The Enantioselective Binding of Mefloquine Enantiomers to P-Glycoprotein Determined Using an Immobilized P-Glycoprotein Liquid Chromatographic Stationary Phase

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INTRODUCTION

Mefloquine (MQ), α-2-piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol (Fig. 1), is an antimalarial agent widely used to treat chloroquine-resistant malaria (1). The agent is administered as a racemic mixture of erythro-isomers, (+)-[11R,2′S]-MQ {(+)-MQ} and (−)-[11S,2′R]-MQ {(−)-MQ}. In humans, there is an enantioselective distribution of MQ with higher plasma and brain concentrations of (−)-MQ (2,3).

MQ has also been shown to inhibit the activity of the drug efflux transporter P-glycoprotein (Pgp) (4–7). Shao *et al*. demonstrated that MQ increased the intracellular accumulation of the Pgp substrate daunomycin in the P388/ADR leukemia cell line (4). In addition, when MQ was used concomitantly with the Pgp-substrate vinblastine (VBL), the two agents interacted with each other synergistically in a noncompetitive manner.

However, MQ has also been shown to increase the intracellular accumulation of VBL (6,7). In rat brain capillary endothelial GPNT cells, the inhibition of VBL transport was enantioselective with (+)-MQ displaying up to an eightfold greater effect than (−)-MQ (6,7). In Caco-2 human colon carcinoma cells, both (−)-MQ and (+)-MQ significantly increased cellular accumulation of VBL, but the effect was not enantioselective (6,7).

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ABBREVIATIONS: Pgp: P-glycoprotein; VBL: vinblastine; CsA: cyclosporin A; MQ: mefloquine.

The objective of this study was to examine the molecular basis of the observed effect of MQ on VBL transport in light of the observed combined effects of MQ and VBL on daunomycin intracellular accumulation. The experimental approach examined the effect of (+)-MQ and (−)-MQ on the Pgp binding affinities of [^{3}H]-VBL and [^{3}H]-cyclosporine A ([^{3}H]-CsA). The studies were carried out using an immobilized Pgp liquid chromatographic stationary phase (Pgp-SP) (8). The Pgp-SP was constructed using membranes derived from a human cell line transduced with a retroviral vector directing the expression of MDR1 (8). This phase has been used to determine ligand-Pgp binding affinities and to investigate ligand-ligand binding interactions on Pgp (8,9).

The experimental approach was competitive binding studies using (+)-MQ and (−)-MQ as the displacers and [^{3}H]-VBL or [^{3}H]-CsA as the ligand. The results demonstrate that the addition of either (+)-MQ or (−)-MQ completely suppressed the binding of [^{3}H]-VBL to Pgp, in a manner indicative of an anticooperative allosteric interaction. There was no observed enantioselectivity in this process. However, when [^{3}H]-CsA was the marker ligand, (+)-MQ competitively displaced this marker, whereas (−)-MQ had no effect. This indicates that, under the experimental conditions, MQ enantioselectively binds [(+)-MQ > (−)-MQ] at a site at which [^{3}H]CsA binds to Pgp. This is the first observation of enantioselective binding to human-derived Pgp.

MATERIALS AND METHODS

Materials

[^{3}H]VBL and [^{3}H]CsA were purchased from Amersham Life Science Products (Boston, MA). VBL, CsA, CHAPS, glycerol, and benzamidine were from Sigma Chemical Co. (St. Louis, MO). (+)-MQ and (−)-MQ were kindly provided by Hoffmann La Roche (Basel, Switzerland). Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT). The chromatographic backbone (immobilized artificial membrane PC stationary phase, IAM.PC) was obtained from Regis Chemical Co. (Morton Grove, IL). The HR5/2 glass column was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Preparation of the Pgp-SP

The Pgp-SP was prepared as previously described (8,9). In brief, the Pgp-positive membranes were obtained from the MDA435/LCC6^{MDR1} human breast cancer cell line (10). The cultured cells (8×10^7 cells) were harvested in 10 ml of buffer (Tris-HCl [50 mM, pH 7.4], 50 mM NaCl, 2 μM leupeptin, 2 μM phenylmethanesulfonyl fluoride, and 4 μM pepstatin). The suspension of cells was homogenized for 2 × 30 s, centrifuged at 1,000 × g for 10 min, and the supernatant was collected and centrifuged at 150,000 × g for 30 min.

