer (PRESTIGE® 24i, PZ Cormay S.A.), using the respective kit and following the manufacturer instructions.

P-glycoprotein expression assessment at the intestinal barrier

Distal ileum collection and brush border membrane preparation

Animals were maintained in standard cages and groups allowing social interactions. Animals were fasted for 4 h before administration of TX5 hydrochloride (10 mg/kg, b.w.) or water (vehicle), by gavage. Each animal was subjected to a brief inhalatory anesthesia with isoflurane to reduce the discomfort associated to the administration. TX5 hydrochloride was prepared at the day of use in ultrapure water. A maximum volume of 1 ml was administered *per* 200 g b.w. Control animals received ultrapure water using equivalent administration volumes of TX5-treated animals. Animals were fasted for the last 12 h prior experiments, but water supplemented with 1 % sugar was given *ad libitum*. Twenty-four hours after TX5 or water administration, rats were anesthetized with a mixture of ketamine and xylazine (90/10 mg/kg b.w., respectively, ip) and placed in *decubito supino* position. The abdomen was opened and the distal portion of the ileum was selected as the last 20 cm close to the ileocecal valve. The segments were gently rinsed with ice-cold saline solution (0.9 % NaCl) containing 0.4 % PMSF 40 μg/ml, and immediately used for the isolation of brush border membranes (BBM). To obtain BBM, the mucosal tissue was obtained by hand scraping, homogenized and processed,

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using the methodology described by Kessler and colleagues (Kessler et al., 1978) for the preparation of brush border membranes from small intestine, with *minor* modifications introduced and validated by Mottino and co-authors (Mottino et al., 2000). Briefly, the intestinal segments were opened lengthwise, the mucus layer was carefully removed and the mucosa was scraped from the wall. The tissue thus obtained was used for BBM preparation. Mucosa samples were homogenized in buffer [buffer A: 50 mM mannitol, 2 mM Tris/HCl, cOmplete™ Protease Inhibitor Tablets (one tablet *per* 25 ml buffer)] and BBM were prepared from total homogenate by a divalent cation precipitation method followed by differential centrifugation (21 000 x *g*, 1 hour at 4 °C). The final pellet was resuspended in buffer (buffer B: 300 mM mannitol, 10 mM HEPES, 10 mM Tris base, cOmplete™ Protease Inhibitor Tablets). Aliquots of BBM preparations were used for Western blot analysis. Protein concentration in membrane preparations was measured using BSA as standard (Lowry et al., 1951).

Western blot analysis of P-glycoprotein

The western blot analysis of P-gp expression was performed on BBM of the distal portion of the ileum of control and TX5-treated rats obtained after exposure of animals to TX5 (10 mg/kg b.w.) for 24 h. This dose was selected after a preliminary safety assessment of TX5 (Rocha-Pereira et al., 2019). Detection of P-gp was performed using an anti-P-gp C219 mouse monoclonal primary antibody from Calbiochem (Calbiochem®, Merck KGaA, Darmstadt, Germany) validated for use in immunoblotting.

After assessment of the total protein content, using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and BSA as standard, samples were diluted to equal protein concentration, and 120 µL were mixed with 30 µL SDS-PAGE reducing buffer [11.5 % SDS (w/v), 62.5 mM Tris HCl (pH 6.8), 50 % glycerol (v/v), 1 % bromophenol blue (w/v) and 25 % β-mercaptoethanol (v/v)]. Seventy micrograms of protein were loaded and separated in 7.5 %

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SDS/polyacrylamide gels, at a constant voltage of 150 mV, using a running buffer [25 mM Tris Base, 192 mM glycine and 0.1 % SDS (w/v), pH 8.6]. Gels were allowed to equilibrate in transfer buffer [20 % methanol (v/v) in 25 mM Tris Base and 192 mM glycine, pH 8.3] and then transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshine, United Kingdom), at a constant amperage of 290 mA, for 90 min. Membranes were then rinsed in Tris-buffered saline solution with Tween 20 [TBS-T: 20 mM Tris base, 300 mM NaCl and 5 % Tween 20 (v/v), pH 8.0], and nonspecific sites were blocked for 1 h, at room temperature, in blocking buffer [5 % skim milk (w/v) in TBS-T]. Membranes were then incubated with primary antibodies (overnight at 4 °C): C219 mouse monoclonal anti-P-gp (1:1000) and mouse polyclonal anti-tubulin (1:2500, Sigma-Aldrich, Saint Louis, Missouri, USA), prepared in 3 % skim milk (w/v) in TBS-T blocking buffer. Tubulin was used as a protein loading control. After washing three times (10 min each) with TBS-T, the membranes were incubated with the anti-mouse peroxidase secondary antibody (1:2000, Amersham Pharmacia Biotech), prepared in 3 % skim milk (w/v) in TBS-T blocking buffer, for 2 h, at room temperature, under mild shaking. Following three washes (10 min each) with TBS-T, immunoreactive bands were detected using the Clarity™ Western ECL Substrate (Bio-Rad Laboratories), according to the supplier`s instructions, and digital images were acquired using a Molecular Imager® ChemiDoc™ XRS + System (Bio-Rad Laboratories) and analyzed with ImageLab™ 6.0 Software (Bio-Rad Laboratories, Berkeley, CA, USA).

