Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 346 (2008) 169–172
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Note

Protein concentration and pH affect the apparent P-glycoprotein–ATPase activation kinetics

Aki T. Heikkinen *, Jukka Mönkkönen

Department of Pharmaceutics, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland

Received 24 April 2007; received in revised form 29 May 2007; accepted 29 May 2007
Available online 3 June 2007

Abstract

Reliable predictions of the role of P-glycoprotein in the pharmacokinetics are needed already at the early stage of drug development. In order to obtain meaningful *in vitro–in vivo* scaling factors, it is essential to know the factors affecting the *in vitro* results. In this study, the apparent P-glycoprotein–ATPase activation kinetics were determined using the cell membrane fraction of human MDR1-transfected insect cells. The apparent affinities to P-glycoprotein of basic verapamil and quinidine were higher at pH 7.4 than at pH 6.8. However, this shift in pH did not have a significant effect on the apparent affinity of acidic monensin. The protein concentration used in the assay did not affect the apparent activator affinities, but was inversely related to the maximum activation achieved. Thus, pH and protein concentration should be taken into account when interpreting the Pgp–ATPase data.

Keywords: P-glycoprotein; MDR1; ABCB1; ATPase activity; Kinetic parameter

1. Introduction

P-glycoprotein (Pgp) is an ATP-dependent efflux transporter expressed in several pharmacokinetic barriers of the body, such as intestinal epithelia and blood brain barrier. Pgp has an important role in the pharmacokinetics of some of the Pgp substrate drugs (Fromm, 2003). Therefore, drug candidates are screened for their Pgp interaction at the early stage of drug development, and reliable predictions of the role of Pgp in the pharmacokinetics are desired (Balimane et al., 2006).

Interaction between Pgp and drug molecules can be detected with several *in vitro* methods, but the ability to predict whether the interaction is significant *in vivo* is currently poor. Several methods are used for identifying Pgp interacting compounds and for determining kinetic parameters of Pgp-mediated transport (Adachi et al., 2001; Doppenschmitt et al., 1999; Troutman and Thakker, 2003). However, different methods may result in very different kinetic parameters, and the evaluation of *in vivo* relevance of these *in vitro* determined parameters is difficult. Therefore, it is essential to know which factors affect the *in vitro* results before meaningful *in vitro* to *in vivo* extrapolations can be achieved.

The Pgp–ATPase activation method has been used for screening Pgp interacting drugs (Polli et al., 2001) and also for determining kinetic parameters (Adachi et al., 2001; Xia et al., 2006). However, the kinetic parameters obtained with the Pgp–ATPase method varies between laboratories. In this study we tested the effect of pH and protein concentration to the Pgp–ATPase activation assay. Our results show that these factors result in variation in Pgp–ATPase activity measurements and thus may partly explain the variation in the parameters that has been reported.

2. Materials and methods

Verapamil was purchased from ICN Biomedicals (Aurora, OH, USA) and all the other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the chemicals were of analytical grade or better.

The Pgp–ATPase assay was performed using the cell membrane fraction from High Five cells expressing human Pgp (GENTEST/BD Biosciences, Woburn, MA, USA). The assay was performed according to the manufacturer's protocol with modifications in protein concentration and in buffer pH. Fifty

* Corresponding author. Tel.: +358 17 163 465; fax: +358 17 162 252.
E-mail address: Aki.Heikkinen@uku.fi (A.T. Heikkinen).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.05.063

microlitres of membrane suspension (20, 40 or 60 μg of protein/well) in ATPase buffer containing 50 mM Tris, 50 mM MES, 2 mM EGTA, 2 mM dithiothretiol, 50 mM potassium chloride and 5 mM sodium azide, pH 6.8 or pH 7.4 as indicated, was preincubated with or without test compound for 5 min at 37 °C. Ten microlitres of 30 mM ATP magnesium salt in ATPase buffer was added (final ATP concentration was 5 mM). After 20 min of incubation at 37 °C, 30 μl 10% of SDS solution supplemented with Antifoam A was added to stop the reaction. Subsequently, 150 μl of detection solution containing 8% of ascorbic acid, 3 mM of zinc acetate and 7 mM of ammonium molybdate was added and these mixtures were incubated at 37 °C for 20 min. The absorbance of each mixture was measured at 660 nm using Victor^2 (Perkin Elmer/Wallac, Turku, Finland). All the experiments were performed using the same membrane lot to avoid the possible effect of membrane lot to lot variation.

