Bioorganic & Medicinal Chemistry Letters 18 (2008) 3741–3744

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Effect of some P-glycoprotein modulators on Rhodamine-123 absorption in guinea-pig ileum

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ARTICLE INFO

Article history:
Received 8 April 2008
Revised 9 May 2008
Accepted 10 May 2008
Available online 20 May 2008

Keywords:
Rhodamine-123
Guinea-pig ileum
P-Glycoprotein

ABSTRACT

Several reference compounds such as Cyclosporin A, Tamoxifen, Verapamil, and our compound **1**, known as P-gp modulators, have been tested for their P-gp modulating activity in isolated organ bath. Compound **1** showed the best result in organ bath experiment (EC_{50} = 14.7 μM), Cyclosporin A and Tamoxifen displayed EC_{50} = 25.2 and 39.4 μM, respectively.

The systemic availability of food and drugs is modulated by a physical and biochemical barrier, the intestinal mucosa.[1–5] The role of this barrier significantly depends on specific membrane transport systems and intracellular enzymes. Among these transporters, P-glycoprotein (P-gp) is the most widely studied and probably the most important efflux pump that regulates the absorption of drugs.[6–10] P-gp is a transmembrane protein (170 kDa) localized in the apical membrane of the intestinal mucosa, canalicular membranes of the liver, proximal tubule of the kidney, and endothelial cells of the Blood Brain Barrier (BBB).[11,12] P-gp is an ATP-ase pump that transports various hydrophobic compounds out of the cell using the energy from ATP hydrolysis.[13] This pump belongs to the ATP Binding Cassette transporter family (ABC superfamily). ABC transporters have two highly conserved ATP-binding catalytic domains, NBD-1 and NBD-2 (Nucleotide Binding Domain) and two relatively variable transmembrane domains that act together as a unit.[13] ABC pumps transported substrates include sugars, amino acids, vitamins, lipids, sterols, peptides, toxins, and drugs.[11,12] To date, the following binding sites and the corresponding substrates for ABC transporters have been reported: H, Hoechst 33342; P, Progesterone/Prazosine; R, Rhodamine-123/Anthracycline; M, central modulation.[14] Drugs interacting with P-gp have been classified as substrates, inhibitors, or inducers.[15–17] Moreover, there are compounds displaying simultaneously substrate and inhibitor activity toward P-gp (e.g., Quinidine, Verapamil), inhibitor and inducer activity (e.g., Atorvastatin, Mefloquine), mixed substrate, inhibitor and inducer activity (e.g., Saquinavir). The recent FDA guidelines on drugs interactions recommend to evaluate if new drug candidates are substrates, inhibitors, and inducers of P-gp in order to assess their potential for clinical drug-drug interaction.[18]

To date, several in vitro cultured cell-based and ex vivo approaches available to predict the cell permeation, absorption, and gastrointestinal metabolism of molecules have been reported.[8,19] Among in vitro cultured cell-based methods, Caco-2 cell monolayer assay is widely used to predict the intestinal bioavailability. Ex vivo approaches, employing rat ileum in Using diffusion chamber, were reported in few papers.[20,21] However, Caco-2 cell monolayer method presents several biological limitations: (*i*) the expression level of P-gp is not constant, but it depends on the source of the cells; (*ii*) the monolayer preparation takes 21–28 days for confluence, and 17–27 days for P-gp to become functional. On the other hand, the intestinal P-gp expression level is dependent on the intestinal region. In particular, P-gp expression increases from the proximal to distal regions of the small intestine.

In order to study the role of P-gp in intestinal food and drugs absorption, we selected the final tract of guinea-pig small intestine, that is, the ileum, to investigate if this isolated organ bath could serve as a model in the prediction of food and drug absorption.

In contrast to the Caco-2 cell monolayer method, the isolated ileum assay does not present biological limitations such as the variable P-gp expression level and it is less time-consuming and labour-intensive.

Previously, we developed several methods on guinea-pig isolated bath organ to study the activity of some compounds such as sigma, serotonergic 5-HT_{1A}, adrenoceptor β_3 ligands.[22–26] This

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doi:10.1016/j.bmcl.2008.05.055

knowledge prompted us to investigate the P-gp role and its modulation by known pump inhibitors.