The resulting pellets were added to 6 ml of Tris-HCl (50 mM, pH 7.4) containing 500 mM NaCl, 15 mM CHAPS, 2 mM DTT, and 10% glycerol. After 3 h at 4°C, the solution was mixed with 100 mg of dried IAM PC stationary phase, stirred for 1 h at room temperature, and then dialyzed against Tris-HCl (10 mM, pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 1 mM benzamidine for 36 h at 4°C (1.5 l/12 h).

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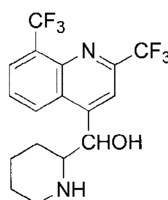

Fig. 1. The structure of mefloquine.

The resulting Pgp-SP was packed into a 0.5 (id) × 0.8 cm HR5/2 glass column. The resulting column was equilibrated with Tris-HCl (50 mM, pH 7.4) at room temperature for 3 h.

Frontal Chromatographic Studies Using Online Flow Scintillation Detection

The chromatographic system has been described (8,9). Detection of the marker ligands, [^{3}H]-VBL and [^{3}H]-CsA, was accomplished using an online flow scintillation monitor (Radiometric FLO-ONE® Beta 500 TR instrument, Packard Instruments). All chromatographic experiments were conducted at room temperature using a flow rate of 0.5 ml/min. The running buffers used in these studies were composed of Tris-HCl buffer (50 mM, pH 7.4) with and without the concomitant addition of 3 mM ATP.

The marker ligand, either 1.0 nM [^{3}H]-VBL or 2.0 nM [^{3}H]-CsA, was applied to the Pgp-SP in sample volumes of 25–50 ml. Before the initiation of a new series of studies, column performance was assessed by applying 1.0 nM [^{3}H]-VBL to the Pgp-SP. The appearance of specific frontal curves with a reproducible retention volume (~30 ml) indicated that there had been no degradation of column performance.

The solutions containing the marker ligands were supplemented with a range of concentrations of either cold VBL, CsA , (+)-MQ, or (−)-MQ. Elution profiles were obtained showing front and plateau regions. The observed elution volume data were used for calculation of ligand dissociation constants. The K_d value of (+)-mefloquine was calculated by nonlinear regression using Prism (GraphPad Software) and a one-site binding (hyperbola), Equation 1 (9,11).

$$Y = Bmax \bullet X/(K_d + X) \quad (1)$$

where X is the concentration of marker ligand (in the present experiment, it is VBL or CsA); Y is equal to [ligand] (V − Vmin), where Vmin is the elution volume of VBL or CsA under conditions where specific interactions are completely suppressed; and V is the retention volume of VBL or CsA at different concentrations of MQ (0.2–1.0 μM).

RESULTS AND DISCUSSION

The Effect of (+)-MQ and (−)-MQ on VBL Binding

When [^{3} H]-VBL was added to the running buffer and chromatographed on the Pgp-SP, frontal and plateau regions were obtained, Fig. 2A, trace 1, and Fig. 2B, trace 1. The relatively flat or shallow increase in the curve during the initial elution volume (0 to 30 ml) followed by a sharp increase in concentration of the marker (breakthrough) followed, in turn, by a plateau is indicative of a specific binding interaction between [^{3} H]-VBL and the immobilized Pgp.