The Pgp–ATPase activity (A) was calculated according to the equation:

$$A = \frac{(\mathrm{Pi}_{(t)} - \mathrm{Pi}_{(0)}) - (\mathrm{Pi}_{(t)\mathrm{vanadate}} - \mathrm{Pi}_{(0)\mathrm{vanadate}})}{tn} \quad (1)$$

where $\mathrm{Pi}_{(t)}$ and $\mathrm{Pi}_{(0)}$ are the amount of liberated phosphate at time t and 0, respectively, and $\mathrm{Pi}_{(t)\mathrm{vanadate}}$ and $\mathrm{Pi}_{(0)\mathrm{vanadate}}$ are the amount of liberated phosphate in the presence of 100 μM sodium orthovanadate at time t and 0, respectively. The activities were normalized to the protein amount n. $\mathrm{Pi}_{(t)\mathrm{vanadate}}$ was not affected by any of the studied molecules (data not shown) indicating that studied molecules did not interfere with the background ATP hydrolysis or with the detection of liberated phosphate.

To estimate the maximum Pgp–ATPase activity with particular activator ($A_{\max}$) and the activator concentration resulting in half-maximum activation (EC_{50}), the data were fitted to the equation:

$$A = A_0 + (A_{\max} - A_0)\frac{C}{C + \mathrm{EC}_{50}} \quad (2)$$

where A_0 is the Pgp–ATPase activity at activator concentration $C = 0$. GraphPad Prism (Version 4.03) was used for data fitting.

3. Results and discussion

The fitted parameters, EC_{50} and $A_{\max}$, determined using different assay protocols are presented in Figs. 1 and 2. The apparent EC_{50} of the basic compounds verapamil and quinidine

