Development of a new screening assay to identify proteratogenic compounds using Zebrafish *Danio rerio* embryo combined with an exogenous mammalian metabolic activation system (*mDart*)

vorgelegt von
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1 Introduction

1.1 Background

Following the worldwide thalidomide sanitary catastrophe in the late fifties of the 20th century, many efforts were put on the development of methods detecting the teratogenic potential of substances (e.g., chemicals, drugs or pesticides). Currently, developmental toxicity and embryotoxicity are tested almost exclusively in vivo, using rats and rabbits as rodent and non-rodent model species respectively. In these in vivo tests, the substance is administered to pregnant animals and embryotoxic effects are further monitored. According to a report from the German ministry namely BMELV (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz) more than 4000 animals were used in 2006 to test developmental toxicity.

In June 2007 a new chemical regulation system came into force within the European Union: REACH (Registration, Evaluation, and Authorization of Chemicals). Following REACH, about 30,000 chemicals put on the market before 1981 will be reassessed for toxicity, including potential embryotoxic and teratogenic activity, within the next 11 years. The number of laboratory animals needed for these studies will be tremendous and will raise a justified public concern. These apprehensions were already shared by scientists in the late 1950’s with the concept of 3Rs (Reduce, Refine and Replace) (Russel and Burch, 1959) were then pursued through governmental authorities such as ECVAM (European Centre for the Validation of Alternatives Methods), ICCVAM (Interagency Coordinating Committee on the Validation of Alternatives Methods) or ZEBET (Die Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch) in Germany.

Currently, there are few alternative methods investigating the embryotoxicity and/or teratogenicity of substances. In the 1970ies, the rat whole embryo culture was developed, (New, 1978; Webster et al., 1997) followed later on with other animal models (e.g., mouse, rabbit). In the late eighties of the last century, cell cultures (i.e., embryonic stem cells test, micromass test) and organ culture (i.e., lung, sex organs) systems completed this toolbox. However, it is highly disadvantageous that some of those models (i) still need animals (rats) to obtain the test systems (Piersma, 2004) (ii) in general don’t cover the whole period of embryo/fetogenese and (iii) don’t address a possible metabolic activation of the test materials (Spielmann et al., 2006).
Nowadays, it is recognised that teratogenic activity is not always due to the parent compound but may be caused by one or more metabolites formed by maternal metabolism (Fantel, 1982; Webster et al., 1997). These parent compounds, termed proteratogens can be enzymatically bioactivated to highly toxic electrophiles or free radicals intermediates (Wells et al., 2005). Therefore, when the chemicals are unknown or poorly characterized, the addition of a mammalian metabolic activation system (e.g., S9-mix, microsomes, hepatocytes) to detect proteratogenic potency has been proposed using whole embryo systems (Fantel et al., 1979; Zhao et al., 1993).

The zebrafish *Danio rerio* embryo, a vertebrate model, combines the advantages of cell and embryo culture systems. In the last 40 years, extensive studies have been performed on this *in vitro* model and relevant ecotoxicological and toxicological applications have been validated and guidelines written (O.E.C.D., 2006). Furthermore, zebrafish *D. rerio* embryo has been promoted as a general model for assessing embryotoxicity and teratogenicity, *DarT* (*Danio rerio* Teratogenic assay). A scoring system was described to assess compounds that trigger teratogenicity (Bachmann, 2002; Nagel, 2002). However, the absence of mammalian metabolic systems in the *in vitro* assays has been a major limitation to their applicability (Spielmann et al., 2006).

During this thesis we successfully developed the combination of *DarT* with a mammalian metabolic activation system (microsomes), which is presented as a new alternative method for complete developmental toxicity testing (*mDarT*).
1.2 Teratogenesis

1.2.1 Origins

The word teratogenesis literally means the formation of a monstrous or misshapen organism and comes from the Greek *teras* or *teratos*, meaning monster or marvel. In contemporary usage, teratogenesis refers to the appearance of malformations in a developing foetus. Teratology is the branch of biology that studies causes, mechanisms and manifestations of abnormal development.

In ancient time in the past and in various cultures, the born of unusual or deformed infants was attributed to causes such as hybridization between humans and gods or humans and demons. Among the Ancient Greek’s and Romans, there was a tendency to consider monstrous infants divine, and some mythologic figures appear to have been derived from terata. In the Europe of the fifteenth and sixteenth centuries, malformations were believed to be the result of association with demons, witches, or other evil creatures, and both the mother and the infant were put to death (Hodgson and Levi, 1997).

In the 1940ies the work of Warknay and his colleagues demonstrated that environmental factors such as maternal dietary deficiencies and irradiation could affect the intrauterine growth and development of mammals (Hodgson and Levi, 1997). In the late 1950ies, thalidomide was pulled from the general market because of its catastrophic adverse effect of teratogenesis such as phocomelia (see Thalidomide effects in humans in Fig 1.1).
For a substance to be a teratogen, it must increase significantly the occurrence of abnormalities, either structural or functional, in the offspring after being administered to either parent before conception, to the female during pregnancy, or directly to the developing foetus (Hodgson and Levi, 1997). The substance can be a chemical (see ethanol effects in humans in Fig. 1-2), a drug (e.g., cocaine), an infectious agent, environmental exposure (e.g., Carbon Monoxide) or maternal conditions (e.g., starvation) (Shepard, 1995).
Nowadays, it is recognised that teratogenic activity is not always due to the parent compound but may be caused by one or more metabolites formed by maternal metabolism (Fantel, 1982; Webster et al., 1997). These parent compounds, termed proteratogens can be enzymatically bioactivated to highly toxic electrophiles or free radicals intermediates (Wells et al., 2005). For example, cyclophosphamide (see effects in humans in Fig. 1.3) is considered to be a potent human proteratogen.

Figure 1-3 Cyclophosphamide major malformations in humans (Sender).
Legend: Left: baby with cyclophosphamide major malformations (e.g., cleft palate); Right: baby after surgical operation.

1.2.2 General principles

Teratology is based on the following fundamental principles:

- **Species sensitivity**

Teratogenicity is species specific, as thalidomide example illustrates. Indeed, humans are sensitive to thalidomide, whereas most other mammals are quite resistant and only some specific animal strains (certain breeds of the rabbit and 8 of 9 primate species) with high doses and at a specific time showed concordant malformations to that of humans (Shepard, 1995).
• **Critical periods**

The type of teratogenic response is determined by the specific developmental stage of the foetus (Hodgson and Levi, 1997). For example, a teratogen is more likely to provoke anatomic defects during organogenesis.

• **Initiating mechanisms:**

The maternal metabolism plays an important role for the teratogenic potential of a compound, since it can reduce or potentize its toxic effects. A number of xenobiotics can be enzymatically bioactivated by maternal pathways and become highly toxic intermediates that bind covalently to embryonic cellular macromolecules or initiate the formation of reactive oxygen species (ROS) that can lead further to teratogenicity (Wells et al., 2005) (Fig. 1-4).

The initiating mechanisms of a compound can have consequences at different levels of an embryo: (i) on the molecular level, there are gene mutations, chromosomal abnormalities, lack of precursors or substrates, enzyme inhibitions or cell membrane damages. (ii) On the cellular level, these mechanisms lead to cell death and/or lack of cell interaction or migration. (iii) On the organism level, if a critical mass of cells is destroyed, there may be too few cells to develop normal embryos.

• **Access to embryo and foetus**

The toxicokinetics of xenobiotics to the embryo and foetus is facilitated when the molecule is lipophilic and nonionized at physiological pH. Toxicokinetics are further described in chapter 1.4.

• **Dose-response relationships**

Most teratogens appear to have a threshold or “no-effect” level below which no malformations are observable (Hodgson and Levi, 1997).
1.2.3 Test methods

1.2.3.1 In vivo methods

Teratogenicity or developmental toxicity testing of chemicals is addressed by several international accepted guidelines (e.g., OECD, n°414). This guideline defines developmental toxicity as "the study of adverse effects on the developing organism that may result from exposure prior to conception, during prenatal development, or postnatally to the time of sexual maturation". According to OECD guideline n°414, "the major manifestations of developmental toxicity include (i) death of the organism, (ii) structural abnormality, (iii) altered growth and (iv) functional deficiency."

Developmental toxicity testing is performed using pregnant laboratory animals of both (i) rodent species (e.g., rat) and (ii) non-rodent species (e.g., rabbit). The test substance is administered daily to pregnant animals from implantation to one day prior to scheduled kill.

According to OECD guideline n°414, four groups (three test items and one control group) of 20 animals are required for a test substance. The highest dose should induce maternal toxicity but not death or severe suffering. One intermediate dose level should produce developmental toxicity effects and the
lowest dose should not produce any evidence of either maternal or developmental toxicity (O.E.C.D., 2001).

A clear advantage of this test is that it covers a relatively long period, from the implantation to the end of the foetogenesis, in which teratogens, and probably proteratogens, can be detected. However, it is acknowledged that the existing in vivo assays for developmental toxicity are very time consuming, laborious, expensive and conflicting with the current needs for rapid screenings of potential drugs (Daston, 1996). Furthermore, it involves the use of many laboratory animals which disagrees with an increasing political and public demand for a reduction in the use of laboratory animals (Walmod et al., 2004).

1.2.3.2 **In vitro methods**

1.2.3.2.1 Overview

Alternative methods for developmental toxicity testing can be divided into three types of systems with increasing complexity: cell cultures, organ cultures, and whole-embryo cultures (WEC). Cell cultures have the disadvantage of simplicity with the advantages of ease of performance and minimal or no animal use. On the other end of the spectrum WEC are more laborious and require the use of more animals.

1.2.3.2.2 Micromass assay

The Micromass (MM) assay may use cultures of limb bud cells, neuronal cells or both. Cells are plated at high density and undergo differentiation into chondrocytes or neurons without additional stimulation. As a routine in vitro teratogenesis screening test, chondrocytes are cultivated in microtiter plates with substances and chondrogenesis and cell proliferation are simultaneously assessed after six days (Renault et al., 1989). However, MM culture systems still require the primary culture of cells isolated from embryos (Huggins, 2003).
1.2.3.2.3 Embryonic stem cell test

The embryonic stem cell lines can be established from mammalian blastocysts and maintained in an undifferentiated state in the presence of feeder layers and/or purified leukemia inhibitory factor.

In 1997 Spielmann and his colleagues developed an embryonic stem cell (EST) test composed of two permanent mouse cell lines, 3T3 fibroblasts and embryonic stem cells (D3). Using three endpoints (inhibition of differentiation of ES cells into cardiac myoblasts and cytotoxicity of ES cells and 3T3 cells) a classification scheme for chemicals embryotoxicity was assigned to three classes: not embryotoxic, moderately embryotoxic and strongly embryotoxic (Spielmann et al., 1997)(Huggins, 2003). However, EST lacks so far metabolic activation system.

1.2.3.2.4 Whole-embryo culture assay

Mammalian embryo cultures are derived from mice, rats or rabbits although rats are the most studied species. In this test, the conceptus is cultured in medium under defined gassing conditions for 24-48 hours. A number of endpoints can be measured such as effects on development of the visceral yolk sac vascularisation and circulation; effects on haematopoiesis; embryonic growth and differentiation; and dysmorphogenic effects (Brown et al., 1995; Flick and Klug, 2006).

The ECVAM had indicated that the rat whole embryo culture is a valuable tool considering its extensive use in many laboratories, its ability to detect dysmorphogenesis in embryos and its capacity to incorporate metabolic activation systems. They caution, however, that the system is limited in that it is complex, covers only a part of organogenesis and requires a fairly high level of sophistication to perform (Huggins, 2003).
1.2.3.2.4.1 Frog embryo teratogenesis assay (Xenopus)

The frog embryo teratogenesis assay (Xenopus) (FETAX) evaluates the effect of toxicants on the development of embryos from a non-mammalian species, the South African clawed frog (Xenopus Laevis). FETAX measures mortality, malformations and growth inhibition in 96 hours (Fort et al., 1988).

The rationale behind using FETAX as a monitor for teratogenic events in mammals is that embryonic development during the first 96 hours in Xenopus parallels many of the major processes of human organogenesis and that metabolic activation systems can be incorporated (Huggins, 2003).

1.3 The Zebrafish D. rerio

1.3.1 Animal model description

The zebrafish D. rerio, a popular aquarium fish (Fig. 1-5), is a member of the family Cyprinidae and is native from Bengal to the Coromandel Coast of India (Hizaoka and Battle, 1958). It is ideally suited to study the fundamental processes underlying embryonic development. In recent years, it has become an important model organism in genetic, developmental biology, ecotoxicology and toxicology. The development of the zebrafish is very similar to the embryogenesis in higher vertebrates, including humans.