Assessment of P-glycoprotein transport activity in everted intestinal sacs

The TX5 effect on P-gp activity was evaluated, *ex vivo*, by using two distinct protocols, *i.e.*, either 24 h after TX5 hydrochloride administration (10 mg/kg b.w., by gavage) or after an acute and direct contact with TX5 (20 μM), using everted intestinal sacs and rhodamine 123 (RHO 123) as a P-gp fluorescent substrate.

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Assessment of P-glycoprotein transport activity in everted intestinal sacs of Wistar Han rats pre-exposed to TX5 for 24 h

Animals were maintained in standard cages and groups allowing social interactions. Animals were fasted for 4 h before administration of TX5 hydrochloride (10 mg/kg, b.w.) or water (vehicle), by gavage. Each animal was subjected to a brief inhalatory anesthesia with isoflurane to reduce the discomfort associated to the administration. TX5 hydrochloride was prepared at the day of use in ultrapure water. A maximum volume of 1 ml was administered *per* 200 g b.w. Control animals received ultrapure water using equivalent administration volumes of TX5-treated animals. Animals were fasted for the last 12 h prior experiments, but water supplemented with 1 % sugar was given *ad libitum*. Twenty-four hours after TX5 or water administration, rats were anesthetized with a mixture of ketamine and xylazine (90/10 mg/kg b.w., respectively, ip) and placed in *decubito supine* position. The abdomen was opened, the ileocecal valve was identified and the distal portion of the ileum was removed (20 cm approximately). The segments were gently rinsed with ice-cold saline solution (0.9 % NaCl) and immediately used to prepare everted intestinal sacs using an ice-cold plate. Each intestinal sac (10 cm approximately) was placed in a chamber containing Krebs-Henseleit buffer (40 mM glucose, pH 7.4), continuously aerated (95 % O_2 – 5 % CO_2) and at 37 °C, in the presence, or absence, of verapamil (100 μM), a P-gp inhibitor. After a 10-min equilibration period, everted sacs were filled (serosal side) with 1 ml of the same buffer containing RHO 123 (300 μM). The serosal (basal) to mucosal (apical) transport of RHO 123 was evaluated by sampling aliquots of 100 μL of the buffer every 5 min for a 45-min period. The volume collected at each sampling moment was replaced by an equal volume of buffer solution, at the same temperature. At the end, the sacs were gently dried and weighted. Rhodamine 123 concentration was determined by spectrofluorometry (excitation/emission wavelength = 485/528 nm) in samples of mucosal medium, using a RHO 123 standard curve prepared in the same buffer solution. The excreted amounts of RHO 123 into the mucosal side were plotted against the incubation time and the

mucosal efflux rates were estimated from the slope (m) of the linear regression, and expressed in pmol of RHO 123 transported *per* mg of tissue.

Assessment of P-glycoprotein transport activity in everted intestinal sacs in the presence of TX5

The procedure was the same as described before, with *minor* alterations. Briefly, a set of 12 hour-fasted Wistar Han rats was used. After anesthesia with a mixture of ketamine and xylazine (90/10 mg/kg b.w., respectively, ip), rats were placed in *decubito supino* position and the abdomen was opened. The duodenum or the ileocecal valve was identified and the proximal or the distal portion of the ileum was removed (20 cm approximately), gently rinsed with ice-cold saline solution (0.9 % NaCl) and immediately used to prepare everted intestinal sacs using an ice-cold plate. The everted intestinal sacs were placed in a chamber containing Krebs-Henseleit buffer (40 mM glucose, pH 7.4), continuously aerated (95 % O_2 – 5 % CO_2) and at 37 °C. Some sacs (mucosal side) were exposed to TX5 (20 μM), while controls were exposed to 0.4 % DMSO (vehicle), both with or without the addition of verapamil (100 μM). Everted sacs were allowed to equilibrate and then filled (serosal side) with 1 ml of the buffer containing RHO 123 (300 μM). A direct contact between the drugs and the intestinal sac occurred throughout the experimental period. The serosal (basal) to mucosal (apical) transport of the fluorescent substrate was evaluated by sampling aliquots of 100 μL of the buffer every 5 min for a 45-min period. At the end, the sacs were gently dried and weighted. Rhodamine 123 concentration was determined by spectrofluorometry (excitation/emission wavelength = 485/528 nm) in samples of mucosal medium, using a RHO 123 standard curve prepared in the same buffer solution. The excreted amounts of RHO 123 into the mucosal side were plotted against the incubation time and the mucosal efflux rates were estimated from the slope (m) of the linear regression, and expressed in pmol of RHO 123 transported *per* mg of tissue.

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Assessment of P-glycoprotein transport activity *in vivo after intraduodenal administration of TX5*

After the *ex vivo* experiments, we assessed P-gp activity *in vivo* after intraduodenal administration of TX5. For that purpose, we used digoxin as a P-gp substrate and evaluated its intestinal absorption in the presence or absence of TX5. After anesthesia with a mixture of ketamine and xylazine (90/10 mg/kg b.w., respectively, ip), the abdomen of the 12 hour-fasted rats was opened, the choledochal was blocked and both the duodenum and the portal vein were cannulated (Ghanem et al., 2011). Drugs (digoxin 0.025 mg/kg b.w. or digoxin+TX5 20 μM) were given intraduodenally in a final volume of 5 ml saline solution. The rat body temperature was controlled throughout the experimental period (37 °C). To quantify the portal content of digoxin, 400 μL of blood was sampled every 10 min for a 60-min period after digoxin administration. After each sampling, an equal volume of saline was administered. Blood was centrifuged (10 000 x *g,* 15 min, room temperature) and the obtained plasma was used to determine digoxin concentration using a COBAS INTEGRA system (Roche Diagnostics).

