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Research Paper

The Antiepileptic Drug Topiramate is a Substrate for Human P-glycoprotein but Not Multidrug Resistance Proteins

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Purpose. Resistance to antiepileptic drugs (AEDs) is the major problem in the treatment of epilepsy. One of the candidate mechanisms of pharmacoresistance is the limitation of AED access to the seizure focus by overexpression of efflux transporters, including P-glycoprotein (Pgp) and multidrug resistance proteins (MRPs).In this respect, it is important to know which AEDs are substrates for such drug transporters in humans.
Methods. In the present study, we used polarized kidney cell lines (LLC, MDCK) transfected with human drug transporters (Pgp, MRP1, MRP2 or MRP5) to evaluate whether the AED topiramate is a substrate for any of these transporters. Known Pgp and MRP substrates were used for comparison.
Results. Basolateral-to-apical transport of topiramate, which could be counteracted with the Pgp inhibitor, tariquidar, was determined in Pgp overexpressing LLC cells, whereas topiramate was not transported by any of the MRPs. A comparison with previous experiments in the same transport assay showed that topiramate exhibited the most pronounced Pgp-mediated efflux transport among the AEDS that have been studied as yet.
Conclusions. Thus, these data indicate that brain levels of topiramate may be affected by overexpression of Pgp as determined in patients with intractable epilepsy.

KEY WORDS: blood-brain barrier; epilepsy; multidrug transporters; pharmacoresistance.

INTRODUCTION

The antiepileptic drug (AED) topiramate is approved for the treatment of epilepsy and prophylaxis of migraine, but is also used for treatment of bipolar disorder, neuropathic pain, depression, obesity, alcoholism and post-traumatic stress disorder (1). As with other AEDs, a substantial fraction of patients with epilepsy is resistant to treatment with topiramate (2). The mechanisms underlying this pharmacoresistance are only incompletely understood but may involve both pharmacodynamic and pharmacokinetic factors (3). In this respect, two plausible hypotheses have gained considerable favor in recent years, the *target hypothesis*, which suggests that intrinsic (genetic) or acquired (disease-related) alterations to the structure and/or functionality of AED targets in epileptogenic brain regions lead to reduced drug effects, and the *transporter hypothesis*, which suggests that AED levels are decreased at their brain targets because of intrinsic or acquired overexpression of drug efflux transporters, such as P-glycoprotein (Pgp), in epileptogenic brain regions (4,5).

In a model of AED-resistant epilepsy in rats, i.e., phenytoin-resistant kindled rats, resistance extended to topiramate and various other AEDs (6), which reflects the clinical situation in most patients with pharmacoresistant epilepsy, indicating that resistance is mediated by unspecific, pharmacokinetic factors rather than by specific drug-target alterations (3). Phenytoin-resistant kindled rats exhibit a significant upregulation of Pgp at brain capillary endothelial cells in the epileptogenic focus region (7), which would restrict the penetration of all drugs that are Pgp substrates into this region, thus representing a plausible mechanism of multidrug resistance (4). Such overexpression of Pgp has also been determined in patients with pharmacoresistant epilepsy (3). Furthermore, several members of the multidrug resistance protein (MRP) family, namely MRP1, MRP2 and MRP5, were found to be upregulated in epileptogenic brain tissue of patients with intractable epilepsy (8). This prompted us to study whether topiramate is a substrate of human Pgp, MRP1, MRP2 or MRP5. For this purpose, we used a highly sensitive assay (concentration equilibrium transport assay; CETA) in polarized kidney cell lines (LLC, MDCK) transfected with human efflux transporters. By using this assay, we demonstrated recently that several major AEDs are substrates for human Pgp (9). The present study shows for the first time that topiramate is transported by human Pgp.

