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New model of pharmacoresistant seizures induced by 3-mercaptopropionic acid in mice

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A B S T R A C T

About 30% of the patients with epilepsy do not respond to clinically established anticonvulsants, despite having effective concentrations of the antiepileptic drug in plasma. Therefore, new preclinical models of epilepsy are needed to identify more efficacious treatments. We describe here a new drug-resistant seizure model in mice to be used at the early stages of pre-clinical trials. This model consists in inducing daily generalized seizures for 23 consecutive days by administration of 3-mercaptopropionic acid (MP). As a result, 100% of animals become resistant to phenytoin and 80% to phenobarbital. Such resistance is strongly associated with the overexpression of P-glycoprotein (Pgp), observed in cerebral cortex, hippocampus and striatum while resistance to Pgp nonsubstrate drugs such as carbamazepine, diazepam and levetiracetam is not observed.

This model could be useful for screening novel anticonvulsant drugs with a potential effect on pharmacoresistant seizures treatment.

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1. Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent and spontaneous seizures (Meldrum, 1984). About 30% of the patients with epilepsy do not respond to clinically established anticonvulsants despite effective antiepileptic drug (AED) plasma concentrations, defining a multidrug resistance (MDR) phenotype (Kwan and Brodie, 2000). Therefore, there is a genuine need to incorporate new models of refractory epilepsy (RE) at the preclinical stage of drug development, in order to identify new AEDs that overcome the problem of drug resistance (White, 2003).

The inability of the AEDs to reach their molecular targets and the high frequency and severity of seizures have been proposed as explanations to drug resistant epilepsy (Lazarowski et al., 2007; Potschka, 2010; Rogawski, 2013). Drug concentrations in several tissues (including the brain) are regulated by drug efflux transporters of the ATP-binding cassette (ABC) superfamily, such as P-glycoprotein (Pgp), the multidrug resistance associated proteins (MDRPs), and Breast Cancer Resistance Protein (BCRP) (Kuteykin-Teplyakov et al., 2009). Back in 1995, Tishler observed increased expression of Pgp in drug-resistant patients, suggesting that the loss of response may be caused by limited brain bioavailability of the AEDs (Tishler et al., 1995). Thereafter, several reports indicated high levels of Pgp expression in epileptogenic brain tissue from patients with RE (Sisodiya et al., 2002; Lazarowski et al., 1997; Aronica et al., 2004; Löscher and Potschka, 2005a, b).

A diversity of experimental models of pharmacoresistant epilepsy in rats has demonstrated the inducible Pgp overexpression at the blood brain barrier (BBB) which is associated to the MDR phenotype (Seegers et al., 2002; Rizzi et al., 2002; Jing et al., 2010; Volk and Löscher, 2005; Bankstahl and Löscher, 2008). In these models, Pgp levels in vessel-related cells and neurons correlated with the loss of protective effects of phenytoin (PHT), a proven Pgp substrate (Zhang et al., 2012). These models require several weeks of treatment and only about 40% of the surviving animals become resistant to drugs (Löscher et al., 1993; Löscher, 2011; Potschka, 2012). These

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characteristics make the use of these models difficult at the first stages of the preclinicial assays of new AEDs due to the high number of animals needed to test a considerable number of candidate drugs.

Brain Pgp expression pattern has previously been studied in a rat model of seizure induced by 3-mercaptopropionic acid (MP) (Lazarowski et al., 2004). MP is a classic seizure-inductor as a consequence of the inhibition of gamma aminobutyric acid (GABA) synthesis. (De Sarro et al., 2003; Giraldez et al., 1999; Giraldez and Girardi, 2000; Girardi et al., 1989, 2004; Sprince et al., 1969). The repetitive seizure activity induced by daily administration of MP in rats during 7 days induces an increase in Pgp expression in capillary endothelial cells of the blood brain barrier, neurons, and glial cells from cortex, striatum, and hippocampus (Lazarowski et al., 2004). This situation is associated with astrocyte hypertrophy, characterized by an increase in number and thickness of branching and in soma size (Girardi et al., 2004) and lower brain penetration of PHT (Hocht et al., 2007).

Although the repetitive administration of MP in rat represents a good experimental model to reproduce pharmacoresistant seizures, an important disadvantage is that experiments in rats require high amount of the experimental drugs.Concerning this issue, mouse represents an attractive cost-efficient model.

The aim of this study was the development of a new model of pharmacoresistant seizures linked to Pgp overexpression induced by repetitive administration of MP in mice. This model could be used to screen for drugs capable of controlling RE in short time-periods.

The model was validated by evaluating the protective effects of standard AEDs: PHT and Phenobarbital (PB), (proven Pgp substrate AEDs (Zhang et al., 2012)) and Carbamazepine (CBZ), Levetiracetam (LEV) and Diazepam (DZP) (Pgp nonsubstrates). Based on the hypothesis that there is an overexpression of Pgp in animals treated with MP, we expect to observe drug resistance to Pgp-substrate AEDs, and a protective response to those AEDs that are not Pgp substrate. The effect of co-administration of nimodipine (NIMO) (a Pgp blocker) with PHT and PB was also studied.

2. Materials and methods

2.1. Animals and drugs

We employed Swiss albino male mice (25–35 g) provided by the Faculty of Veterinary, National University of La Plata. The animals were housed in colonies of ten, under a regime of 12 h light/dark with water and food ad libitum. Every effort was made to minimize animal stress. The animal care for this experimental protocol was conducted in accordance with the NIH guidelines for the Care and Use of Laboratory Animals and it was approved by the Ethical Committe of Exact Sciences Faculty of University of La Plata.

MP was acquired from Merck (Hohenbrunn, Germany). LEV was provided by Glaxosmithline. CBZ and NIMO were a generous gift from Bagó Laboratories. DZP was acquired from Roche, PB from Serva (Heidelberg, Germany) and PHT from Sigma-Aldrich.