When 0.2 μM (+)-MQ or 0.2 μM (−)-MQ were added to the running buffer, the specific frontal regions were lost from the [^{3} H]-VBL chromatograms (Fig. 2A, trace 2, and Fig. 2B, trace 2, respectively). These results indicate that the specific binding interaction between [^{3} H]-VBL and the immobilized Pgp had been lost. If MQ had competitively displaced [^{3} H]-VBL, the chromatographic traces would have mirrored the frontal curves seen in trace 1 (Figs. 2A and 2B), but the breakthrough volumes would have been reduced. The change in the shapes of the curves is consistent with an anticooperative allosteric interaction. In this process, MQ binds to a contiguous or separate site on the Pgp molecule producing secondary effects at the VBL binding site that reduce the ability of VBL to bind to that site.

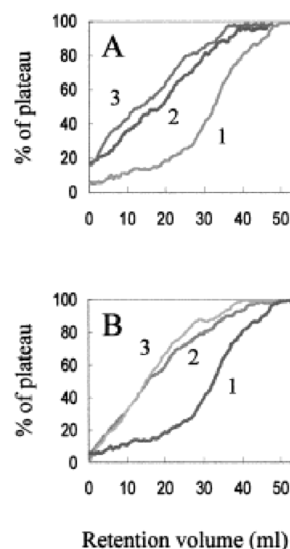

Fig. 2. The effect of MQ enantiomers on the frontal affinity chromatography of 1.0 nM [^{3}H]-VBL on the Pgp-SP where: **(A)** Trace 1: [^{3}H]-VBL alone; Trace 2: 200 nM (+)-MQ added to the running buffer, Trace 3: 200 nM (+)-MQ plus 3 mM ATP added to the running buffer. **(B)** Trace 1: [^{3}H]-VBL alone; Trace 2: 200 nM (−)-MQ added to the running buffer, Trace 3: 200 nM (−)-MQ plus 3 mM ATP added to the running buffer. The running buffer was 50 mM Tris-HCl (50 mM, pH 7.4).

Anticooperative allosteric effects on the binding of VBL to immobilized Pgp have been observed after the addition of 3 mM ATP to the running buffer (9). In the present study, the addition of 3 mM ATP to a running buffer containing either 0.2 μM (+)-MQ or 0.2 μM (−)-MQ produced no significant change in the observed VBL chromatograms (Fig. 2A, trace 3, and Fig. 2B, trace 3, respectively). These results support the observation that the effect produced by the addition of MQ to the running buffer was allosteric in nature and not competitive.

Shao *et al.* (4) demonstrated that MQ and VBL cooperate to reduce daunomycin transport and that VBL did not significantly affect the K_i of MQ relative to daunomycin transport. The authors concluded that MQ and VBL act at physically different sites on the Pgp molecule. The hypothesis developed in the current study that MQ affects VBL through an anticooperative allosteric interaction is consistent with these conclusions. In addition, previous studies with the Pgp-SP (9) have demonstrated that VBL competitively displaced doxorubicin, a compound closely related to daunomycin. Thus, the two-site synergism of MQ and VBL relative to

daunomycin transport can be explained by a competitive displacement produced by VBL at the site at which daunomycin binds to Pgp and an anticooperative allosteric interaction produced by MQ binding to a different site.

At the MQ concentrations used in this study (0.2–1.0 μM), both (−)-MQ and (+)-MQ had equivalent effects on VBL binding and no enantioselectivity was observed. The lack of enantioselectivity is consistent with the results from the studies in Caco-2 cells but not with the eightfold greater effect of (+)-MQ observed in rat brain capillary endothelial GPNT cells (6,7). The source of the difference in MQ enantioselectivity between the Caco-2 and GPNT cell lines is not clear and may simply reflect a species-dependent variation in Pgp (12).

The Effect of (+)-MQ and (−)-MQ on CsA Binding

Previous studies with the Pgp-SP have demonstrated that the presence of 3 mM ATP in the running buffer is required to produce specific binding interactions between CsA and the immobilized Pgp (9). This effect appears to be produced through a cooperative allosteric interaction. Therefore, in the present investigation, when [^{3}H]-CsA was the marker ligand, all of the chromatographic experiments were conducted with 3 mM ATP in the running buffer.