Statistical analysis

Area under the curve (AUC) was calculated using the Phoenix® WinNonlin® software (Certara®, Princeton, USA). Statistical analysis was performed with the GraphPad Prism software program version 6 (San Diego, California, USA) and data presented as the means ± standard error of the mean (SEM). Outliers were identified using the ROUT test. The Shapiro–Wilk normality test was conducted before group comparison. Statistical comparison between groups was estimated using the parametric Student *t*-test, the non-parametric Mann-Whitney test or the two-way ANOVA parametric method followed by the Holm-Sidak or Tukey's multiple comparisons *post hoc* test. A *p* value lower than 0.05 was considered to denote statistically significant differences.

RESULTS

P-gp was activated in vivo after TX5 administration by gavage

We started the study by assessing the effect of TX5 over P-gp activity *in vivo* in a preliminary study. For this, a dose of 30 mg/kg, b.w., of TX5 hydrochloride associated to the prototypical P-gp substrate digoxin (0.25 mg/kg, b.w.) was administered, by gavage, to Wistar Han rats. In this experimental approach, blood collected from the caudal vein at eight distinct time points for a seven-hour-period was used to quantify digoxin. Significant differences were found in digoxin plasma concentrations between TX5-treated and control rats (Figure 1), as showed by the significantly lower area under the curve (AUC) of digoxin in TX5-treated rats, when compared to controls (1463 ± 467 ng.min/ml *versus* 2238 ± 224.5 ng.min/ml, respectively).

TX5 did not induce P-gp expression in the distal ileum of the rat intestine

Since P-gp expression varies along the intestine, being the highest in the distal portion of the ileum (Doherty and Charman, 2002, Ghanem et al., 2011), we evaluated the effect of TX5 hydrochloride (10 mg/kg b.w.) on P-gp expression levels in this portion of the rat ileum, 24 hours after its administration by gavage. As can be observed in Figure 2, no differences on P-gp expression were found between BBM isolated from the distal portion of the intestine of control and TX5-exposed rats, 24 hours after TX5 administration.

P-gp activity was not altered 24 h after TX5 administration in the distal ileum of the rat intestine

After assessing the effect of TX5 over P-gp expression levels in the distal portion of the rat ileum, we studied the putative effect of TX5 over P-gp activity in the same conditions, *i.e.*, 24 hours after the administration of TX5 hydrochloride (10 mg/kg b.w.) by gavage. For that purpose, the rat everted intestinal sac was used as a reliable *ex vivo* model. The secretion of RHO 123, a typical P-gp fluorescent substrate, into the mucosal compartment of intestinal sacs, was evaluated at different time points. According to the obtained results, P-gp transport activity was unaffected in the distal ileum of rats pre-exposed to TX5 (Figure 3).

TX5 increased P-gp activity in the distal ileum after a direct contact, but this effect was not observed in the proximal ileum

To study whether TX5 was able to cause functional alterations in P-gp activity after a short-term contact, we used everted intestinal sacs prepared from the distal ileum and a direct and immediate contact with TX5 (20 μM) took place by adding the compound directly to the buffer-containing chamber. Since an activating effect was observed and because several other efflux carriers are known to exist in the ileum, the experiment was carried out in the presence and in the absence of verapamil, a P-gp inhibitor. As can be observed in Figure 4, TX5 hydrochloride caused an almost two fold more increase in the efflux of RHO 123 (68.9±10.1; 90.9±14.4; 106.9±17.2; 125.1±21.0 pmol/mg of tissue) when comparing with control sacs (39.5±5.3; 50.3±7.3; 60.7±8.7; 72.7±9.9 pmol/mg of tissue), effect that occurred from minute 30 on and that was inhibited by verapamil.

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In order to verify if the activating effect observed in the distal portion of the rat ileum also occurred in the proximal portion, the same experimental approach was carried out by using everted intestinal sacs prepared from the proximal portion of the small intestine. The results obtained show that TX5 did not cause P-gp activation in the proximal portion (Figure 5A) and that the overall efflux of RHO 123 was significantly lower in this portion when compared to the distal portion (Figure 5B).

P-gp was not activated in vivo after the intraduodenal administration of TX5

To determine if the TX5-mediated activating effect observed *ex vivo* in the distal portion of the ileum can elicit consequences in the bioavailability of drugs or other xenobiotics that are P-gp substrates, the time-course changes in portal digoxin concentration was assessed after intraduodenal administration of a TX5 bolus. However, *in vivo*, no differences were found in digoxin plasma concentrations between control and TX5-treated rats (Figure 6).

