



Workshop 5.15

In vitro metabolism: Applications in pharmacology and toxicology

Metabolic Activation for In Vitro Systems

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Summary:

A significant portion of substances is not directly toxic, but must be activated to reactive metabolites. For most compounds it is unknown if metabolic activation plays a role. *In vitro* systems presently can only determine the toxicological potential of the parent drugs, and not that of potentially toxic metabolites. The development of metabolic systems which can be incorporated within a particular *in vitro* technique is therefore of high priority. Ideally, the *in vitro* system should express all relevant enzymatic activities, because it is a priori not known which enzyme(s) are involved. Also, the metabolic system must be compatible with the *in vitro* assay. Main emphasis is placed on liver preparations as the liver exhibits the highest amount and complexity of metabolic enzymes.

Keywords: metabolic activation, liver preparation, *in vitro* system

Introduction

In the planned REACH project about 30,000 available chemicals have to be registered, evaluated and authorised regarding their properties, including their toxic, carcinogenic, mutagenic, and teratogenic potential. The determination of all required data using *in vivo* experiments would mean the use of an immense number of animals. Hence, especially with regard to the concept of the 3Rs, *in vitro* methods are regarded to be a much wanted alternative.

However, a significant number of substances show toxic effects *in vivo* but not *in vitro*. Well known examples are polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene), cyclophosphamide, aflatoxin B1, Vitamin A and acrylamide. These substances are not directly toxic, but must be activated to reactive metabolites, which then exert their toxic potential by reacting with constituents of the cell, especially proteins and nucleic acids. Aflatoxin B1, for instance, is metabolised to aflatoxin-8,9-epoxide by CYP 3A4 and CYP 1A2 in a phase I reaction. This intermediate product can then be detoxified by a subsequent phase II reaction to the GSH-conjugate or undergo covalent binding to the DNA and thus be mutagenic (see fig. 1). Although

for the given examples metabolic activation pathways are well established, for most compounds it is unknown whether metabolic activation plays a role.

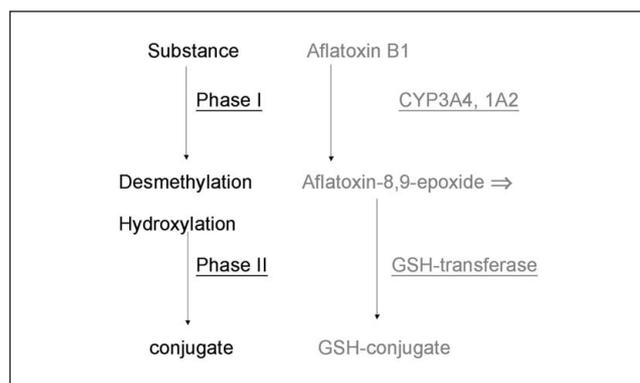


Fig. 1: Metabolic activation of aflatoxin B1.

Aflatoxin B1 is metabolised to aflatoxin-8,9-epoxide by CYP 3A4 and CYP 1A2 in a phase I reaction. This intermediate product can then be detoxified by the subsequent phase II reaction to the GSH-conjugate or undergo covalent binding to DNA, thus exerting its mutagenic effects.

To date *in vitro* systems can only determine the toxicological potential of the parent drugs and not that of potentially toxic metabolites. The development of metabolic systems which can be incorporated within a particular *in vitro* technique is therefore of high priority. Ideally, the metabolic *in vitro* system should express all the relevant enzymatic activities, because during screening of large numbers of substances it is *a priori* not known which enzyme(s) are involved. Also, the metabolic system must be compatible with the *in vitro* assay. The relevance of this approach consists in the adjustment of the existing *in vitro* systems to the *in vivo* situation. The aim is to reveal indirectly acting toxic or teratogenic substances which is presently not possible.

In this paper different target systems for teratogenic substances, as well as different metabolic activation systems, are introduced and their benefits and handicaps discussed. For exemplification the F9 test system is described in more detail.

