TARGETING PROSTAGLANDIN E₂ EP1 RECEPTORS PREVENTS SEIZURE-ASSOCIATED P-GLYCOPROTEIN UP-REGULATION ‡

Anton Pekcec, Bernadette Unkrüer, Juli Schlichtiger, Jonna Soerensen, Anika M.S. Hartz, Björn Bauer, Erwin A. van Vliet, Jan A. Gorter, Heidrun Potschka

Institute of Pharmacology, Toxicology, and Pharmacy, Ludwig-Maximilians-University, Munich, Germany (A.P., B.U., J.S., J.S., H.P.)
Department of Biochemistry and Molecular Biology, Medical School, University of Minnesota, Duluth, MN, USA (A.M.S.H.)
Department of Pharmaceutical Sciences, College of Pharmacy, University of Minnesota, Duluth, MN, USA (B.B.)
Epilepsy Institute of The Netherlands, Heemstede, The Netherlands; Center for Neuroscience, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands (E.A.v.V., J.A.G.)
Running Title Page

Running Title: EP1 receptor and P-glycoprotein regulation

Corresponding Author: Dr. Heidrun Potschka
Institute of Pharmacology, Toxicology, and Pharmacy
Ludwig-Maximilians-University Munich
Koeniginstr. 16; 80539 Munich, Germany
Phone: +49-89-21802662; Fax: +49-89-342316
Email: potschka@pharmtox.vetmed.uni-muenchen.de

Text pages: 34
Tables: 0
Figures: 5
References: 54
Abstract: 246 words
Introduction: 534 words
Discussion: 2209 words

Abbreviations: COX, cyclooxygenase; COX-2, cyclooxygenase-2; TBS, tris-buffered saline;
P-gp, P-glycoprotein; BBB, blood-brain barrier; PGE2, Prostaglandin E2; EEG,
Electroencephalogram; NMDA, N-methyl-D-aspartic acid; CNS, central nervous system

Section: Neuropharmacology
Abstract

Up-regulation of the blood-brain barrier efflux transporter P-glycoprotein in CNS disorders results in restricted brain access and limited efficacy of therapeutic drugs. In epilepsies, seizure activity strongly triggers expression of P-glycoprotein. Here, we identified the prostaglandin E2 receptor, EP1, as a key factor in the signaling pathway that mediates seizure-induced up-regulation of P-glycoprotein at the blood-brain barrier.

In the rat pilocarpine model, status epilepticus significantly increased P-glycoprotein expression by 92% to 197% in the hippocampal hilus and granule cell layer as well as the piriform cortex. The EP1 receptor antagonist, SC-51089 (8-Chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-[1-oxo-3-(4-pyridinyl)propyl]hydrazide hydrochloride), abolished seizure-induced P-glycoprotein up-regulation and retained its expression at control level. The control of P-glycoprotein expression despite prolonged seizure activity suggests that EP1 receptor antagonism will also improve antiepileptic drug efficacy.

Data which give preliminary evidence for this concept have been obtained using a massive kindling paradigm during which animals received a sub-chronic SC-51089 treatment. Following withdrawal of the EP1 receptor antagonist, a low dose of the P-glycoprotein substrate phenobarbital resulted in an anticonvulsant effect in this pre-treated group, whereas the same dosage of phenobarbital did not exert a significant effect in the respective control group.

In conclusion, our data demonstrate that EP1 is a key signaling factor in the regulatory pathway that drives P-glycoprotein up-regulation during seizures. These findings suggest new intriguing possibilities to prevent and interrupt P-glycoprotein over-expression in epilepsy. Future studies are necessary to further evaluate the appropriateness of the strategy to enhance the efficacy of antiepileptic drugs.
Introduction

Blood-brain barrier efflux transporters restrict the access of various CNS therapeutics to the target tissue (Loscher and Potschka, 2005). Whereas basal expression levels can already constitute a limiting factor, the situation worsens when pathophysiology-associated transcriptional activation of efflux transporters further strengthens the barrier function in different CNS diseases. In epilepsy, seizures strongly induce expression of the efflux transporter, P-glycoprotein (Loscher and Potschka, 2005). This over-expression has been shown to correlate with reduced brain penetration of antiepileptic drugs and a reduction or even complete loss of pharmacosensitivity (Rizzi et al., 2002; van Vliet et al., 2007; Wen et al., 2008). Based on these data, P-glycoprotein over-expression is discussed as one factor accounting for the high rate of failure in epilepsy therapy with lack of satisfactory seizure control in up to 40% of patients (Kwan and Brodie, 2006).

An obvious strategy to overcome transporter-mediated pharmacoresistance is interference with efflux transport by competitive or non-competitive transporter inhibitors. On an experimental level, add-on treatment with a P-glycoprotein inhibitor conclusively resulted in almost complete seizure control in rats with pharmacoresistance to the antiepileptic drug phenobarbital (Brandt et al., 2006). However, direct transporter inhibition also affects basal transporter function throughout the body, thereby limiting the protective function of efflux transport and increasing exposure of sensitive tissues to harmful xenobiotics (Fromm, 2004). Taking these caveats into consideration, targeting the regulatory pathways that drive efflux transporter over-expression during disease could be a highly promising alternative approach. Thus, elucidation of the mechanistic links that connect seizure activity to increased P-glycoprotein expression holds the promise to identify new therapeutic targets for preventing seizure-induced transporter over-expression and improving antiepileptic drug therapy. Recently, we reported that excessive glutamate release during epileptic seizures acts as an initial trigger of a signaling cascade in brain capillary endothelial cells that drives P-
glycoprotein up-regulation (Bauer et al., 2008). We demonstrated that glutamate signaling through the NMDA receptor and cyclooxygenase-2 (COX-2) leads to P-glycoprotein over-expression in response to seizures (Bauer et al., 2008). Importantly, inhibition of COX-2 abolished the glutamate effect on P-glycoprotein expression, suggesting that COX-2 inhibition could be used to improve delivery of antiepileptic drugs to the target sites in epileptogenic brain regions and to restore pharmacosensitivity. However, using this approach one needs to bear in mind potential COX-2 inhibitor side effects including increased risk for cardio- and cerebrovascular events. Therefore, it is of interest to identify downstream signaling proteins that might be alternative targets to prevent P-glycoprotein up-regulation in the epileptic brain.