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DISCUSSION

In recent years, our research group has being dedicated to disclose new compounds able to induce and/or activate P-gp in important barrier tissues, in order to find enhancers of safety of xenobiotics with potential to be used in intoxication scenarios. In fact, P-gp presents a wide range of structurally unrelated substrates and has a privileged location in crucial tissues, which underlies its involvement in the modulation of pharmaco(toxico)kinetic processes. As such, P-gp often limits drug absorption and penetration into crucially important organs, such as the brain (Gameiro et al., 2017; Silva et al., 2015b). For this reason, under the toxicological point of view, ABC efflux transporters, namely P-gp, are relevant tools to take potentially harmful xenobiotics out of the cells, consequently reducing their toxicity (Gameiro et al., 2017). In this regard, we recently demonstrated the ability of some (thio)xanthonic compounds to increase both the expression levels and the activity of P-gp in the Caco-2 cell line, a human epithelial colorectal adenocarcinoma line that has been widely used as a model of the intestinal epithelial barrier, since it presents many properties typical of absorptive enterocytes (Sambuy et al., 2005).

Five thioxanthones (TX1–5) and five dihydroxylated xanthones (X1–5) were shown to be, in the Caco-2 cell model, P-gp inducers and activators (Silva et al., 2015a; Silva et al., 2014b; Silva et al., 2015b). Particularly, Silva and colleagues showed that TX1-5 can promote the *de novo* synthesis of P-gp, as well as increase its efflux function, reducing, in consequence, the cytotoxicity of the harmful substrate, paraquat, towards Caco-2 cells (Silva et al., 2015a). It is known, however, that P-gp induction and activation not always occur simultaneously and that an increase in P-gp expression is not necessarily accompanied by a proportional increase in the pump activity. This independence was shown, for example, for colchicine (Silva et al., 2014a) and nonsteroidal anti-inflammatory drugs (Takara et al., 2009) in the Caco-2 cell model. Similarly, it is also possible the existence of an activating effect without any changes in the expression pattern of the protein. In fact, inducers enhance the transporter expression levels,

while activators enhance the transporter activity, although one compound can have overlapping mechanisms of action (Gameiro et al., 2017; Wessler et al., 2013).

Considering the promising results obtained with TX5 (Silva et al., 2015a), which proved to be able to increase, in Caco-2 cells, P-gp expression by 208 % and activity by 198 %, as revealed by flow cytometry, we decided to further study the effects of this compound both *ex vivo* and *in vivo*. Furthermore, a relationship between expression and activity was shown to occur since the activity of P-gp was increased by 156 % in cells pre-exposed to TX5 for 24 h (Silva et al., 2015a).

The evaluation of the potential of a new compound to modulate P-gp activity *in vivo* is an integral part of drug development and is recommended by regulatory agencies (Zhou et al., 2019). Thus, we first conducted a preliminary *in vivo* study to assess the efficacy of TX5 hydrochloride by administering, by gavage, a single high dose of the compound (30 mg/kg b.w.) to Wistar Han rats, evaluating its effect on the pharmacokinetics of digoxin (0.25 mg/kg b.w.). In fact, digoxin is a well-known P-gp substrate, which pharmacokinetics is well established, and thus has been being widely used in *in vivo* experiments (Bai et al., 2019; de Lannoy and Silverman, 1992; Fromm et al., 1999; Li et al., 2014; Oda and Murakami, 2017; Yang et al., 2016). Our *in vivo* study suggested a P-gp activator effect mediated by TX5, since the AUC of digoxin was significantly lower in TX5-treated rats when comparing to controls (1463 ± 467 ng.min/ml *versus* 2238 ± 224.5 ng.min/ml, respectively). The lower AUC of digoxin found in the plasma of rats administered with TX5 suggests a higher efflux of digoxin at the intestinal barrier after its administration by gavage. This effect might be related with a higher activity of P-gp expressed in the intestine as consequence of a direct and immediate activation of the efflux protein by TX5. Overall, this effect was translated in a significantly lower AUC of digoxin in TX5-treated animals when compared to control animals.

Given the *in vivo* confirmation of the TX5-mediated P-gp activating effect previously observed *in vitro* (Silva et al., 2015a), we proceeded to a preliminary safety assessment of the compound (Rocha-Pereira et al., 2019).

Despite the absence of apparent signs of discomfort or toxicity, we found TX5 (30 mg/kg b.w.) to cause relevant toxicity after a complete histopathological analysis. An extensive hepatic necrosis, splenic parenchyma hyperemia, signs of red pulp hemorrhage, slight myocardium hyperemia, small intestine inflammation, as well as renal tubular edema and slight hyperemia were observed (Rocha-Pereira et al., 2019). These findings led to a dose reduction of TX5 to be used in subsequent safety assessment assays. This selected lowest dose (10 mg/kg b.w.) did not induce considerable toxicity in the biological matrices studied (Rocha-Pereira et al., 2019), which justified its use in the functional studies described in the present study.

It should be noted that the initial evaluation of the effect of the compound *in vivo,* using the 30 mg/kg dose described in this study, occurred immediately after administration, by gavage, of TX5 or TX5 plus digoxin, and lasted for 12 hours. As such, the activation effect was observed shortly after administration and, therefore, unaffected by the potential toxicity of the compound at this dose (30 mg/kg b.w.) (Rocha-Pereira et al., 2019).