PHT was dissolved in saline and the pH = 11.2 was adjusted with NaOH. Carboxymethylcellulose 1% (CMC) was used as vehicle for CBZ, NIMO and PB. Finally, DZP and LEV were dissolved in saline. All AEDs were evaluated at their time of maximum response. All drugs except MP were administered at 10 ml/kg.

The study was not performed blinded to treatment groups. Animals were randomized to treatment.

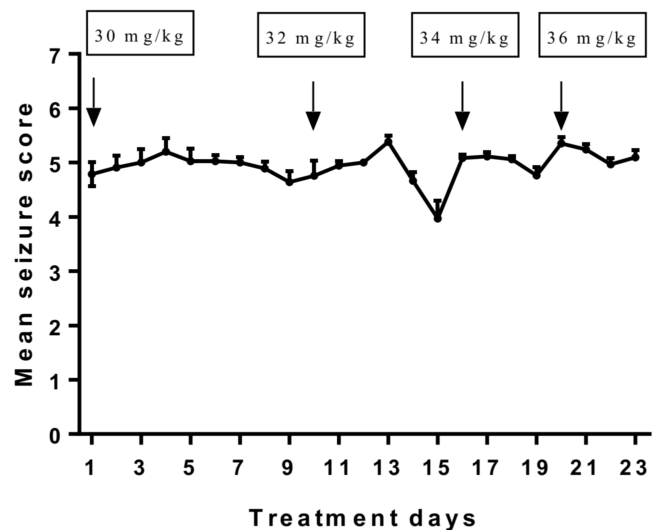

Fig. 1. *MP treatment.* Swiss albino mice were administered once a day with a variable dose of MP from 30 to 36 mg/kg. The dose was increasing according to animal behavior in order to maintain the generalized seizure response. Mean seizure severity score ± SEM vs MP treatment days (n = 47).

2.2. 3-Mercaptopropionic acid (MP) treatment

Initially, mice received a daily administration of saline (0.1 ml intraperitoneal [i.p.]) during 7 days. This procedure allows the animals' habituation to handling, avoiding down regulation of GABA receptors induced by acute handling, which could affect the susceptibility to convulsions (Skilbeck et al., 2010). Thereafter they were daily administered with i.p. MP for 23 consecutive days. MP dose was increased through the treatment, starting from 30 mg/kg on day 1 to 36 mg/kg on day 23 in order to obtain generalized seizures throughout the 23 days (Fig. 1). The MP starting dose (30 mg/kg) is the necessary dose to induce generalized seizures without causing the death of the animals and it was chosen from preliminary dose-response experiments. 23 MP treatment days was the optimal time to avoid PHT anticonvulsant effect. (See Section 2.2.1).

MP was daily prepared in saline (5 ml/kg) and neutralized with trizma base immediately before its administration. After each MP injection, the mice were placed individually in transparent Plexiglass cages and they were observed for 30 min. Seizure activity was classified according to a behavioral scale adapted to the MP induced seizures (Racine, 1972):

0- no response.
1- ear and facial twitching, nodding, piloerection, chewing.
2- myoclonic jerks.
3- forelimb clonus.
4- kangaroo position with bilateral clonic jerks of the forelimbs.
5- generalized clonic seizures with falling.
6- sudden running fits and generalized clonic seizures.
7- generalized tonic-clonic seizures.

2.2.1. Determination of the initial dose of MP and treatment time

To determine initial MP dose, 3 different mice groups (n = 4) were administered with 27, 30 and 33 mg/kg during 4 days. The percentages of animals with generalized seizures and mortality were recorded each day.

MP convulsant dose was administered i.p. once a day for 0, 7, 10, 13, 19 or 23 consecutive days to determine the necessary treatment time to develop PHT resistance. MP19 and MP23 groups were injected from Monday to Friday; the other groups were administered from Monday to Sunday. 24 h after the last administration of MP, PHT 18 mg/kg, i.p., and 2 h later, MP convulsant dose were

administered. The animals were observed for 30 min. Seizure score were recorded. Animals that had generalized seizure (stage 4 or higher) were considered resistant.

2.2.2. Evaluation of Pgp overexpression induced by repetitive MP administration

MP23 group (n = 22): Mice were manipulated as described in Section 2.2. during 23 days. 24 h later the last MP administration, they were sacrificed by cervical dislocation. Four of them were used for immunohistochemistry studies (see below). The remaining eighteen mice were used for western blot studies (see below).

Control group (n = 22): Mice were managed as described for the MP23 group, but they received saline (0.15 ml) instead of MP during 23 days. Four mice were used for immunohistochemistry studies and eighteen mice were used for western blot studies.

2.2.3. Evaluation of PHT resistance induced by repetitive MP administration

These experiments were focused to determine if the repetitive administration of MP induces resistance to PHT, which is a recognized Pgp susbtrate (Stepien et al., 2012). On day 24, MP was administered at time of maximum response to PHT.

MP23-PHT group (n = 6): after habituation, mice were daily injected with MP during 23 days as described previously. 24 h after the last MP injection, mice received PHT (18 mg/kg, i.p.) and 2 h later MP (36 mg/kg, i.p.). At this dose, preliminary experiments from our laboratory showed that PHT was able to prevent stage 4–7 of seizures induced by a single administration of MP in control mice. After MP administration, mice were observed during 30 min. Seizure severity and latencies were registered.

MP23-PHT + NIMO group (n = 6): animals were managed as described for the MP23-PHT group, but they received NIMO (3.5 mg/kg, i.p., a Pgp blocker) 30 min before PHT administration.

MP23-NIMO group (n = 6): mice were manipulated as described above for the MP23-PHT + NIMO group, except that they received vehicle instead of PHT.

MP23-vehicle group (n = 6): animals were handled as described above for the MP23-PHT, except that they received vehicle instead of PHT on day 24. This group represented the seizure control group.

S23 − PHT group (n = 6): mice were managed as described above for the MP23-PHT group except that they received saline instead of MP for 23 consecutive days. On 24th day mice were administered with PHT (18 mg/kg, i.p.) and, 2 h later, MP (30 mg/kg, i.p.).