MATERIALS AND METHODS

Cell Lines and Cell Cultures

LLC-PK1 cells transfected with human *MDR1* (LLC-MDR1) and respective wildtype (Wt) LLC cells as well as MDCK

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type II cells transfected with human MRP1 (MDCK-MRP1), MRP2 (MDCK-MRP2) or MRP5 (MDCK-MRP2) and respective MDCKII wildtype cells were kindly provided by Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands). After obtaining the cells, they were cultured as described in detail recently (10,11). Cells were used within ten passages or less after thawing from liquid nitrogen, and at a maximum of 13 passages after receiving them from Prof. Borst. Because transfected LLC cells may lose the transporter cDNA in the absence of a selection agent such as vincristine, they were regularly tested for vincristine resistance (640 nmol) before being used for transport experiments (for details see Baltes *et al.* (11)).

Transcellular Transport Assays

Cells were seeded on transparent polyester membrane filters (Transwell-Clear®, 24 mm diameter, 0.4 μm pore size, Corning Costar Corporation, Cambridge, MA, USA) at a density of 0.3×10^6 cells/cm^2 (LLC) or 0.4×10^6 cells/cm^2 (MDCK), cultured for 1–2 days to confluence and used for transport assays between days 5 and 7 after confluence (for details see Baltes *et al.* (10,11)). Transport studies were performed with the filter inserts in Transwell® multiwell culture plates that allow studying drug transport between an apical and basolateral compartment. For the present experiments, 6-well plates were used. Before starting the transport experiments, the medium was replaced with Opti-MEM® (Gibco™/Invitrogen Corporation, Eggenstein, Germany), and the transwells were pre-incubated for one hour (with or without transport inhibitor, respectively; see below). This reduced serum medium was used without any additives according to the protocol of the laboratory that provided the cell lines (Prof. P. Borst) in order to minimize protein binding of the drugs. At the beginning of the experiment ($t=0$), the pre-incubation medium was replaced by fresh Opti-MEM® containing the drug in both chambers (see below). The volumes in the upper and lower compartment were 2,000 μl and 2,700 μl, respectively. For drug analysis, samples were taken at 60, 120, 240, and 360 min (in some experiments also 480 and 600 min). The transport assays including pre-incubation were performed at 37°C in a humidified incubator (5% CO_2) with shaking the transwells gently at 50 rpm. Monolayers were checked for integrity by measuring transepithelial electrical resistance (TEER) of the polarized cells before and after each transport experiment and by using [^{14}C]-mannitol (in separate wells) as described recently (9). Experiments in which TEER values decreased by more than 15% compared to initial readings were discarded. Less than 1% of mannitol diffusion per hour and an apparent permeability (P_{app}) of [^{14}C]-mannitol lower than 12 nm/sec were used as indicators of integrity of the monolayer. To check for functional Pgp or MRPs in the apical membrane of cell monolayers, transport of the reference substrates digoxin, calcein and CMFDA (see below) was tested in separate wells in each experiment with topiramate.

Each transport study was initiated by adding the drug to both (apical and basolateral) sides of the monolayer, so that initial drug concentration was the same in both compartments (for details see Luna-Tortós *et al.* (9)). In experiments with transport inhibitors, the respective inhibitor was also added to both chambers. For drug analysis, aliquots were collected from both compartments over the course of an experiment (100 μl basolateral and 130 μl apical, in order to avoid influences by hydrostatic pressure). Except the first experiment with topiramate in LLC-MDR1 cells (see Results), each experiment was performed in triplicate and, except for topiramate in MRP2 and MRP5 transfected cells, repeated at least once.