Prostaglandin E2 is the major product of COX-2 signaling in the brain. It acts on four different G-protein-coupled receptors (EP1, EP2, EP3 and EP4), each of which with a very distinct signal transduction profile and often opposing cellular actions (Hata and Breyer, 2004). While blocking EP2, EP3, and EP4 can aggravate neurodegeneration (Bilak et al., 2004; McCullough et al., 2004; Ahmad et al., 2005), blocking EP1 has neuroprotective effects (Suganami et al., 2003; Kawano et al., 2006). Thus, EP1 represents the most interesting target on which we focused our experimental efforts. Using rodent epilepsy models, we tested our hypothesis that EP1 receptors play a key role in signaling events that drive P-glycoprotein expression in the epileptic brain and that antagonism of EP1 receptors prevents P-glycoprotein induction.
Methods

Animals

Seventy-three female Wistar Unilever rats (200-220g, Harlan-Winkelmann, Netherlands, Horst, The Netherlands) were used for the pilocarpine model. To study if SC-51089 pretreatment increases the efficacy of phenobarbital on seizure thresholds by controlling P-glycoprotein expression, 54 male NMRI mice were used (Harlan-Winkelmann, Netherlands, Horst, The Netherlands). Animals were kept under controlled environmental conditions (24-25°C, 50-60% humidity, 12-hour dark/light cycle) with free access to tap water and standard feed. Before experiments animals were allowed to adapt to the new environment for at least 1 week. All animal protocols were approved by the Institutional Animal Care and Use Committees of the University of Minnesota and the University of Munich, and were in compliance with the European Communities Council Directive, 86/609/EEC, the German and USDA Animal Welfare Acts, as well as guidelines by the German Research Foundation, DFG, and the National Institutes of Health, NIH.

Induction of a status epilepticus by pilocarpine

To study the role of the EP1 receptor in seizure-induced P-glycoprotein up-regulation we used the lithium-pilocarpine status epilepticus model. Female Wistar Unilever rats received in total four i.p. injections in 12-hour intervals of either vehicle (Aqua ad injectabilia) or SC-51089 (Biomol, Playmouth, PA, USA; 10 mg/kg i.p.) starting 30 min before the first pilocarpine injection. To induce a status epilepticus, lithium chloride (127 mg/kg i.p., Sigma, Taufkirchen, Germany) was administered 14 h and methyl-scopolamine (1 mg/kg i.p., Sigma; Taufkirchen, Germany) was administered 30 min before pilocarpine dosing. As described previously (Glien et al., 2001), pilocarpine (Sigma, Taufkirchen, Germany) was given by i.p. injection (10 mg/kg) every 30 min until the onset of ongoing generalized convulsive seizures (status epilepticus). The total number of pilocarpine injections was limited to a maximum of
12 per animal. Seizure activity was continuously monitored following pilocarpine administration. Control rats received saline injections instead of pilocarpine and methylscopolamine. Seizures were terminated 90 min after onset of continuous generalized seizure activity with diazepam (10 mg/kg, i.p.); diazepam dosing was repeated after 3 min if seizure activity continued. Only rats that displayed continuous convulsive seizure activity during status epilepticus were used for further analysis.

**Tissue preparation and P-glycoprotein immunohistochemistry**

Rats were decapitated 48 h after induction of status epilepticus, brains were immediately removed, embedded in Tissue Freezing Medium® (Jung, Nussloch, Germany), frozen in liquid nitrogen, and stored at –80°C. Brain tissue was cut in 14 and 40 µm serial slices using a cryostat (HM 560; Microm, Walldorf, Germany) and sections were mounted on HistoBond® adhesion slides (Marienfeld, Lauda-Koenigshofen, Germany). 40 µm serial brain sections were used for Nissl-staining with thionin to visualize neurodegeneration in the hippocampus. P-glycoprotein was stained in 14 µm brain sections by overnight incubation with C219 monoclonal mouse antibody (Calbiochem, Darmstadt, Germany; 1:100) at 4°C as described previously (Volk et al., 2004b; Bauer et al., 2008). The next day, brain tissue sections were washed and incubated for 1.5 h in antiserum containing biotinylated donkey anti mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA; 1:200). Sections were washed and subsequently incubated for 1.5 h in horseradish peroxidase-conjugated streptavidin (1:375, Dako Cygomatics, Hamburg, Germany). Brain tissue sections were rinsed again prior to nickel-intensified diaminobenzidine staining (0.05% 3,3-diaminobenzidine, 0.01%, nickel ammonium sulphate; both from Sigma, Taufkirchen, Germany, and 0.01% H₂O₂ (Lukasiuk et al., 2006). Finally, brain sections were washed, air dried, dehydrated, and coverslipped with Entellan (Merck, Darmstadt, Germany).
Histological Evaluation and Image Analysis

P-glycoprotein staining of brain sections was analyzed using a computer-assisted image analysis system as described previously (Bauer et al., 2008; Pekcec et al., 2008b). The hardware consisted of an Olympus BH2 microscope with a Plan-Neofluar objective (Zeiss, Göttingen, Germany), a CCD color camera (Axiocam; Zeiss, Göttingen, Germany), and an AMD Athlon™ 64 processor-based computer with an image capture interface card (Axiocam MR Interface Rev.A; Zeiss, Göttingen, Germany). Brain sections were analyzed at a 400x magnification. Captured images were 1300x1030 pixels in dimension and were processed using KS400 image analysis software (Windows Release 3.0; Carl Zeiss Vision, Halbergmoos Germany). Detailed image analysis methodology has been published previously (Volk et al., 2004a; Volk et al., 2004b). Briefly, prior to image analysis, a spatial calibration was performed and a signal threshold value was defined to exclude background signals. Data reported reflect pixel density above this threshold, which was used to analyze all sections within the same experiment. P-glycoprotein immunostaining was analyzed in the hilus, dentate gyrus, and CA-3 region of the hippocampus, and the parietal and piriform cortex. The area labeled for P-glycoprotein was evaluated using either 3 fields (in the hilus) or 10 fields (dentate gyrus, CA-3 region, parietal, and piriform cortex) of 43434 µm² per subfield.