S23-NIMO group (n = 6): mice were managed as described above for MP23-NIMO group except that they received saline instead of MP for 23 consecutive days.

These procedures are summarized in Fig. 2

2.2.4. Evaluation of PB resistance induced by repetitive MP administration

PB has previously been reported as Pgp substrate (Stepien et al., 2012). This experiment aims to assess whether the resistance to PHT is also extended to PB. On day 24, MP was administered at time of maximum response to PB.

MP23-PB group (n = 10): after habituation, mice were daily injected with MP during 23 days as described previously. 24 h after the last MP injection, mice received PB (15 mg/kg, i.p.) and 1 h later MP (36 mg/kg, i.p.). Preliminary experiments from our laboratory showed that PB 15 mg/kg was able to prevent seizures corresponding to stage 2 or higher. After MP administration, mice were observed during 30 min. Seizure severity and latencies were registered.

MP23-PB + NIMO group (n = 10): animals were managed as described for the MP23-PB group, but they received NIMO (3.5 mg/kg, i.p.) 30 min before the PB administration.

MP23–vehicle group (n = 6): animals were handled as described as others groups but received vehicle on day 24. It was a seizure control group.

S23-PB group (n = 10): mice were managed as described above for the MP23-PB group except that they received saline instead of MP for 23 consecutive days. On day 24 mice were administered with PB (15 mg/kg, i.p.) and, an hour later, MP (30 mg/kg, i.p.)

S23-vehicle group (n = 6): mice were managed as described above for the MP23-PB group except that they received vehicle for 23 consecutive days as pretreatment.On day 24 mice were administered with vehicle and, an hour later, MP (30 mg/kg, i.p.).

These procedures are summarized in Fig. 3.

2.2.5. Evaluation of the anticonvulsant effects of Pgp non-substrate AEDs after repetitive administration of MP

CBZ, DZP and LEV have been reported as Pgp nonsubstrates when they were evaluated in *in vivo* animal models (Owen et al., 2001; Mealey et al., 2010; Zhang et al., 2012). These experiments were designed to evaluate if repetitive MP administration induces resistance to these AEDs. On day 24, MP was administered at time of maximum response to each AED.

MP23-CBZ group (n = 9): after habituation, mice were daily injected with MP during 23 days as described previously. 24 h after the last MP injection, mice received CBZ (25 mg/kg, i.p.) and 15 min later, MP (36 mg/kg, i.p.).

MP23-DZP group (n = 6): MP was daily injected during 23 days, 24 h after the last MP administration mice received DZP (0.5 mg/kg, i.p.) and, an hour later, MP (36 mg/kg, i.p.).

MP23-LEV group (n = 5): MP was daily injected during 23 days, 24 h after the last MP administration mice received LEV (33 mg/kg, i.p.) and, an hour later, MP (36 mg/kg, i.p.).

MP23-saline group (n = 9): MP was daily injected during 23 days, 24 h after the last MP administration mice received saline and, an hour later, MP (36 mg/kg, i.p.).

S23-CBZ, S23-DZP and S23-LEV (control groups; n = 6): For each respective group animals received saline during 23 days. On day 24 they received CBZ (25 mg/kg) or DZP (0.5 mg/kg) or LEV (33 mg/kg) and after the time indicated above for each drug, MP (30 mg/kg, i.p.).

S23-saline group (n = 6): Saline was daily injected during 23 days. On 24th day they received saline and, an hour later, MP (30 mg/kg, i.p.).

These procedures are summarized in Fig. 4.

2.3. Evaluation of Pgp expression by immunohistochemistry

This technique was applied as previously described (Gori and Girardi, 2013) with certain modifications.

Brains were removed and immediately embedded in 1, 2-dichloroethane (Merck Millippore) and snap frozen in dry ice. Frozen brains were stored at −80 °C for at least overnight. Coronal brain sections (20 μm) were cut by means of a Leica CM 1850 cryostat, mounted on gelatin-coated slides and fixed in cold 100% acetone for 10 min.

Brain sections of both treated and control groups were simultaneously processed for Pgp-170 immunostaining. In order to inhibit endogenous peroxidase activity, tissue sections were incubated in 0.5% v/v H_2O_2 in phosphate buffer saline (PBS) for 30 min at room temperature. Brain sections were blocked during 1 h with 3% v/v normal sheep serum and 0.3% triton in PBS, then the sections were incubated for 24 h at 4 °C with 1:100 anti-Pgp-170 primary monoclonal antibody C219 (Calbiochem) or C494 (Signet Laboratories, Dedham, MA). After washing with PBS-X (PBS 0.02 5% Triton X-100) tissue sections were incubated for 1 h at room temperature with 1:100 anti mouse biotinilated antibody (Sigma-Aldrich) and then for 1 h with 1:200 streptavidin-peroxidase com-

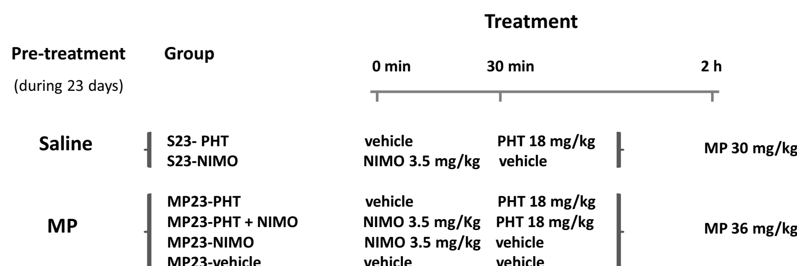

Fig. 2. *Treatment scheme*: PHT activity in mice treated with MP or saline during 23 days.