Drugs

Topiramate was kindly provided by Johnson & Johnson (Raritan, NJ, USA) and studied at a concentration of 10 μg/ml (30 μM), which is within the plasma concentration range following administration of therapeutic doses of this drug in patients with epilepsy (12). As a reference substrate for Pgp, [^{3}H]-digoxin (40 Ci/mmol; PerkinElmer, Rodgau-Jügesheim, Germany) was diluted with unlabeled digoxin (Sigma-Aldrich; Taufkirchen, Germany) to give an activity of 0.05 μCi/ml and a final concentration of 10 nM in the assay. For inhibition of Pgp, tariquidar (kindly provided by Xenova, Slough, Berkshire, U.K.) was used at a concentration of 0.5 μM. To avoid that expression of endogenous Pgp by MDCK cells interfered with MRP-mediated drug transport, tariquidar (0.5 μM) was also added in all experiments with MRP-transfected MDCK cells (except the experiments with CMFDA; see below). In MDCK-MRP1 cells, calcein acetoxymethyl ester (calcein-AM; 1 μM; Invitrogen, Karlsruhe, Germany) was used as a sensitive and specific tool for measurement of MRP1 activity (13,14). The lipophilic calcein-AM diffuses into cells where it is cleaved by intracellular esterases, resulting in fluorescent calcein, which is actively extruded by MRP1 (14,15). Tariquidar (0.5 μM) was included in the assay to inhibit transport of calcein-AM by Pgp. In MDCK-MRP2 cells, vinblastine was used as a reference substrate (11). For this purpose, [^{3}H]-vinblastine sulphate (9.8 Ci/mmol; Amersham, Buckinghamshire, U.K.) was diluted with unlabeled vinblastine sulphate (Alexis Biochemicals, Lörrach, Germany) to give an activity of 0.025 μCi/ml and a final concentration of 2 μM in the assay. Tariquidar (0.5 μM) was included in the assay to inhibit transport of vinblastine by Pgp (11). In MDCK-MRP5 cells, 5-chloromethylfluorescein diacetate (CMFDA [CellTracker™ Green]; 2.5 μM; Invitrogen) was used as a reference substrate (17). CMFDA is a nonfluorescent, membrane-permeable compound that is hydrolyzed by esterases intracellularly to a thiol-reactive, fluorescent, negatively charged intermediate that is membrane-impermeable but extruded from the cell by MRP5 (16,17). Preliminary experiments indicated that the Pgp inhibitor tariquidar does not affect the efflux of the CMFDA metabolite in MDCK-MRP5 cells (not illustrated), so that the Pgp inhibitor was not included in the present experiments with CMFDA. For inhibition of MRP-mediated efflux, MK571 [*(E)*-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl] phenyl]-[[3-dimethylamino-3-oxopropyl]thio]methyl]thio]-propanoic acid, sodium salt], obtained from Alexis Biochemicals, was used at a concentration of 50 μM in the experiments with MRP1, MRP2 and MRP5 expressing MDCK cells.

Topiramate was dissolved in purified water and then diluted in the cell culture medium. Vinblastine sulphate was dissolved in purified water. Digoxin, tariquidar, calcein-AM and CMFDA were dissolved in DMSO (<0.1% DMSO in final solution), and MK571 was dissolved in medium.

Drug Analysis

Topiramate was analyzed by liquid chromatography with a mass specific detector (LC-MS-MS) with an API-2000 triple quad MS (Applied Biosystems, Applera GmbH, Darmstadt, Germany). Details are described elsewhere (12).

The radioactivity in samples from experiments with [^{3}H] vinblastine or [^{3}H]digoxin was quantified using a scintillation counter. Calcein and the fluorescent CMFDA metabolite were quantified using a Fluoroscan II (LabSystems® Oy; Helsinki, Finland) with 485 nm excitation and 538 nm emission filters.

Statistics

Results of the individual transport assays are presented for each chamber as the percentage of the initial drug concentration *versus* time. An exception are the experiments with calcein-AM and CMFDA, for which results are presented as absolute fluorescence of calcein or the CMFDA metabolite per chamber. The statistical significance of differences between drug concentrations in the two chambers was calculated by two-way analysis of variance (ANOVA) for repeated measures, followed by Bonferroni post-tests.

RESULTS

In MDR1-transfected LLC cells with apical expression of Pgp, concentrations of the Pgp substrate digoxin rapidly increased in the apical chamber, but decreased in the basolateral chamber as a result of basolateral-to-apical transport by Pgp (Fig. 1A). This Pgp-mediated transport could be completely prevented by the Pgp inhibitor tariquidar. Some transport by endogenous Pgp, which could be inhibited by tariquidar, was also determined in LLC wildtype cells.