Neurodegeneration was evaluated in the following hippocampal subregions of Nissl-stained brain sections: CA1, CA2, CA3a, CA3c/CA4, and dentate gyrus. Neuronal damage was assessed by a previously described semiquantitative grading system (Pekcec et al., 2008a): score 0, no obvious damage; score 1, lesions involving 20–50% of neurons; score 2, lesions involving >50% of neurons (note that neuronal loss must exceed 15-20% before it is reproducible by visual inspection (Fujikawa et al., 2000)). Visual inspections were carried out in an observer-blinded fashion.

Compared to the extent of cell loss occurring after a SE in CA3 and CA1, which can be easily scored by microscopic examination, loss of neurons in the dentate hilus is more difficult to
assess without cell counting. Therefore, in a second step, polymorphic neurons (i.e., mossy cells and interneurons) were counted in the dentate hilus of the hippocampal formation. Neuronal loss in the hilus was quantified with the computer-assisted imaging system StereoInvestigator 6.0 (Microbrightfield Europe, Magdeburg, Germany). The hardware consists of a Leica DMLB microscope (Leica, Bensheim, Germany), a Plan-Neofluar lens (Leica, Bensheim, Germany), a single chip charge coupled device (CCD) color camera (CX9000, Microbrightfield Europe, Magdeburg, Germany), and an AMD Athlon (tm) 64 Processor. An experimenter blinded to the treatment conditions traced the extent of the hippocampal hilus and performed the counting of cells using the optical fractionator method. In slide-mounted serial sections the area of the dentate hilus was traced and within each traced contour a step grid was placed. Counting frames were automatically and randomly placed along the grid. The thickness of the counting frame was equal to the thickness of the section (minus guard zones from the top and bottom of the section). Only cells which appeared within the counting frame and came into focus were counted. The number of counted cells was used to estimate the total number of hilar cells of the dentate hilus (West et al., 1991).

Electrode implantation and kindling procedure in mice

Mice were anesthetized with chloralhydrate (400 mg/kg, i.p.) prior to stereotactic implantation of a teflon-isolated bipolar stainless steel electrode into the right amygdala (stereotaxic coordinates in millimeter relative to Bregma: AP -1.0 L -3.2 DV -5.3. One screw, placed above the left parietal cortex served as the indifferent reference electrode. Anchor screws were fixed to the skull to secure all mounted material and the BLA-electrode was embedded in dental acrylic cement. After surgery, the animals were allowed to recover for a period of at least 2 weeks.

Kindling of mice and determination of initial kindling after-discharge thresholds (ADT) using an ascending stair step procedure were performed as described previously for rats (Potschka et
al., 2004; Pekcec et al., 2007). In 1 min intervals, the initial current of 8 µA was increased in steps of about 20% of the previous current until after-discharges were elicited. Twenty-four hours later, kindling was started using an individual stimulation current that was 20% above the determined initial ADT (1 ms, monophasic square-wave pulses, 50 Hz for 1 s). Electrical stimulation of the amygdala was performed once daily and five times per week. After each amygdala stimulation, seizure severity was scored according to the Racine scale (Racine, 1972); 1, immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus; 2, head nodding associated with more severe facial clonus; 3, clonus of one forelimb; 3.5, bilateral clonus without rearing; 4, bilateral clonus accompanied by rearing; 4.5, generalized clonic seizures without rearing and falling (e.g. because of direct loss of balance); 5, rearing and falling accompanied by generalized clonic seizures. In addition to seizure severity, seizure duration and after-discharge duration (ADD) were recorded following each amygdala stimulation. After 10 fully developed generalized seizures mice were considered to be fully kindled and kindling was discontinued.

Anticonvulsine efficacy of Phenobarbital

Kindling induced seizures are known to increase P-glycoprotein expression at the blood-brain-barrier (Volk et al., 2004b). To test whether prevention of seizure-induced P-glycoprotein up-regulation by EP-1 receptor antagonism might enhance the anticonvulsant efficacy of an antiepileptic drug, we used a massive kindling protocol with frequent induction of kindled seizures to induce a pronounced endothelial P-glycoprotein expression. Therefore, fully kindled mice were stimulated five times a day with an interval of 1 hour over nine consecutive days in total. In a subgroup of animals without treatment we determined the degree of P-glycoprotein induction by massive kindling (n=10) in comparison with animals which were fully kindled but were not elicited to a subsequent massive kindling paradigm (n=9).
To test for the effect of SC-51089 the animals received repeated twice daily injections of the EP-1 receptor antagonist SC-51089 (10 mg/kg, i.p.; n=7) or the respective vehicle solution (Aqua ad injectabilia, 10ml/kg, i.p.; n=4) during the stimulation paradigm (1st to 9th experimental day). Injections were done thirty minutes prior to the first daily stimulation and directly after the 5th kindling stimulation of the day. To test whether this pretreatment with an EP1 receptor antagonist during a phase with repeated seizures is suitable to increase the efficacy of an anticonvulsive compound, the effect of the P-glycoprotein substrate phenobarbital was studied on subsequent days. On the 10th experimental day, all mice received an injection of vehicle solution (saline 10 ml/kg i.p) 30 minutes prior evaluation of the generalized seizure threshold. Seizure thresholds were determined using the ascending stair step procedure as described above. On the next day (11th experimental day), all mice were treated with a low dose of phenobarbital (6 mg/kg i.p.) 30 minutes prior evaluation of seizure thresholds. The phenobarbital dose was selected based on pretests with different dosages of phenobarbital in kindled mice which had not received a pretreatment. The dosage was chosen as it did not exert an anticonvulsive effect on seizure thresholds in kindled mice and is thus suitable to evaluate increased efficacy by blood-brain-barrier transporter modulation.