A single female can lay up to 400 eggs per week under laboratory conditions and spawn throughout the year (Laale, 1977). Zebrafish develops outside the female body and the shell of their eggs (chorion) is completely transparent enabling the detailed observation of the developing embryo very easily (Fig. 1-6). Additionally, the embryos themselves are transparent during the first few days of their lives – yielding a unique possibility to examine the formation of internal organs "live" inside the living organism. The embryonic development of zebrafish is very rapid: In the first 24 hpf (hours post fertilization), all major organs are formed and by the third day of development the fish hatch (Dahm and Geisler, 2006).

In contrast to larger species, the size of the larval and adult zebrafish minimizes costs through low quantities of dosing solutions and thereby creates limited volumes of waste for disposal and minimizes quantities of labware and
chemicals for performing various assays and histological assessments (Hill et al., 2005).

These advantages – large number of offspring, rapid embryonic development, transparency of the embryos and small size – make the zebrafish an ideal model organism to study various aspects of the development process in vertebrates.

![Zebrafish](image)

**Figure 1-5** Zebrafish (*D. rerio*) females (upper individual) can easily be differentiated from males (lower individual) by their extended bellies and the lack of reddish tint along the silvery longitudinal lines (Braunbeck et al., 2005)

### 1.3.2 Embryonic development

The different stages in zebrafish embryonic development until hatching are given in Table 1-1 and relevant embryonic stadium were outlined in Figure 1-6.
Table 1-1 Stages of embryonic development of the zebrafish *D. rerio* at 26 ± 1°C (Kimmel *et al.*, 1995)

<table>
<thead>
<tr>
<th>Time (hpf)</th>
<th>Stage</th>
<th>Characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Fertilization</td>
<td>Zygote</td>
</tr>
<tr>
<td>0</td>
<td>Zygote period</td>
<td>Cytoplasm accumulates at the animal pole, 1 cell-stage</td>
</tr>
<tr>
<td>0.75</td>
<td>Cleavage period</td>
<td>Discoidal partial cleavage</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1. median vertical division: 2-cell-stage</td>
</tr>
<tr>
<td>1.25</td>
<td></td>
<td>2. vertical division: 4-cell-stage</td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td>3. vertical and parallel to the plane of the first: 8-cell-stage</td>
</tr>
<tr>
<td>2</td>
<td>Blastula period</td>
<td>Start of blastula stage</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Late cleavage; blastodisc contains approximately 256 blastomers</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>Flat interface between blastoderm and yolk</td>
</tr>
<tr>
<td>5.25</td>
<td>Gastrula period</td>
<td>50% of epibolic movement; blastoderms thin and interface between periblast and blastoderm becomes curved</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>75% of epibolic movement</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>Epibolic movement ends, blastopore is nearly closed</td>
</tr>
<tr>
<td>10.5</td>
<td>Segmentation period</td>
<td>First somite furrow</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>Somites are developed, undifferenciated mesodermal component of the early trunk, tail segment or metamere</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>Side to side flexutures; otoliths</td>
</tr>
<tr>
<td>24</td>
<td>Pharyngula period</td>
<td>Phylotypic stage, spontaneous movement, tail is detached from the yolk, early pigmentation</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>Reduced spontaneous movement; retina pigmented, cellular degeneration of the tail end; circulation in the aortic arch 1</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>Tail pigmentation; strong circulation; single aortic arch pair, early motility; heart beating starts</td>
</tr>
<tr>
<td>72-96</td>
<td>Hatching period</td>
<td>Regular heart-beat; yolk extension beginning to taper; dorsal and ventral stripes meets at tail; segmental blood vessels; thickened sacculus with 2 chambers; foregut developmental; neuromasts</td>
</tr>
</tbody>
</table>
Introduction

Figure 1-6 Inverted microscope observation of important developing stages of the zebrafish *D. rerio* at 26°C

**A.** 8 cells, 1 hpf. **B.** Somites, 12 hpf. **C.** Tail detached, 24 hpf. **D.** Pigmentation, 48 hpf. **Abbreviations:** *E*: Eye; *ET*: End Tail; *hpf*: hours post fertilization; *Ht*: Heart*; *SC*: Spinal Cord; *S/O*: Sacculi/Otoliths*; *SM*: Somites*; *T*: Tail; *YS*: Yolk sac*; *YSE*: Yolk sac extension*; *: See Glossary.
1.3.3 The Zebrafish *D. rerio* in Ecotoxicology and Toxicology

1.3.3.1 Zebrafish *D. rerio* embryo assays in Ecotoxicology

1.3.3.1.1 Alternative to the acute fish test

The zebrafish is worldwide used to test the acute toxicity of chemicals to fishes (O.E.C.D., 1992). In 1994, Schulte and Nagel proposed the embryo test as an alternative to the fish acute toxicity test in order to reduce animal tests (Schulte and Nagel, 1994). Furthermore, toxicity results obtained with the embryo test are in good accordance with the ones obtained with the juvenile or adult fish acute toxicity test. (Nagel, 2002). In May 2006, the fish embryo test was described in a draft guideline from OECD for the testing of chemicals (see Fig. 1.7).

1.3.3.1.2 A model in routine waste water control

The “Goldsrorfentest” was used as a standard method to assess waste water toxicity using adult Goldorf fishes. In 1995, Friccius and his coworkers tested the applicability of the zebrafish embryo test in 29 samples of industrial effluents from 11 different sewage plants (Friccius et al., 1995; Nagel, 2002). It was shown that the fish embryo test was as or even more sensitive than the “Goldorfentest”. This led to the replacement of the “Goldsrorfentest” by the zebrafish *D. rerio* embryo test (D.I.N, 2003) in Germany, where it came into force the 1st January 2005 (see Fig. 1-7).
1.3.3.2 Zebrafish *D. rerio* embryo assays in Toxicology

1.3.3.2.1 *DarT*, a model for teratogenic assay

The *Danio rerio* teratogenic assay, or *DarT*, was developed in 2000 (Bachmann, 2002). 41 mammalian teratogens and non-teratogens were evaluated with this model. From the 41 teratogenic and non-teratogenic substances tested, the results of 88% of the substances were in agreement with findings in mammals. From the left 12%, 4 were false positives (acetone, 2,5-Hexandion, Isobutyl-ethyl-valproic acid and 1-Methoxy-2-propanol) and 2 were false negatives (salicylic acid and thalidomide). Even for teratogens requiring metabolic activation, malformations could be detected with *DarT*, however the effects, if any, were not as strong as assumed (Nagel, 2002).
1.3.3.2.2 Cardiotoxicity

Cardiovascular effect is an important sublethal endpoint can be measured within DarT. It was shown that drugs known to provoke anomalies on the heart-beat were selected to investigate their effects on zebrafish embryos heart-beat. From these drugs, two were reducing and the other two were increasing the heart-beat frequency. For all the drugs, the results of the zebrafish embryo test were in concordance with their known mode of action. (Nagel, 2002; Zeller, 1995).

1.3.3.2.3 Pigmentation

Fish embryos are suitable to detect pigmentation toxicity. Pigmentation was found to be reduced in the zebrafish embryos by several anilines and phenols (Schulte, 1997). p-tert-Butylphenol which is known to influence the pigmentation in humans was also tested with the zebrafish embryos and gave similar results (Maiwald, 1997; Nagel, 2002).

1.4 Toxicokinetics

1.4.1 Principles

The fate of a xenobiotic in the human body can be characterized by the ADME (absorption, distribution, metabolism and excretion) scheme (see Fig. 1-8). If not injected directly into the circulation, a xenobiotic must be absorbed from an absorption site (e.g., gastrointestinal fluid or intramuscular or subcutaneous injection site). After entering the systemic circulation, the xenobiotic is distribute via the blood stream to the tissues and will eventually reach its site(s) of action. To a certain extent, most xenobiotics become reversibly bound to plasma proteins and/or tissues constituents. A xenobiotic may be degraded by metabolism (biotransformation) and ultimately it is excreted from the body. During the ADME process, the xenobiotic has to permeate biological membranes. Xenobiotic cross membranes mainly by passive diffusion according to Fick’s law. Furthermore, it depends on the lipid
solubility of the substance in question. For an ionizable xenobiotic, lipid diffusion depends on its pK_a value and the pH of body fluids (Fichtl, 1999).

The two major excretion pathways in humans are via bile and via urine. For volatile compounds, exhalation can represent the dominant mechanism of excretion. In some cases, secretion into the mother’s milk may be of toxicological significance.

Both renal and biliary excretions require water-soluble substrates and become increasingly efficient with rising polarity of the compound to be excreted. While some xenobiotics are sufficiently water-soluble for excretion, many lipophilic components cannot be directly excreted and would accumulate if not biotransformed to more polar derivatives (see Fig. 1-9) (Oesch and Arand, 1999).
Enzymes involved in biotransformation are mostly located in the liver, which makes hepatic metabolism an important topic in drug discovery (Kumar and Surapaneni, 2001). However, other organs (e.g., lungs, kidneys, gut) also contain metabolizing enzymes. The reactions catalyzed by metabolizing enzymes are divided into two phases, Phase I and Phase II.

Phase I metabolic reactions are functional reactions, which usually introduce a polar functional group to a parent molecule to form a metabolite. The most common Phase I functional reactions include, but are not limited to, hydrolysis, reduction, and oxidation. The most important enzyme family in Phase I metabolism is the CYP family (Guengerich, 2003).

Phase II reactions are usually conjugating reactions, which conjugate a polar moiety to the parent compound or its Phase I metabolite. Therefore, the metabolites resulting from Phase II are usually much more polar and hydrophilic than the parent molecule, and are readily excreted from urine and/or bile. The common conjugation reactions for drugs are glucuronidation and sulfation as described in chapter 1.4.3.
1.4.2 Phase I enzyme systems

1.4.2.1 Cytochrome P450s

Cytochrome P450s (CYPs) are the most important drug metabolizing enzymes for numerous xenobiotics (e.g., drugs, agricultants, pollutants and dietary components) as well as some endogenous substrates (e.g., bile acids, steroids, and cholesterol) in animals and humans. CYP enzymes are present in many mammalian organs, such as kidneys, lungs and intestines with the majority in the liver. Structurally, CYPs are hemoproteins composed of the coenzyme protoporphyrin IX and a variable moiety of ca. 50kDa (Testa and Krämer, 2007).

Based on amino acid sequence homology, CYP superfamily members are divided into various subfamilies, which are further divided into various isoforms. More than 25 CYPs subfamilies are currently identified and only four subfamilies (i.e., CYP1, CYP2, CYP3 and CYP4) are defined as drug metabolizing enzymes. Major isoforms involved in the biotransformation of xenobiotics in humans are CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Fig. 1-10) (Guengerich, 2008). Among these isoforms, CYP3A4 is the most abundant isoform and accounts for approximately 30 to 40% of the total CYPs content in human liver and small intestine. CYP3A4 is estimated to metabolize between 50-70% of the currently used drugs. CYP isoforms exhibit significant substrate specificity during the biotransformation process, such as regio- and stereo-selectivity. Furthermore, CYP enzymes can be inhibited, as well as induced by several specific xenobiotics, which possess the potential for drug-drug interactions (Lin et al., 2003).
Figure 1-10 Contribution of the major human Cytochrome P450s to the Phase I metabolism of all drugs currently marketed (Guengerich, 2003)

The generally accepted catalytic cycle for CYP reactions is shown in Fig. 1-11. However, the point should be made that this is a simplified version and that the system is dynamic, and the steps do not necessarily proceed in a linear order around the cycle. Moreover, rate-limiting steps in CYP reactions probably vary considerably, depending upon the reaction and the in vitro experimental setting (Guengerich, 2008).
1.4.2.2 Epoxides Hydrolases

Epoxides hydrolases (EHs) are a group of functionally related enzymes that catalyse the hydrolytic cleavage of oxirane rings. The two mammalian EHs with implication for xenobiotic metabolism are microsomal epoxide hydrolase (mEHs) (see Fig. 1-12) and soluble epoxide hydrolase (sEH). mEHs are known to be involved in the biotransformation of benzo[a]pyrene and are strongly expressed in animal and human livers (Prestwich et al., 1985).
1.4.2.3 Flavin containing Monooxygenases

Flavin Monooxygenases (FMOs) are a gene family of five enzymes (FMO1 to FMO5) involved in the metabolism of numerous nucleophilic heteroatom-containing xenobiotics (e.g., N-, S-, P-) (Cashman, 1995). Among the five FMO isoforms, FMO3 is found primarily in human liver (Ziegler, 1993), (Tynes and Philpot, 1987). Like CYPs, FMOs (i) are primarily localized in the endoplasmic reticulum, (ii) require the coenzyme NADPH and molecular oxygen for activity and (iii) are found in all mammalian species in nearly all tissues (Halpert et al., 1998), but primarily in livers. Species differences and genetic polymorphism in FMO activities are exhibited (Ziegler, 1998). Since CYPs have the similar capability to metabolize nucleophilic heteroatom-containing molecules, the differentiation of the oxidation activity between FMOs and CYPs can be achieved by chemical inhibitors, heat sensitivity (Mushiroda et al., 2000), and recombinant isoforms (Lin et al., 2003).
1.4.3 Phase II enzyme systems

1.4.3.1 UDP-Glucuronosyltransferases

UDP-Glucuronosyltransferases (UGTs) are a superfamily of membrane-bound enzymes that catalyze the transfer of α-D-UDP-glucuronic acid (UDPGA) to a large number of endogenous compounds and xenobiotics to form glucuronide metabolites (Fig. 1-13) (Cashman et al., 1996). In most cases, glucuronic acid conjugation substantially increases the hydrophilicity of a substrate and favouring its excretion.