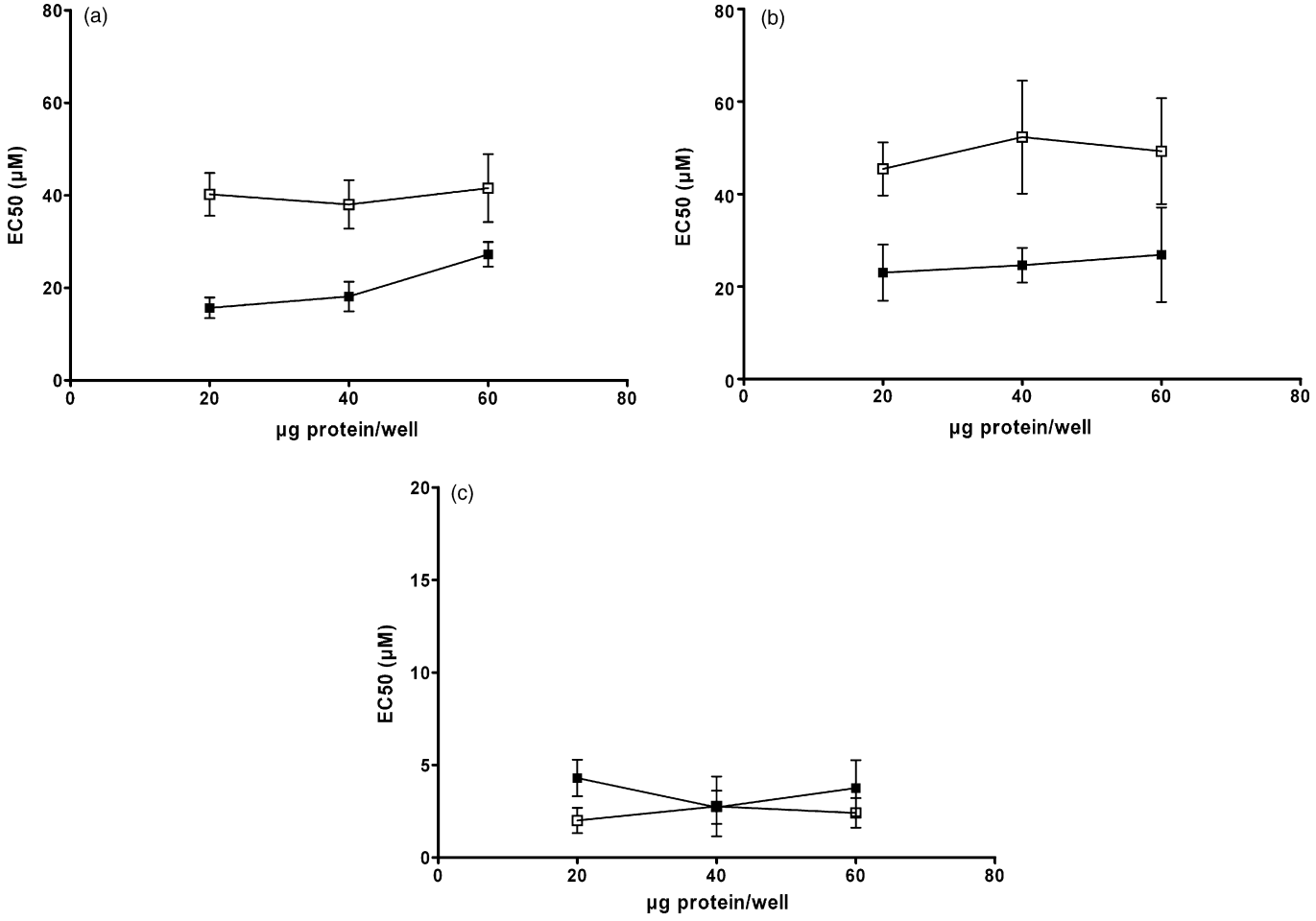

Fig. 1. Apparent EC_{50} values of verapamil (a), quinidine (b) and monensin (c) at pH 6.8 (open symbols) and at pH 7.4 (closed symbols) when assays were performed using 20, 40 or 60 μg of protein/well. Symbols represent the parameters fitted on data from 4 measurements at 5 to 7 concentrations per compound ± standard error of fitted parameters.

