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Screening of newly synthetized xanthones and potential P-glycoprotein modulation at intestinal barrier- *in vitro* and *ex vivo* studies

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INTRODUCTION

P-glycoprotein (P-gp) is an efflux pump belonging to the ATP-binding cassette (ABC) transporter superfamily and has an ubiquitous and constitutive distribution throughout the body [1].

Due to its wide distribution, namely to its polarized expression in barrier and excretory tissues, to its varied range of substrates and to its large efflux capacity, P-gp is vital in the pharmacokinetic processes of absorption and distribution of toxic substrates, reducing their intracellular accumulation and, consequently, their toxicity [2,3]. This **defense mechanism** is particularly important at the **intestinal level**, significantly reducing the intestinal absorption of xenobiotics, limiting its access to the target organs, resulting in a decrease in their toxicity [4].

Thus, P-gp can be faced as a **potential antidotal pathway**, when **induced** and/or **activated** [1,5].

AIM

The **aim of the present study** was to investigate, **in a human colorectal adenocarcinoma cell line (SW480 cells), six newly synthetized xanthone derivatives (Xs)**, since xanthones are known to **interact with P-gp through modulation mechanisms such as induction and/or activation** [6-8]. Additionally, for the most promising compounds, ***ex vivo* studies** were conducted for the same purpose.

MATERIALS AND METHODS

In vitro studies:

- **The cytotoxicity** of the tested xanthones (0 - 50 µM) was evaluated by the **Neutral Red** (NR) and **MTT** uptake assays, 24 h after exposure, **to select a noncytotoxic concentration** to be used in the subsequent studies.
- The effect of the tested xanthones on **P-gp expression** was evaluated by **flow cytometry**, using a **P-gp monoclonal antibody** (**UIC2**) conjugated with Phycoerythrin (PE), 24 h after exposure
- **P-gp activity** was measured through **two different protocols**, both **using Rhodamine 123** (**RHO 123,** 5 µM) as a **fluorescent P-gp substrate**. In the **first** protocol, the **accumulation of RHO 123** was evaluated in **SW480 cells previously exposed to the tested Xs** (20 µM) for **24 h**, assessing to **eventual alterations in P-gp activity due to the possible effects on P-gp expression** caused by the xanthones. In the **second** protocol, the **accumulation of RHO 123** (5 µM) was evaluated in **the presence of the tested Xs** (20 µM), allowing a **direct detection of alterations in P-gp activity** without affecting protein expression.

Ex vivo studies:

- The effect of **X12** on **P-gp activity** was evaluated, *ex vivo*, at the distal portion of the ileum of adult Wistar-Han male rats. After gently washed in an ice-cold saline solution, **the intestine portions** were **everted** and the corresponding everted intestinal sacs were placed in a chamber containing 5 mL Krebs-Henseleit (KH) buffer (40 mM glucose, pH 7.4), continuously aerated (95% O_2 – 5% CO_2) and at 37ºC, with or without the addition of **X12** (20 µM), and in the presence or absence of Zosuquidar (10 µM), a known P-gp inhibitor. The serosal compartment was filled with 1 mL of KH buffer containing **300 µM RHO 123**, which was used as P-gp substrate. Serosal to mucosal transport was evaluated by sampling aliquots of the buffer every 5 min for a 45-min period. RHO 123 concentration was determined spectrofluorometrically (measured at excitation/emission wavelengths of 485/528 nm, in a multi-well plate reader) in all samples taken from mucosal medium.

RESULTS

In vitro results

Xanthones cytotoxicity assays

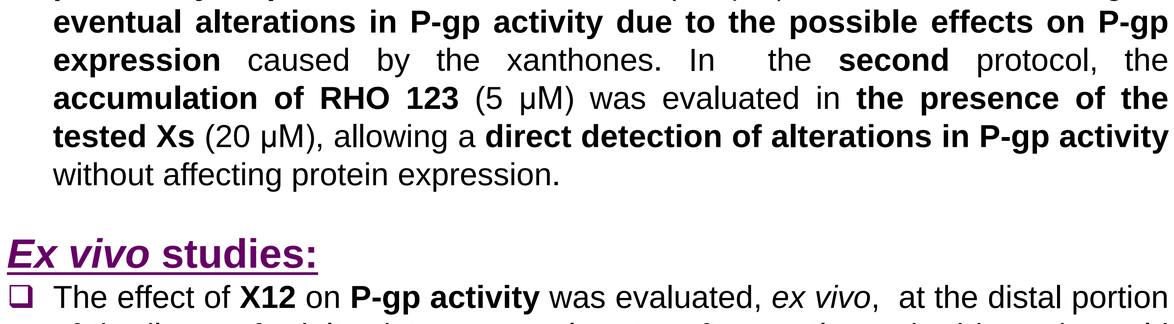

Figure 1. Xanthones (0 – 50 µM) cytotoxicity in Caco-2 cells evaluated by the NR uptake assay 24 hours after exposure, [*p < 0.05; **p < 0.01;****p < 0.0001 vs. control].