Although, we evaluated phenobarbital efficacy following SC-51089 withdrawal with this protocol, we were keen to exclude any acute interaction of both drugs. All mice received phenobarbital (6 mg/kg, i.p.) 30 min prior evaluation of the generalized seizure thresholds. To test if SC-51089 acutely enhances phenobarbital efficacy, the animals received an injection of SC-51089 (10 mg/kg i.p., n=5) or of the corresponding vehicle solution (Aqua ad injectabilia, n=5) immediately before phenobarbital administration. Threshold data obtained following drug administration were compared with data from a vehicle control experiment performed the day before. To prevent bias of the results by inter-individual differences, this experiment was repeated after several weeks in the same animals in a cross-over design.
Tissue Preparation and EP1 Western blotting

The data obtained with the EP1 receptor antagonist in the epilepsy models raised the question whether the effects are due to an interference with signaling events in brain capillaries. Recent data, demonstrated that glutamate/NMDA-receptor/COX-2 signaling in brain capillaries contributes to the transcriptional activation. The present in vivo data suggest EP1 receptors as downstream effectors in these endothelial signaling events. However, this hypothesis is based on the assumption that EP1 receptors are expressed in brain capillaries. To test for endothelial EP1 receptor expression, rat brain capillaries were isolated as previously described (Bauer et al. 2008, Zibell et al., 2009). Brain capillaries were homogenized in lysis buffer containing protease inhibitor; differential centrifugation was used to obtain crude plasma membranes from capillary lysates. Western blots of brain capillary membranes were performed using the Invitrogen NuPage™ electrophoresis and blotting system (Invitrogen, Carlsbad, CA, USA). Blotted membranes were incubated overnight with EP1 antibody (1:1000, 1 μg/ml, Cayman Chemical, Ann Arbor, MI). Blotting membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated ImmunoPure® secondary antibody (1:15,000, Pierce, Rockford, IL, USA). Proteins were detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and visualized with a BioRad Gel Doc™ XRS imaging system (BioRad, Hercules, CA, USA).

For determining P-glycoprotein expression the parahippocampal cortex was dissected. Tissue samples were homogenized in lysis buffer containing per 20 ml: 200 μl 1 M Tris pH 8.0; 1 ml 3 M NaCl; 2 ml 10% NP-40; 4 ml 50% glycerol; 800 μl Na-orthovanadate (10 mg/ml); 200 μl 0.5 M EDTA pH 8.0; 400 μl protease inhibitors; 200 μl 0.5 M NaF; 11.2 ml H₂O. Fifty μg total protein per lane was separated by SDS-PAGE, (7.5% acrylamide) and transferred to nitro-cellulose by electroblotting (BioRad, Transblot SD, Hercules, USA). Blots were incubated with primary antibodies (C219; Alexis Biochemicals, San Diego, CA, USA, 1:500);
mouse anti-β-actin, clone AC-15, Sigma, 1:50000) and the secondary antibody, anti-mouse labeled with horseradish peroxidase (1:2500, Dako, Glostrup, Denmark). Immunoreactivity was visualized with lumi-light plus western blotting substrate (Roche Diagnostics, Mannheim, Germany) and the blots were digitized using a Luminescent Image Analyzer, LAS-3000 (Fuji Film, Japan). The optical density of each sample was measured using Scion Image (Scion Corporation, release beta 3b, MD, USA) software. For each sample the optical density of the P-glycoprotein was calculated relative to the optical density of β-actin.

Statistical Analysis

Data are given as mean ± SEM. Statistical differences in P-glycoprotein expression between controls and treated groups were analyzed using the Mann-Whitney U-test. Data of the phenobarbital experiments with kindled mice were analyzed by the Wilcoxon matched pairs test. Fisher Exact test was used to compare the number of animals that developed a status epilepticus in the different treatment groups as well as mortality rates between groups. Differences in seizure parameters during the massive kindling procedure and Western blot analysis were calculated using the Student’s t-test. All statistical tests were performed two-tailed. Differences between means were considered to be statistically significant when P < 0.05.
Results

Lack of an effect of EPI receptor antagonism on status epilepticus

Depending on the experimental conditions, COX-2 antagonism can result in anticonvulsant effects (Kim and Jang, 2006) but also in seizure aggravation (Baik et al., 1999). These data raise concern whether seizure susceptibility might also be affected when targeting downstream events in the COX-2 associated signaling cascades. Therefore, we carefully analyzed whether EPI receptor antagonism exerts effects on the development of a status epilepticus in the fractionated pilocarpine model. Forty-eight percent of SC-51089 treated animals (n = 13 out of 27) developed a status epilepticus in response to repeated injections of the cholinomimetic pilocarpine. In vehicle treated controls, a status epilepticus was successfully induced in 46% of the animals (n = 17 out of 37). No significant difference between both groups was observed in the amount of pilocarpine required to induce a status epilepticus. SC-51089 treated rats received 6.18 ± 0.75 pilocarpine applications (each 10 mg/kg) prior to onset of status epilepticus. Vehicle treated rats required 5.69 ± 0.75 pilocarpine applications. Importantly, the severity of status epilepticus in SC-51089 treated animals was not different from vehicle treated animals. Once a second generalized seizure was observed, rats exhibited ongoing generalized seizure activity until administration of diazepam. Comparison of the mortality rate during status epilepticus revealed no significant differences (p = 0.3414) between the SC-51089 treated (n=4 out of 13) and the vehicle treated (n=11 out of 17) group.

EPI receptor antagonism prevents the induction of P-glycoprotein by status epilepticus

Immunolocalization of the P-glycoprotein antibody was observed in microvessel endothelial cells of all rats regardless of the treatment paradigm (Fig.1A-C). The DAB staining method was chosen for the present experiments as it exclusively labels endothelial P-glycoprotein. Therefore, the P-glycoprotein-labeled areas determined by computer-assisted analysis can be completely attributed to endothelial P-glycoprotein expression (Volk et al., 2005).
As previously demonstrated, prolonged seizure activity induced by pilocarpine represents a very strong trigger for transcriptional activation of P-glycoprotein expression. In line with these data, pilocarpine-induced status epilepticus significantly increased P-glycoprotein labeling in the hippocampal hilus, the hippocampal granule cell layer, and in the piriform cortex by 91.67% to 197.14% (Fig. 1B, D-F). In all three brain regions treatment with the EP1 receptor antagonist SC-51089 efficaciously prevented the seizure-associated induction of endothelial P-glycoprotein expression and kept P-glycoprotein expression at control level (Fig. 1D-F).