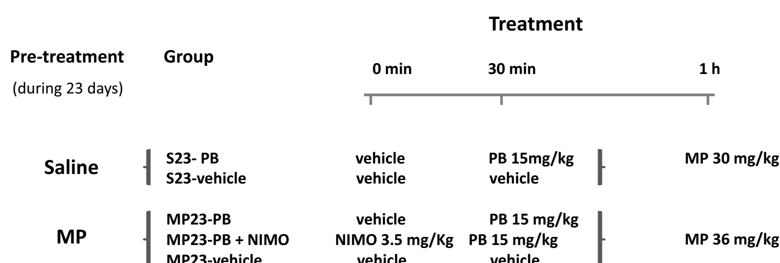

Fig. 3. *Treatment scheme*: PB activity in mice treated with MP or saline during 23 days.

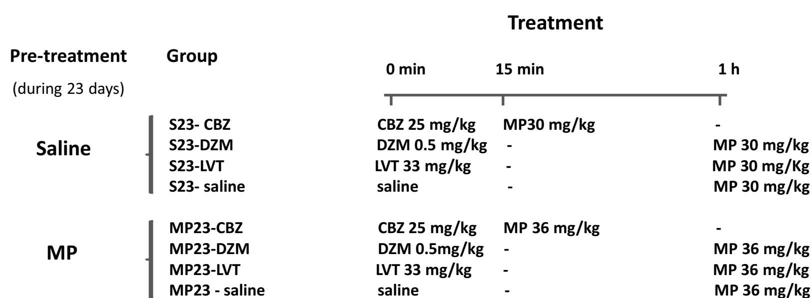

Fig. 4. *Treatment scheme*: CBZ, DZP and LEV activity in mice treated with MP or saline during 23 days.

plex (Sigma-Aldrich). Reaction was carried out with 0.035% w/v 3, 3-diaminobenzidine plus 2.5% w/v nickel ammonium sulphate and 0.1% v/v H_2O_2 dissolved in acetate buffer. Sections were cover-slipped using DPX (Fluka) for light microscopic observation.

Negative controls were processed simultaneously by omitting the primary antibodies.

All antibodies, as well as streptavidin complex, were dissolved in PBS containing 1% v/v normal sheep serum and 0.3% v/v triton X-100, pH 7.4.

Images of inmunoperoxidase sections were obtained using an Axiophot Zeiss light microscope, equipped with a video camera (Olympus Q5). Images obtained from the light microscope were analyzed with ImageJ software (National Institute of Health, USA). The resolution of each pixel was 256 gray levels (8 bits). Immunoblot values represent the means of 4 mice per each experimental condition. The percentages of marked surface in twenty to twenty five fields from each region were measured. The whole hippocampus, cerebral cortex and striatum were measured.

2.4. Evaluation of Pgp expression by western blot

Hippocampus, striatum and cerebral cortices (pooled from two mice) were dissected in cold environment. Tissue was homogenized in a solution containing 0.32 M sucrose neutralized with Tris base solution (0.2 M) pH 7.2 and 0.05 ml/g proteinase inhibitor cocktail at 10% (w/v), in a Teflon glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 900 x *g* for 10 min at 4 °C. The supernatants were spun at 100,000 x *g* for 30 min. The resulting pellets containing the crude membranes were resuspended in 20 mM Tris–HCl, 0.25 M sucrose, and 0.5 mM EDTA (Auzmendi et al., 2009). Protein concentration was determined using Bradford reagent (Sigma) with bovine serum albumin as standard (Bradford, 1976).

For gel electrophoresis, aliquots of tissue sample were diluted 4 fold with dodecyl sulfate (SDS) sample buffer and denatures at 100 °C. 30 μg of protein was loaded on each lane. After separation by SDS-PAGE 8%, the resolved proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Hybond-P GE Healthcare). Membranes were blocked with 4% non-fat milk and 2% Glycine in buffer phosphate saline (PBS-Tween 0.05%) and then incubated with anti-Pgp (1:300 C219 antibody Calbiochem) overnight at 4 °C. After three washes whit PBS-T the membranes were incubated with horseradish peroxidase-conjugated secondary antibody in buffer (1:500 Amersham GE Healthcare) for one hour and them again washed three times with PBS-T. To visualize immunoreactivity, membranes were incubated with the ECL chemiluminescense detection system (Amersham ECL Western blotting detection reagents and analysis system from GE Healthcare, Buckinghamshire,UK) and exposed to X-ray blue films (Agfa, Argentina). Developed films were scanned and optical density quantified by Image Studio Light software of Li-Cor.

As a protein load control, a polyclonal anti-β-actin antibody (Sigma) was used at a dilution 1:1000 in the same membranes after stripping.

Table 1
Determination of MP treatment time. Percentage of mice that presented generalized seizure after different times of MP treatment (n = 6 in all groups).

Days of MP treatment	%PHT-resistant mice
0	0
7	0
10	16.7
13	33.33
19	100
23	100

Densitometry of immunoreactive bands was performed using computer-assisted software (Image Studio Lite) and normalized with reproved actine signal on the same membranes. Values represent the means of 9 samples (pool of 2 mice) per each experimental condition.

2.5. Statistics

Inmunohistochemistry. Statistical comparisons of percentage of stained area (mean ± SEM) were performed using student's *t*-test. Statistical significance was set to $p < 0.05$.

Western Blot: Results are expressed as mean ± SEM of percentage increased compared to the control. Statistical comparisons were performed using student's *t*-test. Statistical significance was set to $p < 0.05$.

Anticonvulsant effect: Results obtained from the evaluation of convulsive behavior after MP administrations are expressed as score mean ± SEM. In all experiments one-way ANOVA followed by Tukeyís post hoc test were performed. We used Fisher's exact test to compare categorical variables.

3. Results

3.1. MP repetitive administration: mice behavior

Each MP administration induced piloerection, nodding and myoclonus with a latency of 3.11 ± 0.55 min. Eventually, animals developed forelimb clonus and kangaroo position with bilateral clonic jerks of the forelimbs. Subsequently, animals presented seizures characterized by sudden circle running, ending with a tonic-clonic seizure with a latency of 4.52 ± 0.94 min. This type of seizure activity was induced during the 23 days of MP treatment. On day 23 all animals had a seizure score 5–7 with a survival rate of 80%.

