UNIVERSITY OF MEDICINE AND PHARMACY OF TIRGU MURES
DOCTORAL SCHOOL

PhD thesis

The use of *in vitro* models in the research of epilepsy and antiepileptic drug development

PhD Candidate: **Zsolt Gáll**
Scientific Advisor: **Prof. Dr. Tibor Szilágyi, PhD**

Abstract

Background

Epilepsy is one of the oldest neurological disorders, which affects about 65 millions of people worldwide. There is no etiologic treatment available for this multifaceted disease, currently the anticonvulsants are considered first-line therapy. However, about 30% of the patients are resistant to pharmacotherapy and despite the introduction of 17 new compounds in the last 25 years a breakthrough was not achieved until now. The majority of our knowledge about the underlying causes and possible pharmacological targets was gained by using experimental models of epilepsy. Until recently, *in vivo* models have been more commonly used for drug screening, but *in vitro* techniques are also considered for this purpose. Neuroscientists have contributed significantly to the understanding of the pathogenesis of epilepsy by using brain slices and this popular tool aroused the curiosity of pharmacologists also. Nevertheless, animal testing cannot be completely replaced.

Objectives

The main purpose of this thesis was the evaluation of the utility of brain slices to obtain (1) experimental models of epilepsy for drug screening (and testing newly developed anticonvulsants – lacosamide, rufinamide – along with carbamazepine) and (2) an *in vitro* pharmacokinetic model to characterize protein binding and intracerebral distribution of compounds. Along with brain slices, brain homogenate was also used to assess the protein binding of tested drugs.

In order to support the use and interpretation of *in vitro* data, (3) an *in vivo* pharmacokinetic study was conducted for rufinamide and (4) the complementary pharmacological actions (e.g. anxiolytic/anxiogenic) of newly introduced anticonvulsants (lacosamide, rufinamide) were also evaluated.

Methods

Electrophysiological recordings were obtained using hippocampal slices from immature Wistar rats (P7-13). Low-magnesium and 4-aminopyridine (4AP) induced *in vitro* models of epilepsy were used to study the anticonvulsant effects of several antiepileptic drugs (rufinamide, lacosamide, and carbamazepine) and antidepressants (fluoxetine and imipramine) on seizure-like events and interictal bursts recorded from the CA3 pyramidal layer.

Neuropharmacokinetic characterization was performed by incubating acute brain slices with certain drugs, (i.e. lacosamide, rufinamide, carbamazepine, fluoxetine and imipramine) in artificial cerebrospinal fluid and after reaching equilibrium the amount of drugs in slices and incubation media was determined. By using a specific formula, the unbound volume of distribution in brain was calculated for each compound, which in combination with other parameters (e.g. brain-to-plasma ratio, unbound fraction of drug) served for the calculation of active site concentration. Rapid equilibrium dialysis was used for determining protein binding of drugs to plasma and brain homogenate.

An *in vivo pharmacokinetic study* was employed to determine the brain exposure to rufinamide by calculating brain-to-plasma ratio in rats. Beside that, the primary pharmacokinetic parameters were also described after oral and i.v. administration.

Ethological studies implied the analysis of the behavior of animals on the elevated plus maze (EPM), a test based on the assumption that rodents avoid open spaces. It is a validated tool for evaluation of potential anxiolytic activity of drugs. Selected classical and novel antiepileptic drugs were studied along with first-line anxiolytic agents.

Results

As all the above mentioned studies required high analytical performance, precise, selective and rapid quantification methods using liquid chromatography-mass spectrometry (LC-MS) were developed for each compound. Two bioanalytical methods using LC-MS were fully validated and published.

In vivo pharmacokinetic parameters of rufinamide and brain-to-plasma ratio in rats were published for the first time in scientific literature. These showed dose-dependent pharmacokinetics in rats, similar to clinical observations – long absorption phase, less than proportional exposure with dose. The observed increase of clearance with dose suggested a nonlinear drug disposition after oral administration.

Low-magnesium and 4AP *in vitro* models of epilepsy reflected the anticonvulsant action of each tested compound, however, some proconvulsant-type actions were also observed. Carbamazepine was not capable to reduce significantly the induced epileptiform activities, while rufinamide and lacosamide were proved to be more efficient. Furthermore, our results support the new approach toward antidepressants regarding their use in epileptic patients, an anticonvulsant action of fluoxetine, similar to lacosamide, being observed in *in vitro* conditions.

The neuropharmacokinetic parameters of newer anticonvulsants – lacosamide, rufinamide – differed from that of carbamazepine in terms of protein binding. The unbound concentration being responsible for pharmacodynamic action, this would suggest a slight advantage for the former, but investigating the potential role of efflux transport at the blood-brain barrier by combining *in vitro* and *in vivo* pharmacokinetic results, it was found that these drugs might be actively pumped out of rat brain.

The behavioral studies revealed that the EPM test generates controversial results when non-GABAergic agents are tested: fluoxetine did not show any anxiolytic effect after acute and chronic administration; buspirone was proved to be anxiogenic after acute but not after chronic administration. A potential anxiolytic effect of anticonvulsants would confer them clinical advantages by treating the epilepsy related anxiety of patients: after acute administration an evident anxiolytic effect of carbamazepine and valproic acid was observed. However, the effects of rufinamide and lacosamide on anxiety were not detectable with the EPM test.

Conclusions

In vitro techniques can contribute to a better understanding of the underlying processes related to the drugs' action by completing the preclinical profile of compounds. Our experimental data obtained by using electrophysiological recordings from brain slices and pharmacokinetic characterization with brain slices and homogenate (protein binding, intracellular distribution) offered new insights into the complexity of epilepsy and anticonvulsant action.

In different phases of drug discovery several models are used for the screening of lead compounds. Our results revealed that even in case of validated animal models (i.e. EPM) some predictive validity issues may be encountered when testing novel compounds. So the development of new experimental models is mandatory for the advancement of drug discovery and development. The *in vitro* models studied in this thesis had sufficiently high throughput, reliability and validity in order to be considered for drug screening.

Keywords: *in vitro* models of epilepsy; brain slice method; electrophysiology; antiepileptics; pharmacokinetics; brain penetration and exposure; anxiety; behavioral studies.