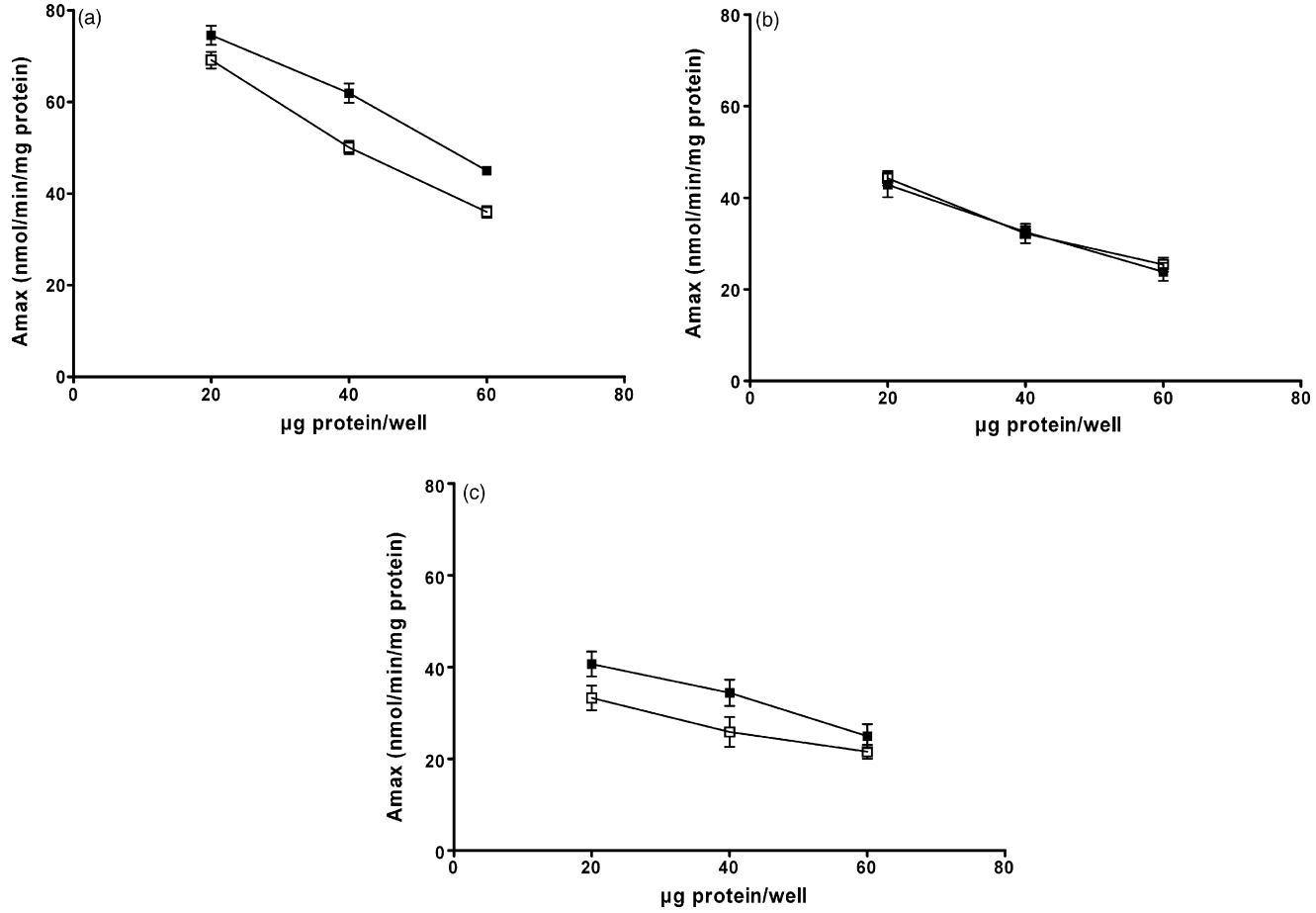

Fig. 2. Apparent A_{max} values of verapamil (a), quinidine (b) and monensin (c) at pH 6.8 (open symbols) and at pH 7.4 (closed symbols) when assays were performed using 20, 40 or 60 μg of protein/well. Symbols represent the parameters fitted on data from 4 measurements at 5 to 7 concentrations per compound ± standard error of fitted parameters.

were lower at pH 7.4 than at pH 6.8 with all protein concentrations used. Most of the known Pgp substrates are weak bases and their distribution between aqueous and lipid phases varies according to pH at the physiological pH range. As Pgp binds at least some of its transported substrates inside the lipid bilayer (Shapiro et al., 1997), it is expected that basic substrates reach the binding site more readily at higher pH, resulting in lower EC_{50} values. However, the reversed effect was not clearly seen with the acidic monensin. This is probably due to the fact that the pH area used was too far away from the pK_a of monensin (Table 1), thus the concentration at the binding site of Pgp was not significantly affected. Further, pH shift did not affect the Pgp–ATPase activation of the neutral compounds digoxin and testosterone (data not shown), suggesting that the pH shift did not affect on the Pgp function *per se*.

Table 1
Physicochemical properties of the Pgp–ATPase activators used in the study

	pK_a [a]	log *D* at pH 6.8[a]	log *D* at pH 7.4[a]
Quinidine	9.04[b]	1.25	1.81
Verapamil	8.97[b]	1.77	2.33
Monensin	4.26[c]	1.2	0.67

[a] Values were calculated by the ACDLABS ACD/pKa/logD program (Version 6.00).
[b] Basic.
[c] Acidic.

In addition to EC_{50}, the pH shift slightly affected A_{max} values of verapamil and monensin. According to the Sigma product information sheet, the pK_a of MgATP is 6.5, therefore the studied pH shift also affects the ionization of ATP. The affinity to Pgp of different forms of ATP may vary and could cause the observed shift in A_{max} parameters. However, if this was the reason the effect would be seen independent of the activator used. With quinidine, digoxin and testosterone there was no sign of pH effect on A_{max} (digoxin and testosterone data not shown). Thus, the reason for the variable A_{max} values of verapamil and monensin in different pH environments remains unclear.

The protein concentration used in the assay did not significantly affect EC_{50}, suggesting that the concentration of activators at the binding site was not significantly affected by the variation in the protein concentration used.