3.1.1. Determination of the initial dose of MP and treatment time

The high dose (33 mg/kg) induced generalized seizure in 100% of mice with a mortality of 50% at first day. This group was not evaluated the 3 following days. 27 mg/kg induced generalized seizure only in 50% of mice. 30 mg/kg was the optimal dose due to it induced generalized seizure in 100% of mice and 0% mortality at 4th day.

PHT resistance increased across treatment days (Table 1). 100% of mice displayed PHT resistance after 19 and 23 days of MP treatment. However, after 19 days of treatment seizure score was 4.33 ± 0.02, while after 23 days it was 5.66 ± 0.02 ($p < 0.05$) (Fig. 5). Therefore 23 days of MP treatment was chosen.

3.1.2. Repetitive administration of MP in mice induces Pgp overexpression in brain

Pgp immunoreactivity in the control group was weakly positive in blood vessels from cerebral cortex, hippocampus and striatum (Fig. 6A and B) and western blot studies showed Pgp expression in these areas (Fig. 7A). Brain sections from MP23 animals showed an intense Pgp immunoreactivity in blood vessels from cerebral cortex, hippocampus, and striatum. Parietal, piriform and perirhinal

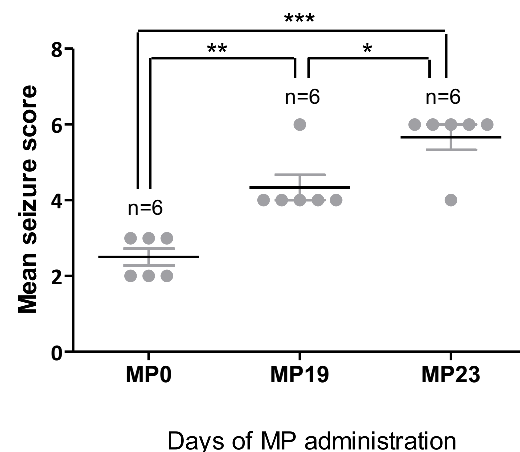

Fig. 5. *Seizure score.* MP0 group did not receive MP treatment. MP19 and MP23 groups received 19 and 23 MP administrations respectively. (n = 6 in all groups). *Mean seizure score* ± SEM vs treatment days was graphed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

cortices were measured showing an increase in the immunostaining of 1025% in the whole cerebral cortex. Hippocampus showed immunostaining in all the subareas with an increase of 508% while striatum values increased 2700% respect to control ($p < 0.001$) (Fig. 6A–C). Western blot technique revealed a significant increase in Pgp expression in all the studied MP brain areas, when compared with control group: cerebral cortex (38%; $p < 0.05$), hippocampus (63%; $p < 0.01$) and striatum (40%; $p < 0.05$) (Fig. 7A and B).

The immunohistochemistry results were identical either with C494 or C219 antibodies; all figures presented in this investigation are of C219 staining.

3.1.3. Repetitive MP-induced seizure trigger PHT resistance

When vehicle was administered to MP23 (MP23-vehicle group) 100% of mice presented generalized seizures with score 5, indicating that MP23 mice were responsive to MP on day 24. In S23-PHT group none of the mice had generalized seizures. They presented partial seizures with a score between 0 and 3 (1.6 ± 0.62; $p < 0.001$ vs. MP23-PHT), so that PHT had anticonvulsant effect in S23 mice. All animals (100%) from MP23-PHT group showed generalized seizures with score 6 after the last MP administration; therefore PHT did not show anticonvulsant effect. Concerning the animals receiving NIMO before PHT (MP23-PHT + NIMO), they did not show neither generalized nor partial crisis, with score 0 or 1 (0.4 ± 0.36; $p < 0.001$ vs. MP23-PHT), indicating that acquired resistance to PHT can be reversed by prior administration of a Pgp blocker. 100% of mice from MP23-NIMO and S23-NIMO groups showed generalized seizures with score between 5 and 7 (6.4 ± 0.36 and 5 ± 0 respectively); NIMO itself had no anticonvulsant effect in neither MP23 nor S23 mice. (Fig. 8A and B)

3.1.4. Repetitive MP-induced seizure elicit PB resistance

S23-PB group showed anticonvulsant effects in 100% of mice ($p < 0.001$ vs S23-vehicle group) with a score of 0 or 1 (mean of 0.18 ± 0.12; $p < 0.001$ vs S23-saline group). Score 1 included nodding, eye blinking and immobility; from these results, the criterion on resistance is the presence of seizures with score ≥ 2.

80% of mice from MP23-PB group showed score ≥ 2 ($p < 0.01$ vs S23-PB group) with a mean of 3.4 ± 0.68 ($p < 0.001$ vs S23-PB group), being resistant to the PB anticonvulsant effects.

Animals that received NIMO and PB (MP23-PB + NIMO) had seizure score 2.7 ± 0.78, being 60% ≥ 2 ($p < 0.05$ vs MP23-vehicle group). This group did not differ from the MP23-PB group. (Fig. 9A and B)