In these experiments, increasing concentrations of MQ from 0.2 to 1.0 μM were added to the running buffer. This concentration range reflects maximum steady-state plasma concentrations of (−)-MQ (~4.0 μM) and (+)-MQ (~0.7 μM) (3). In addition, MQ concentrations of >10 μM saturated the Pgp-SP.

The addition of increasing concentrations of (+)-MQ to the running buffer produced corresponding reductions in [^{3}H]-CsA retention volumes. Although the retention volumes decreased, the specific frontal chromatograms were retained (Fig. 3), signifying the existence of a competitive binding interaction between (+)-MQ and [^{3}H]-CsA. Using Eq. 1, the K_d value for (+)-MQ was calculated to be 471 ± 146 nM, which is consistent with the K_i value of 410 nM determined for the effect of MQ on daunomycin accumulation in P388/MDR cells (4).

Addition of increasing concentrations of (−)-MQ did not effect the retention volume of [^{3}H]-CsA, nor was there a change in the specific frontal chromatograms (data not shown). This indicates that (−)-MQ was not able to competitively displace [^{3}H]-CsA. Therefore, there is an enantioselective difference in the affinities of the MQ enantiomers, (+)-MQ > (−)-MQ, at a site at that [^{3}H]-CsA binds to Pgp. To our knowledge, this is the first report of enantioselective binding to Pgp.

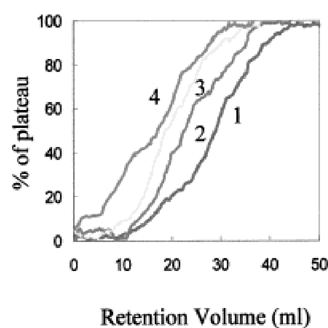

Fig. 3. The effect of (+)-MQ on the frontal affinity chromatography of 2.0 nM [^{3}H]-CsA on the Pgp-SP where: 1) represents the elution profile of [^{3}H]-CsA without (+)-MQ in the running buffer; 2) after addition of 0.5 μM (+)-MQ; 3) after addition of 0.8 μM (+)-MQ; 4) after addition of 1.0 μM (+)-MQ. The running buffer was Tris-HCl (50 mM, pH 7.4) containing 3 mM ATP.

CONCLUSIONS

Enantioselectivity at a site at which CsA binds to Pgp presents two avenues for further investigation of this site. Because the physicochemical properties of enantiomers are equivalent, (−)-MQ can be used to determine nonspecific interactions with Pgp allowing for a clearer picture of the specific binding interactions between (+)-MQ and Pgp. In addition, enantioselective binding at this site also provides a three-dimensional probe that can be used in pharmacophore modeling.

The data from this study also demonstrate that MQ and VBL bind to separate but allosterically interconnected sites on the Pgp-SP. This model is consistent with previous data obtained with the Pgp-SP demonstrating that CsA and VBL have separate, but closely linked binding sites on the Pgp molecule (9). Because the affect of MQ on VBL binding was anticooperative and not enantioselective, (+)-MQ, (−)-MQ, or the racemic mixture could be used to produce the synergistic effects on Pgp transport reported by Shao *et al.* (4). However, this does not hold for CsA Because (+)-MQ had a greater effect on CsA binding to Pgp than (−)-MQ. Thus, the enantioselectivity of the MQ interactions with Pgp present a number of possible clinical options for paired inhibitors. For example, if VBL is the target, then one might use a combination of (−)-MQ and CsA and, conversely, if CsA is the target, one might use (+)-MQ and VBL. The results suggest that competitive binding experiments on the Pgp-SP may be a rapid method for the identification of synergistic pairs for specific clinical targets.

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