In a first experiment with topiramate in LLC-MDR1 cells, a clear asymmetrical (basolateral-to-apical) transport was observed, indicating that this drug is a substrate for human Pgp (Fig. 1B). This was substantiated in a follow-up experiment (Fig. 1C), in which the transport of topiramate in LLC-MDR1 cells could be completely counteracted by tariquidar. In LLC-wt cells, no transport was observed.

In MRP1-transfected MDCK cells with basolateral expression of MRP1, a marked apical-to-basolateral transport

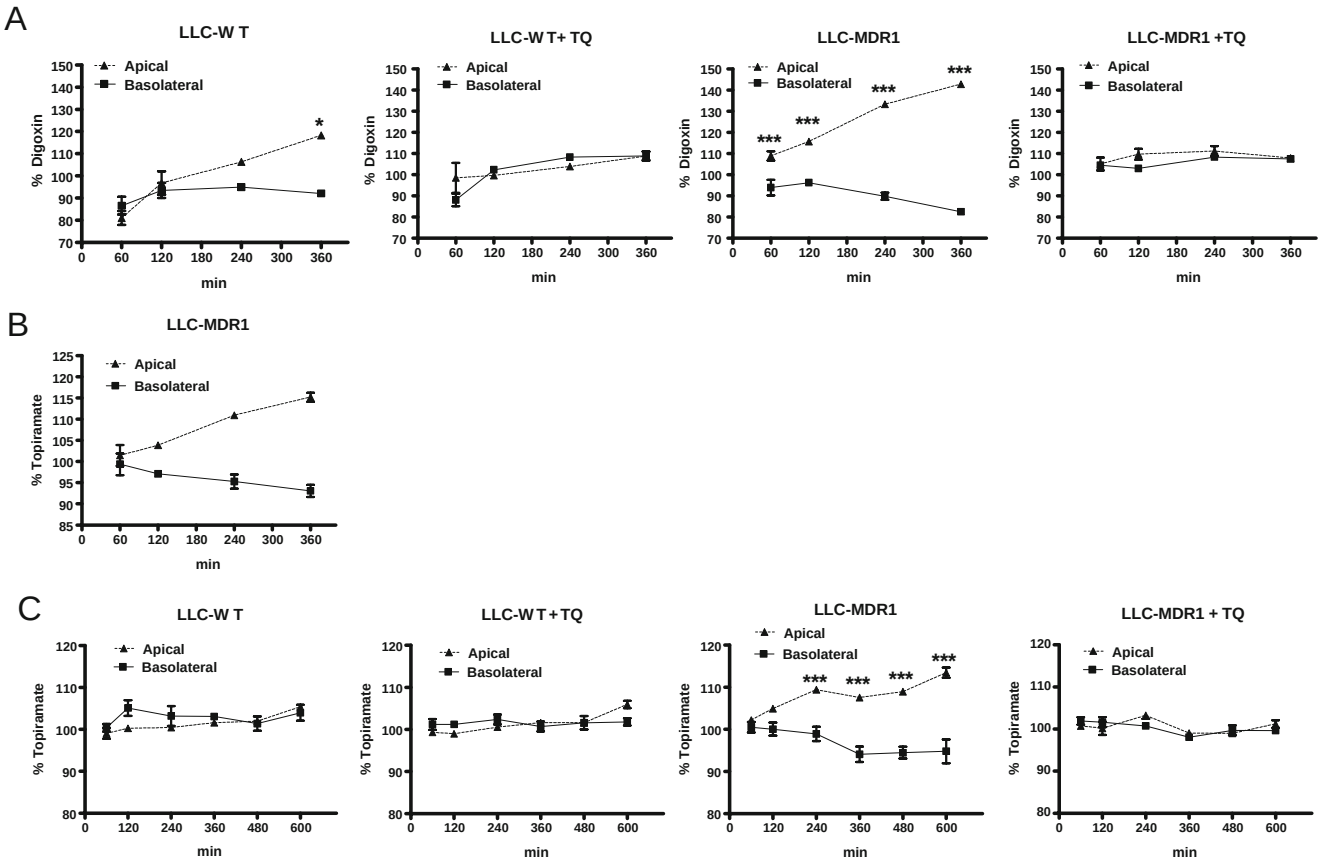

Fig. 1. Representative experiments with digoxin (**A**) and topiramate (**B**, **C**) in LLC cells transfected with human *MDR1* and wildtype (Wt) LLC cells. Experiments were performed by concentration equilibrium transport assay (CETA), in which the drug is added to both (apical and basolateral) sides of the monolayer, so that the initial drug concentration is the same in both compartments. Data are given as the percentage of the initial drug concentration (= 100%) in either the apical or basolateral chamber *versus* time. Significant differences between the two chambers are indicated by asterisk (*P<0.05; **P<0.01; ***P<0.001). In all experiments, except the first experiment with tariquidar shown in "C," it was tested whether directional transport can be inhibited with the Pgp inhibitor tariquidar (TQ). Except for the experiment shown in "B," which was performed in duplicate (thus excluding statistical analysis), all experiments were performed in triplicate and values are shown as means ± SEM. When no error bar is visible, the deviation was within the size of the symbols.