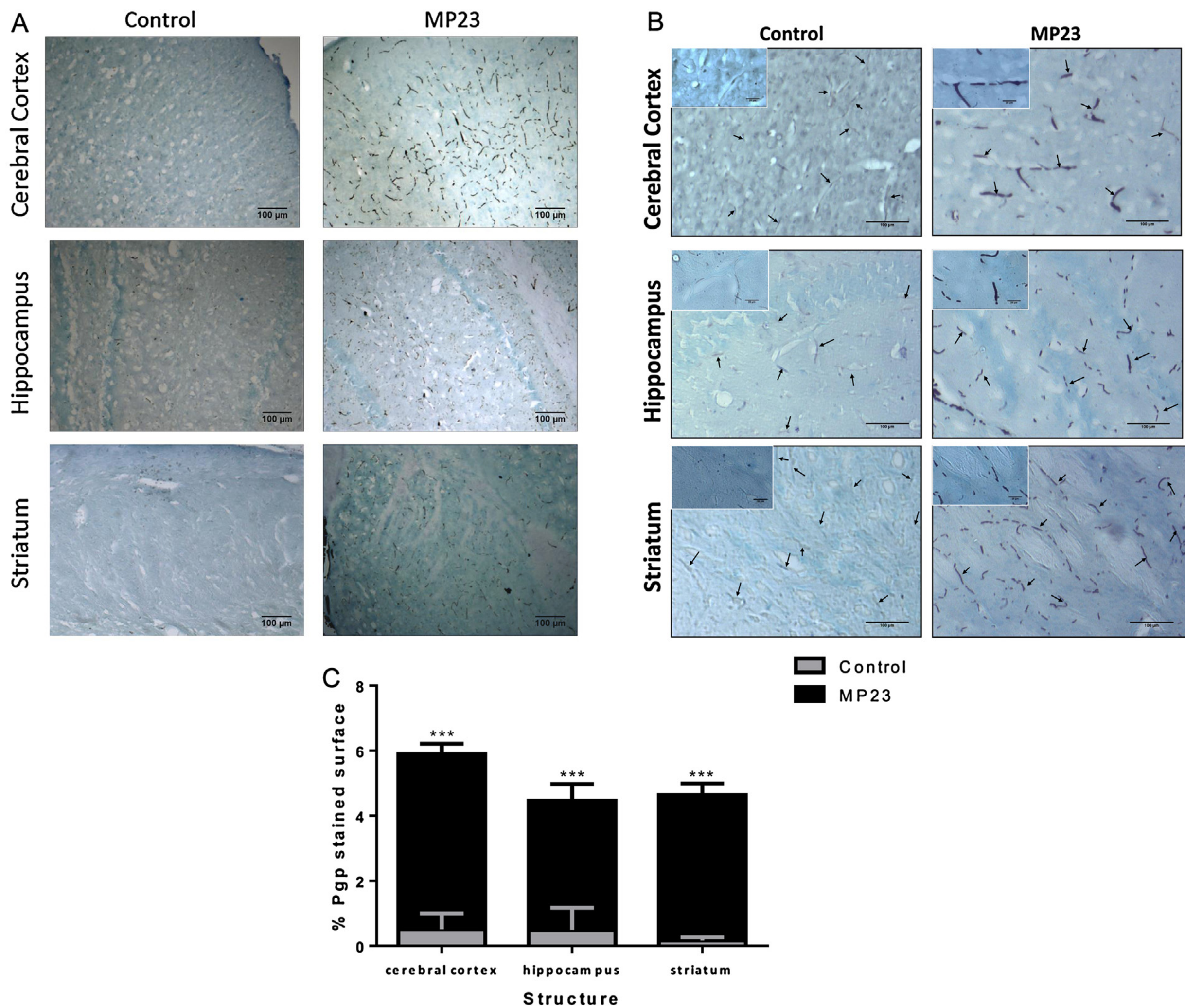

Fig. 6. *Pgp expression in cerebral cortex, hippocampus and striatum by immunohistochemistry*. A- Immunostaining pictures from different brain areas in MP23 and control mice. (Primary magnification 100×). Parietal cerebral cortex, CA1 hippocampal subarea and striatum are shown. B- Primary magnification 200×. Arrows indicate Pgp stained vessels. Insets show magnification at 400×. C- The percentage of stained surface ± SEM in MP23 and control mice was graphed. *** $p < 0.001$.

3.1.5. Repetitive MP-induced seizure does not decrease the anticonvulsant effect of non-Pgp substrate AEDs

MP23 and S23 mice administered with saline on day 24 showed generalized seizure (score 5 or 6; 5 ± 0 and 5.11 ± 0.11 respectively) in all mice after MP administration. CBZ administration on S23 (S23-CBZ group) protected the mice from generalized seizure at evaluated doses showing a significantly lower mean seizure score respect to S23-saline (1.4 ± 0.3; $p < 0.001$). In the same way, MP23-CBZ decreased the score respect to MP23-saline group (2.11 ± 0.35; $p < 0.001$) (Fig. 10). These results indicate that CBZ can induce anticonvulsant effects despite the observed Pgp overexpression.

LEV and DZP did not show anticonvulsant effect on S23 groups at evaluated doses but they reduced the seizure score in both MP23-LEV (2.4 ± 0.2; $p < 0.05$ vs. MP23-saline) and MP23-DZP (1 ± 0.44; $p < 0.001$ vs. MP23-saline) groups indicating a greater anticonvulsant activity on MP23 compared to S23 brains (Fig. 10). While the MP23-DZP group showed a significant difference compared to S23-DZP group, MP23-LEV group did not differ from the S23-LEV group.

4. Discussion

The overexpression of Pgp has previously been shown to be a consequence of seizures (Bauer et al., 2008; Miller, 2008, 2015), thus uncontrolled seizures in patients represent a high risk factor to develop RE. Pgp expression can be induced in RE experimental models, in order to investigate the potential advantage of novel drugs compared with clinically established AEDs (Potschka, 2012). In this investigation we have developed a mice model with Pgp overexpression by means of daily administration of the convulsant agent MP, achieving a high percentage of resistant mice.

This seizure model requires the progressive increase of MP dose (30–36 mg/kg) through the experimental procedure to induce daily generalized seizures during 23 consecutive days. This experimental procedure is in agreement with previous reports from Világi et al. (2009). They employed an increasing dose of 4 amino- pyridine to produce seizure stage 5 according to Racine scale. The need to increase the MP dose could be related to a rised seizure threshold (tolerance to convulsant stimuli); in contrast to the progressive decrease in seizure threshold observed in kindling models (Mason and Cooper, 1972; Leclercq et al., 2014; Racine, 1972).