Metabolic activation systems

Different metabolic systems appear suitable for such a task (Rueff et al., 1996), and the main emphasis is placed on liver preparations, as in most cases the liver exhibits the highest number and complexity of metabolic enzymes:

- S9 (liver 9.000 x g) preparation as used in the Ames test
- Hepatocytes or liver slices
- Genetically engineered cells expressing relevant metabolic enzymes

Subcellular liver fractions (S9-mix / microsomes)

These fractions are easy to handle "standard methods". S9-mix is used in the Ames test to detect genotoxic substances that need to be metabolically activated. However, for the present application many more mechanisms need to be considered than for the mutagenic response of bacteria. Hence, a number of possible limitations must be pointed out: all co-factors must be added. A substantial number of animals is necessary to obtain the subcellular liver fractions and differences have been analysed when subcellular and cellular systems were compared. Thus it is difficult to obtain physiologically relevant data. A further disadvantage when working with microsomes is given by the absence of most phase II enzymes. On the other hand, no additional (cell) culture is needed and the systems can be automated.

Hepatocytes and liver slices

Precision-cut liver slices are easy to prepare but exhibit only a short culture time. Hepatocytes can be obtained freshly isolated or cryopreserved. Both preparations cover all relevant pathways and contain all relevant enzymes and cofactors at physiological levels. Although liver slices and fresh cells can only be obtained directly after liver resection or removal, cryopreserved cells can be stored for long time periods and are thus always available. However, they show a decrease in phase II activity and phase I inducibility when compared to fresh cells. Another adverse property is the varying quality of hepatocytes and liver slices from different isolation experiments.

Genetically engineered cells

These cell lines, e.g. transfected V79, show two major advantages compared with hepatocytes: they are easy to handle and always available. However, they cover only a limited amount of enzymes, mostly phase I enzymes, which are not expressed at physiological levels.

Target systems

Embryonic Stem cell Test (EST)

For this assay, the pluripotent mouse stem cell line D3 is used. Under routine conditions the cells grow undifferentiated. If appropriate conditions are applied, they can e.g. differentiate into contracting cardiomyocytes. The cells are cultured with different concentrations of the test substance, and the number of contracting clones is monitored to determine an ID₅₀ value (50% inhibition of differentiation). Also an IC₅₀ (50% inhibition of