was determined for calcein (Fig. 2). This transport could be largely reduced by the MRP inhibitor MK571. In contrast to calcein, no apical-to-basolateral transport was determined for topiramate (Fig. 2).

In MRP2-transfected MDCK cells with apical expression of MRP2, a significant basolateral-to-apical transport was determined for vinblastine (Fig. 2). This asymmetrical transport was almost completely prevented by MK571. No basolateral to apical transport was observed with topiramate (Fig. 2).

In MRP5-transfected MDCK cells with basolateral expression of MRP5, a significant apical-to-basolateral transport was determined for the fluorescent CMFDA metabolite (Fig. 2). As in the other MRP-transfected cell lines, this transport could be blocked by MK571. In contrast to the CMFDA metabolite, no apical-to-basolateral transport was determined for topiramate (Fig. 2). It should be noted that, except for the experiments with CMFDA, tariquidar (0.5 μM) was present in all experiments with MRP-transfected MDCK cell lines to prevent any transport by endogenous Pgp.

DISCUSSION

The present experiments showed that topiramate is transported by human Pgp in a polarized porcine kidney cell line (LLC) that is widely used as an *in vitro* model of the blood-brain barrier (BBB) for drug transport studies (10,11,18–20). Transport of topiramate in LLC-MDR1 cells could be completely prevented by the Pgp inhibitor tariquidar, and no asymmetric transport was observed in LLC wildtype cells, demonstrating the selectivity of the transport mediated by human Pgp in the transfected cells. At higher concentrations than those needed to inhibit Pgp, tariquidar also inhibits breast cancer resistance protein (BCRP;