It is known that there is a regional difference of P-gp activity in the intestinal tract and that the expression levels of P-gp greatly vary along the small intestine, being the highest in the distal ileum and the lowest in the proximal portion (Doherty and Charman, 2002; Iida et al., 2005; Li et al., 2015). For that reason, we decided to start the study of the effects of TX5 on P-gp expression levels using the portion of the ileum where the expression pattern of the transporter is the most expressive. In the present study, we did not observe any effect of the compound on the carrier expression levels. This effect was evaluated by western blot in BBM of the rat distal ileum, 24 hours after the administration, by gavage, of TX5 hydrochloride (10 mg/kg b.w.). In contrast, a considerable inducing effect of P-gp expression was previously

found *in vitro*, 24 hours after exposure to TX5, an effect observed by flow cytometry and subsequently corroborated by western blot (Silva et al., 2015a).

Although the mechanism of action of P-gp inducers remains unclear, it is known that P-gp induction is complex and regulated by distinct nuclear factors. Some of them are the pregnane X receptor (PXR), constitutive androstane receptor (CAR), nuclear factor erythroid-derived 2-related factor (Nrf2), Y-box binding protein-1 (YB-1), nuclear factor Y (NF-Y), nuclear factor-κB (NF-κB), liver X receptor (LXR), farnesoid X receptor (FXR) and peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ) (Gameiro et al., 2017). Possibly, a time-point of 24-hours after TX5 hydrochloride administration may not be sufficient to observe *in vivo* changes in the P-gp expression pattern. The choice of this time-point was based in the results obtained *in vitro*, for which a prominent inducing P-gp effect was observed (Silva et al., 2015a). In fact, 24 hours of incubation of cells were needed to observe a potent *in vitro* TX5-mediated inducing effect (Silva et al., 2015a). However, it will be legitimate to expect the need for a longer period to observe this induction effect, *in vivo*. Accordingly, the study performed by Martignoni *et al.* showed that the expression of cytochrome P450 enzymes can be similarly induced both *in vitro* and *in vivo*, although a greater time of exposure to the inducer is required when the effect is seen *in vivo* (6 to 24 hours *versus* 3 days, respectively) (Martignoni et al., 2004). Additionally, it is known that there are interspecies differences in P-gp expression levels (Han et al., 2018; Myllynen et al., 2010). As such, we can consider the existence of putative differences in the pattern of expression of P-gp between the human Caco-2 cell model and the rat everted intestinal sac that could ultimately explain our results. Nevertheless, the results of the expression herein presented were in agreement with the results obtained in BBM, 24 hours after exposure of the animals to TX5 hydrochloride (10 mg/kg b.w., gavage). The absence of inducing effects in the BBM prepared from the distal portion of the ileum was accompanied by a corresponding absence of functional alterations at the level of the transporter activity in that same ileum portion. Indeed, no differences on the serosal to

mucosal cumulative efflux of RHO 123 were registered between TX5-treated and control everted intestinal sacs. In fact, changes in carrier activity due to expression levels changes are expected to occur, although, as previously mentioned, a causal relationship between both might not exist. The study performed by Ghanem *et al.* described an increased P-gp functionality in the distal ileum in response to an inducing effect advocated by acetaminophen (Ghanem et al., 2011). Accordingly, the study carried out by Silva and co-authors also demonstrated the influence of a previous increase of P-gp expression on the activity of the transporter. These TX5-triggered effects therefore resulted in less paraquat-mediated cytotoxicity in Caco-2 cells (Silva et al., 2015a). However, the results of the study herein presented, while consistent, indicate the absence of TX5-induced changes in both P-gp expression and activity, 24 hours after exposure to the compound. Considering that inducers and activators act by distinct mechanisms of action, the absence of inducing effects of P-gp expression observed in the present study did not exclude the possibility of a TX5-mediated activating effect. As previously stated, the regulation of the expression of a given protein is a very complex process, while the activation implies the binding of a compound to the carrier, enabling the binding of the substrate to another site and promoting, in consequence, its transport across the membrane (Gameiro et al., 2017). This is, apparently, a much simpler process that involves the nucleotide binding domains, not implying the participation of any nuclear factor.

Thus, we used the same experimental *ex vivo* approach to study the putative TX5-mediated P-gp activating effect both in the distal and the proximal portions of the rat ileum, after a short-term and direct contact between the compound and the everted intestinal sac. Again, we started by testing the effect of TX5 in the distal portion of the rat ileum, where P-gp expression levels are highest. We intended to evaluate the immediate modulatory effect of TX5 in the activity of P-gp expressed at the apical membrane of rat ileum enterocytes, using everted intestinal sacs as an *ex vivo* model (Tian et al., 2019; Yigitaslan et al., 2016). Since a direct

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contact between the compound and the intestinal sac was desired, the same concentration of TX5 tested *in vitro* (20 μM) was chosen (Silva et al., 2015a). Very importantly, and in accordance with our previous *in vitro* data, TX5 (20 μM) caused a significantly higher serosal to mucosal secretion of RHO 123 in the distal portion of the rat ileum, as a result of a direct P-gp activation. This effect was completely abolished by the P-gp inhibitor verapamil (100 μM). However, this increased transport activity was time-dependent since it became apparent only from minute 30 on. This fact suggests the need for a certain time of contact with the activator before the effect becomes measurable. A similar observation took place in another study, where the acetaminophen-mediated P-gp activating effect in the rat distal ileum was registered 20 min after filling the sacs with RHO 123 (Ghanem et al., 2011).

In the present study, and in opposition to the distal portion, no TX5 activating effects occurred in the proximal portion. This finding highlights the possibility for a differential activating effect mediated by the compound in the two portions of the ileum. Furthermore, considering the overall smaller efflux of RHO 123 in the proximal portion of the intestine, it becomes more difficult to see potential differences elicited by TX5 as consequence of its activating effect.