Surprisingly, the A_{max} and the protein concentration were inversely related (Fig. 2). However, the basal Pgp–ATPase activity (3.7 ± 1.5 nmol/min mg protein) was not detectably affected by the protein concentration. The ATP concentration remained above 3 mM during all incubations. Three millimolars of initial ATP concentration was enough to give practically maximum

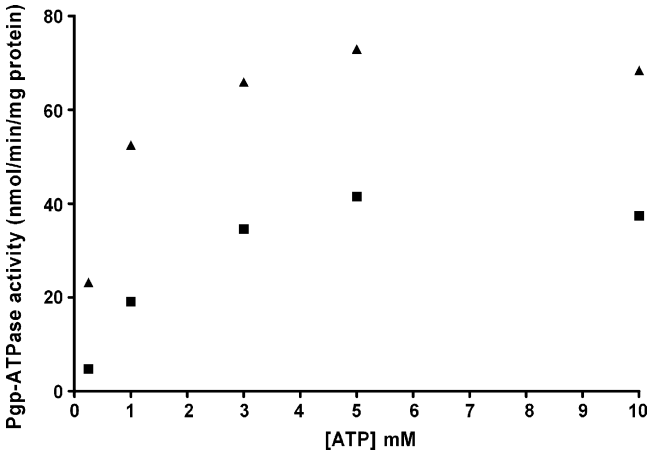

Fig. 3. Kinetics of the Pgp–ATPase activity at pH 7.4 in the presence of 500 μM verapamil. Pgp–ATPase activity was measured at various ATP concentrations using 20 μg protein/well (triangles) and 60 μg protein/well (squares). Symbols represent the average of two measurements.

Pgp–ATPase activity (Fig. 3), suggesting that apparently lower Pgp–ATPase activity with higher protein concentrations is not due to ATP depletion. Pgp–ATPase activity has been shown to be inhibited by ADP, and also substrate inhibition of Pgp–ATPase by high ATP concentrations has been suggested (Sharom et al., 1995). End product and substrate inhibition could explain the apparently lower Pgp–ATPase activities when higher protein concentrations are used.

4. Conclusions

In Pgp–ATPase assay, the possible effect of pH on activators lipid distribution should be taken into account when analyzing ionizable compounds.

Apparent Pgp–ATPase activity is lower when high protein concentrations are used, possibly due to ADP accumulation during the incubation.

Acknowledgement

This study was financially supported by the Finnish Funding agency for Technology and Innovation (TEKES).

References

Adachi, Y., Suzuki, H., Sugiyama, Y., 2001. Comparative studies on in vitro methods for evaluating in vivo function of MDR1 P-glycoprotein. Pharm. Res. 18, 1660–1668.

Balimane, P.V., Han, Y.H., Chong, S., 2006. Current industrial practices of assessing permeability and P-glycoprotein interaction. AAPS J. 8, E1–E13.

Doppenschmitt, S., Spahn-Langguth, H., Regardh, C.G., Langguth, P., 1999. Role of P-glycoprotein-mediated secretion in absorptive drug permeability: an approach using passive membrane permeability and affinity to P-glycoprotein. J. Pharm. Sci. 88, 1067–1072.

Fromm, M.F., 2003. Importance of P-glycoprotein for drug disposition in humans. Eur. J. Clin. Invest. 33 (Suppl. 2), 6.

Polli, J.W., Wring, S.A., Humphreys, J.E., Huang, L., Morgan, J.B., Webster, L.O., Serabjit-Singh, C.S., 2001. Rational use of in vitro P-glycoprotein assays in drug discovery. J. Pharmacol. Exp. Ther. 299, 620–628.

Shapiro, A.B., Corder, A.B., Ling, V., 1997. P-glycoprotein-mediated Hoechst 33342 transport out of the lipid bilayer. Eur. J. Biochem. 250, 115–121.

Sharom, F.J., Yu, X., Chu, J.W., Doige, C.A., 1995. Characterization of the ATPase activity of P-glycoprotein from multidrug-resistant Chinese hamster ovary cells. Biochem. J. 308, 381–390.

Troutman, M.D., Thakker, D.R., 2003. Efflux ratio cannot assess P-glycoprotein-mediated attenuation of absorptive transport: asymmetric effect of P-glycoprotein on absorptive and secretory transport across Caco-2 cell monolayers. Pharm. Res. 20, 1200–1209.

Xia, C.Q., Xiao, G., Liu, N., Pimprale, S., Fox, L., Patten, C.J., Crespi, C.L., Miwa, G., Gan, L.S., 2006. Comparison of species differences of P-glycoproteins in beagle dog, rhesus monkey, and human using Atpase activity assays. Mol. Pharm. 3, 78–86.