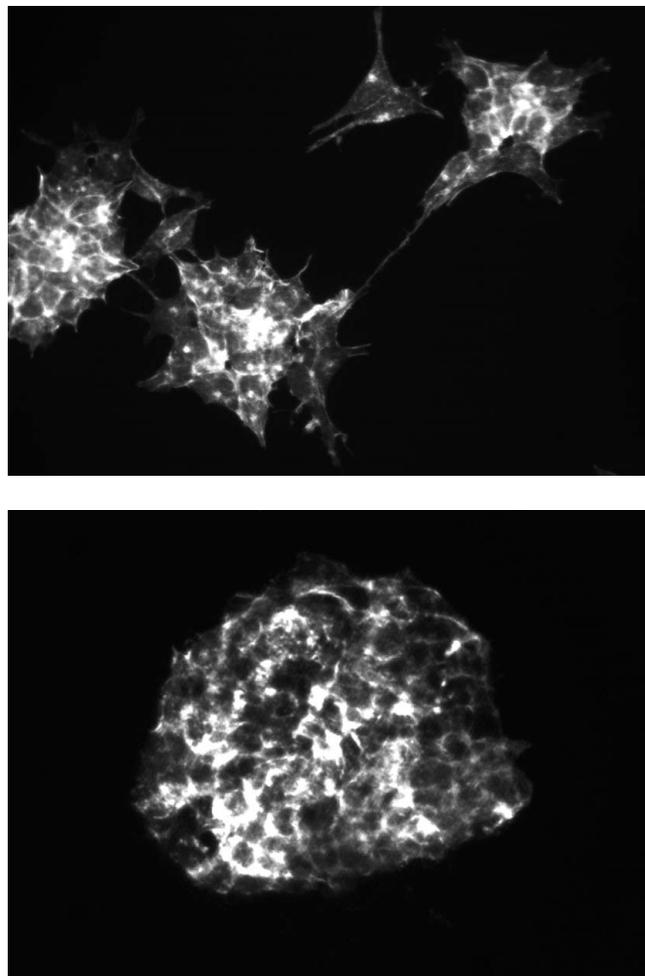


Fig. 2: Undifferentiated (bottom) and differentiated (top) F9 cells. Under routine conditions, F9 cells show an undifferentiated morphology and grow in clumps. However, differentiation to a neuronal-like morphology can be induced by teratogenic substances, e.g. valproic acid (VPA). Blue: nucleus, green: cytoskeleton.



growth) is identified and compared with the IC_{50} of 3T3 Balb/c fibroblasts. Thus the teratogenic as well as the cytotoxic potential of the substance are determined (Spielmann et al., 1997).

Micro Mass culture (MM)

In this assay the limb bud or mid-brain cells of GD 12 mouse embryos are examined. They are cultured with the test substances and their development is monitored by Alcian blue stain. Teratogenic substances lead to morphologic changes in the target cells, exhibiting inhibition of differentiation and growth. As endpoints an ID_{50} (Alcian blue stain) and IC_{50} (cytotoxic effects resulting in reduction of neutral red uptake) are determined (Flint 1993).

Whole Embryo Culture (WEC)

For this assay whole rat embryos of GD 9.5 are cultured with the test substances in roller bottles. Different parameters are monitored as growth (crown-rump-length, yolk sac diameter, protein contents), differentiation (number of somites, morphology), and incidence of abnormalities. To classify no, weak and strong embryotoxic substances the IC_{NOEL} and IC_{max} of the embryos are compared to the IC_{50} of 3T3 fibroblasts (Piersma et al., 1996).

F9 cell line

The F9 cell line is derived from mouse testicular teratocarcinoma cells. Under routine conditions they show an undifferentiated morphology growing in clumps. However, differentiation to a neuronal-like morphology can be induced by teratogenic substances, e.g. valproic acid (VPA) (see fig. 2) (Göttlicher et al., 1998; Lampen et al. 1999). The changes taking place during differentiation closely mimic events of early mouse embryogenesis (Hogan et al., 1983).

VPA (2-n-propylpentanoic acid) is an antiepileptic drug particularly used for the treatment of several forms of epilepsy and bipolar disorders and as migraine prophylaxis (Sörensen, 1988). Unfortunately, it proved to have considerable teratogenic poten-

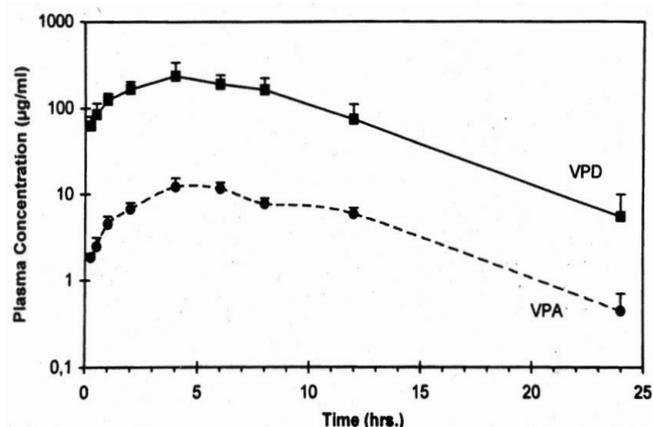
tial in humans, inducing neural tube defects. A number of VPA analogues with varying teratogenic activity in mice have been synthesised which show that the teratogenic potential depends strictly on the structure of the compound (Nau et al., 1991). The teratogenic analogues bear a free carboxylic group and an α -hydrogen atom at the branching point of carbon atom C-2, but no further branching may be present (Nau and Löscher, 1986; Nau et al., 1991). Furthermore, the teratogenic effect of VPA and its derivatives is stereospecific. The (S)-enantiomer of 4-yn-VPA, for instance, is highly teratogenic in mice while the (R)-enantiomer shows little to no effect (Nau et al., 1991).