In order to verify if P-gp sensitivity to inhibitors in guinea-pig ileum was comparable to Caco-2 cells, we employed Caco-2 cell monolayer as in vitro standard experimental model.[27] Moreover, we investigated if both experimental models (guinea-pig ileum and Caco-2 cells) could be considered complementary in the prediction of the P-gp activity in human intestine.

Rhodamine-123 and [^{3}H]vinblastine have been employed in guinea-pig ileum and Caco-2 cells, respectively, as P-gp substrates. In each procedure we confirmed the P-gp activity involvement by testing known P-gp modulators such as Verapamil, Cyclosporin A,[28] Tamoxifen,[29] and our ligand 2-{2-[2-(3-methoxy-phenyl)-ethyl]-phenoxymethyl}-pyridine (compound **1**)[30] (Fig. 1) as a reference.

In Fig. 3 Rhodamine-123 fluxes between buffer and ileum epithelium at the *stationary state* in the absence (A) and in the presence (B) of a P-gp inhibitor are reported.

The time-course for different Rhodamine-123 doses was performed by equilibrating the fluorescent probe with guinea-pig ileum in 20 mL isolated organ bath. These preliminary investigations led us to optimize Rhodamine-123 concentration and the time to reach the equilibrium between two opposite fluxes: (1) Rhodamine-123 passive flux from buffer into the ileum epithelium (J_{pass}in); (2) Rhodamine-123 active efflux P-gp mediated (J_{act}) and Rhodamine-123 passive flux (J_{pass}out) from epithelium to buffer.[31]

The *equilibration time* has been determined monitoring the fluorescence emission, that initially decreased due to Rhodamine-123 passive flux into the ileum epithelium until a constant value. This constant fluorescent value corresponded to two opposite events: Rhodamine-123 flux (passive and P-gp mediated) from ileum into the buffer, and Rhodamine-123 passive flux from buffer into the ileum epithelium.

Rhodamine-123 fluorescence emission remained unchanged for 50 min after the *equilibration time* and we defined this time (50 min after the *equilibration time*) as *stationary state*.

The fluorescence determination in buffer solution (see Fig. 3) was performed by spectrofluorimetric measurement. Rhodamine-123 emission spectrum was recorded at $\lambda = 510 \pm 30$ nm (excitation) and at λ = 528 nm (emission) as depicted in Figure 2A. The calibration curve in the same medium was made starting from 1 nM to 100 nM (Fig. 2B). In this buffer we calculated the quantum yield ($\Phi = 0.52$).[32,33] All tested compounds at 100 μM did not quench 20 nM Rhodamine-123 emission since Rhodamine-123 emission spectrum did not overlap Cyclosporin A, Tamoxifen, Verapamil and compound **1** excitation spectra.

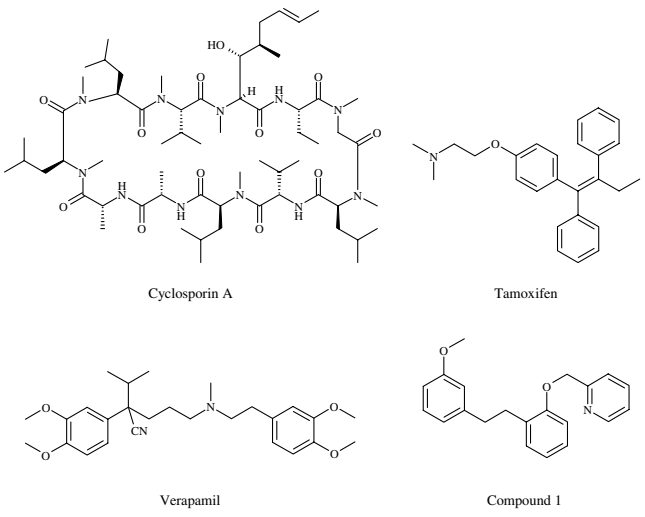

Figure 1. P-gp modulators.

