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Chronic administration of phenytoin induces efflux transporter overexpression in rats

Silvana Alvariza [a], Pietro Fagiolino [a], Marta Vázquez [a], Iris Feria-Romero [b], Sandra Orozco-Suárez [b,*]

[a] Pharmaceutical Sciences Department, Faculty of Chemistry, Universidad de la República, Montevideo, Uruguay
[b] Medical Research Unit for Neurological Diseases, Speciality Hospital, 21st Century National Medical Center of the Mexican Institute of Social Security, Mexico City, Mexico

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ABSTRACT

Background: Efflux transporters overexpression has been proposed as one of the responsible mechanism for refractory epilepsy by preventing access of the antiepileptic drug to the brain. In this work we investigated whether phenytoin (PHT), could induce efflux transporters overexpression, at different biological barriers and to evaluate the implication it could have on its pharmacokinetics and therapeutic/toxic response.
Methods: Forty-two adult females Sprague Dawley divided in five groups were treated with oral doses of 25, 50 and 75 mg/kg/6 h of PHT for 3 days and two additionally groups were treated with intraperitoneal (*ip*) doses of 25 mg/kg/6 h or 100 mg/kg/24 h. At day 4 PHT plasma concentrations were measured and, obtained several organs, brain, parotid gland, liver and duodenum in which were analyzed for the Pgp expression. At day 4 PHT plasma concentrations were measured and several tissues: brain, parotid gland, liver and duodenum were obtained in order to analyze Pgp expression. In order to evaluate the oral bioavailability of PHT, two groups were administered with oral or intraperitoneal doses of 100 mg/kg and plasma level were measured.
Results: An induction of the expression of efflux transporter mediated by phenytoin in a concentration-and-time dependent manner was found when increasing oral and *ip* doses of phenytoin, One week after the interruption of *ip* treatment a basal expression of transporters was recovered.
Conclusions: Overexpression of efflux transporters can be mediated by inducer agents like PHT in a local-concentration dependent manner, and it is reversible once the substance is removed from the body. The recovery of basal Pgp expression could allow the design of dosing schedules that optimize anticonvulsant therapy.

Introduction

Drug transporters are important in the absorption and disposition of endogenous substances and xenobiotics, including drugs. Transporters work in concert with drug-metabolizing enzymes to eliminate drugs and their metabolites. They can mediate in various cell types, tissue-specific drug distribution (drug targeting) and they may also serve as protective barriers to particular organs and cell types [1].

The efflux transporters (Pgp, MRPs, and BCRP) and enzymes such as the ones of the cytochrome P450 family located along the intestine and the liver play an important role in the clearance and bioavailability (BA) of several drugs, and can determine the extent of absorption and elimination of their substrate molecules [2]. Many of these drugs may also induce or inhibit the synthesis, expression or activity of these proteins, causing different types of non-linearities in their pharmacokinetic response.

On the other hand, overexpression of these efflux transporters has been proposed as one of the mechanisms responsible for refractory epilepsy by preventing access of the antiepileptic drug (AED) to the pharmacological targets [3,4]. Phenytoin (PHT) is a first-line anti-epileptic drug used in the treatment of different types of epilepsy and whose effectiveness in the *status epilepticus* is well established. However, this AED does not escape from the emergence of drug resistance during chronic treatment in some susceptible patients [5].

There is evidence that suggests that some drugs, including AEDs themselves, may be responsible for efflux transporter overexpression [6,7]. If this is the case, it is possible that increasing doses

* Corresponding author.
E-mail addresses: sorozco5@hotmail.com, sorozco5@yahoo.com.mx (S. Orozco-Suárez).

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during chronic treatment may lead to a greater overexpression, making the response even worse.

It has been seen in experimental animals that overexpression was limited only to certain brain areas, while elsewhere the efflux transporter expression was neither increased nor diminished and thus local drug concentrations were not affected [8,9]. This specific overexpression allows the coexistence of efficacy and toxicity, leading to a difficult situation for dose adjustments. The aim of this work was to investigate whether PHT could induce efflux transporter overexpression at different biological barriers as well as brain and splanchnic tissues by means of a dose-dependent mechanism, and to evaluate the implication it could have on its pharmacokinetic and therapeutic/toxic response.

Materials and methods

Chemicals

Phenytoin was purchased from Pfizer laboratories (EPAMIN™ Oral suspension and EPAMIN™ SP, solution for injection).

All other chemicals used were of analytical grade and were used as supplied.

Animals

Forty-two adult female Sprague Dawley rats initially weighing 250–300 g were used in the present study. They were individually housed at 22 °C and maintained on a 12-h light/dark cycle. Rats had free access to food and water. Procedures involving animal care were conducted in agreement with the Mexican Official Standard (NOM-062-ZOO-1999) and the Ethical Committee of the National Commission Scientific Research-IMSS.