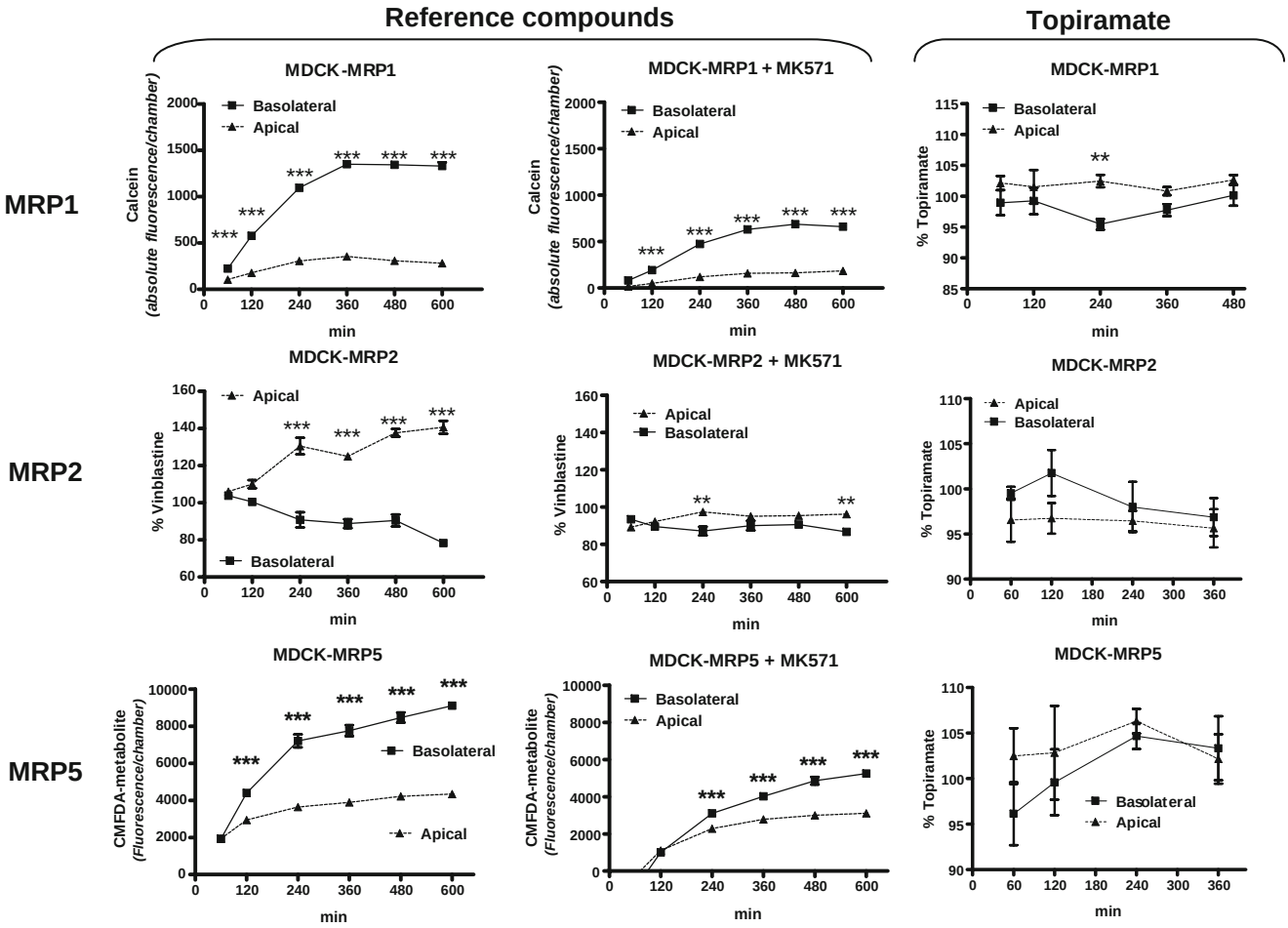

Fig. 2. Representative experiments with known MRP substrates and topiramate (B,C) in MDCK cells transfected with human MRP1, MRP2 or MRP5. Experiments were performed by concentration equilibrium transport assay (CETA), in which the drug is added to both (apical and basolateral) sides of the monolayer, so that the initial drug concentration is the same in both compartments. For vinblastine and topiramate, data are given as the percentage of the initial drug concentration (= 100%) in either the apical or basolateral chamber *versus* time. For calcein and the fluorescent CMFDA metabolite, data are shown as absolute fluorescence per chamber. Significant differences between the two chambers are indicated by asterisk (*$P<0.05$; **$P<0.01$; ***$P<0.001$). In all experiments, except those with CMFDA, the Pgp inhibitor tariquidar (0.5 μM) was added to block endogenous Pgp in the MDCK cells. In the experiments with known MRP substrates (calcein-AM for MRP1, vinblastine for MRP2, CMFDA for MRP5), it was tested whether directional transport can be inhibited with the selective MRP inhibitor MK571. All experiments were performed in triplicate and values are shown as means ± SEM. When no error bar is visible, the deviation was within the size of the symbols.