UGTs can be divided into two families (UGT1 and UGT2) and are resident proteins of the ER with the orientation of the catalytic center towards the ER lumen. When \textit{in vitro} UGTs-catalyzed reactions are performed with liver microsomes, permeabilization of the membranes with detergent is required to give the enzyme free access to its substrate and cofactor.

![Figure 1-13 Conjugation of a nucleophile (i.e., R-X-H) with α-D-UDP-glucuronic acid to form the glucuronidation metabolites (Lin et al., 2003)](image)

Figure 1-13 Conjugation of a nucleophile (i.e., R-X-H) with α-D-UDP-glucuronic acid to form the glucuronidation metabolites (Lin et al., 2003)
1.4.3.2 Sulfotransferases

Sulfotransferases (STs) are dimeric enzymes that biotransform many drugs and xenobiotics by sulfation to more hydrophilic metabolites readily excreted from the body (Fig. 1.14). PAPS (3'-Phosphoadenosine 5'-phosphosulfate) is the "sulfate donor" for STs during the biotransformation. STs are either cytosolic or microsomal, however the most common STs involved in Phase II metabolism for drugs and xenobiotics are located in cytosol (Lin et al., 2003).

![Figure 1-14 Sulfation catalyzed by Sulfotransferases (Lin et al., 2003)](image)

Legend: ST: sulfotransferase; PAPS: 3'-Phosphoadenosine 5'-Phosphosulfate; PAP: 3'-Phosphoadenosine 5'-Phosphate.
1.4.4 *In Vitro* systems

1.4.4.1 Overview

Since hepatic and extra-hepatic metabolism represents a major biotransformation pathway for xenobiotics, extensive efforts are made to thoroughly investigate their metabolism during development phases (Hariparsad *et al.*, 2006). Therefore, *in vitro* models containing Phase I and/or Phase II enzyme systems were developed. Figure 1-15 summarizes the hierarchy of resemblance between the *in vivo* situation with the *in vitro* models.

![Figure 1-15](image)

<table>
<thead>
<tr>
<th>Complexities</th>
<th>supransomes</th>
<th>microsomes + cytosol</th>
<th>S9 - mix</th>
<th>(Transgenic) Cell lines</th>
<th>Primary Hepatocytes</th>
<th>Liver slices</th>
<th>Perfused liver</th>
<th>In vivo animal model</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resemblance on true <em>in vivo</em> situation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1-15 *In vitro* and *in vivo* model used in the development of new drugs, ranging from human to isolated enzymes, in order to *in vivo* resemblance (Brandon *et al.*, 2003)
1.4.4.2 Sub cellular fractions

1.4.4.2.1 Liver Microsomes

Liver microsomes are small membranous vesicles derived from the ER of hepatocytes as a result of tissue homogenization and are isolated by ultracentrifugation (Fig. 1-16). The microsomal fraction consists of membranous vesicles with free ribosomes, glycogen granules, and fragments of other subcellular structures such as mitochondria and Golgi apparatus and also contain CYPs, FMO, mEHs and UGTs. Their activity can be measured by various model substrates (Hodgson and Levi, 1997).

Liver microsomes account for the most popular in vitro model, providing an affordable way to give a good indication of the CYPs and UGT metabolic profile. Also, the influence of specific isozymes can be studied in the presence of specific inhibitors (Birkett et al., 1993). NADPH regenerating system (NRS) or NADPH is required to supply the energy demand of the CYPs and FMOs. Similarly, UGTs require UDPGA and alamethicin for activity.

Figure 1-16 Isolation of microsomes from primary hepatocytes (Cooper, 2000)

Microsomes can be prepared from all species such as rats or humans:

Human liver microsome activities can vary substantially between individuals. This issue, however, can be successfully solved by the application of pooled microsomes, which results in a representative enzymatic activity (Araya and Wikvall, 1999). Individual human liver microsomes can also be used to screen for the interindividual variability of the biotransformation of a drug. It is
also possible to identify the critical CYP involved in the biotransformation of a drug using individual human liver microsomes by correlating the enzyme activity of a particular CYP, using a bank of human donors, to the metabolism of the drug. The influence of gender on drug biotransformation can be investigated with gender-specific human liver microsomes pools (Brandon et al., 2003).

Different animal liver microsomes (e.g., mouse, rat, monkey) are available. The CYPs content can be artificially raised in animal livers using general inducing agents. This raise is obtained by treating laboratory animals with prototypical CYPs enzymes inducers (see Table 1-2) before collecting the animal liver.

The induction mechanism involves synthesis of new enzyme at the transcriptional or post-transcriptional level. The effects of inducers on the metabolic activity of hepatic microsomes subsequently isolated from treated animals have often been reported. Although, the comparison with the in vivo situation is modified, the specificity and the sensitivity can be tremendously enhanced (Hodgson and Levi, 1997).

Table 1-2 Inducing agents of CYPs in rats

<table>
<thead>
<tr>
<th>CYP</th>
<th>CYP 1A1/1A2</th>
<th>CYP 2B1</th>
<th>CYP 3A4</th>
<th>CYP 2E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Naphthoflavone</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>β-Naphthoflavone &amp; Phenobarbital</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aroclor1254</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The major advantage of microsomes is their simplicity in use. They are well-characterized in vitro systems for drug biotransformation research. However, some drawbacks exist. First, it should be noted that results obtained with microsomes must be extrapolated, because these enzymes are enriched in the microsomal fraction and there is no competition with other enzymes. This results in higher biotransformation rates in microsomes compared to the in vivo situation, but also compared to other in vitro systems (Sidelmann et al., 1996). Additionally, the absence of other enzymes (e.g., Phase II enzymes:
acetyltransferase (AT), glutathione S-transferase (GST) and STs) and cytosolic cofactors can leave metabolites formed in intact liver cells unnoticed (Crommentuyn et al., 1998).

**1.4.4.2.2 Cytosolic fraction**

The liver cytosolic fraction contains the soluble Phase II enzymes, e.g., AT, GST, and STs. It is obtained by differential centrifugation of whole-liver homogenate. For the catalytic activity of the Phase II enzymes, supplementation of exogenous cofactors is necessary, e.g., acetyl coenzyme A (acetyl CoA) for AT, PAPS for ST, and glutathione (GT) for GST.

The main characteristic of this system is the presence of only three enzymes in the cytosolic fraction at higher concentrations compared to liver S9-mix. The biotransformation capacity of AT, ST, and GST can be studied separately or in combination depending on the cofactors added (Brandon et al., 2003).

**1.4.2.3 S9-mix**

The liver S9-mix contains both microsomal and cytosolic fractions. Similar to microsomes, a NRS or NADPH solution is required to supply the energy demand of the CYPs and FMOs. For the catalytic activity of Phase II enzymes, addition of exogenous cofactors is also necessary. S9-mix is used for genotoxicity testing such as the Ames test, which is a simple and rapid *in vitro* method for detecting the mutagenicity of chemicals (Maron and Ames, 1983).

Compared with microsomes and cytosol, S9-mix offers a more complete representation of the metabolic profile, as it contains both Phase I and Phase II activities. However, a disadvantage is the overall lower enzyme activity in the S9-mix compared to microsomes or cytosol, which may leave some metabolites unnoticed.
1.4.4.3 Cell cultures

Primary hepatocytes are a popular *in vitro* system for drug biotransformation research due to their strong resemblance of *in vivo* liver. Detailed reviews dealing with this model have been published previously (Hewitt *et al.*, 2007). Hepatocytes of various animal species can be isolated from the liver by collagenase perfusion (Howard *et al.*, 1967). However, this method is not applicable for human hepatocytes. Therefore, a method for human liver parts has been developed (Guyomard *et al.*, 1990).

Once isolated, hepatocytes can be held viable in suspension for only a few hours, or they can be maintained in monolayer culture for a maximum of 4 weeks. Both, cultured hepatocytes (Chenery *et al.*, 1987) and suspensions of primary hepatocytes (Bayliss *et al.*, 1999) have repeatedly proven to be powerful tools to analyze the specific metabolic profile of a variety of drugs with good *in vitro–in vivo* correlations (Bayliss *et al.*, 1999) (see Fig 1-17).

However, it has been widely recognized that cultured hepatocytes are subject to a gradual loss of liver-specific functions, with special reference to a decreased CYP expression. This loss is different for the specific CYP isoforms; for some isoforms it becomes evident after a few days of culture (CYP 2E1 and CYP 3A4), while others remain nearly unaffected by the isolation and culturing processes (CYP 1A2 and CYP 2C9) (George *et al.*, 1997).
Advantageous of isolated hepatocytes is the possibility of cryopreservation. Cryopreserved hepatocytes have been shown to retain the activity of most Phase I and Phase II enzymes (Silva et al., 1999) and are now commercially available. Due to their widespread use in drug biotransformation research, isolated hepatocytes have become a well-established and well-characterized *in vitro* model and, with special techniques, isolated hepatocytes can be made viable for up to 3 weeks (Tuschl, G., Personal communication - see Fig. 1-18). However, it should be noted that prolonged culture conditions result in a more complex data interpretation, since outcomes partly depend on culture system factors.
Primary hepatocytes are well characterized, contain both Phase I and II and are viable for few weeks under specific culture conditions. However, this in vitro model is extremely lab-intensive and there is considerable interindividual variation with human hepatocytes. Nevertheless, this can be overcome by using mixtures of hepatocytes from multiple donors to mimic average enzyme content (Brandon et al., 2003).
1.4.4.4 Liver slices

Cultures of tissue slices were developed in the 1920s by Otto Heinrich Wartburg. Today, the incubation of liver slices in nutrient enriched media offers a powerful tool to study biotransformation in vitro. The development of high-precision tissue slicers set the stage for the "renaissance" of liver slices in in vitro biotransformation studies. The thin slices obtained (250 µm thickness) with the Krumdieck and the Brendel-Vitron slicers realistically and reliably represent the in vivo situation and have been used to study the biotransformation of many compounds (Ekins, 1996). However, the difficulties of long-term storage of liver slices complicate their commercialization. Dynamic organ culturing (DOC) was developed to prolong the limited viability period of liver slices. In this technique, the slice is continuously exposed to both culture medium and gas atmosphere (Smith et al., 1985a). Some variants of DOC were developed later (Olinga et al., 1997).

One main advantage of liver slices is the nonrequirement for digestive enzymes and thus the intact cellular tissue architecture, allowing for biotransformation studies in nonhepatocytes. Another main advantage is the possibility to study the induction of CYPs isoforms by new drugs.

The most prominent disadvantages include inadequate penetration of the medium into the inner part of the slice, damaged cells on the slice outer edges with impaired biotransformation, and the short viability time period of 5 days. Also, the optimal incubation method is highly dependent on the applications of the liver slices. All in all, tissue slice cultures are a powerful tool to study biotransformation in vitro, but the drawbacks mentioned still prevent its large-scale application (Brandon et al., 2003).
1.5 Test materials

1.5.1 Cyclophosphamide

Figure 1-19 Cyclophosphamide hydrate CAS#: 6055-19-2

1.5.1.1 Physicochemical Properties

Cyclophosphamide (CPA) (Fig. 1-19) is an odorless, fine white crystalline powder with a molecular weight of 279.1 g/mol. It is soluble in water and ethanol. Its LogK_{O/W} is 0.63. CPA reacts with strong oxidizing agents, is sensitive to moisture and light and is hydrolyzed in aqueous solutions above 30°C.