Oral administration

Three groups of 6 rats each underwent oral administration of PHT by swallowing doses of 25 (G1), 50 (G2) and 75 (G3) mg/kg/6 h, for four days. A control group (CG) ($n = 6$) was administered with 2 mL of a dextrose solution (8%) every 6 h. At the fourth day of treatment blood samples (150 μL) were withdrawn from the lateral caudal vein and collected in tubes containing EDTA as anticoagulant (Minivacutainer™), every 20 min up to 4 h and then more spaciously. Each group was divided into three pairs. Blood samples were withdrawn from each subgroup in an alternate manner and the respective plasma fractions stored at −80 °C until analysis. Thus, subgroup 1 provided samples at the following scheduled times: 1st, 4th, 7th, . . .; subgroup 2 at: 2nd, 5th, 8th, . . .; and subgroup 3 at: 3rd, 6th, 9th, . . . times.

After sampling, three animals of each group were sacrificed and brain hippocampus, parotid gland, right hepatic lobe and duodenum were removed and cryoprotected with isopentane and frozen in ice dry and stored at −80 °C until immunochemistry process.

The three remaining animals were sacrificed one week after treatment interruption.

In order to determine the BA of the oral solution, two other groups (3 rats each) were assayed. They were treated with oral (G4) and intraperitoneal (G5) single doses of 100 mg/kg of PHT (EPAMIN™ Oral suspension, Pfizer Laboratories or EPAMIN™ SP, solution for injection Pfizer Laboratories). Blood samples (150 μL) were collected every 20 min for 3 h and hourly up to 6 h in tubes containing EDTA as anticoagulant (Minivacutainer™), and their respective plasma fractions stored at −80 °C until analysis.

Intraperitoneal administration

To determine how the administration route and dosing schedule influenced efflux transporters induction, two groups (G6 and G7) of six rats each were treated intraperitoneally with 25 mg/kg/6 h or 100 mg/kg/24 h respectively, for 4 days. At the fourth day of treatment, animals were sacrificed and samples of brain (right hippocampus), parotid gland, right hepatic lobe and duodenum were extracted and stored for the immunochemistry process.

Phenytoin analysis

PHT plasma concentrations were determined by a high performance liquid chromatography method, based on the procedure developed by Savio et al. [10] with minor modifications. Ten microliters of internal standard solution (Nitrazepam [NTZ] 16 μg/mL in methanol) were added to 50 μL of plasma. The extraction was performed with 250 μL of ethyl acetate and vortexed for 1 min. After centrifugation, the supernatant was separated and dried under nitrogen stream at 37–40 °C. Dry residue was dissolved with 50 μL of mobile phase and 20 μL injected into a Dionex Ultimate 3000 Series chromatograph. A Phenomenex® Luna C18 (5 μm, 100 Å, 150 mm × 4.6 mm) column was used as reversed stationary phase. The mobile phase was a mixture of water/methanol/acetonitrile (43/47/10) pumped with a flow rate of 1.0 mL/min. The column compartment was kept at 40 °C, and the wavelength detection was 220 nm. Under these conditions the retention times of analytes were: 4.5 and 5.8 min for PHT and NTZ, respectively.

The HPLC method was linear between 0.5 (the lower limit of quantification: LLOQ) and 25.0 mg/L of PHT. Inter and intra-day coefficients of variation (CVs) for PHT were below 15% and the accuracy of the method was between 85 and 115%.

Immunohistochemistry

All blocks of tissue were cut in cryostat at 12 μm (Leica GM 1510S, Germany) and mounted onto poly-L-lysine-coated slides and fixed with cold acetone. Then, the sections were washed in 0.12 M phosphate buffer saline (PBS, pH 7.2–7.6), and incubated for 30 min at room temperature with blocking solution, 1% normal horse serum (Vector Lab) in PBS 0.12 M. Later, the sections were incubated with Anti-P Glycoprotein [JSB-1] antibody (Abcam Lab. ab3366) diluted in PBS (1:200) for 24 h at 4 °C. After that, the sections were washed with PBS 0.12 M for 15 min, and incubated for 2 h at room temperature with the goat anti-mouse IgG Alexa 488 (Molecular Probes, In Vitrogen Lab. A21121) diluted in PBS. In the next step, the sections were counterstained with propidium iodide for 1 min and a final washing was performed in PBS 0.12 M for 10 min, gently dried, and cover-slipped with Vectashield (Vector Lab) mounting medium. The samples were observed in a fluorescence microscope (Carl Zeiss), with refrigerated camera (Evolution) and an image analyzer Image Pro Plus 7 (Media Cybernetics).

Analysis of Pgp expression

The intensity analysis was made from the images which were acquired in order to quantify the pixel intensity in green channel corresponding to the Alexa 488. The accumulated histogram was obtained from the intensity values contained in the image's bitmap, previously calibrating intensity and spatial scale to establish the intensity values as integrated intensity of each image. The optical density was determined in relation with the control group and expressed as pixels/mm^2.

Pharmacokinetic and statistical analysis of data

Areas under the plasma concentration–time curves (AUC) were calculated from zero to the last sampling time ($AUC_{trapeze}$), by