Remarkably, only *in vivo* teratogenic VPA-derivatives induce cell differentiation of F9 cells, whereas non-teratogenic VPA-derivatives do not show this effect (Lampen et al., 1999). The differentiation cannot only be monitored morphologically but also by the increased expression of differentiation markers. As F9 cells differentiate to a neuronal-like morphology, the expression of the NCAM-gene (neuronal cell adhesion molecule) is induced by teratogenic test substances (Lampen et al., 2005). This effect can further be demonstrated in a luciferase reporter gene assay coupled to viral promoter sequences. F9 cells are transiently transfected with a plasmid comprising the viral (RSV) promoter-driven luciferase gene. The promoter is only activated and thus the reporter gene only expressed by the teratogenic derivatives of VPA. Luciferase activity can therefore only be measured in F9 cells cultured with substances showing a teratogenic potency. The reporter activity highly corresponds to the teratogenic potential of the derivative (Lampen et al., 1999).

Combinations

In contrast, two other analogues, valpromide (VPD) and 2-pentyl-4-pentynoic hydroxamic acid, which exhibit a teratogenic potency *in vivo*, cannot be detected with the F9 cell system. Both of these substances need to be metabolically activated: VPD to VPA (see fig. 3) (Radatz et al., 1998) and the hydroxamic acid to its corresponding carbonic acid.

First experiments have been done using S9-mix as metabolic activation system. Two approaches can be distinguished: direct addition of S9-mix to the F9 cells and pre-incubation of S9-mix with the test substances followed by incubation of the F9 cells with the extracted medium (see fig. 4). Direct addition showed two problems: a high amount of S9 is toxic to the F9 cells, but with less S9 too little metabolite is formed to induce differentiation of the target cells. Hence, direct addition is not suitable for the given system. For the pre-incubation, in contrast, no limit of the applicable amount of S9-mix is observed. VPD metabolism with human, rat (Aroclor induced) and mouse S9-fractions was compared. Rat S9 metabolised the highest proportion of VPD but, as *in vivo*, VPA is further metabolised and not available for differentiation of the F9 target cells. Human S9 and mouse S9 metabolise similar proportions of VPD, but with human S9 more VPA is found than with mouse S9 (see tab. 1). The formed VPA is first extracted to be concentrated and subsequently added to F9 culture medium to induce differentiation. Due to the cyto-



Radatz et al. 1998

Fig. 3: Metabolic activation of valpromide (VPD) to valproic acid (VPA).

In vivo measurement of VPD metabolism and VPA formation in NMRI mice treated with 3 mmol/kg BW VPD (Radatz et al., 1998).

toxic potential of the S9-mix further studies to improve this procedure need to be done. However, these first *in vitro* results incorporating the metabolic activation correspond well with the *in vivo* results. For the given experiment, human S9 is best suited to mimic the *in vivo* situation and eliminate interspecies differences.

Further studies to test the well known proteratogen cyclophosphamide were done combining metabolic activation systems with target systems, e.g. hepatocytes with chicken brain cultures (Bruinink et al., 2002), or whole embryo culture (Ozolins et al., 1995), or genetically engineered cells (V79) with the D3 stem cell line (Bremer et al., 2002). Bruinink et al. (2002) used primary hepatocytes to metabolise cyclophosphamide and isophenphos and tested the neurotoxic effect of the metabolites with embryonic chicken brain cells. The cells were able to discrimi-

nate between the toxicity of the parent drugs and their metabolites and between metabolites with an unspecific cytotoxic activity (cyclophosphamide) and metabolites with a high potential to damage specific nerve cell populations (isophenphos). Ozolins et al. (1995) co-cultured murine whole embryos with primary maternal hepatocytes and exposed them to cyclophosphamide. They showed that the hepatocytes were necessary for the expression of embryotoxicity, which was concentration-dependent. Bremer et al. (2002) cultivated the D3 stem cell line with supernatants of the genetically engineered mammalian cell line V79, transfected with CYP 2B1 cDNA to metabolise cyclophosphamide. The combined system was able to detect the embryotoxic potential in a reporter gene assay for developmental cardiotoxicity.