1.5.1.2 Application and mode of action

CPA is an alkylating antineoplastic agent that acts against a wide variety of oncologic and nononcologic conditions (e.g., transplantation prophylaxis, severe rheumatoid disorders) in various therapeutic categories. The drug prevents cell division by cross-linking DNA strands and decreasing DNA synthesis. It is cell-cycle-phase nonspecific. The mechanism by which this occurs is apparently through its metabolites phosphoramid mustard and acrolein (Schardein and Macina, 2007).

Since the parent drug shows no cytotoxicity, it requires bioactivation (Fig. 1-20) via ring oxidation by CYPs to finally yield the alkylating metabolite phosphoramid mustard and the acrolein (Bohenstengel et al., 1996).
1.5.1.3 Developmental toxicity

In *in vivo* teratogenicity studies (e.g., mice, rats, rabbit, primates), CPA elicited a full spectrum of developmental toxicities including multiple malformations (growth retardation and exencephaly). In humans, a specific syndrome of defects was identified to include congenital malformations of the digit, palate, ears, face, skin and in some cases the skull was affected (see Fig. 1-3) (Schardein and Macina, 2007).

---

**Figure 1-20** Metabolism of cyclophosphamide. The inactivation pathways are depicted horizontally, while cyclophosphamide activation is shown vertically. (De Jonge *et al.*, 2005).
1.5.2 Ethanol

\[ \text{C}_2\text{H}_5\text{OH} \]

Figure 1-21 Ethanol CAS#: 64-17-5

1.5.2.1 Physical and Chemical Properties

Ethanol is a colorless versatile solvent (Fig. 1-21), miscible in all proportions with water, some organic solvents and light aliphatic compounds and has a molecular weight of 46.07 g/mol. Its LogK\(_{\text{OW}}\) is -0.30.

1.5.2.2 Application and mode of action

Ethanol acts as a central nervous system depressant with intoxicating properties (Schardein and Macina, 2007).

Alcohol-induced oxidative stress is linked to the metabolism of ethanol. Three metabolic pathways of ethanol have so far been described in the human body, which involve the following enzymes: alcohol dehydrogenase (Fig. 1-22), microsomal ethanol oxidation system (Fig. 1-23) and catalase (Fig. 1-24). Each of these pathways could produce free radicals that affect the antioxidant system (Das and Vasudevan, 2007).

The classical pathway of ethanol metabolism, which is catalysed by alcohol dehydrogenase to form acetaldehyde results in the formation of free radicals (Fig. 1-22).

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ \rightarrow \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+ 
\]

Figure 1-22 Alcohol Dehydrogenase mode of action with ethanol (Das and Vasudevan, 2007)

The microsomal electron transport system also participates in ethanol oxidation via catalysis by the CYP isoenzymes. The enzymes in this family
include the 2E1, 1A2 and 3A4 isoforms, which vary in their capacity to oxidize ethanol (Fig. 1-23).

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{CH}_3\text{CHO} + \text{NADP}^+ + 2 \text{H}_2\text{O}
\]

Figure 1-23 CYPs mode of action with ethanol (Das and Vasudevan, 2007)

Peroxisomal activity also contributes to ethanol oxidation in the liver (Fig. 1-24).

\[
\text{RCH}_2\text{CH}_2\text{COSCoA} + \text{O}_2 \xrightarrow{\text{AcylCoA oxidase}} \text{RCH}==\text{CHCOSCoA} + \text{H}_2\text{O}_2
\]

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} \text{CH}_3\text{CHO} + 2 \text{H}_2\text{O}
\]

Figure 1-24 Catalase mode of action with ethanol (Das and Vasudevan, 2007)

1.5.2.3 Developmental toxicity

Laboratory animal studies (e.g., mouse and rat) clearly demonstrated potent developmental toxicity after ethanol administration, with defects in craniofacial development being the most predominant.

The developmental toxicity associated with ethanol was first documented in human foetuses in 1968. Fetal Alcohol Syndrome (FAS) was rediscovered in 1973 by Jones and Smith and refers to a pattern of defects in the offspring of alcoholic women consisting of pre- and postnatal growth deficiency, physical malformations, motor dysfunction and mental retardation (Abel and Dintcheff, 1978). The most typical abnormalities are associated with the central nervous system, craniofacial development, cardiac system and the skeleton (see Fig. 1-2) (Schardein and Macina, 2007).
1.5.3 Benzo[a]pyrene

![Benzo[a]pyrene CAS#: 50-32-8](image)

**1.5.3.1 Physicochemical Properties**

Benzo[a]pyrene (B[a]P) (Fig. 1-25) is a member ubiquitously distributed in organic compounds known as polycyclic aromatic hydrocarbons (PAHs). It is a crystalline powder with a molecular weight of 252.3 g/mol. B[a]P is practically insoluble in water (max. 10 nM at 25°C) and very lipophilic (LogK_{OW} = 6). B[a]P is sensitive to light.

**1.5.3.2 Application and mode of action**

B[a]P as a product of incomplete combustion - at temperatures between 300 and 600°C - is present in tobacco smoke, polluted air and in contaminated food and water.

The ultimate carcinogens arising from the metabolic activation of B[a]P are stereoisomers of B[a]P 7,8-diol-9,10-epoxide (Fig. 1-26). These metabolites arise by prior formation of the 7,8-epoxide, which gives rise to the 7,8-dihydrodiol through the action of EHs. This is further metabolized by the CYPs to the 7,8-diol-9,10-epoxides, which are both potent mutagens and unsuitable substrates for the further action of the EHs. Stereochemistry is important in the toxicity of the final product. Of the four possible isomers, the (+)-B[a]P diol epoxide-2 is the most active carcinogen (Hodgson and Levi, 1997).
Figure 1-26 Schematic representation of the metabolism of bento[a]pyrene into the carcinogenic 7,8-dihydrodiol 9,10-epoxide by mEH and CYP P450. (Lin et al., 2003).

Legend: mEH: microsomal epoxide hydrolase; P450; Cytochrome P450.

1.5.3.3 Developmental toxicity

B[a]P has been shown to be carcinogenic to experimental animals. Moreover, B[a]P is embryotoxic and teratogenic in mice; the inducibility of aryl hydrocarbon hydrolase activity in dams and foetuses is an important factor in determining these effects. A reduction in fertility in both male and female offspring was observed in mice following exposure to B[a]P in utero (Shepard and Lemire, 2004).

There is no data available to reference B[a]P as a human teratogen.
1.5.4 Thalidomide

\[
\alpha\text{-Phtalimidoglutarimide or thalidomide (Thal) (Fig. 1-27) is a neutral racemic compound derived from glutamic acid. It consists of R- and S-enantiomers. Thal is a white crystalline powder sparingly soluble in water (< 0.1 g/L) with a LogK\text{(O/W)} of 5.0. It is stable in the solid form but spontaneously hydrolyses in solution at pH 6.0 or higher to produce at least 12 hydrolysis products. It is stabilized in aqueous media through acidification (Teo et al., 2004).}
\]

1.5.4.1 Physicochemical Properties

Although much careful work has been done in an attempt to understand the mechanism of action, the reason for the chemical and species
specificity remains a pharmacologic riddle. Many hypotheses have been suggested such as (i) DNA-intercalating pathway or (ii) disruption mechanism in angiogenesis or (iii) expression inhibition of adhesion receptors (D’amato et al., 1994; Neubert et al., 1996).

1.5.4.3 Developmental toxicity

Thal effects are species specific. On the one hand, some species such as dog, cat, rabbit and monkey were sensitive to Thal and displayed concordant malformations (limb). The most sensitive species to Thal are three primates species (the baboon, the stump-tailed monkey and the bonnet monkey) all of which showed developmental toxicity at a dose of 5 mg/kg/day (Hendrickx and Newman, 1973). On the other hand laboratory species such as rats and mice were the least sensitive. Indeed, with mice (A and Swiss strains) (Dipaolo, 1963) and rats (Sprague-Dawley and Wistar strains) (King and Kendrick, 1962), Thal elicited embryolethality, growth retardation and nonconcordant malformations at a dose level of $\approx 30$ mg/kg. In contrast, rabbits were more responsive to Thal than the other rodents and elicited concordant (limb) malformations and embryolethality at 25 mg/kg (Staples and Holtkamp, 1963).
2 Aims and objectives

Zebrafish *D. rerio* embryos are routinely used as a teratogenic screening test, namely DarT. However fish eggs lack biotransformation in early embryonic stages. To fill this gap, we combined zebrafish *D. rerio* embryos with an exogenous metabolic activation system (MAS) and tested this model to prototypical proteratogenic compounds (test materials).

To reach this aim, three possible models were considered.

- **The direct co-culture model**

  The direct co-culture model (see Fig. 2-1) involves a close contact of fish eggs with a MAS together with the test material. The advantage of this model is that it allows high reactive, often unstable, new metabolites a direct and prompt contact with fish embryos (Hodgson and Levi, 1997). However, there are different incubation conditions for fish eggs and MAS (see chapter 4.1.1.1).

![Figure 2-1 The direct co-culture model with the fish embryo, a metabolic activation system and the test material.](image)

Legend: blue: buffer; yellow: microsomes; green: test material; red: reactive metabolite.
Aims and objectives

- **The indirect co-culture model**

  As displayed in Figure 2-2, in the indirect co-culture, the well is divided into two compartments separated by a semi-permeable membrane. This model could reduce a possible toxicity of MAS to fish eggs. However one must consider the short-lived metabolites of products and the different incubation parameters for the fish eggs and MAS (see chapter 4.1.1.1).

![Figure 2-2 The indirect co-culture model with the fish embryo, a metabolic activation system and the test material. Legend: blue: buffer; yellow: microsomes; green: test material; red: reactive metabolite.](image)

- **The three-step model**

  The three-step model (see Fig. 2-3) consists of three phases, namely (i) **incubation** of test material with MAS; (ii) **extraction** of metabolite(s) from MAS; and (iii) **exposure** of metabolite(s) with fish eggs for 48 hours. Although,
incubation requirements for fish eggs and MAS are optimal, extraction of new metabolite(s) could be a limiting-step due to their unstability.
Figure 2-3 The three-step model with the fish embryo, a metabolic activation system and the test material.

Legend: blue: buffer; yellow: microsomes; green: test material; red: reactive metabolite.

From these three models, the direct co-culture would be favourite as it is the only one that (i) allows a close contact of fish embryos with MAS and the test material during the whole incubation time and (ii) would be easy to handle. Therefore, the direct co-culture seems the most appropriate model.

The choice of the *in vitro* metabolic activation system (e.g., subcellular fractions, cell culture) is made considering its ability (i) to have a low toxicity for fish eggs and (ii) to be easy to handle and to obtain.
Subcellular fractions, respectively S9-mix and microsomes from rat liver would be the most probable activation mechanism to be included in the direct co-culture model. Even though they are not as complex as cell culture, they are very easily applicable and have a high Phase I and II enzymes content. Moreover, as a simple model, the incubation conditions for metabolic activity are more flexible compared to cell culture. Although S9-mix contain Phase I and II, its toxicity was previously reported when combined with cell cultures (Jorgensen et al., 2004). This could irreversibly damage fish embryo development. Therefore, rat liver microsomes were chosen as the metabolic activation system.
3 Materials and Methods

3.1 Equipment and Materials

Animal Care
Cyclop-eeze ® (aquatic nutrient) Argent Laboratories, U.S.A.
TetraMin ® (aquatic nutrient) Tetra-GmbH, Germany
Aquarium (Glass, silicon sealed, 80 l) Zoo-Markt Meyer, Darmstadt, Germany
Pump Zoo-Markt Meyer, Darmstadt, Germany
Foam insert (Fluval ® “1Plus”) Art.# A-180 Hagen, Germany
Air diffuser (Hobby ® 30 x 15 x 15 mm) DOHSE aquaristik KG, Germany
Boxes Kunststoffwerkstatt Merck KGaA, Germany

Equipement
Precision incubator, model INE 500 Memmert GmbH & Co. KG, Germany
Precision balance, Sartorius, model BP211D Sartorius AG, Germany
Balance Navigator Elektronische Messgeräte GmbH & Co, Germany
Laboratory pH-meter, 766-Knick IKA®-Werke GmbH & Co.KG, Germany
Magnetic stirrer, IKAMAG ® 2 KMO2 Basic GFL-mbH, Germany
Shaking water bath - 1092 Scientific industries, U.S.A.
Vortex-Genie 2 ® GFL-mbH, Germany
Water bath - 1002 Zeiss, Germany
Stereo microscope Olympus, U.S.A.
Inverted microscope, CKX41 Olympus, U.S.A.
Software analySIS Origin® 7.0 SR0 v 7.0220 (B220) OriginLab Corporation, U.S.A.
Software Adobe ® Photoshop ®