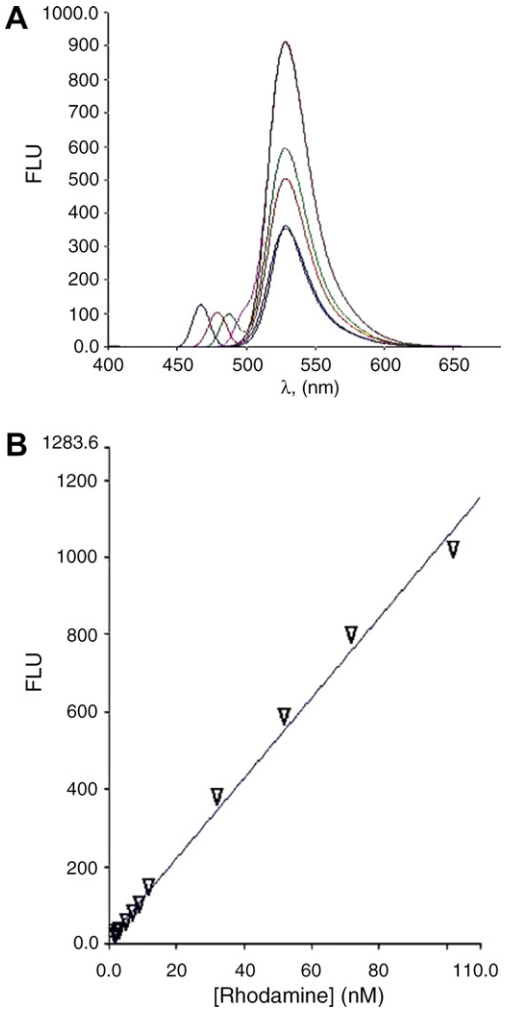

Figure 2. Rhodamine-123 emission spectrum at λ = 528 nm exciting from λ = 490 nm until λ = 530 nm by increasing 10 nm (A); Rhodamine-123 calibration curve in Krebs solution (B).

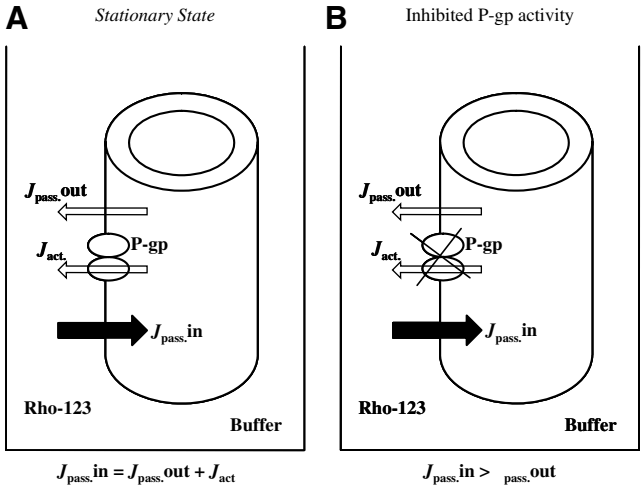

Figure 3. Rhodamine-123 fluxes in isolated guinea-pig ileum at the *stationary state* in the absence (A) and in the presence (B) of a P-gp inhibitor.

After the *stationary state*, the ileum reactivity was probed by adding 50 nM Carbachol. In fact, this cholinergic agonist compound induced a rapid contractile response demonstrating the ileum biological vitality. The results recorded within 50 min starting from the *equilibration time* were considered significant if the contractile response to Carbachol, after this time, was positive.

After the *equilibration time*, during the *stationary state*, all tested compounds were added as single dose (from 1 to 100 μM) and the fluorescence was evaluated every 10 min. For Cyclosporin A, the maximal decrease of fluorescence units was at 50 μM at the end of the *stationary state*. As depicted in Figure 4, the maximal decrease of fluorescence units by Cyclosporin A was 60% of the initial value ($P < 0.05$). In the same experimental conditions, Tamoxifen (Fig. 4) was tested and for this compound the maximal decrease in fluorescence units was observed at 100 μM (50%, $P < 0.05$).

Compound **1** displayed reduction of fluorescence emission reaching the maximal effect at 100 μM (70%, $P < 0.05$) as depicted in Figure 4. In the presence of 1 μM Verapamil, a dramatic ileum relaxation was observed and this effect was due to its antagonist activity versus Ca^{++} 'L-type' channels.

All compounds, excepting Verapamil, were tested at 200 μM displaying the same fluorescence emission found at 100 μM.

