Szakács Tünde

In vitro módszerek alkalmazása a gyógyszerfejlesztésben

Budapesti Műszaki és Gazdaságtudományi Egyetem,
MTA Kémiai Kutatóközpont, Kémiai Intézet
Farmakobiokémiai Osztály
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Az *in vitro* módszerek alkalmazása a gyógyszerkutatásban és fejlesztésben egyre nagyobb jelentőségre tesz szert. A P450 enzimek kutatása már az 1940-es években elkezdődött és a szervezetbe kerülő idegen és endogén anyagok eltávolításában játszott szerepükre is rövid időn belül fény derült. Ezért - és különösen ember esetében, ahol nincs lehetőség erkölcsi okokból a toxikológiai vizsgálatok elvégezésére - nő meg az *in vitro* primer májsejtkultúrával és májsejt frakcióval (citoszol, mikroszóma, S9 frakció) végzett vizsgálatok jelentősége.

A dolgozatban egyrészt a gyógyszerkutatás szempontjából lényeges, xenobiotikumok által létrehozott kölcsönhatásokat tanulmányoztuk *in vitro* módszerek felhasználásával. Másrészt az *in vitro* adatokból becsült *in vivo* farmakokinetikai paraméterek és az irodalomban található *in vivo* mért adatok korrelációját vizsgáltuk.

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Tünde Szakács

In vitro methods in drug development

PhD. theses

Budapest University of Technology and Economic,
Chemical Research Center, Hungarian Academy of Sciences, Department of Pharmacobiochemistry
Budapest
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Introduction

The *in vitro* tests have great importance in the modern drug development, because the data based on these experiments may predict the metabolism, toxicity and the pharmacokinetics of the drug, and its interactions with other molecules. If we know these data, the time and the costs of drug development can be reduced. Hepatocyte-based systems are widely used at early stage of development.

The following *in vitro* tests were used:

1. Metabolism and kinetic studies by primary suspension culture of hepatocytes and different hepatocyte fractions.
2. The effect of pretreating rats with inducers on metabolism and the role of the enzymes (cytochrome P450) in the metabolism were studied.
3. To study the role of the (cytochrome P450) enzymes in the metabolism, selective inhibitors were used.

In the first part of the work the effect of α-methyldopa on the metabolism of antipyrine was studied. Our aim was to answer the following questions:

1. Whether α-methyldopa has inhibitory effect on any cytochrome P450 enzymes?
2. Whether this drug inhibits the metabolism of antipyrine?

The second part deals with a modeling problem. The data based on *in vitro* metabolism experiments may predict the *in vivo* clearance and bioavailability. The current method for prediction of the parameters mentioned is the determination of the intrinsic clearance of the liver. In the course of our work vinpocetine metabolism was studied. The *in vivo* clearance and bioavailability of this drug have been measured in several species by several research groups, but no *in vitro* experiments were carried out.

1. We used the „well-stirred" model to assess whether the prediction of *in vivo* parameters (clearance, bioavailability) based on *in vitro* substrate loss experiments is reasonable.
2. The effect of protein binding on the pharmacokinetic parameters was also studied.
3. The explanation for the difference observed in bioavailability of tested species was searched using data from our *in vitro* experiments and also from the literature.

Objectives

The *in vitro* methods in drug research and development have growing importance. The research concerning P450 enzymes started in the 1940's and the role of these enzymes in removing endogen and foreign compounds from the organisms was suggested. The *in vitro* tests carried out with hepatocytes and hepatocyte fractions (cytosol, microsomes, 10000 x g fraction) have even greater importance in case of human, since no *in vivo* toxicological experiments can be done.

In this work some interactions among xenobiotics (I.) and *in vitro-in vivo* correlation (II.) were studied by several *in vitro* tests.

I.) α-methyldopa is an antihypertensive drug. Our work concerning this compound is based on two findings published in the literature. The first one was described by Szabó et al. (1995), who studied the changes of half-life of antipyrine in the presence of different inducers and α-methyldopa. Pretreatment of rat with spironolactone reduced the half-life of antipyrine by 50%. When spironolactone was combined with α-methyldopa no change of half-life was observed compared to half live obtained with untreated animals. Phenobarbital pretreatment caused the same decrease in the half life as caused by spironolactone but the combination of phenobarbital and α-methyldopa resulted in no change compared to the value obtained by phenobarbital alone. The change in half-life can be caused by reduced or enhanced rate

of metabolism therefore we started to study the *in vitro* metabolism of antipyrine. To study the effect of inducers and α-methyldopa on the metabolism of antipyrine microsomal fractions prepared from the livers of untreated and phenobarbital,- or spironolactone treated rats were used. The formation rate of the three main oxidative metabolites of antipyrine was investigated by the use of specific cytochrome P450 inhibitors. The effect of α-methyldopa on cytochrome P450 enzymes and the metabolism of antipyrine were also studied.

The second observation that α-methyldopa can covalently bind to microsomal protein in the presence of NADPH was taken into consideration as well (Dybing et al., 1976). This covalent binding can affect the rate of metabolism of drugs. For studying this possibility two microsomal reactions were tested: aminopyrine N- demethylation and 7-pentoxyresorufin O-dealkylation.

II.) Besides the *in vitro* metabolism studies the *in vitro* farmacokinetic experiments have also very important role in the process of drug development. From the data of *in vitro* tests *in vivo* kinetic parameters can be assessed. These parameters allow prediction of *in vivo* behaviour of the drug and also predict the value of the bioavailability.

Vinpocetine (Cavinton®) was choosen for the experiments, because the *in vivo* pharmacokinetic parameters have been measured in several species but no *in vitro* experiments were carried out.