Metabolic Activation System
NADPH, Cat No: N1630 Sigma, U.S.A.
NADPH Regenerating System Solution A & B GENTEST, U.S.A.
Hydrochloric Acid, 1.09063.1000 Merck KGaA, Germany
Tris(hydroxymethyl)-aminomethan, 1.08382.0100 Merck KGaA, Germany

Test materials
Cyclophosphamide monohydrate, Cat No: 239785 Calbiochem, Germany
Thalidomide, Cat No: T 144 SIGMA, U.S.A.
### Materials and Methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo[a]pyrene, Cat No: 48564</td>
<td>SUPELCO, U.S.A.</td>
</tr>
<tr>
<td>Ethanol, 1.11727.4000</td>
<td>Merck KGaA, Germany</td>
</tr>
<tr>
<td>DMSO, Cat No: D 2650</td>
<td>SIGMA, U.S.A.</td>
</tr>
<tr>
<td>3-Aminobenzoic acid ethyl ester (MS-222), Cat No: A5040</td>
<td>SIGMA, U.S.A.</td>
</tr>
<tr>
<td>DMEM/F12 Serum, Cat No: 11330-032</td>
<td>GIBCO, Germany</td>
</tr>
<tr>
<td>Protein Inhibitor Cocktail set III, Cat No: 539134</td>
<td>Calbiochem, Germany</td>
</tr>
<tr>
<td>Titriplex® III, Cat No: 1.08418.0250</td>
<td>Merck KGaA, Germany</td>
</tr>
</tbody>
</table>

**Microsomes (XenoTech, LLC, U.S.A.)**

- Male Sprague Dawley Rat Liver Microsomes, Phenobarbital and β-Naphthoflavone-treated rats, R1081
- Male Sprague Dawley Rat Liver Microsomes, Phenobarbital-treated rats, R 1078
- Male Sprague Dawley Rat Liver Microsomes, β-Naphthoflavone-treated rats, R 1083
- Male Sprague Dawley Rat Liver Microsomes, Isoniazid-treated rats, R 1088
- Male New Zealand Rabbit Liver Microsomes, L 1000

**Microsomes (Celsis - In Vitro Technologies, Inc, U.S.A.)**

- Sprague Dawley Rat Liver Microsomes; Aroclor1254-treated. M 10001
- Sprague Dawley Rat Liver Microsomes; Isoniazid-treated. M 50001
- Sprague Dawley Rat Liver Microsomes; β-Naphthoflavone-treated. M 20001

**Laboratory Glass Ware**

- Petri Dishes (Ø 185 mm)                                                  | VWR, Germany              |
- Test tubes (Ø 16 mm)                                                    | VWR, Germany              |
- Amber Vial (1, 5, 10 ml)                                                | VWR, Germany              |
- Graduated cylinder (50, 500 ml)                                         | VWR, Germany              |

**Disposables**

- 24-well plates                                                         | Nunc GmbH, Germany        |
- Pasteur pipette non sterile graduated (3 ml)                           | VWR, Germany              |
- Polypropylene test tubes (Ø 16 mm)                                     | BD Biosciences, Germany   |
3.2 Test Organism

3.2.1 Origins

Species: Parents: Zebrafish, *D. rerio*, originated from West-Aquarium
Test organisms: Embryos of *D. rerio*

Breeder: Institute of Toxicology, Merck KGaA

3.2.2 Animal care and egg production

A breeding stock of unexposed and healthy mature zebrafish older than 6 months was used for egg production. Spawners were maintained in aquaria with a loading capacity of a minimum of 1 L water per fish. Lighting was controlled by a timer to provide a 12-hour-light and 12-hour-dark regime. Females and males were continuously held together in a ratio of 1:2. Dry flake food was fed twice a day and live food (white mosquito larvae) was fed once a day (O.E.C.D., 2006; Westerfield, 2000).

Mating and spawning took place within 15 min after turning on the lights in the morning. To prevent adult zebrafish from egg predation, egg traps were covered with a stainless steel mesh with a grid size of 2 mm. Plastic plant imitations serving as spawning substrate were fastened onto the mesh. About 20-30 min after the onset of light the egg traps were removed and the eggs were collected (O.E.C.D., 2006). At 26°C, fertilized eggs undergo the first cleavage after approximately 15 min and consecutive synchronous cleavages form 4, 8, 16, and 32 cell blastomeres. At these stages (4-32 cells) eggs can be identified clearly as fertilized and only these were used for further experimentation (Nagel, 2002; Schulte and Nagel, 1994).
3.3 **Fish Embryo Assay with Metabolic Activation (mDarT)**

3.3.1 **Standard protocol**

3.3.1.1 **Temperature**

The optimal temperature of mammalian microsomes is 37°C. In contrast, fish embryos develop optimally at a temperature of 26-28.5°C. However, they tolerate an eight degree range, between 25°C and 33°C. Incubating them for long periods above or below these extremes may produce abnormalities (Braunbeck *et al.*, 2005; Kimmel *et al.*, 1995). Thus, mammal microsomes were incubated at 32°C to achieve the highest metabolic activation together with normal fish embryo development.

3.3.1.2 **Fish medium**

As fish medium (FM), reconstituted water was used in accordance with the DIN 38415-6. Fish medium composition is summarized in Table 3-1.

<table>
<thead>
<tr>
<th>Salts composition</th>
<th>Concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$ * 2 H$_2$O</td>
<td>2</td>
</tr>
<tr>
<td>MgSO$_4$ * 7 H$_2$O</td>
<td>0.5</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.7</td>
</tr>
<tr>
<td>KCl</td>
<td>0.07</td>
</tr>
</tbody>
</table>

These salts are indispensable for correct functioning of Na$^+$/K$^+$ and Mg$^{2+}$/Ca$^{2+}$ ions pump for zebrafish embryos osmolarity equilibrium. The proportion Ca$^{2+}$:Mg$^{2+}$ is 4:1 and Na$^+$:K$^+$ ions is 10:1. The pH was kept between 6.8 and 8.0. The fish medium was aerated to oxygen saturation and was kept above 80%.
3.3.1.3 Metabolic activation system

Tris was dissolved in fish medium and neutralized with HCl. Tris-HCl buffer concentration was 0.1 M, pH 7.6 at 25°C to achieve pH 7.4 at 32°C. Microsomes vial was thawed at 25°C in a water-bath for 5 min. Thereafter microsomes were diluted in Tris-buffer to an induced rat liver microsomal protein final concentration of 0.7 mg/ml assayed by the Bicinchoninic Acid (BCA) protein assay (Smith et al., 1985b). NADPH stock solution was dissolved in Tris-buffer to a final concentration of 1 mM. The end volume of MAS was 2000 µl.

3.3.1.4 Exposure of the eggs

3.3.1.4.1 Study design

For each test material a concentration range finding experiment was conducted (three to four concentrations) with a constant spacing factor of 2. In general, two controls were used in each experiment: (i) a negative control consisting of the highest concentration of the proteratogen without MAS and (ii) the MAS control consisting of the MAS alone. For protocol details, see Annex A. Typically, twenty fish embryos were used per concentration group.

3.3.1.4.2 Eggs selection

Fish embryos were washed twice in glass Petri dishes with the Tris-buffer (Fig. 3-1). Within the first hour post fertilization, the fecundated fish embryos (from 4 to 32 cell blastomeres - Fig. 1-6A) were sorted under a stereomicroscope and collected in a plastic Petri dish containing Tris-buffer. From the pooled fecundated fish embryos, twenty eggs were randomly transferred into a 2 ml vial for each group. All these steps were performed at 26°C.
For cyclophosphamide and ethanol, MAS was prepared in a polypropylene test tube stored on ice, filled with Tris-HCl buffer (0.1 M), microsomes and the test material (i.e., premix). NADPH was weighted in a brown glass, dissolved with Tris-HCl buffer and stored on ice.

A 5 min preincubation step was performed at 32°C and 100 rpm for the test tubes containing the premix and the NADPH stock solution (10 mM). Afterwards, the twenty fish embryos were transferred together in 820 µl into a test tube using a pipette 5000 and thereafter reaction was initiated by NADPH at the latest 2 hpf. The incubation was performed for 60 min in a shaking water bath at 32°C, 100 rpm.
For benzo[a]pyrene and thalidomide, the MAS was prepared in a polypropylene test tube stored on ice, filled with Tris-HCl buffer (0.1 M), microsomes (i.e., premix). NADPH was weighted in a brown glass, dissolved with Tris-HCl buffer and stored on ice.

A 5 min preincubation step was performed at 32°C and 100 rpm for the test tubes containing the premix and the NADPH stock solution (10 mM). Afterwards, the twenty fish embryos were transferred together in 820 µl into a test tube using a pipette 5000 and thereafter DMSO and/or the test material were added. Reaction was initiated by NADPH at the latest 2 hpf. The incubation was performed for 60 min in a shaking water bath at 32°C, 100 rpm.

3.3.1.4.3 Eggs development

The exposure was stopped by transferring the fish embryos into different Petri dishes filled with fish medium (1 Petri dish/group). The fish embryos were then exposed individually in 24-well plates containing 2 ml fish medium per well and left for 48 h at 26°C with a 12 h light – 12 h dark cycle in a precision incubator. After 48 hpf, the fish embryos were anesthetized and killed with a MS-222 solution (0.3%).

3.3.2 Evaluation

3.3.2.1 Scoring

At different time points, i.e., 8, 24 and 48 hpf, fish eggs were evaluated and scored for lethal or teratogenic effects using an inverted microscope with phase contrast optics, a mounted time-lapse recorder and the analySIS software. The different lethal or teratogenic endpoints are summarized in Table 3-2. The effects observed were documented under the Quality Management System (QMS) established at the Merck preclinical development division.
**Table 3-2** Lethal and teratogenic effects observed in zebrafish *D. rerio* embryos depending on the observation time (Bachmann, 2002; Nagel, 2002)

<table>
<thead>
<tr>
<th>Category</th>
<th>Physiological / dysmorphogenic effect</th>
<th>8 hpf</th>
<th>24 hpf</th>
<th>48 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lethal effect</strong></td>
<td>Coagulated egg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Teratogenic effects</strong></td>
<td>Malformation of head</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malformation of tail&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malformation of endtail&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malformation of sacculi/otoliths&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malformation of heart&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Modification of the spinal chord structure&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scoliosis</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rachischisis</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deformity of yolk&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Growth retardation&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

**Note.**

<sup>a</sup>The lethal effect was assessed when there was no more clear organs structure recognition.

<sup>b</sup>Malformation of the tail was assessed when the tail was bended or not completely detached from the yolk sac.

<sup>c</sup>Malformation of the endtail was assessed when the spike was bended or twisted.

<sup>d</sup>Growth retardation was assessed in comparison to the control eggs. Different parameters were included: The global size of the fish egg, the eye and the sacculi/otoliths position, the degree of pigmentation, the tail not detached and the frequency of spontaneous movement. *See Glossary.
3.3.2.2 Validity parameters

1) The fertilization rate of the fish embryos must be higher than 50%.

2) The assay is considered to be valid if the viability of the control eggs (MAS and negative) exceeds or is equal to 90% after 48 hpf (no teratogenic or lethal effects).

3.3.2.3 Fingerprint endpoints

Major malformations were defined teratogenic fingerprints if it was concentration-dependent and observed in ≥ 50% of the impaired embryos. Following the detection of specific teratogenic fingerprint, the evaluation of in vitro test systems was based on the comparison of effectiveness in the test system with documented in vivo effects (Piersma, 2004).

3.3.2.4 Statistics

A one-way analysis of variance (ANOVA) test was run and a Tukey's Post Hoc was performed when significant differences were reported.
4 Results

4.1 Development of the mDarT

4.1.1 Impact of Parameters on fish embryo development without MAS

4.1.1.1 Overview of the two systems

The mDarT protocol was developed taking into consideration normal fish egg development and optimal rat liver microsome incubation conditions. Every parameter was considered to be acceptable if a minimum of 90% of fish eggs developed normally. In Table 4-1 the optimal incubation parameters for zebrafish *D. rerio* embryos and rat liver microsomes are summarized.