Tab. 1: *In vitro* measurement of VPD metabolism and VPA formation after pre-incubation with S9-mix.

Rat S9 metabolised the highest proportion of VPD but, as *in vivo*, VPA is further metabolised and not available for differentiation of the F9 target cells. Human S9 and mouse S9 metabolise similar proportions of VPD, but with human S9 more VPA is found than with mouse S9.

S9-mix	VPD metabolism	VPA formation
human	30-45%	25-35%
rat (Aroclor induced)	50-80%	10-20%
mouse	30-50%	10-15%

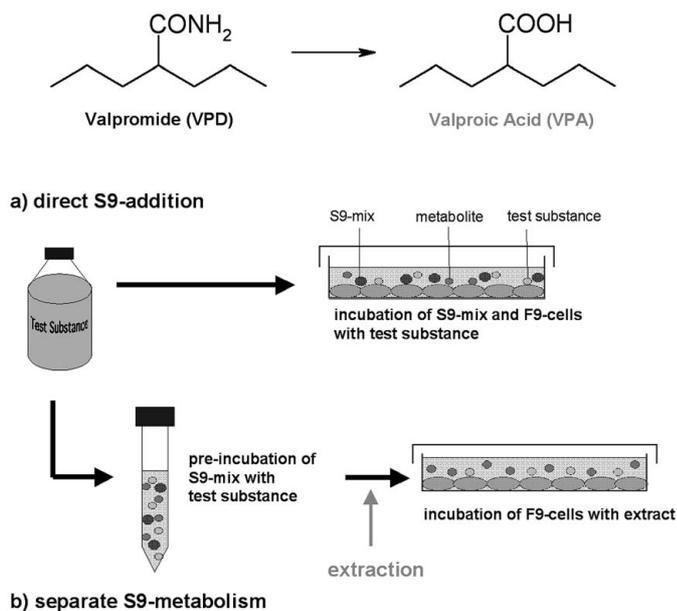


Fig. 4: Metabolic activation using S9-mix.

Two approaches can be distinguished: direct addition of S9-mix to the F9 cells and pre-incubation of S9-mix with the test substances followed by incubation of the F9 cells with the extracted medium.

Discussion and conclusion

A reliable *in vitro* system for the identification of the embryotoxic potential of substances should reproduce all aspects of embryogenesis. Therefore, also the maternal metabolism and foeto-placental interaction have to be taken into account, because some proteratogens require bioactivation to provide active molecules that interact with the developing embryo. The described examples show that the current approaches of combining existing *in vitro* assays with metabolic activation systems are feasible to extend the usefulness of the *in vitro* test procedures. The addition is necessary to exclude false negative results regarding adverse effects when characterising a substance. However, substantial differences in the results were observed in the same indicator system when cellular or subcellular hepatic activating systems were used or when subcellular preparations from rats and humans were compared (Rueff et al., 1996). Ideally, the metabolic system should express all relevant enzymes, because during screening of many substances it is *a priori* not known which enzyme(s) may be involved. Also, the needed cofactors should be present in physiological concentrations. Generally, intact cells of liver origin seem to satisfactorily mimic the metabolic activation that occurs *in vivo* and they reflect the *in vivo* genotoxicity better than S9-mix (Rueff et al., 1996).

The possible advantages of using hepatocytes for activation are several: (1) human cells can be used to avoid species differences; (2) they contain enzymes and cofactors at physiological levels; (3) high activity of both phase I and phase II enzymes is present. These metabolic activation systems are now being developed for incorporation into *in vitro* systems for development of robust testing systems which can be transferred to other laboratories. To be practicable they have to be simple to handle, reproducible and cost-effective.

The relevance of these approaches consists in the adjustment of the existing *in vitro* systems to be more reflective of the complex *in vivo* situation with the aim to reveal indirectly acting toxic or teratogenic substances which is presently not possible.



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