Given the P-gp activating effect triggered by TX5 in the distal portion of the rat ileum, we decided to go further by studying the putative effect of the compound over P-gp activity *in vivo*. Indeed, given the relevance of P-gp at the intestine, where it limits drug absorption, we hypothesized that its direct activation at the intestinal barrier *in vivo* could constitute a valuable therapeutic approach to reduce xenobiotics absorption, namely those with relevant toxicity. However, our expectations were not confirmed since no differences in the concentrations of digoxin in the portal vein plasma were found between anesthetized rats administered only with digoxin and those where digoxin and TX5 hydrochloride were co-administered directly in the duodenum. This methodology was previously used by Ghanem and colleagues, allowing the detection of differences in digoxin plasma concentration in response to the treatment with acetaminophen, as soon as 5 min after its intraduodenal

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administration (Ghanem et al., 2011). This effect was justified by the acetaminophen-mediated inducing effect of P-gp expression on both intestinal portions, although particularly prominent in the proximal region. Likewise, and despite the absence of P-gp inducing effects triggered by TX5 in our experimental conditions, we may consider that the lack of functional effects on P-gp activity *in vivo* is closely related to the heterogeneity of the intestine with respect to P-gp expression (Li et al., 2017). In fact, we should consider the possibility that the observed effect is exclusively a consequence of exchanges in the proximal portion of the small intestine, where basal P-gp expression levels are the lowest and where no inducing or activating effects were observed. Alternatively, the differentiated modulation mediated by TX5 observed *ex vivo* may mask some activating effect *in vivo*. These hypotheses require further research. Nevertheless, it is important to emphasize that the conditions used in this *in vivo* study were different from that used in the *in vivo* preliminary study, namely the methodology used, the time points considered and the dose of the compound tested. All these factors may be contributing to the differences found.

Taken together, our data might indicate a TX5-mediated activation effect only observable in the distal portion of the rat ileum and when a direct contact between the compound and the everted intestinal sac takes place. The translation of this finding to humans will, however, require further investigation since it is known that there are species differences in P-gp abundance, already demonstrated for humans and rats (Li et al., 2017). Additionally, it is known that the absorption of the majority of drugs is initiated in the duodenum (Shugarts and Benet, 2009), which may condition the impact of the activating effect observed in the most distal portion of the ileum. Nevertheless, our findings i) suggest that TX5 can differently modulate P-gp activity in the rat intestinal barrier; ii) demonstrate that TX5 can differently modulate P-gp activity in distinct portions of the rat ileum *ex vivo*; iii) support a greater P-gp activity under TX5 stimulation in the distal portion of the rat ileum. These findings may open new perspectives for the exploration of this particular territory in the context of the

development of new antidotal strategies or therapeutic adjuvants in the pharmacological approach to intoxications caused by P-gp substrates.

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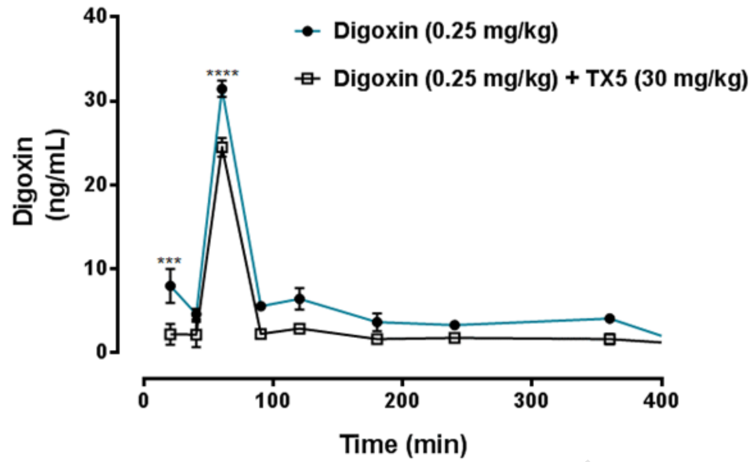

Figure 1 – Digoxin plasma concentration in control and TX5-treated rats. Animals were administered, by gavage, with digoxin (0.25 mg/kg) or digoxin (0.25 mg/kg) + TX5 hydrochloride (30 mg/kg). Digoxin concentration in different time points was quantified in plasma samples obtained from blood collected from the caudal vein, using an AutoAnalyzer (PRESTIGE 24i, PZ Cormay S.A.). Data are presented as means ± SEM of five rats *per* group. Statistical comparisons were made using the two-way ANOVA followed by the Holm-Sidak multiple comparisons test (*** $p< 0.001$; **** $p< 0.0001$ for TX5-treated *versus* control animals).