Table 4-1 Optimal incubation conditions for each system

<table>
<thead>
<tr>
<th></th>
<th>Zebrafish <em>D. rerio</em> embryo</th>
<th>Rat liver microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>26°C 37°C</td>
<td>2 h 100-150 rpm</td>
</tr>
<tr>
<td>Time</td>
<td>48 h 2 h</td>
<td></td>
</tr>
<tr>
<td>Vessels</td>
<td>24-well plate Test tubes</td>
<td></td>
</tr>
<tr>
<td>Agitation</td>
<td>n.d. 100-150 rpm</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>Fish Medium Tris or Phosphate-buffer</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>8 7.4</td>
<td></td>
</tr>
<tr>
<td>Osmolarity</td>
<td>100 mOsm/kg n.d.</td>
<td></td>
</tr>
<tr>
<td>Cofactors</td>
<td>n.d. NADPH or NRS</td>
<td></td>
</tr>
<tr>
<td>Additives</td>
<td>n.d. MgCl₂, EDTA</td>
<td></td>
</tr>
<tr>
<td>Organic solvent</td>
<td>DMSO 1% DMSO 0.2%</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* The following terms were abbreviated: n.d.: no data; NRS: NADPH Regenerating System; EDTA: ethylenediaminetetraacetic acid; DMSO: Dimethyl Sulfoxide; rpm: rotation per minute.
4.1.1.2 Temperature

The optimal temperature for mammal microsomes is 37°C. In contrast, fish embryos appear to develop normally if they are kept within an eight degree range, between 25°C and 33°C. Incubating them for long periods above or below these extremes may produce abnormalities (Kimmel et al., 1995). Incubation of microsomes is usually performed from 30 to 60 min and is not recommended for more than 120 min at 37°C.

To investigate the influence of temperature on zebrafish *D. rerio* development, 20 fish embryos were incubated at three different temperatures (26°C, 32°C and 37°C) for 60 and 90 min respectively. Results are displayed in Tab. 4-2

Table 4-2 Influence of temperature and incubation time on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>Temperature groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>26°C 32°C 37°C</td>
</tr>
<tr>
<td></td>
<td>120 min 60 min 90 min 60 min 120 min</td>
<td></td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>95</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 24-well plate, fish medium, 2 hpf.

**Note.** The following terms were abbreviated: hpf: hours post fertilization; -: No embryo effects; Σ: Sum.

At 37°C, 50% of the fish eggs were coagulated independent of the incubation time while at 32°C there was ≤ 10% of impaired embryos at either 60 min or 90 min. Therefore, temperature had a direct influence on fish embryo development, whereby increasing temperature resulted in decreased incidence of normal eggs.
4.1.1.3 Vessels and agitation

4.1.1.3.1 Vessels

Vessels may influence fish embryo development and enzyme activity depending upon its material and its form. Using Petri dishes or Erlenmeyer flasks with their large surface area may enhance O\textsubscript{2} transfer between the air-medium interface for CYP activity and thereby increasing biotransformation. Test tubes are regularly used for microsome studies, glass test tubes being generally recommended. However, polypropylene test tubes limit microsomal protein and lipophilic xenobiotic adhesion onto the test tubes surface. These different vessels were tested by incubating with 20 fish eggs. Results are displayed in Tab. 4-3.

Table 4-3 Influence of vessels on zebrafish D. rerio embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>Vessels groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-well plates</td>
<td>Petri dishes Ø 20 mm</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>Number of experiments</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

Incubation conditions: 90 min, 2 hpf, 32°C, fish medium.

Note. The following terms were abbreviated: Ø: Diameter; PP: Polypropylene; hpf: hours post fertilization; -: No embryo effects; Σ: Sum.

Lethality and teratogenicity were ≤ 10% in the control and the vessels groups. Therefore, the vessel had little impact on fishes embryonic development.
4.1.3.2 Agitation

The aqueous Boundary Layer (also called the unstirred water layer) is a “stagnant” water layer adjacent to each side of a membrane barrier (e.g., microsomes, chorion) (Kansy et al., 2004). Its thickness depends on the bulk-solution stirring. Rate processes, such as xenobiotic biotransformation and metabolite uptake depend on the probability of CYPs active site access and ability to cross membranes.

Microsome agitation is usually performed at 100 or 150 rpm (Guengerich, 2001). The shaking impact on fish embryos development has not been investigated so far. Therefore, fish eggs were incubated at either 100 or 150 rpm. Tab. 4-4 summarizes the results.

Table 4-4 Influence of agitation on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>Agitation groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fish medium</td>
<td>100 rpm</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>95</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 90 min, 2 hpf, 32°C and PP test tubes in fish medium.

**Note.** The following terms were abbreviated: rpm: rotation per minute; hpf: hours post fertilization; -: No embryo effects; Σ: Sum.

Out of the control and agitation groups, there was ≤ 10% of fish embryos exhibiting teratogenic or lethal effects. Thus, agitation had limited impact on fish embryonic development although a trend of decreased normal eggs was observed.
4.1.1.4 Buffer systems

4.1.1.4.1 Background

Solvent and pH value are important to prevent protein aggregation and keep microsomal activity stable (Schein, 1990). When studying an enzyme one must consider (i) enzyme optimum pH (in our case 7.4), (ii) non-specific buffer effects on the enzyme, and (iii) interactions with substrates or metals. Since the buffering capacity is maximal at the pK$_a$, buffers should be used close to this value.

4.1.1.4.2 Phosphate buffer

Phosphate buffer (pK$_a$ value 7.2) might be an ideal buffer system for microsomes (Stoll and Blanchard, 1990). However, dissolution of phosphate buffer with fish medium resulted in precipitation and thus fish medium was substituted for Ultrapure water. Three concentrations were tested (10, 25 and 50 mM) with the latter being the preferred for microsome activity (as recommended by XenoTech and BD Biosciences). The buffer capacity may be identical for the three concentrations tested but by using high buffer concentration, the ionic strength of the medium can promote stimulatory effects on some CYPs activities (Kelley et al., 2005; Yun et al., 1998).

To investigate the impact on zebrafish *D. rerio* development, 20 fish eggs were incubated at different concentrations of Phosphate buffer. Results are summarized in Tab. 4-5.
### Table 4-5 Influence of Phosphate buffer (pH 7.4) on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>Phosphate buffer groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ultrapure Water</td>
<td>Phosphate 10 mM</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>90</td>
<td>80</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 90 min, 32°C, 2 hpf, 100 rpm and PP test tubes.

**Note.** The following terms were abbreviated; hpf: hours post fertilization; -: No embryo effects; Σ: Sum.

In all buffer groups, there was > 10% impaired embryos. Moreover, lethal effects were concentration dependent. Therefore, phosphate buffer was considered not to be appropriate for normal embryonic development of fish.

Osmolarity tolerance for zebrafish embryos is up to 100 mOsm/kg. Above this value, fish embryos have been shown to exhibit high lethality (Lange *et al.*, 1995). To verify this, the osmolarity was measured for 50 mM Phosphate buffer, which was 150 mOsm/kg.
4.1.1.4.3 Tris buffer

Tris has a poor buffer capacity below pH 7.5 and its pKₐ is temperature dependent. However, Tris buffer is nonpolar, dissolves totally in fish medium and is regularly used in protocols and guidelines focusing on microsome studies (Stoll and Blanchard, 1990). Three different concentrations (25, 50 and 100 mM) of Tris-buffer were tested. Tris was neutralized with HCl at pH 7.6 and 25°C to reach pH 7.4 at 32°C (there is a decrease of 0.03 pH units for °C increment).

To investigate the impact on zebrafish *D. rerio* embryonic development, 20 fish eggs were incubated at different concentrations of Tris buffer. Results are summarized in Tab. 4-6.

Table 4-6 Influence of Tris-HCl (pH 7.4) on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>Tris-buffer groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fish medium</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 mM</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 90 min, 32°C, 2 hpf, 100 rpm and PP test tubes.

**Note.** The following terms were abbreviated: hpf: hours post fertilization; -: No embryo effects; Σ: Sum.

No lethal or teratogenic effects were observed at any concentration. Up to 100 mM, Tris-HCl fulfilled the validation criterion.
4.1.1.4.4 Cell culture medium

Primary hepatocytes were considered as an alternative to subcellular fractions using an indirect co-culture model. 20 fish eggs were exposed to hepatocyte cell culture medium (DMEM/F12) with or without serum. Results are displayed in Tab. 4-7.

Table 4-7 Influence of DMEM/F12 with or without serum (pH 7.4) on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>Cell culture medium groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fish medium</td>
<td>DMEM/F12 with serum</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 120 min, 2 hpf, 26°C and 24-well plate.

**Note.** The following terms were abbreviated: hpf: hours post fertilization; DMEM: Dulbecco’s modified eagle medium; -: No embryo effects; Σ: Sum.

For both cell culture medium groups, all fish embryos were coagulated at 96 hpf. As for Phosphate buffer, high osmolarity may explain the high lethality. The cell culture medium osmolarity without serum was 280 mOsm/kg. Diluting cell culture medium (1/10, 1/2) was not considered as it would drastically reduce primary hepatocytes vitality and thus limit xenobiotic biotransformation potential.
4.1.2 Impact of MAS on fish embryo development

4.1.2.1 Characterization of microsomes from different suppliers

Two microsome suppliers were selected to investigate their impact on zebrafish *D. rerio* embryonic development. The two suppliers do not use similar CYP substrates, meaning specific CYP activities cannot be directly compared. Therefore, only CYP concentrations were compared in Table 4-8.

<table>
<thead>
<tr>
<th>Liver microsome fractions</th>
<th>Rat inducers</th>
<th>Rabbit**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ARO* βNF- PB*</td>
<td>INH</td>
</tr>
<tr>
<td>In Vitro Technologies</td>
<td></td>
<td>1.03</td>
</tr>
<tr>
<td>(nmol/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XenoTech</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>(nmol/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP families induced</td>
<td>All</td>
<td>All</td>
</tr>
</tbody>
</table>

Note. The following terms were abbreviated: ARO: Aroclor1254; βNF: β-Naphthoflavone; PB: Phenobarbital; INH: Isoniazid; DEX: Dexamethasone; hpf: hours post fertilization; -: no data.

*Microsomes treated with Phenobarbital together with β-Naphthoflavone provide a global rise in CYP activities in rat liver microsomes similar to Aroclor1254.

**non-induced rabbit liver microsomes.

The CYP concentrations from XenoTech microsomes were higher compared to In Vitro Technologies microsomes.
4.1.2.2 Incubation time and microsome concentration

4.1.2.2.1 In Vitro Technologies microsomes

Increasing incubating periods of microsomes with fish embryos may increase test materials biotransformation rate and may cover a critical period of fish embryo development (i.e., organogenesis) and thus potentially provoke substantial teratogenic effects.

In Vitro Technologies (IVT) Aroclor1254 induced rat liver microsomes cover a broad range of CYP activities, thus allowing a wide xenobiotic screening. The influence of incubation time combined with Aroclor1254 microsomes concentration was evaluated: (i) 60 min with two high microsomes concentrations (0.7 and 0.8 mg/ml), (ii) 90 min with two intermediate concentrations (0.2 and 0.4 mg/ml) and (iii) 240 min with two low concentrations (0.01 and 0.1 mg/ml). Results are displayed in Tab. 4-9.

Table 4-9 Influence of Aroclor1254 induced rat liver microsome concentration and incubation time on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Control group</th>
<th>IVT rat liver microsome groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>240 min</td>
<td>60 min</td>
</tr>
<tr>
<td></td>
<td>Fish medium</td>
<td>MAS 0.7 mg/ml</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>90</td>
<td>80</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, NADPH (1 mM), PP test tubes and Aroclor1254 induced rat liver microsomes.

**Note.** The following terms were abbreviated: **MAS:** Metabolic Activation System; **Aro:** Aroclor1254; **hpf:** hours post fertilization; -: No embryo effects; **Σ:** Sum; **PP:** polypropylene.
None of the rat liver microsomes groups were within the defined validity parameters (≤ 10% of impaired embryos). Toxicity increased with increasing microsome concentration and incubation time.

4.1.2.2.2 XenoTech microsomes

Microsomes from XenoTech induced with Phenobarbital together with β-Naphthoflavone provide a global increase in CYP activities in rat liver microsomes in a similar manner to Aroclor1254. The influence of incubation time combined with microsome concentration was evaluated: i) 60 min with three high microsomes concentrations (0.35, 0.7 and 0.8 mg/ml) and ii) 90 min with two lower concentrations (0.35, and 0.5 mg/ml). Results are displayed in Tab. 4-10.

Table 4-10 Influence of β-Naphthoflavone and Phenobarbital induced rat liver microsome concentration and incubation time on zebrafish D. rerio embryonic development

| Incubation conditions: | 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, NADPH (1 mM), PP test tubes and β-Naphthoflavone-Phenobarbital induced rat liver microsomes.

Note. The following terms were abbreviated: MAS: Metabolic Activation System; βNF: β-Naphthoflavone; PB: Phenobarbital; hpf: hours post fertilization; -: No embryo effects; Σ: Sum; PP: polypropylene.