Figure 2. Xanthones (0 – 50 µM) cytotoxicity in Caco-2 cells evaluated by the MTT uptake assay 24 hours after exposure, [****p < 0.0001 vs. control].

- In the **MTT reduction assay**, no significant cytotoxicity was detected for any of the tested concentrations (0 - 50 µM) and up to 24 hours of exposure to **X2**, **5**, **6** and **16**. For **X1** and **X12**, no significant effects were observed for concentrations up to 20 µM, but a significant decrease was observed with a concentration of 50 µM.
- Concerning the **NR uptake assay**, no significant effects were observed after 24 hours of exposure to **X2**, **6** and **12**, and for all the tested concentrations. However, for **X1**, **5** and **16**, a small but significant reduction in the NR uptake was observed only for the highest tested concentration (50 µM).
- The **20 µM** concentration was selected as a **noncytotoxic concentration** to be used in the subsequent studies

P-gp expression

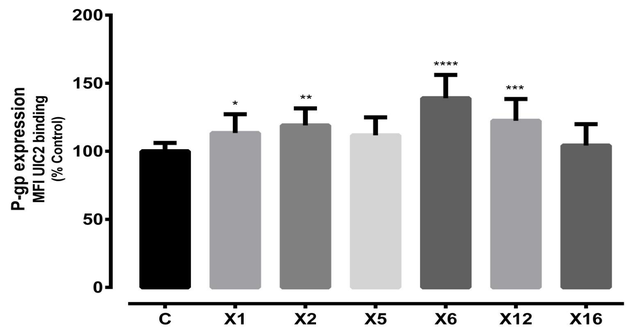

Figure 3. P-glycoprotein expression evaluated by flow cytometry in SW480 cells, 24 hours after exposure to the tested xanthones (20 µM), [*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 vs. control].

P-gp activity (24 hours)

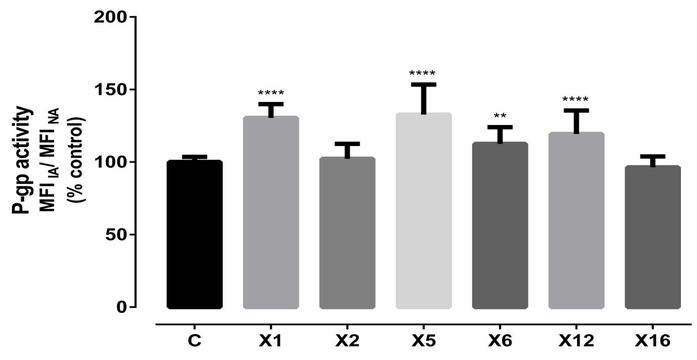

Figure 5. P-glycoprotein activity evaluated by fluorescence spectroscopy in SW480 cells exposed to the tested xanthones (20 µM) for 24 hours, [**p < 0.01; ****p < 0.0001 vs. control].

P-gp activity

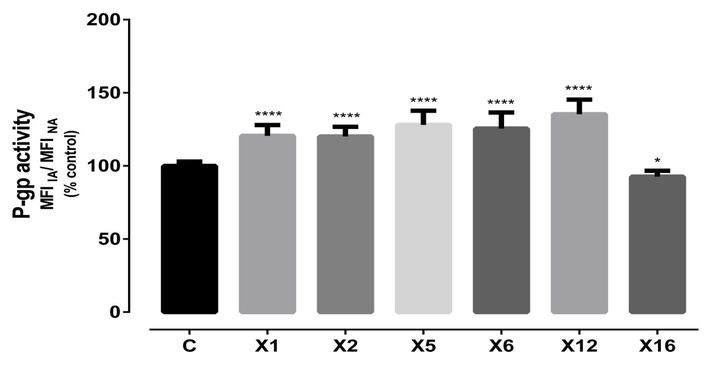

Figure 4. P-glycoprotein activity evaluated by fluorescence spectroscopy in SW480 cells exposed to the tested xanthones (20 µM) only during the 90 minutes incubation period with the fluorescent substrate (5 µM RHO 123), [*p < 0.05; ****p < 0.0001 vs. control].