It is worth mentioning that the increase of MP dose along treatment might be due to extrusion of this convulsant drug by overexpressed Pgp, however there is no data about this at present.

We have observed an Pgp overexpression after repetitive seizures, by immuno histochemical and western blot studies, associated with a low response to PHT and PB anticonvulsant effect, which was reversed by NIMO (a Pgp blocker), according with previous studies on rat brain (Hocht et al., 2007, 2009).

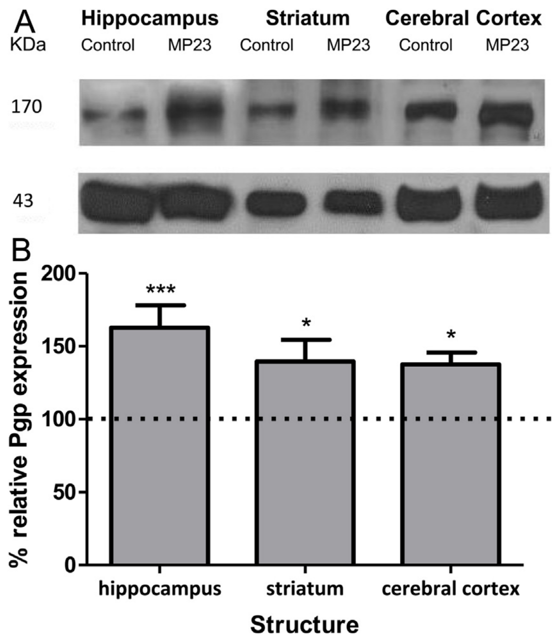

Fig. 7. *Pgp expression in cerebral cortex, hippocampus and striatum by western blot* (A) Western blot for Pgp (170 KDa) and α- actine (43 KDa). Each lane represents 30 μg of pooled membrane proteins. (B) The bar graphs illustrate% of relative expression respect to control ± SEM. *p < 0.05; ***p < 0.001.

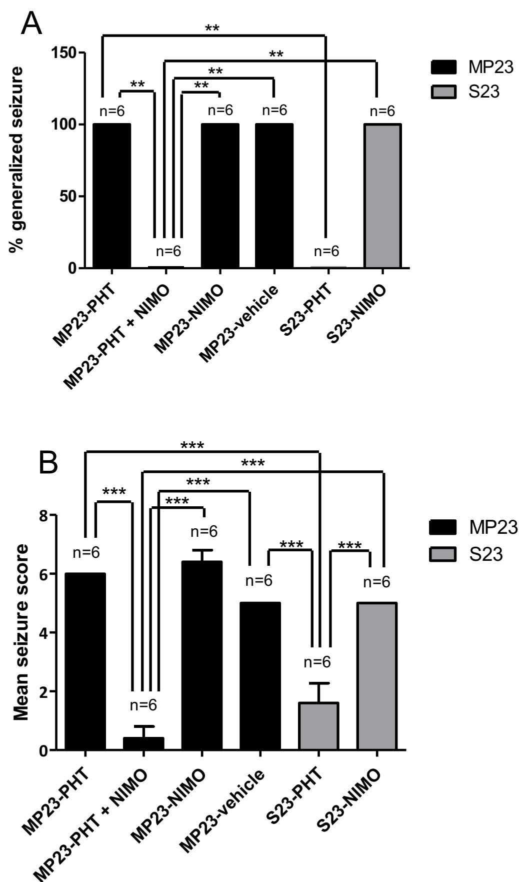

Fig. 8. *Repetitive MP induced seizure trigger PHT resistance.* A) Percentage of mice with generalized seizures vs. treatment. B) Seizure score mean ± SEM vs. treatment. ** p < 0.01; *** p < 0.001.

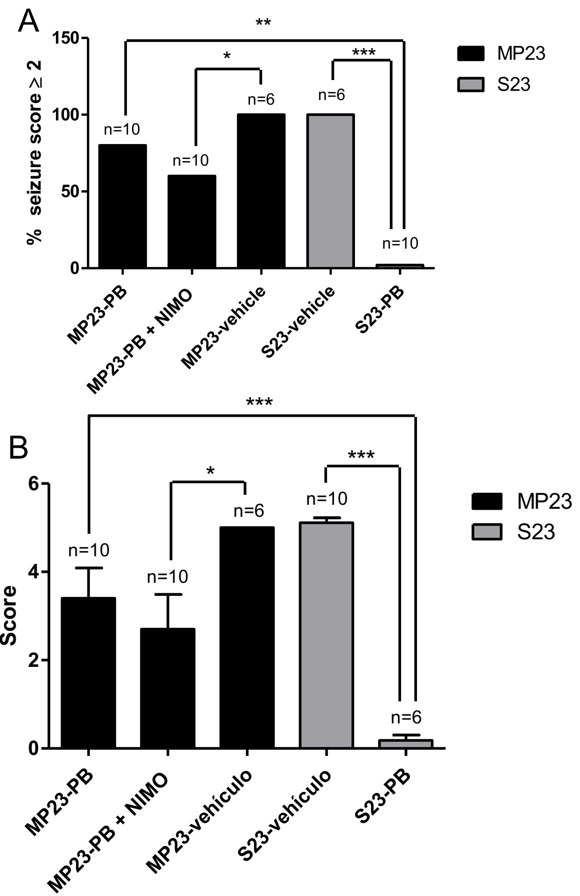

Fig. 9. *Repetitive MP induced seizure elicit PB resistance.* A) Percentage of mice with seizure score ≥2 vs. treatment. B) Seizure score mean ± SEM vs. treatment. *p < 0.05; **p < 0.01; ***p < 0.001.

NIMO administration before PB reduced partially resistance to PB, but NIMO was needed to see PB protective effect in MP23 mice. The low percentage of PB resistance reversion by NIMO may be due to expression of other transport proteins such as MRPs which recognize PB as substrate besides Pgp (Luna Tortós et al., 2008).