Fish embryos can be incubated either for 60 min with 0.7 mg/ml βNF-PB induced rat liver microsomes or for 90 min with 0.35 mg/ml induced βNF-PB
rat liver microsomes and fulfil the validation criterion ($\leq 10\%$ of impaired embryos). As it was observed with the In Vitro Technologies microsomes, toxicity increased with higher microsome concentrations and incubation times.

### 4.1.2.3 Different CYP family inducers

#### 4.1.2.3.1 Aims

Specifically induced rat liver microsomes are of relevance when the CYP family is known for the xenobiotic under investigation (see Table 1-2 and Table 4-8). However, it has been reported that the production of ROS, mediated by liver microsomes was influenced by specific CYP families. Thus, in rat liver microsomes, CYP2E1 was found to be more active in producing $\text{H}_2\text{O}_2$ (by-product of CYP cycle) compared with several other CYPs families (Puntarulo and Cederbaum, 1998), meaning Isoniazid rat liver microsomes may be more toxic than Aroclor1254 rat liver microsomes using the same concentration. To verify this theory, microsomes induced by different inducers were investigated.
4.1.2.3.2 In Vitro Technologies microsomes

20 Fish eggs were incubated with three different induced rat liver microsomes: a mix of Aroclor1254 and Isoniazid (50:50), Isoniazid alone and β-Naphthoflavone alone. Each microsome fraction was tested at two different concentrations. Results are displayed in Tab. 4-11.

Table 4-11 Influence of different CYP inducers and rat liver microsome concentration on zebrafish D. rerio embryonic development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Control group</th>
<th>IVT rat liver microsomes groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fish medium</td>
<td>MAS ARO / INH</td>
</tr>
<tr>
<td></td>
<td>0.15 mg/ml</td>
<td>0.6 mg/ml</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>90</td>
<td>70</td>
</tr>
</tbody>
</table>

Incubation conditions: 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, NADPH (1 mM) and PP test tubes.

Note. The following terms were abbreviated: MAS: Metabolic Activation System; ARO: Aroclor1254; βNF: β-Naphthoflavone; INH: Isoniazid; hpf: hours post fertilization; PP: polypropylene; -: No embryo effects; Σ: Sum.

None of the rat liver microsome groups were within the defined validation criteria (≤ 10% of impaired embryos). Microsome toxicity was concentration and inducer dependent. The β-Naphthoflavone rat liver microsomes fraction was the most toxic and the Isoniazid rat liver microsome fraction was the least toxic. Surprisingly, the Isoniazid rat liver microsomes fraction (high concentration of CYP2E1) was not as toxic as previously reported (Puntarulo and Cederbaum, 1998).
4.1.2.3.3 XenoTech microsomes

20 Fish eggs were exposed with three different induced rat liver microsomes and one non-induced rabbit liver microsomes. For each fraction, one unique concentration (0.7 mg/ml) was tested. Results are displayed in Tab. 4-12

Table 4-12 Influence of different liver microsome fractions on zebrafish D. rerio embryonic development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Control group</th>
<th>XenoTech liver microsome groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAS βNF-PB 0.7 mg/ml</td>
<td>MAS βNF 0.7 mg/ml</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>90</td>
<td>95</td>
</tr>
</tbody>
</table>

Incubation conditions: 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, NADPH (1 mM) and PP test tubes.

Note. The following terms were abbreviated: MAS: Metabolic Activation System; βNF: β-Naphthoflavone; PB: Phenobarbital; INH: Isoniazid; DEX: Dexamethasone; hpf: hours post fertilization; -: No embryo effects; PP: polypropylene; Σ: Sum.

The control group and the rat liver microsome groups fulfilled the validation criteria (≤ 10% of impaired embryos). Fish embryos can be incubated for 60 min at 0.7 mg/ml with any of the different XenoTech rat liver microsome fractions or rabbit liver microsome fraction.
4.1.2.4 Microsome toxicity

Microsome toxicity is mainly driven by lipid peroxidation. It is the initiating reaction in a cascade of events (see Fig. 4-1), starting with the oxidation of polyunsaturated fatty acids present in the microsome vesicles by radicals or electrons to form lipid hydroperoxides. These break down and yield a variety of toxic products, mainly aldehydes such as malondialdehyde (Amunom et al., 2007; Hodgson and Levi, 1997).

Figure 4-1 Schematic illustrating lipid peroxidation and destruction of membranes (Hodgson and Levi, 1997).

Legend: R: Radical; R*: Free Radical; ROO*: reactive oxygen species; RH: Radical complexed with one Hydrogen atom.

Lipid peroxidation initiation is reported to be either enzymatic - via NADPH-P450 reductase or CYPs - or non-enzymatic when it is strongly affected by transition metals, such as endogenous Fe$^{2+}$ that can be present in the original microsome suspension (Marmunti et al., 2004) (Aviram et al., 1999) (Puntarulo and Cederbaum, 1998). However, other parameters may explain microsome toxicity. During liver homogenization, phospholipases and proteases are released and can be present in microsome preparations if appropriate care is not taken.

Inhibition of non-enzymatic lipid peroxidation and protease activity may limit toxicity and increase the percentage of normal embryos when incubated with microsomes.
4.1.2.5 Influence of additives

4.1.2.5.1 Ethylenediaminetetraacetic acid

Lipid peroxidation can be stimulated by inorganic iron compounds, but to a greater extent if adenine nucleotides, containing iron compounds as a contaminant, are also present (Wills, 1969). Therefore, a chelating agent such as ethylenediaminetetraacetic acid (EDTA) was investigated to test for reduced microsome toxicity (Hayes, 2001).

EDTA impact was evaluated using two concentrations (0.5 mM and 1 mM) with 20 fish eggs incubated with the MAS (see Tab. 4-13).

Table 4-13 Influence of EDTA on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>EDTA groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAS ARO</td>
<td>MAS ARO</td>
</tr>
<tr>
<td></td>
<td>0.7 mg/ml</td>
<td>0.7 mg/ml</td>
</tr>
<tr>
<td></td>
<td>0.5 mM EDTA</td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>85</td>
<td>75</td>
</tr>
</tbody>
</table>

*Incubation conditions:* 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, In Vitro Technologies liver microsomes, NADPH (1 mM) and PP test tubes.

*Note.* The following terms were abbreviated: **MAS:** Metabolic Activation System; **ARO:** Aroclor1254; **EDTA:** ethylenediaminetetraacetic acid; **hpf:** hours post fertilization; **-:** No embryo effects; **Σ:** Sum.

The control group (MAS alone) was below the validation criterion (85% instead of ≥ 90% normal embryos). The MAS with 0.5 mM of EDTA triggered normal development of 75% eggs and only 55% were normally developed with 1 mM EDTA in MAS. The addition of EDTA did not reduce the number of impaired embryos but, in contrast, increased the final toxicity.
4.1.2.5.2 Protein inhibitor cocktail

The presence of proteases or phospholipases may increase microsome toxicity. Therefore, induced rat liver microsome fractions were incubated with a protein inhibitor cocktail (PIC) specific for mammal proteolytic enzymes (i.e., serine, cysteine, aspartic proteases).

PIC impact was investigated using two concentrations - 1X and 2X (see Note). The cocktail was incubated with 20 fish eggs and the MAS. Results are displayed in Tab. 4-14.

Table 4-14 Influence of a protein inhibitor cocktail on zebrafish D. rerio embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>PIC groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAS ARO 0.7 mg/ml</td>
<td>MAS ARO 0.7 mg/ml</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>85</td>
<td>55</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, In Vitro Technologies liver microsomes, NADPH (1 mM) and PP test tubes.

**Note.** The following terms were abbreviated: MAS: Metabolic Activation System; ARO: Aroclor1254; PIC 1X: Protein Inhibitor Cocktail (4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride – 1 mM; Aprotinin – 0.8 µM; Bestatin – 50µM; E-64 Protease Inhibitor – 15 µM; Pepstatin A – 10 µM); PIC 2X: Protein Inhibitor Cocktail with double concentration; hpf: hours post fertilization; -: No embryo effects; Σ: Sum.

The control group (MAS alone) was below the validation criterion (85% instead of ≥ 90% normal embryos). 55% fish embryos exposed with MAS and PIC 1X developed normally and only 35% when fish embryos were exposed to PIC 2X. None of the PIC groups limited the toxicity of the MAS. In fact, the fish embryo toxicity increased with the addition of PIC.
4.1.2.5.3 Dimethyl Sulfoxide

Dimethyl Sulfoxide (DMSO), a small amphiphilic molecule, is widely used as an organic solvent when xenobiotics with low water solubility need to be dissolved. It has been estimated that more than 30% of drug discovery compounds have an aqueous solubility of less than 10 µM (Di et al., 2006). Moreover, DMSO is not only known as a potent organic solvent, it may also modify membrane structure by thinning it or creating pores (Gurtovenko and Jamshed, 2007).

DMSO toxicity was previously assessed in zebrafish D. rerio embryos and no effect on exposed eggs was recorded with DMSO concentrations up to 2% (v/v) (Hallare et al., 2006). However, teratogenic effects were not investigated and moreover synergistic effects may occur between DMSO and Tris buffer. Therefore, fish embryos (20 each) were exposed to different concentrations of DMSO in Tris buffer. Results are summarized in Tab. 4-15.

Table 4-15 Influence of DMSO concentration on zebrafish D. rerio embryonic development

<table>
<thead>
<tr>
<th>96 hpf</th>
<th>Control group</th>
<th>DMSO groups / Tris-HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO 0.5% (v/v)</td>
<td>DMSO 1% (v/v)</td>
<td>DMSO 2% (v/v)</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 60 min, 100 rpm, 32°C, 2 hpf, Tris-HCl (0.1 M) at pH 7.6 and PP test tubes.

**Note.** The following terms were abbreviated: DMSO: Dimethyl Sulfoxide; hpf: hours post fertilization; -: No embryo effects; Σ: Sum; v/v: volume/volume.

Up to 2% DMSO (v/v) the normal development of fish eggs was ≥ 90%. DMSO is also known to inhibit several CYP families and may further be
metabolized to a toxic metabolite namely DMSO$_2$ \cite{Chauret et al., 1998} \cite{Gerhards and Gibian, 1967; Hickman et al., 1997} \cite{Jia and Liu, 2007}.

20 Fish eggs were incubated with different concentrations of DMSO and liver microsome from rat or rabbit. Results are displayed in Tab. 4-16.

Table 4-16 Influence of DMSO concentration on zebrafish \emph{D. rerio} embryonic development with different liver microsomes.

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Control groups</th>
<th>MAS-DMSO groups</th>
<th>MAS-DMSO groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAS $\beta$NF</td>
<td>MAS INH</td>
<td>MAS Rabbit</td>
</tr>
<tr>
<td>0.2%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>1%</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>19</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>95</td>
<td>95</td>
<td>90</td>
</tr>
</tbody>
</table>

\textit{Incubation conditions}: 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, XenoTech liver microsomes (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

\textit{Note}. The following terms were abbreviated: \textbf{MAS}: Metabolic Activation System; $\beta$NF: $\beta$-Naphthoflavone; \textbf{PB}: Phenobarbital; \textbf{INH}: Isoniazid; \textbf{DMSO}: Dimethyl Sulfoxide; \textbf{hpf}: hours post fertilization; \textbf{-}: No embryo effects; Σ: Sum.

All control groups (MAS alone) fulfilled the validation criteria ($\leq$ 10\% of impaired embryos). DMSO combined with the MAS was toxic to fish eggs when its percentage was 1\% (v/v). At 0.2\% (v/v) the validation criterion was fulfilled for $\beta$NF-induced rat liver microsomes and rabbit liver microsomes.

The percentage of normal eggs dropped with Isoniazid-induced liver microsomes (no normal eggs with 1\% of DMSO). Indeed, this inducer raised specifically CYP2E1 that can specifically metabolize DMSO, due to its small active site \cite{Jain and Lyer, 2004}. The final percentage of DMSO for any kind of microsome fractions must be kept $\leq$ 0.2\% (v/v).
4.1.2.6 Multiple thawings

Microsomes are stable indefinitely at or below -70°C and typically thawed in a water-bath at 25°C for 5 min, stored on ice and the remainder refrozen after use (Pearce et al., 1996). One must consider that the enzyme degradation rate in microsomes is proportional to the temperature increase and to multiple thawings.

Therefore it was investigated whether fish egg development was influenced when rat liver microsomes were subjected to as many as 3 cycles of thawing and refreezing. Results are displayed in Tab. 4-17.

Table 4-17 Influence of multiple thawings on zebrafish D. rerio embryonic development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Control group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Thawing-Freezing cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl buffer</td>
<td>1</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Incubation conditions: 90 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, βNF-PB treated XenoTech liver microsomes (0.35 mg/ml), NADPH (1 mM) and PP test tubes.