Ex vivo results

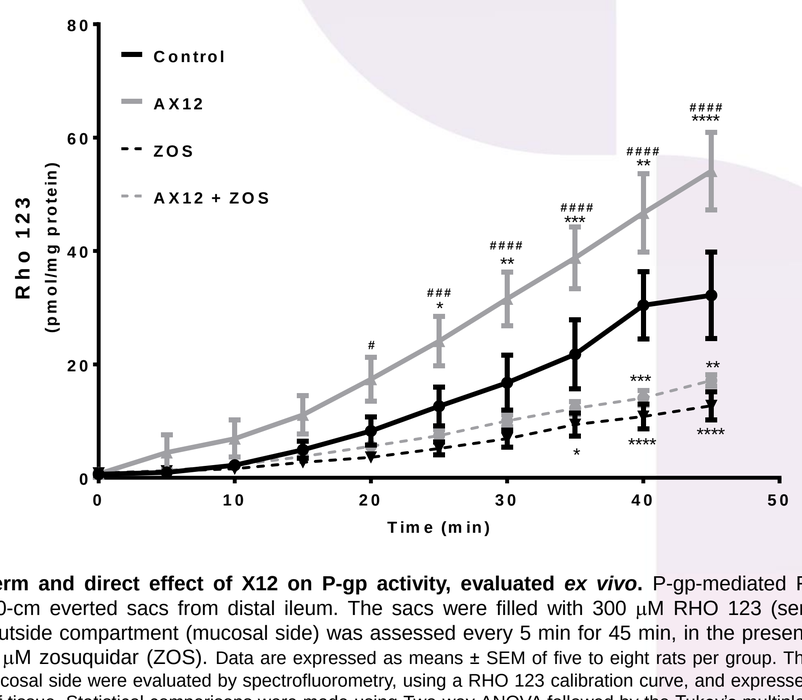

Figure 6. Short-term and direct effect of X12 on P-gp activity, evaluated *ex vivo*. P-gp-mediated RHO 123 efflux was measured using 10-cm everted sacs from distal ileum. The sacs were filled with 300 µM RHO 123 (serosal side). The dye secreted into the outside compartment (mucosal side) was assessed every 5 min for 45 min, in the presence or absence of 20 µM X12 and/or 10 µM zosuquidar (ZOS). Data are expressed as means ± SEM of five to eight rats per group. The excreted amounts of RHO 123 into the mucosal side were evaluated by spectrofluorometry, using a RHO 123 calibration curve, and expressed as pmol of RHO 123 transported per mg of tissue. Statistical comparisons were made using Two-way ANOVA followed by the Tukey's multiple comparisons post hoc test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 versus Control #P<0.05; # # #P<0.001; # # # # P<0.0001 X12 versus X12 + ZOS).

CONCLUSIONS

- As previously reported for other xanthonic derivatives [6-8], the newly synthetized xanthones demonstrated to interact with P-g, *in vitro*.
- Flow cytometry analysis of P-gp expression demonstrated that **X6** and **X12** (20 µM, 24h), **significantly increased P-gp expression**. Compounds **X1** and **X2**, although to a lower extent, also significantly increased P-gp expression.
- **P-gp activity** evaluated 24 h after exposure to the tested xanthones demonstrated that the increase in P-gp activity observed after pre-exposure to **X1**, **X6** and **X12** may result from an increase in the expression of this efflux pump. Although no increases in P-gp expression were observed, **X5** also significantly increased P-gp activity.
- **P-gp activity** was also evaluated with the tested compounds present only during the short RHO 123 incubation period, indicating that **X5**, **X6** and **X12** were the most **efficient P-gp activators**.
- ***Ex vivo*** studies demonstrated **X12** ability to increase the efflux of RHO 123 in everted intestinal sacs. This increase was blocked by **Zosuquidar**, a specific P-gp inhibitor, which proves the involvement of the protein in the efflux of the substrate.
- In conclusion, the ***in vitro*** and ***ex vivo*** results confirmed the potential of these xanthones as **P-gp inducers** and **activators** and, therefore, they can be faced as a **potential therapeutic** approaches in cases of intoxications with toxic substrates.

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