In contrast, MP23 animals were sensitive to Pgp nonsubtrate AEDs, like CBZ, DZP and LEV. The fact that DZP and LEV elicit protection in MP23 animals at doses that they were not effective in control animals suggests that these AEDs have a strong anticonvulsant action on a convulsive brain (which is not observed on animals with seizure triggered by an acute episode). In agreement with this idea, Ohno et al. (2010) observed that LEV, in a similar dose, significantly suppressed the development and acquisition of PTZ kindling, and they found an increase in the hippocampal levels of SV2A protein (a primary action site of LEV) in fully kindled animals (Ohno et al., 2009).

It is important to note that current models of drug resistant epilepsy require extremely long times for its setting up, due to the resistance induction period and the subsequent treatment used for the selection of nonresponders. Models generating epileptic animals having spontaneous seizures after induction of a status epilepticus (through pilocarpine, kainic acid or prolonged electrical stimulation of the basolateral amygdala (BLA)) require 6 weeks after the status induction until the animals exhibit spontaneous seizures and then, another 6 weeks for the selection of resistant animals (Bankstahl and Löscher, 2012). Kindling model requires about 8 weeks after implantation of the electrodes until the kindled state is reached, and between 6 and 8 additional weeks for selection of resistant animals (Löscher, 2006). MP23 model can be an excellent

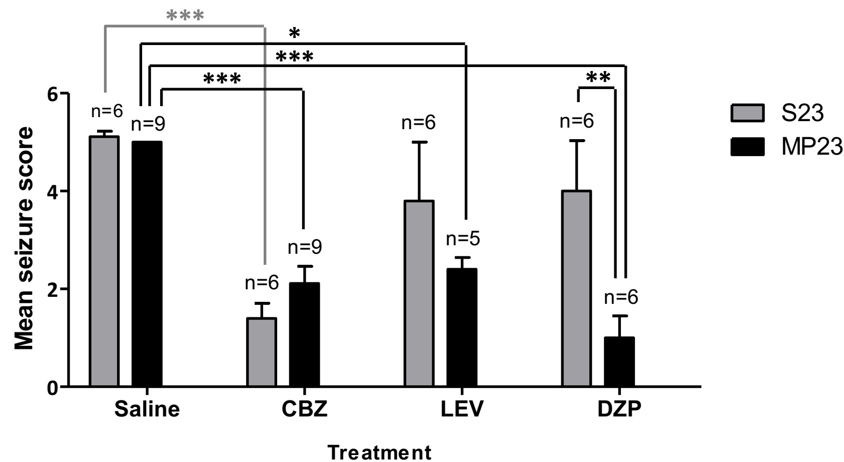

Fig. 10. *Evaluation of Pgp non-substrate AEDs on MP23 and S23 mice.* CBZ, LEV and DZP were evaluated. Mean seizure score ± SEM vs. treatment was represented. *p < 0.05; **p < 0.01; ***p < 0.001.

alternative for evaluating new drugs, considering that it has the advantage of generating animals with pharmacoresistant seizures in only 23 days. 100% of mice are PHT- resistant and 80% are PB-resistant. The animals still remain resistant for at least a week after the end of treatment with 3MP (results not shown).

MP23 have a mortality rate of 20%, other chemoconvulsant models have shown different percentages. Curia et al. have reviewed mortality rate in different laboratories, investigating pilocarpine and litium pilocarpine models. Pilocarpine dose of 320–360 mg/kg had a mean of 37.3% (17–55%) of death and 22% (5–31%) was registered with the dose of 380 mg/kg while in litium-pilocarpine treatment 56% (24–100%) was reported (Curia et al., 2008). In kainic acid mice model Ahmad et al. reported 50% of mortality and only 44% of all mice get an epileptic stage; reducing the animal performance to 22%. These authors mention 20 to 50% of mortality depending on the mice strain (Ahmad et al., 2013).

Electrical kindling models have lower mortality than chemoconvulsant models (Kandratavicius et al., 2014), but not all the animals get a fully kindled stage. All MP23 survival animals are useful for anticonvulsant assays.

Taking into account other epilepsy models with animals treated with a single AED, it is worth mentioning that the models of spontaneous seizures achieve about 35% of PB-resistant animals, considering only the animals which develop spontaneous seizures (Bankstahl and Löscher, 2012; Brandt and Löscher, 2014), while amygdala kindling model achieves between 12 and 23% of PHT-resistant animals (Löscher et al., 1993; Löscher, 2011; Potschka, 2012).

The high percentage of resistance achieved in our model avoids selecting non-responders by administering AEDs before the new candidate evaluation, assuming that all the animals are resistant. This is an advantage because AEDs used in the selection stage could alter the response to the test drug (e.g. due to altered pharmacokinetics induced by the used drug in the selection stage).

Additionally, the new model can be used to study the mechanisms of Pgp overexpression induced by recurrent seizures, as well as for the evaluation of new drugs for RE treatment. For example, it can be employed to evaluate the effectiveness of drugs in a high Pgp expression model (evaluation at 24th day), or to test the drug potential to prevent the development of resistance due to Pgp overexpression (daily administration).

We propose the use of this model in the early stages of screening new Pgp nonsubstrate AEDs, selecting the promising compounds to be evaluated later in RE model. Further studies will be made to elucidate other pharmacoresistance mechanisms.

5. Conclusion

In this investigation we have developed a new mice model of pharmacoresistant seizures, which is capable of identifying drug treatments for the control of Pgp-mediated drug resistant seizures. This new model involves 23 consecutive daily MP administrations; it has 100% PHT resistance and 80% PB resistance at the end of treatment while the Pgp nonsubstrate CBZ, LEV, DZP have anticonvulsant effect.

We have evidenced the Pgp overexpression by means of western blotting assays in cerebral cortex, striatum and hippocampus. An increase of Pgp expression in blood capillaries was observed in all regions studied by immunochemistry.

This model promises to be useful for screening novel anticonvulsant drugs with a potential effect on pharmacoresistant seizures treatment.

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