*Sub-chronic EP1 receptor blockade in a massive kindling paradigm gives first evidence that prevention of P-glycoprotein induction might increase phenobarbital efficacy*

Based on the efficacious control of P-glycoprotein expression despite prolonged seizure activity, we hypothesized that EP1 receptor antagonism may improve antiepileptic drug efficacy. Phenobarbital has been repeatedly described to be a substrate of P-glycoprotein (Potschka et al., 2002; Brandt et al., 2006; Luna-Tortos et al., 2008). The kindling model renders an elegant tool for controlled and repeated induction of seizure activity as well as for the precise assessment of anticonvulsant efficacy. Moreover, it has been repeatedly described that kindled seizures induce P-glycoprotein expression in a significant manner (Volk et al., 2004b). Therefore, we used fully kindled mice to obtain first data whether a sub-chronic treatment with the EP1 receptor antagonist SC-51089 during a phase with repeated elicitation of epileptic seizures (massive stimulation paradigm with 45 stimulations during the 9-day treatment phase) enhances the anticonvulsant efficacy of phenobarbital during a subsequent seizure threshold experiment. In this kindling paradigm, massive kindling proved to result in an increase of P-glycoprotein expression in the parahippocampal cortex by 49% as assessed by Western blot analysis (Fig 2A).
In pretests, different phenobarbital doses (ranging from 4-8 mg/kg) were tested in a separate set of kindled mice to select a dose that does not exert a significant effect when given without pre-treatment. A dose of 6 mg/kg i.p. was chosen based on these experiments.

During the massive stimulation paradigm both groups responded to repeated stimulations in a comparable manner with no significant differences in the seizure parameters. The mean seizure severity reached 4.05 ± 0.18 in vehicle treated mice and 4.31 ± 0.18 in SC-51089 treated mice; vehicle treated mice developed 39.0 ± 4.0 and SC-51089 treated mice 42.7 ± 1.6 (mean ± SEM) generalized seizures. Cumulative duration of motor seizures during the stimulation phase amounted to 1459 ± 64 s in the vehicle treated group and 1513 ± 84 s (mean ± SEM) in the SC-51089 treated group. Cumulative duration of after discharge duration during the stimulation phase amounted to 942 ± 44 s in the vehicle treated group and 1020 ± 72 s (mean ± SEM) in the SC-51089 treated group. Sub-chronic SC-51089 treatment was well tolerated by all animals and was not associated with any adverse effects. Following withdrawal of SC-51089, seizure thresholds proved to be comparable in both groups amounting to 240 ± 60 µA in SC-51089 pretreated mice and 260 ± 12 µA in vehicle pretreated mice.

In line with the data from the rat pilocarpine-model, treatment with the EP1 receptor antagonist SC-51089 efficaciously counteracted the seizure-associated induction of P-glycoprotein expression in the parahippocampal cortex (Fig. 2B).

Phenobarbital administration resulted in an anticonvulsant effect in SC-51089 pretreated animals, in that the seizure threshold was significantly increased by 161.01% (Fig. 3A). In line with the pre-tests, phenobarbital did not exert a significant effect on seizure thresholds in mice that received vehicle injections during the massive stimulation paradigm. The dosage of
6 mg/kg phenobarbital did neither result in behavioral side effects, such as ataxia, in vehicle-treated nor in SC-51089 treated mice. In contrast to SC-51089 pre-treatment, acute SC-51089 co-administration did not potentiate the anticonvulsant effect of phenobarbital (Fig. 3B). In the acute experiments, SC-51089 did neither impact the duration of motor seizures (vehicle treated group 35.7 ± 2.26 s, mean ± SEM; SC-51089 treated group 35.9 ± 2.81 s, mean ± SEM) nor after discharge duration (vehicle treated group 33.7 ± 1.48 s, mean ± SEM; SC-51089 treated group 34 ± 2.92 s, mean ± SEM).

**EP1 receptor blockade exerts no effect on seizure-induced neurodegeneration**

EP1 receptors have been identified as downstream effectors of COX-2 neurotoxicity in a model of ischemic stroke (Kawano et al., 2006). As COX-2 has also been implicated in the neurotoxicity resulting from epileptic seizure activity (Takemiya et al., 2006; Bauer et al., 2008), we hypothesized that targeting EP1 receptors may exert neuroprotective effects in the pilocarpine model. A pronounced and significant decrease (p=0.0002) in the neuronal density of the hippocampal hilar formation was identified in vehicle-treated rats after pilocarpine-induced status epilepticus (4768 ± 772) compared to vehicle-treated control rats (13605 ± 1328) (Fig. 4). SC-51089 treatment did not prevent status epilepticus-associated neurodegeneration in this hippocampal sub-region (4859.08 ± 519.8) (Fig. 4).

**EP1 receptor expression in rat brain capillaries**

To demonstrate EP1 receptor expression in brain tissue and isolated brain capillaries, Western blot experiments have been performed. Blot analysis indicated expression of the EP1 receptor in rat brain homogenates (Fig. 5). EP1 protein expression was moreover detected in isolated rat brain capillaries and the membrane fraction of isolated brain capillaries (Fig. 5).
Discussion

As an adaptation to changing requirements in tissue protection, the expression of the blood-brain barrier efflux transporter P-glycoprotein is regulated in a highly dynamic manner (Miller et al., 2008). Epileptic seizures represent a very strong trigger of P-glycoprotein transcriptional activation resulting in a pronounced over-expression in the epileptic brain (Loscher and Potschka, 2005). It is intensely discussed that enhanced blood-brain barrier efflux might negatively affect the efficacy of antiepileptic drugs. Increased efflux may limit brain penetration of antiepileptic drugs, thereby lowering the anticonvulsant effect and contributing to pharmacoresistance (Loscher and Potschka, 2005; Kwan and Brodie, 2006). Recently, we reported first evidence that an endothelial glutamate/COX-2 signaling pathway drives P-glycoprotein expression in response to seizure activity (Bauer et al., 2008). Here, we identified EP1 receptors as a key effector in the respective cascade up-regulating P-glycoprotein expression in epilepsies. As demonstrated in the present study, the involvement of EP1 receptors in transcriptional activation of P-glycoprotein opens new intriguing possibilities to control P-glycoprotein expression in the diseased brain and to enhance the efficacy of pharmacotherapy.