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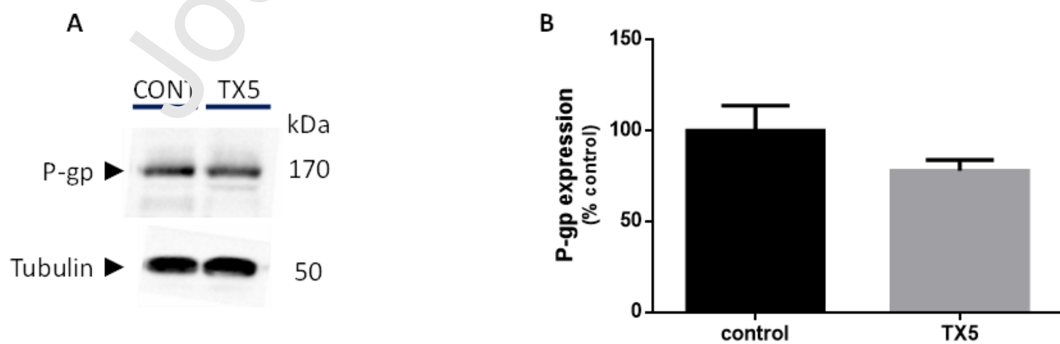

Figure 2. Western blot analysis of P-gp expression levels in the brush border membranes (BBM) of the distal ileum of rats orally exposed to TX5 hydrochloride (10 mg/kg) for 24 h. Western blot study of P-gp was performed using BBM prepared from 20-cm segments of the distal ileum of control and TX5-

exposed rats. Representative images of Western blot analysis of P-gp (170 kDa) immunocontent **(A)** and respective densitometric analysis **(B)** are shown. Densitometric analysis are expressed as percentage of control and presented as means ± SEM of at least four rats *per* condition. Statistical comparisons were made using the non-parametric Mann-Whitney test.

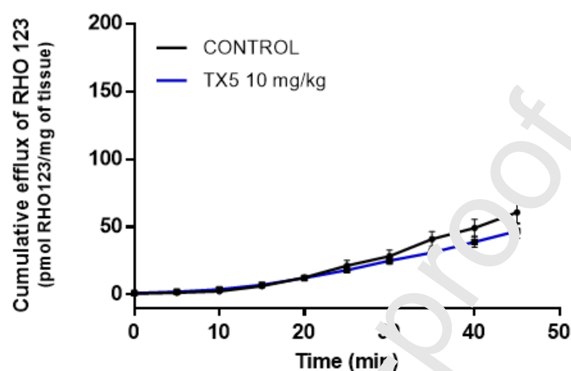

Figure 3. Effect of TX5 on P-gp activity 24 h after administration. P-gp-mediated RHO 123 efflux was measured *ex vivo* using 10-cm everted sacs from distal ileum of control and TX5-exposed (10 mg/kg b.w.) rats (24 h). The sacs were filled with RHO 123 (300 μM; serosal side). The dye secreted into the outside compartment (mucosal side) was assessed every 5 min for 45 min. Data are presented as means ± SEM of at least ten rats *per* group. The excreted amounts of RHO 123 into the mucosal side were plotted against the incubation time and the mucosal efflux rates were estimated from the slope (m) of the linear regression, and expressed in pmol of RHO 123 transported *per* mg of tissue. Statistical comparisons were made using the two-way ANOVA parametric method followed by the Tukey's multiple comparisons post hoc test.

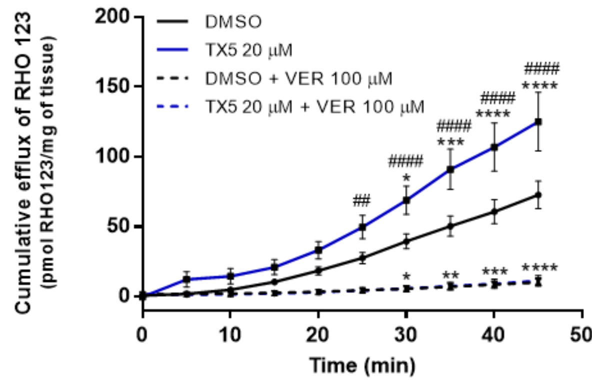

Figure 4. Short-term and direct effect of TX5 on P-gp activity in rat distal ileum. P-gp-mediated RHO 123 efflux was measured *ex vivo* using 10-cm everted sacs from the distal ileum of rats. The sacs were filled with RHO 123 (300 µM; serosal side). The dye secreted into the outside compartment (mucosal side) was assessed every 5 min up to 45 min, in the presence or absence of TX5 (20 µM) and VER (100 µM). Data are presented as means ± SEM of six to eleven rats per group. The excreted amounts of RHO 123 into the mucosal side were plotted against the incubation time and the mucosal efflux rates were estimated from the slope (m) of the linear regression and expressed in pmol of RHO 123 transported *per* mg of tissue. Statistical comparisons were made using the two-way ANOVA parametric method followed by the Tukey's multiple comparisons post hoc test. VER - verapamil. $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001; $^{****}p$ <0.0001 *versus* control; $^{\#\#}p$ <0.01; $^{\#\#\#\#}p$ <0.0001 *versus* TX5 + VER.