ABCG2)(21), but the lack of any asymmetric transport of topiramate in LLC wildtype cells argues against the possibility that endogenous BCRP was involved in the basolateral-to-apical transport of topiramate and its inhibition by tariquidar determined in MDR1-transfected LLC cells. In a previous study in *Mdr1a* knockout mice that lack Pgp at the BBB, the brain-serum concentration ratio for topiramate was about two-fold higher in knockout than in wild-type animals, suggesting that topiramate is a substrate for Pgp-mediated transport in this species (22). However, in view of species differences in substrate recognition by Pgp between mice and humans (10,19,23,24), the study by Sills *et al.* (22) in mice did not conclude that topiramate is also a substrate of human Pgp.

To our knowledge, there is only one previous study that examined whether topiramate is transported by human Pgp (25). In this study, a variety of AEDs were tested for their ability to be transported by Pgp through Caco-2 monolayers using bi-directional (apical-to-basolateral and basolateral-to-apical) studies. No evidence could be found to indicate Pgp-mediated efflux of topiramate in this immortalized line of heterogeneous human epithelial colorectal adenocarcinoma cells, but instead greater apical-to-basolateral transport was observed, which could not be affected by the MRP inhibitor MK571 (25). Caco-2 cells differ from the LLC cells used in the present experiments by the expression of various influx transporters that could conceal active efflux by Pgp, thus limiting the usefulness of Caco-2 cells for discerning the specific role of Pgp in drug transport (26). Furthermore, the conventional bi-directional transport assay with the Transwell system used by Crowe and Teoh (25), in which the drug is applied to either the apical or basolateral chamber for studying bi-directional transport, may fail to identify highly permeable compounds as Pgp substrates, because passive transcellular diffusion forms a bias in such assays by concealing active transport (9,27).

In the modified transport assay (CETA) used in the present experiments, the drug is initially added at identical concentration to both sides of the polarized, transporter-overexpressing cell monolayer, thus minimizing the influence of transcellular diffusion in response to concentration gradients (9). Under these concentration equilibrium conditions, a clear unidirectional (basolateral-to-apical) efflux of topiramate was determined in MDR1-transfected LLC cells in the present study.

To allow a comparison of the magnitude of the Pgp-mediated transport of topiramate with that of the known Pgp substrate digoxin and other AEDs determined by us recently in the concentration equilibrium assay with LLC-MDR1 cells, the area under curve (AUC) in the apical chamber was calculated in percent above the initial concentration × time for all experiments (see Luna-Tortós *et al.* (9) for details). As shown in Fig. 3, topiramate exhibited the most pronounced directional transport among the AEDS that have been studied as yet. However, compared to the prototype Pgp substrate digoxin, the Pgp-mediated transport determined for AEDs appeared weak. However, a number of factors may lead to underestimation of drug transport in *in vitro* assays as used in the present study. First, it has to be considered that even under the conditions of the modified transport assay used in this study, passive diffusion will affect the transport of highly permeable drugs such as most AEDs. The directional transport from the basolateral to the apical compartment, as observed for topiramate and several other AEDs in the equilibrium assay, leads to a concentration gradient between the two compartments and thereby rediffusion from the apical to the basolateral chamber, thus reducing the amount of drug in the apical compartment. As a consequence, directional transport by Pgp will be underestimated. Second, *in vitro* assays are performed over a limited period of time,