For all drugs, EC_{50} values were determined by plotting fluorescence decrease (arbitrary units) vs Log[dose]. The best result was obtained for compound **1** ($EC_{50} = 14.7 \pm 2.5$ μM). Cyclosporin A displayed $EC_{50} = 25.2 \pm 4.0$ μM and Tamoxifen $EC_{50} = 39.4 \pm 3.5$ μM. The corresponding dose–response curves are depicted in Figure 5. P-gp interacting mechanism for compound **1** has been previously determined in vitro using Caco-2 cells monolayer assays.[30]

P-gp modulating activity in Caco-2 cells monolayer was compared to the corresponding activity in guinea-pig ileum and the following results, which were consistent in the two assays, were found: compound **1** displayed $EC_{50} = 14.7 \pm 2.5$ μM in organ bath and $EC_{50} = 27.6 \pm 0.2$ μM in Caco-2 cells;[30] Cyclosporin A displayed $EC_{50} = 25.2 \pm 4.0$ μM in organ bath and $EC_{50} = 45.5 \pm 7.5$ μM in Caco-2 cells; Tamoxifen displayed $EC_{50} = 39.4 \pm 3.5$ μM in organ bath and $EC_{50} = 56.5 \pm 4.5$ μM in Caco-2 cells. Moreover, Verapamil displayed $EC_{50} = 20 \pm 1.0$ μM in monolayer experiment.

Since the results obtained with the two methods are comparable, we suggest the isolated ileum bath model as additional test to define the critical role of P-gp pump in food and drugs absorption. However, several differences should be considered between ileum and Caco-2 monolayer methods such as pharmacokinetic processes, membrane crossing, and the interference with other biological systems (enzymes, other extrusion pumps, channels and receptor proteins). Moreover, the P-gp involved in ileum is a normal transporter and not aberrant as in system overexpressing it such as Caco-2 cells.

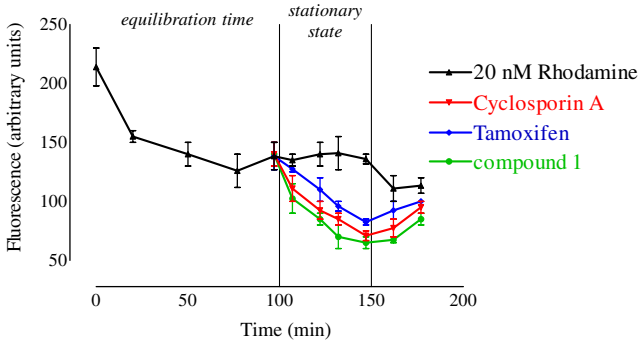

Figure 4. P-gp modulation activity in guinea-pig ileum in the presence of 20 nM Rhodamine-123 and 50 μM Cyclosporin A (red), or 100 μM Tamoxifen (blue), or compound **1** (green). The fluorescence (arbitrary units) has been detected from 5 min to 50 min. Each point represents the mean ± SEM of two independent experiments. $P < 0.05$, significantly different from control.

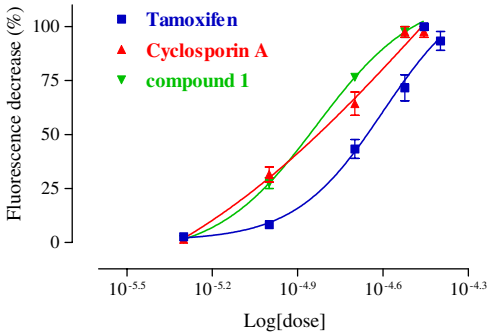

Figure 5. Dose–response curves in guinea-pig ileum by Rhodamine-123 emission measurement at 50 min after compounds incubation. Values are means ± SEM from three experiments. Groups of data were compared with analysis of variance followed by Tukey's multiple comparison test ($P < 0.001$).

In conclusion, P-gp-mediated transport across the intestine was found to be inhibited by tested compounds. The inhibitory potencies of these compounds were comparable in the two studies: ex vivo in guinea-pig ileum, and in vitro in Caco-2 cells.

Thus, it is suggested that the P-gp function and sensitivity to inhibitors should be similar in guinea-pig ileum and Caco-2 cells and that these experimental models could be predictive to further study of P-gp in human intestine. However, one of the functional differences between ileum and Caco-2 cells is the lack, in the latter model, of expression of the cytochrome P450 isozymes and in particular, CYP3A4, which is highly expressed in the intestine.[34] Since in Caco-2 cell monolayer method the drugs metabolism is not estimated, a discrepancy between in vitro and in vivo results could be observed. Thus, bath experiment include the potential drugs metabolism so that results from this assay can be more predictive for the in vivo results.

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