Glutamate released during seizure activity and signaling via endothelial NMDA receptors proved to function as the initial trigger that activates the intracellular cascade of events affecting P-glycoprotein expression (Bauer et al., 2008). With the regional raise in extracellular glutamate concentrations during seizures, these data also rendered an explanation for the localized over-expression of P-glycoprotein in brain regions involved in seizure generation and spread. The signaling pathway proved to involve COX-2 as a downstream effector (Bauer et al., 2008). In isolated brain capillaries, both the COX-2 inhibitor celecoxib as well as genetic deficiency of COX-2 abolished the effect of glutamate exposure on P-glycoprotein. NMDA receptor activation results in Ca\(^{2+}\) influx which is known to activate phospholipase A2 (PLA2)(Rao et al., 2007). PLA2 then releases arachidonic acid from cell
membranes thereby delivering the substrate to the COX-2 enzyme. Prostaglandin E2 represents the major product derived from processing of arachidonic acid by COX-2 in the brain (Balboa et al., 2002; Kawano et al., 2006; Yang and Chen, 2008). PGE2 can exert its effect via four different G-protein coupled receptors (EP1, EP2, EP3, and EP4 receptors) (Hata and Breyer, 2004). In view of our aim to identify alternate targets for control of P-glycoprotein expression in the epileptic brain, we considered tolerability issues when selecting a target candidate among these receptors. As EP2, EP3 and EP4 receptors mediate neuroprotective effects (Bilak et al., 2004; McCullough et al., 2004; Ahmad et al., 2005), antagonism of these receptor subtypes can not be considered as an applicable therapeutic approach. In apparent contrast, EP1 receptors are critically involved in the neurotoxicity associated with activation of NMDA receptors in the diseased brain (Bilak et al., 2004; McCullough et al., 2004; Ahmad et al., 2005). In accordance with this role, EP1 receptor antagonism exhibits neuroprotection (Suganami et al., 2003; Kawano et al., 2006). Therefore, we hypothesized that EP1 receptor antagonism may even result in dual benefit including control of endothelial P-glycoprotein as well as neuroprotection.

SC-51089 is a selective and potent antagonist of EP1 (Malmberg et al., 1994; Hallinan et al., 1996). In the present study, SC-51089 treatment blocked status epilepticus-mediated induction of P-glycoprotein expression. Interestingly, P-glycoprotein expression levels were kept at basal level in SC-51089 treated animals with a status epilepticus. These data suggest a key role for EP1 in the signaling events that drive P-glycoprotein expression in seizures. Moreover, they also suggest that glutamate signaling through the NMDA receptor, COX-2, and EP1 is an exclusive pathway up-regulating P-glycoprotein in seizures. This hypothesis is further supported by the finding that EP1 is expressed in isolated brain capillaries and brain capillary membranes. However, signaling pathways in neighboring cells that might contribute to up-regulation of endothelial P-glycoprotein cannot be ruled out at this point. Regarding the downstream events that mediate the effects of EP1 receptor activation on P-glycoprotein
expression further research is necessary. The transcription factor NFKappaB represents a likely candidate as EP receptor signaling can result in activation of NFKappaB, and NFKappaB in turn can affect P-glycoprotein expression rates (Yu et al., 2008).