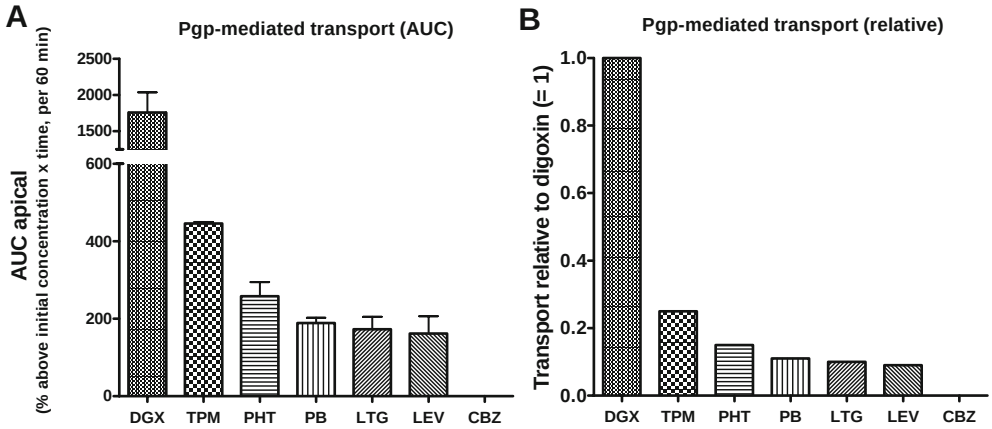

Fig. 3. Comparison of the magnitude of basolateral-to-apical transport of drugs in LLC-MDR1 cells under the conditions of the concentration equilibrium transport assay. For the data shown in "A," transport was estimated by calculating the area under the drug concentration *vs.* time curves (AUC) in the apical chamber in percent above the initial concentration × time (in min)(for more details, see Luna-Tortós *et al.* (9)). Data are shown as means ± SEM. To allow comparing experiments with a duration of either 360 or 600 min, all data are expressed per 60 min. Each experiment was performed in triplicate and repeated at least twice (range 2–8 experiments per drug). "B": Based on AUCs, transport was also calculated relative to the prototype Pgp substrate digoxin (= 1). Except for topiramate, data are from Luna-Tortós *et al.* (9). No Pgp-mediated transport was determined for carbamazepine. Abbreviations (and tested concentrations): DGX, digoxin (10 nM); TPM, topiramate (30 μM); PHT, phenytoin (50 μM); PB, phenobarbital (50 μM); LTG, lamotrigine (20 μM); LEV, levetiracetam (210 μM); CBZ, carbamazepine (30 μM). All AEDs were tested at concentrations within their therapeutic concentration range known from patients with epilepsy.

usually 4–6 h, whereas under *in vivo* conditions, patients are treated with AEDs on a chronic basis, so that AED efflux by Pgp from brain to blood will occur more or less continuously. Third, *in vivo* endothelial cells are continuously exposed to shear stress generated by the flow of blood across their apical surface, whereas *in vitro* transport assays using the Transwell system are static models lacking shear stress that affects the transport of drugs across biological membranes *in vivo* (28). Fourth, *in vivo* the BBB is characterized by extremely low paracellular diffusion, because of the expression of highly specialized tight junctions, what in turn forces most molecules to cross the barrier through transcellular route (29). *In vitro* BBB models are more "leaky" than the *in vivo* BBB and, hence, the interference of high permeability with the active transport is probably more pronounced in the *in vitro vs*. the *in vivo* situation. Thus, data from *in vitro* transport assays as used in the present study can be used as a proof-of-concept to determine whether a drug is a substrate of human Pgp, but only *in vivo* experiments can prove whether this transport affects therapeutic brain concentrations and thus the clinical response to this drug.

In contrast to transport by human Pgp, no evidence could be found to indicate MRP-mediated efflux of topiramate. For this part of the study, a polarized dog kidney cell line (MDCK) transfected with either MRP1, MRP2 or MRP5 was used. Because MDCK cells express substantial amounts of endogenous (dog) Pgp (10,30), all experiments with topiramate were carried out in the presence of the Pgp inhibitor tariquidar. Experiments with known substrates of MRPs and the selective MRP inhibitor, MK571, substantiated the known localization (apical *vs*. basolateral) and functionality of the transporters (31,32).

In conclusion, by using a modified version of the widely used Transwell assay with *MDR1*-transfected LLC cells, the present experiments provide the first direct evidence that topiramate is a substrate of human Pgp. Thus, upregulation of Pgp in the brain, as found in patients with pharmacoresistant epilepsy, may restrict concentrations of topiramate at its brain targets and thereby contribute to resistance. In contrast to Pgp, topiramate was not transported by any of the MRPs examined in the present study. Thus, the overexpression of MRP1, MRP2 and MRP5 found in brain capillary endothelial cells of patients with intractable epilepsy (33) is unlikely to affect brain concentrations of topiramate. Whether other AEDs are transported by MRPs remains to be studied.

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