We used the „well-stirred" model to assess whether the prediction of *in vivo* parameters (clearance, bioavailability) based on *in vitro* substrate loss experiments can give adequate information about the *in vivo* behaviour of the molecule. In studying the *in vitro* elimination kinetics of vinpocetine primary suspension cultures of hepatocytes were used to predict the *in vivo* clearance and the bioavailability of the drug.

The published *in vivo* pharmacokinetic parameters of vinpocetine obtained in several species raised another question. The value of the whole body clearance was high in all of the species tested (rat, dog, human), in contrast to the bioavailabilities, which showed great differences among the species (rat approx. 50%, dog approx. 20%, human approx.6%). We wanted to solve this problem by *in vitro* experiments and throw light on the reason of the phenomenon.

New scientific results

1. The metabolism of antipyrine was studied using liver microsome fractions from untreated and phenobarbital- or spironolactone treated rats. The formation of all three metabolites of antipyrine was effectively catalyzed by CYP2C6/C11 enzymes in case of untreated rats, in contrast in case of animals treated with inducers CYP2B and 3A enzymes also played important role in the metabolism of the drug,

2. For studying the role of enzymes participating in the metabolism of antipyrine specific P450 enzyme inhibitors (troleandomycin, chloramphenicol, cimetidine) and α-methyldopa were used.

Troleandomycin inhibited the formation of norantipyrine and 4-hydroxyantipyrine in the liver microsome fractions from rats treated with P450 enzyme inducers, suggesting role of CYP3A. In contrast the role of CYP3A in the metabolism of antipyrine is negligible in case of untreated rats.

Cimetidine in low concentration (0.05 mM) inhibited the constitutive CYP2C6/11 enzymes only and decreased the formation of all of the three oxidative metabolites tested, suggesting that these enzymes have significant role in formation of all three metabolites. When cimetidine concentration was increased (0.25 mM) N-demethylation in microsomes from phenobarbital induced rats seemed to be much more susceptible to further inhibition than that in microsomes from spironolactone induced animals, suggesting the catalytic role of CYP2B.

Chloramphenicol significantly inhibited formation rates of all three antipyrine metabolites in microsomes from uninduced and induced rats as well, since it is selective inactivator for CYP2B, 2C11 and 3A enzymes.

α-Methyldopa also affected the formation rate of antipyrine metabolism. The molecule probably inhibited the formation of 4, 4' dihydroxyantipyrine causing accumulation of 4-hydroxyantipyrine.

3. Antipyrine is metabolized by several enzymes (CYP2C11/C6, 2B, 3A), so the inhibition of one of these enzymes can not cause the accumulation of the molecule in the body.

4. Good correlation was found between the loss of 7-pentoxyresorufin O-dealkylation or aminopyrine N-demethylation activity and the extent of lipid peroxidation, respectively. The difference between the loss of the two enzyme activities, reflects the different sensibility for the lipid peroxidation. This phenomenon may be explained by the different location of the enzymes in the membrane.

5. The protecting effect of α-methyldopa on enzyme acivities became appreciable at micromolar concentration. One explanation for this finding is that α-methyldopa reacts with reactive oxygen species, which contribute to the initiation of lipid peroxidation.

6. Assessment of *in vivo* parameters (clearance, bioavailability) for vinpocetine from *in vitro* experiments was studied by primary suspension culture of hepatocytes and also by different cell free fractions in case of three different species. In case of human and dog hepatocytes good correlation was found between the *in vitro* and *in vivo* results, in contrast to vinpocetine clearance in rat, which was underestimated. In this species not only the liver contributes to the metabolism, kidneys have also important role. Other organs: lung, small intestinal mucosa, serum have also minor role in the metabolism of vinpocetine.

7. Cell free fractions from rat, dog and human liver were also studied. In case of dog and human the activity was found in the cytosol in contrast to rat, where the activity was found in the microsomal fraction and the 10000 x g sediment.

8. The estimated values for *in vivo* clearance and bioavailability showed good agreement with *in vivo* measured values in each case if the free drug ratio was assumed to equal 1.

Summary

The importance of the *in vitro* tests in the modern drug research and development is still growing. In this work the effect of α -methyldopa in several *in vitro* test systems was studied. In addittion pharmacokinetic parameters obtained from *in vitro* experiments were compared with data measured *in vivo* to assess the predictive power of the *in vitro* test used.

The *in vitro* metabolism of antipyrine was investigated by analysing the formation rate of the three main oxidative metabolites of the drug using liver microsome fractions from untreated and inducer treated rats in the presence of α-methyldopa or selective cytochrome P450 inhibitors. α-Methyldopa inhibited the formation of norantipyrine and increased the formation rate of 4-hydroxyantipyrine. The results of the experiments suggest that antipyrine is metabolized by several cytochrome enzymes (CYP2C11/C6, 2B, 3A), so the inhibition of one of these enzymes can not cause the accumulation of the molecule in the body. These findings may explain that serious drug-drug interactions is relatively rare.

Data obtained from experiments concerning lipid peroxidation show that α-methyldopa can reduce the extent of lipid peroxidation and protect the membrane from the degradation.

The significance of *in vitro* pharmacokinetic experiments was demonstrated by data obtained from the *in vitro* metabolism of vinpocetine. It was suggested that the *in vitro* data might reasonably predict the *in vivo* pharmacokinetic behaviour. There was a good correlation between *in vitro* and *in vivo* clearance and bioavailability of vinpocetine using human and dog hepatocytes. In contrast in case of rat the *in vivo* clearance was underestimated since in this species the metabolism of vinpocetine is not entirely determined by the liver, kidneys also play important role in it. In summary, the role of every organ participating in the metabolism have to be taken into consideration.

Publications

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