At the first glance, recent data that reported an increase in COX-2 expression levels and a decrease in efflux transporter gene expression levels 2 or 24 hours following a pilocarpine-induced status epilepticus might argue against our hypothesis of glutamate/NMDA receptor/COX-2/EP1 receptor signaling driving P-glycoprotein expression (Kuteykin-Teplyakov et al., 2009). However, in this study brain homogenates of different brain regions were analyzed without cellular separation, thus, not allowing conclusions about an association between inflammatory enzymes and transporter expression in endothelial cells. Moreover, the study was limited to RNA levels, and did not explore protein levels, which might not develop in parallel due to posttranscriptional regulatory mechanisms. In previous studies, we clearly demonstrated that COX-2 inhibition or its genetic deficiency efficaciously counteracts glutamate or seizure-associated increases in brain capillary P-glycoprotein \textit{in vitro} and \textit{in vivo} (Bauer et al., 2008; Zibell et al., 2009). Recent data demonstrated that enhanced COX-2 signaling following seizures is not due to an increase in endothelial COX-2 expression but to enhanced release of the COX-2 substrate arachidonic acid and enhanced substrate feeding of COX-2 (Zibell et al., 2009). In contrast, neuronal COX-2 expression was strongly up-regulated in neuronal cells following status epilepticus (Zibell et al., 2009). Thus, the increase in COX-2 RNA levels reported by Kuteykin-Teplyakov and colleagues (2009) is likely to be solely due to transcriptional activation in neurons.
Prevention of P-glycoprotein over-expression in the model used is especially promising as a status epilepticus induced by the cholinomimetic pilocarpine acts as an extremely strong trigger of P-glycoprotein expression (Hoffmann et al., 2006; Bauer et al., 2008). Based on data from epileptic patients and rodent epilepsy models, there is agreement that efflux transporters are over-expressed in epileptic brain tissue (Loscher and Potschka, 2005; Kwan and Brodie, 2006; Hughes, 2008). However, it is a matter of an ongoing debate if transporter over-expression contributes to pharmacoresistance (Loscher and Sills, 2007). Experimental key findings, including studies that describe a correlation between increased P-glycoprotein expression, reduced antiepileptic drug brain penetration, and limited drug efficacy indicate that P-glycoprotein plays a critical role in antiepileptic pharmacotherapy (Rizzi et al., 2002; Potschka et al., 2004; Volk and Loscher, 2005; van Vliet et al., 2007; Wen et al., 2008). Proof-of-principle for the transporter hypothesis in rodent models came from studies in which efficacy of the antiepileptic drugs phenobarbital or phenytoin was significantly improved by add-on treatment with a selective P-glycoprotein inhibitor (Brandt et al., 2006; van Vliet et al., 2006). Regarding its clinical relevance it is discussed whether putative species differences might hamper conclusions for human patients. Therefore, it is of specific interest, that a correlation between blood-brain barrier penetration and P-glycoprotein expression levels has been demonstrated using specimen from human epileptic patients. In patients with oxcarbazepine-resistant epilepsy, the brain-tissue expression of ABCB1 mRNA encoding P-glycoprotein was inversely correlated with brain levels of the active oxcarbazepine metabolite 10-OHCBZ (Marchi et al., 2005). Using an in vitro blood-brain barrier model with human capillary endothelial cells from either normal or epileptic brain, Cucullo et al. (2007) demonstrated a significantly reduced permeability of phenytoin across the in vitro blood-brain barrier from cells of patients with pharmacoresistant epilepsy. In these experiments, the reduced permeability was partly counteracted by the selective P-glycoprotein inhibitor tariquidar. Recent data indicated that levetiracetam, lamotrigine, and phenobarbital are also
transported by the human P-glycoprotein isoform (Luna-Tortos et al., 2008) and therefore are likely to be affected by P-glycoprotein expression rates in epileptic patients. Further scepticism regarding the clinical role of efflux transporters in pharmacoresistance is based on pharmacogenetic studies which resulted in controversial data regarding an association between the P-glycoprotein encoding gene and pharmacosensitivity (Leschziner et al., 2007; Löscher et al., 2008). However, the lack of a clear and reproducible association in these genetic studies is not surprising as experimental studies suggest that P-glycoprotein over-expression in pharmacoresistant individuals is rather acquired and not intrinsic. Thus, differences are rather expected in events regulating P-glycoprotein expression in response to seizure activity. The identification of complex signalling events now raises new challenges to pharmacogenetic studies as genetic differences may exist in the promoter region of the P-glycoprotein encoding gene but also regarding several factors directly or indirectly involved in the described signalling pathway.

Overall, final conclusions regarding the clinical relevance of transporter over-expression have to await progress with positron emission tomography methods aiming to image P-glycoprotein transport function in individual patients. Moreover, other mechanisms of pharmacoresistance such as alterations in target sites need to be considered as well.

The success of P-glycoprotein inhibition in chronic epilepsy models indicates that prevention of seizure-associated induction of P-glycoprotein should render comparable results. In view of the efficacious control of P-glycoprotein expression by the EP1-receptor antagonist SC-51089, we studied the consequences of sub-chronic treatment with the antagonist in a massive kindling paradigm with frequent induction of seizures. The subsequent testing of the anticonvulsant efficacy of phenobarbital revealed a significant potentiation by SC-51089 pretreatment. These data give preliminary evidence that prevention of P-glycoprotein induction by EP1 receptor antagonism might improve pharmacosensitivity towards P-
glycoprotein substrates. However, further studies are necessary to substantiate these data including thorough dose-response studies or testing in chronic models of pharmacoresistant epilepsy with spontaneous seizures.

Based on our findings in the present study, we suggest EP1 as a target for preventing P-glycoprotein over-expression and improving antiepileptic drug efficacy. As demonstrated, this strategy retains P-glycoprotein at basal levels, which is a major advantage. This is of particular relevance considering the protective function P-glycoprotein has in blood-tissue barriers, excretory organs, and in hematopoetic cells (Fromm, 2004). P-glycoprotein-mediated active efflux transport protects sensitive tissues from xenobiotics and accelerates xenobiotic elimination, thereby reducing exposure times. Thus, interfering with basal P-glycoprotein transport function is a major limitation of direct P-glycoprotein inhibition. Therefore, it would be advantageous to control P-glycoprotein expression levels by targeting its regulatory pathways in the epileptic brain. Since enhanced glutamate release is also a hallmark in ischemic brain damage, P-glycoprotein induction in the ischemic brain (Spudich et al., 2006) may also be caused by glutamate and activation of inflammatory events. Targeting P-glycoprotein regulation might therefore also be successful in this condition.

When suggesting the present data as a departure point to design subsequent translational studies, one needs to carefully analyze tolerability issues. As targeting the upstream effector of EP1 receptors, COX-2, might differently affect seizure thresholds, seizure activity, and its consequences (Baik et al., 1999; Kim and Jang, 2006), we carefully analyzed respective seizure data in animals treated with the EP1 receptor antagonist. This analysis gave no evidence for an impact of EP1 receptor antagonism on seizure thresholds or seizure severity. Thus, EP1 receptors seem to represent a more promising target in the P-glycoprotein regulatory cascade as compared to COX-2. This is further underlined by the enhanced risk for cardiovascular and cerebrovascular complications that has been attributed to the use of
selective COX-2 antagonists. However, in view of the controversial data existing for COX-2 inhibitors in rodent epilepsy models with seizure aggravation in some studies and lack of an effect in others (Baik et al., 1999; Kim and Jang, 2006), this aspect requires further investigational efforts to allow definite conclusions regarding safety concerns.