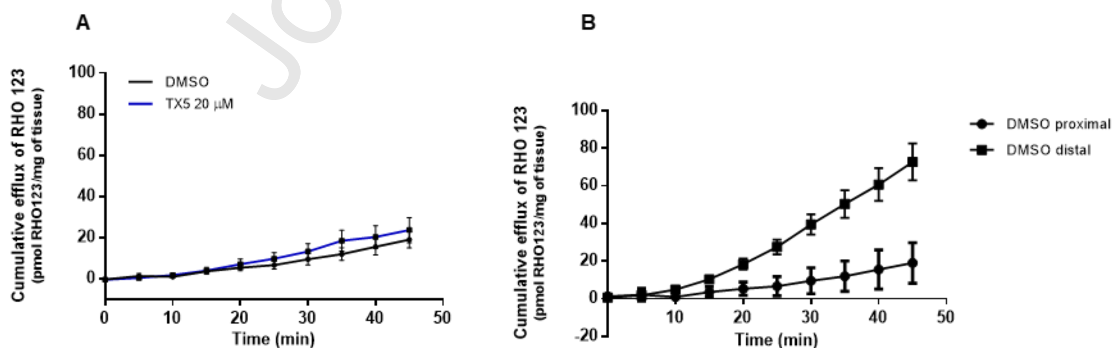

Figure 5. Short-term and direct effect of TX5 on P-gp activity in rat proximal ileum **(A)** and the overall efflux of RHO 123 in the distal and proximal portions of the rat ileum in the absence of TX5 **(B)**. P-gp-mediated RHO 123 efflux was measured *ex vivo* using 10-cm everted sacs from the proximal (A) and

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distal (B) ileum of rats. The sacs were filled with RHO 123 (300 μM; serosal side). The dye secreted into the outside compartment (mucosal side) was assessed every 5 min for 45 min, in the presence or absence of TX5 (20 μM) (A). Data are presented as means ± SEM of seven to ten rats *per* group. The excreted amounts of RHO 123 into the mucosal side were plotted against the incubation time and the mucosal efflux rates were estimated from the slope (m) of the linear regression, and expressed in pmol of RHO 123 transported *per* mg of tissue. Statistical comparisons were made using the two-way ANOVA parametric method followed by the Tukey's or Sidak's multiple comparisons post hoc test.

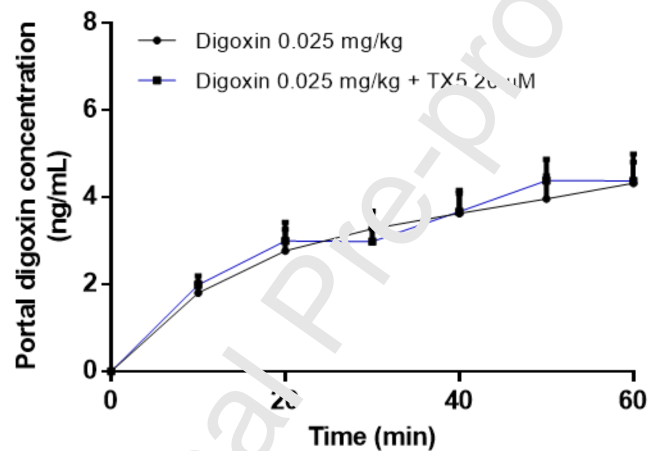

Figure 6. Effect of TX5 on digoxin absorption. Net intestinal absorption of digoxin was indirectly determined by changes in portal plasma concentrations over 60 min after intraduodenal administration of digoxin in anesthetized control (digoxin 0.025 mg/kg) and TX5-treated rats (digoxin 0.025 mg/kg + TX5 20 μM). Data are presented as means ± SEM of eight to nine rats *per* group. Statistical comparisons were made using the two-way ANOVA parametric method followed by the Holm-Sidak multiple comparisons post hoc test.

P-GLYCOPROTEIN ACTIVATION BY 1-(PROPAN-2-YLAMINO)-4-PROPOXY-9*H*-THIOXANTHEN-9-ONE (TX5) IN RAT DISTAL ILEUM: *EX VIVO* AND *IN VIVO* STUDIES

AUTHOR CONTRIBUTIONS SECTION:

Carolina Rocha-Pereira

The author was responsible for the experimental design and for carrying out the *ex vivo* and *in vivo* experiments, as well as the western blot analysis. The author was responsible for the treatment of the results and wrote the manuscript. The author was responsible for the review process.

Carolina Inés Ghanem

The author was a consultant in the implementation of the innovative methodologies described in the manuscript, namely the *ex vivo* and *in vivo* experiments. The author participated in the correction of the final manuscript.

Renata Silva

The author participated in the preliminary *in vivo* experiment in which digoxin and TX5 (30 mg/kg) were administered, by gavage, to Wistar Han rats. Additionally, the author participated in western blot analysis and in the treatment of the results and in the revision process of the manuscript.

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The author participated in the *ex vivo* and all *in vivo* experiments described in the manuscript. The author participated in the experimental design involving animals, namely the volumes administered, anesthesia, refinement techniques used.

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The author participated in the ex vivo and in vivo experiments described in the manuscript.

Emília Sousa

The author was responsible for the synthesis of the compound (TX5) used in this study.

Maria de Lourdes Bastos

The author is the responsible for the Laboratory of Toxicology of the Faculty of Pharmacy of University of Porto. She participated in manuscript writing and correction, as well as in the review process.

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Fernando Remião

The author is the responsible for the research. The author participated in the preliminary *in vivo* experiment in which digoxin and TX5 (30 mg/kg) were administered, by gavage, to Wistar Han rats. He participated in manuscript writing and correction, as well as in the review process.

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The authors declare no conflict of interests.

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- P-glycoprotein (P-gp) is a carrier efflux protein.
- TX5 increases P-gp expression and activity *in vitro*.
- TX5 activates P-gp only at the distal portion of the rat ileum, *ex vivo*.
- TX5 seems to modulate, *in vivo,* the AUC of P-gp substrates.

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