COX-2 is implicated in the neurotoxicity resulting from glutamate release in brain ischemia, traumatic brain injury, and epilepsy. Accordingly, COX-2 inhibition was associated with neuroprotective effects in a variety of experimental studies involving rodent epilepsy models (Takemiya et al., 2006; Bauer et al., 2008). Kawano et al. (2006) identified EP1 as a downstream effector in COX-2 neurotoxicity. In this study, EP1 activation caused NMDA receptor-induced Ca\(^{2+}\) dysregulation and affected the Ca\(^{2+}/Na\(^{2+}\) exchange. Inhibiting EP1 pharmacologically improved brain injury in a mouse focal cerebral ischemia model. Since glutamate excitotoxicity also triggers neuronal cell loss in the epileptic brain (Meldrum, 1993; Mody and MacDonald, 1995), we analyzed the impact of blocking EP1 in the pilocarpine status epilepticus model. In our experiments, we did not find a neuroprotective effect of SC-51089, which is in agreement with data that were obtained in the kainate model (Kawano et al., 2006). Thus, the different outcomes in blocking EP1 might be due to differences in the molecular events leading to neurotoxicity in brain ischemia and epilepsies. However, overall the result is surprising as both, NMDA receptor blockade or COX inhibition, can exert neuroprotective effects in the pilocarpine model (Fujikawa et al., 1994; Zibell et al., 2009). Thus, the lack of neuroprotection in the hippocampal hilus might also be related to kinetic effects, in that neuronal concentrations necessary for cell protection were not reached.

The activation of inflammatory pathways in the epileptic brain has been suggested as a central event in the pathophysiology of epilepsies which might contribute or predispose to the occurrence of seizures and cell death (Vezzani and Granata, 2005). Antiinflammatory drugs are therefore discussed for future therapeutic approaches raising hope for disease modulation.
and inhibition of disease progression or development. The identification of a signaling pathway that involves the inflammatory enzyme COX-2 as well as the EP1 receptor and that drives endothelial P-glycoprotein expression now reveals a novel relevance of brain inflammation. The induction of this inflammatory pathway in brain capillaries may critically impact pharmacosensitivity. Our findings suggest blockade of the pathway by EP1 receptor antagonism as an innovative approach to control P-glycoprotein expression and to enhance antiepileptic drug efficacy.

Acknowledgements

We thank Heidrun Zankl for her excellent technical assistance.
References


Volk H, Potschka H and Loscher W (2005) Immunohistochemical localization of P-glycoprotein in rat brain and detection of its increased expression by seizures are sensitive to fixation and staining variables. *J Histochem Cytochem* 53:517-531.


Footnotes

‡ This research was supported by a grant from the German Research Foundation [PO 681/4-1].
Legends for Figures

Figure 1. SC-51089 prevents seizure-induced P-glycoprotein up-regulation. (A) Representative immunostaining of P-glycoprotein in the hippocampal hilus of a vehicle-treated control rat. (B) P-glycoprotein immunostaining in the hilus of a rat after pilocarpine-induced status epilepticus. Note the striking increase in endothelial P-glycoprotein-expression. (C) P-glycoprotein immunostaining in the hippocampal hilus of a SC-51089-treated rat after pilocarpine-induced status epilepticus. Note the decrease in P-glycoprotein immunoreactivity in capillaries compared to vehicle-treatment after pilocarpine-induced seizures. Quantitative analysis of P-glycoprotein immunostaining in the hippocampal hilus (D), the hippocampal dentate gyrus (E), and the piriform cortex (F). Data are given as mean ± SEM. Statistical comparisons: * significantly higher than controls, p<0.05; # significantly lower than SE + Vehicle, p<0.05. Scale bar = 50µm.

Figure 2. Induction of P-glycoprotein expression by massive kindling and prevention of massive kindling-induced P-glycoprotein up-regulation by SC-51089. (A) The mean ratio (± SEM) of the optical density of P-glycoprotein relative to β-actin expression measured on the Western blot for control mice and mice following massive kindling are given. Note the increase of P-glycoprotein expression following massive kindling. Statistical comparison: * significantly higher than control, p<0.05. (B) The mean ratio (± SEM) of the optical density of P-glycoprotein relative to β-actin expression measured on the Western blot for vehicle treated and SC-51089 treated massive kindled mice is given. Note that treatment with SC-51089 prevents seizure-induced up-regulation of P-glycoprotein expression. Statistical comparison: * significantly lower than vehicle treated mice, p<0.05.
**Figure 3.** Generalized seizure thresholds after phenobarbital injection in kindled mice. The phenobarbital-induced increase of the generalized seizure threshold following a nine day massive kindling paradigm with repeated SC-51089 treatment or respective vehicle treatment is given in (A). In the vehicle-treated group of mice, phenobarbital increased the generalized seizure threshold by 31%. In contrast, SC-51089 treatment resulted in an increase of the generalized seizure threshold by 82% and thereby exceeded the anticonvulsive efficacy of phenobarbital by 161% as compared to the vehicle-treated group. Data are given as mean ± SEM. Statistical comparisons: *significantly higher than controls, p<0.05.

(B) Lack of any acute effects of SC-51089 on phenobarbital efficacy. To exclude any acute interaction of SC-51089 and phenobarbital with subsequent enhanced phenobarbital efficacy, mice received phenobarbital as well as SC-51089 or vehicle respectively. Threshold data obtained following phenobarbital administration were compared with data from a vehicle control experiment performed the day before. To prevent bias of the results by inter-individual differences, this experiment was repeated after several weeks in the same animals in a cross-over design. Note that SC-51089 does neither increase nor decrease phenobarbital efficacy, indicating a lack of any acute drug interactions.

**Figure 4.** SC-51089 has no effect on seizure-induced neurodegeneration. Status epilepticus significantly decreased the density of polymorphic neurons in the hippocampal hilus compared to animals that did not experience a status epilepticus. SC-51089 treatment did not prevent seizure-induced damage of hilar neurons. Data are given as mean ± SEM. Statistical comparisons: *significantly different from means, p<0.05.

**Figure 5.** EP1 is expressed in rat brain capillaries. Western blot showing EP1 expression in rat brain homogenate at about 38 kDa and in brain capillaries and capillary membranes at about 42 kDa.
Figure 3

A

Phenobarbital-induced Increase in Seizure Threshold [%]

Control
SC-51089

B

Phenobarbital-induced Increase in Seizure Threshold [%]

Vehicle
SC-51089
Figure 4

Density of polymorphic neurons in the hippocampal hilus

- Control
- SE + Vehicle
- SE + SC-51089

* indicates significant differences.