Note. <sup>a</sup>Three independent experiments values are represented in each group

The following terms were abbreviated: MAS: Metabolic Activation System; βNF: β-Naphthoflavone; PB: Phenobarbital; hpf: hours post fertilization; -: No embryo effects; Σ: Sum.

Controls and thawing groups fulfilled the validation criterion (≤ 10% of impaired embryos). Multiple thawings of microsomes had no effect on zebrafish D. rerio development. However, the more cycle of thawing and freezing were performed the more toxic were the microsomes.
4.1.2.7 MAS Cofactors

For practical reasons, NADPH was previously used as a standard cofactor source with MAS. However, NRS, composed of NADP+, glucose-6-phosphate and glucose-6-phosphatase dehydrogenase, is generally used to assure maintenance of saturating NADPH concentrations and NADPH-P450 reductase activity (Guengerich, 2001). Indeed: (i) the NADPH/NADP+ ratio is in the favour of NADPH and allow its reaction with the NADPH-P450 reductase, (ii) NADP+ inhibits NADPH-P450 reductase competitively and (iii) NRS is half the cost of NADPH (Guengerich, personal communication).

Therefore, 20 fish eggs were incubated with either NADPH (1mM) or NRS (1X) - see Note. Results are displayed in Tab. 4-18.

Table 4-18 Influence of MAS cofactors on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Control group</th>
<th>Cofactor group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAS βNF-PB NADPH 1 mM</td>
<td>MAS βNF-PB NRS 1X</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>95</td>
<td>80</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 90 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, β-Naphthoflavone-Phenobarbital XenoTech liver microsomes (0.35 mg/ml) and PP test tubes.

**Note.** The following terms were abbreviated: **MAS**: Metabolic Activation System; **βNF**: β-Naphthoflavone; **PB**: Phenobarbital; **NRS 1X**: NADPH Regenerating System using supplier stock solution concentration; **hpf**: hours post fertilization; -: No embryo effects; Σ: Sum.

The control group was within the validation criteria (≤ 10%). When NRS 1X was used with MAS there were 3 lethal eggs and 1 teratogenic egg resulting in ≥ 10% impaired embryos. Despite the obvious NRS advantages, it is not appropriate for cultures with zebrafish *D. rerio* embryos.
4.1.3 Increasing the MAS activity

4.1.3.1 Reference Proteratogen

To investigate MAS activity, CPA was chosen as a reference proteratogen. It is one of the best studied proteratogens and is listed as an excellent candidate compound for use in the validation of *in vitro* teratogenesis assays (Bremer *et al.*, 2002; Flick and Klug, 2006; Fort *et al.*, 1988; Smith *et al.*, 1983). A range finding study with multiple concentration of CPA alone was investigated with 20 fish embryos per group (see Tab. 4-19).

Table 4-19 Influence of CPA on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Internal control</th>
<th>Cyclophosphamide groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl 0.1 M</td>
<td>CPA 3.5 mM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Incubation conditions:* 90 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, and PP test tubes.

*Note:* The following terms were abbreviated: CPA: Cyclophosphamide; -: No embryo effects; N: Number.

Up to 28 mM, there was no lethal or teratogenic effect on the zebrafish eggs.
4.1.3.2 Microsome inducers

4.1.3.2.1 Overview

CPA metabolism by rat tissues has been extensively investigated. It has been found that the rat liver is the principal site of biotransformation. Rat liver microsomes when given NADPH and oxygen, will transform CPA to alkylating and cytotoxic metabolites (Torkelson et al., 1974). In mammals, CPA is oxidatively activated by CYP2C6/2C11 and CYP2B1 (Gut et al., 2000). MAS activity was assessed using lethal and teratogenic eggs.

4.1.3.2.2 PB-induced rat liver microsomes

20 Fish eggs were incubated with CPA and Phenobarbital (PB) induced rat liver microsomes that contained, among other CYPs, a high concentration of CYP2B1 (see Tab. 1-2 and Tab. 4-8). Results are displayed in Tab. 4-20.

Table 4-20 Influence of PB induced rat liver microsomes on zebrafish D. rerio embryonic development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control</th>
<th>MAS control (PB)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA 28 mM</td>
<td>CPA 1.75 mM</td>
<td>CPA 3.5 mM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>-</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

*Percentage of teratogenic eggs based on surviving eggs

**Incubation conditions**: 90 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, Phenobarbital induced rat liver XenoTech microsomes (0.35 mg/ml), NADPH (1 mM) and PP test tubes.
The following terms were abbreviated: **MAS**: Metabolic Activation System; **PB**: Phenobarbital; **hpf**: hours post fertilization; **-**: No embryo effects; **N**: Number.

The control groups (CPA alone and MAS alone) displayed ≤ 10% impaired embryos. The test groups displayed lethal and teratogenic effects on fish embryos although it was not concentration dependent. Indeed, a maximal abnormality and lethality was observed of 7 mM CPA with MAS-PB. Rat liver microsomes induced with PB are therefore not appropriate for CPA biotransformation. Moreover, it was reported that rat liver microsomes induced with PB decreased levels of alkylating metabolites of CPA in animals (Ruzicka and Ruenitz, 1992).
4.1.3.2.3 βNF & PB induced rat liver microsomes

20 Fish eggs were incubated with CPA and β-Naphthoflavone (βNF) - Phenobarbital (PB) induced rat liver microsomes that cover a broad range of CYP activities (see Tab. 1-2 and Tab. 4-8). Results are displayed in Tab. 4-21.

Table 4-21 Influence of βNF-PB induced rat liver microsomes on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative controla</th>
<th>MAS control (βNF-PB)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA 28 mM</td>
<td>1.75 mM</td>
<td>3.5 mM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>- -</td>
<td>3 4</td>
<td>1 3</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20 20</td>
<td>17 16</td>
<td>19 16</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>- -</td>
<td>7 4</td>
<td>6 11</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>40 40</td>
<td>33 35</td>
<td>31 24</td>
</tr>
<tr>
<td>% Teratogenicb</td>
<td>0 0</td>
<td>18 (±6)</td>
<td>11 (±13)</td>
</tr>
<tr>
<td>% Lethal eggsc</td>
<td>0 0</td>
<td>0 (±4)</td>
<td>3 (±11)</td>
</tr>
</tbody>
</table>

Incubation conditions: 90 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, β-Naphthoflavone and Phenobarbital rat liver XenoTech microsomes (0.35 mg/ml), NADPH (1 mM) and PP test tubes.

Note.

a Two independent experiments values are represented in each group

b Mean percentage ± SD of teratogenic eggs based on surviving eggs (two experiments)

c Mean percentage ± SD of lethal eggs based on all eggs (two experiments)

The following terms were abbreviated: CPA: Cyclophosphamide; MAS: Metabolic Activation System; PB: Phenobarbital; βNF: β-Naphthoflavone; -: No embryo effects; Σ: Sum; N: Number; SD: Standard Deviation.

All the control groups fulfilled the validation criteria (≤ 10% impaired embryos). In parallel, all the test groups displayed lethal and teratogenic effects in fish embryos. The highest concentration (28 mM) triggered up to 33%
malformations on zebrafish embryos. However, effects displayed didn't reach 100% lethality or teratogenicity.

βNF-PB induced rat liver microsomes triggered strong teratogenic effects on fish embryos in a concentration-dependent manner. However, the effects observed didn't reach 100% lethality or teratogenicity.
4.1.3.3 Enzyme stability

Enzyme activity is dependent on many factors (e.g., medium, pH, temperature, 3D-configuration). In addition, protein stability in solution is affected by ionic compounds and can affect positively or negatively enzyme reactions. Small anions, such as chlorides, are more destabilizing than citrate or acetate anions (Schein, 1990). Thus, 20 fish eggs were incubated with CPA and MAS using Tris-acetate buffer. Results are displayed in Tab. 4-22.

Table 4-22 Impact of neutralizing agent on CPA biotransformation rate

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control</th>
<th>MAS control (βNF-PB)</th>
<th>Test groups</th>
<th>Test groups</th>
<th>Test groups</th>
<th>Test groups</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA 28 mM</td>
<td>CPA 1.75 mM</td>
<td>CPA 3.5 mM</td>
<td>CPA 7 mM</td>
<td>CPA 14 mM</td>
<td>CPA 28 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>18</td>
<td>15</td>
<td>17</td>
<td>17</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>% Teratogenic eggs*</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>-</td>
<td>10</td>
<td>25</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 90 min, 100 rpm, 32°C, Tris-Acetate (0.1 M) at pH 7.6, 2 hpf, β-Naphthoflavone induced rat liver XenoTech microsomes (0.35 mg/ml), NADPH (1 mM) and PP test tubes.

**Note:**
* Percentage of teratogenic eggs based on surviving eggs.

The following terms were abbreviated: CPA: Cyclophosphamide; MAS: Metabolic Activation System; PB: Phenobarbital; βNF: β-Naphthoflavone; -: No embryo effects; N: Number.

All the control groups fulfilled the validation criteria (≤ 10% impaired embryos). In all the test groups, except for the first concentration (1.75 mM), little effects (lethal and teratogenic) were observed (max. three affected eggs). Lethal and teratogenic effects did not display any concentration-response effect. HCl substitution by acetate did not increase the number of lethal and teratogenic fish embryos.
4.1.3.4 Agitation

Rate processes, such as xenobiotic transformation or metabolite uptake, depend on access to the CYP active site and to their ability to cross membranes. Therefore, different rates of stirring were tested to evaluate their contribution to CPA biotransformation. 20 Fish eggs were incubated with CPA and MAS for different shaking speeds, 100, 125 or 150 rpm. Results are displayed in Tab. 4-23.

### Table 4-23 Influence of agitation on CPA biotransformation rate

<table>
<thead>
<tr>
<th>rpm</th>
<th>100</th>
<th>125</th>
<th>150</th>
<th>100</th>
<th>125</th>
<th>150</th>
<th>100</th>
<th>125</th>
<th>150</th>
<th>100</th>
<th>125</th>
<th>150</th>
<th>100</th>
<th>125</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA 28 mM</td>
<td></td>
<td></td>
<td></td>
<td>CPA 3.5 mM</td>
<td></td>
<td></td>
<td></td>
<td>CPA 7 mM</td>
<td></td>
<td></td>
<td></td>
<td>CPA 14 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPA MAS control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPA MAS control (βNF-PB)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>5</td>
<td>4</td>
<td>-</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>16</td>
<td>18</td>
<td>18</td>
<td>15</td>
<td>14</td>
<td>18</td>
<td>13</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>10</td>
<td>-</td>
<td>25</td>
<td>22</td>
<td>-</td>
<td>24</td>
<td>40</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>-</td>
<td>30</td>
<td>5</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 90 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, β-Naphthoflavone rat liver XenoTech microsomes (0.35 mg/ml), NADPH (1 mM) and PP test tubes.

**Note:** Percentage of teratogenic eggs based on surviving eggs.

The following terms were abbreviated: **CPA**: Cyclophosphamide; **MAS**: Metabolic Activation System; **PB**: Phenobarbital; **βNF**: β-Naphthoflavone; -: No embryo exhibited effects; **rpm**: rotation per minute; **N**: Number.

The controls (CPA and MAS alone) fulfilled the validation criterion i.e., no fish eggs displayed any lethal or teratogenic effects.
For each stirring rate, there was a concentration-dependent response in defected embryos (coagulated and malformed).

For low CPA concentrations (3.5 mM and 7 mM), impaired eggs decreased with increasing agitation while for high CPA concentrations (14 mM and 28 mM) it increased. Therefore high agitation speed could enhance rate processes.
4.1.3.5 Start of exposure

The chorion membrane, a thick three-layer membrane encircling zebrafish embryo, works as a barrier to the external medium composition. Before hardening with time the chorion is still permeable at early stages (≤ 1 hpf) (Adams et al., 2005; Bonsignorio et al., 1996; Gellert and Heinrichsdorff, 2001). Therefore, 20 fish eggs were incubated 1 hpf instead of 2 hpf with CPA and MAS to enhance metabolite uptake into the eggs. Results are displayed in Tab. 4-24.

Table 4-24 Influence of start of exposure on CPA biotransformation rate

<table>
<thead>
<tr>
<th></th>
<th>Negative control</th>
<th>MAS control (βNF-PB)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA 28 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 hpf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hpf, 2 hpf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>% Teratogenic eggsa</td>
<td>-</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>10</td>
<td>-</td>
<td>15</td>
</tr>
</tbody>
</table>

Incubation conditions: 90 min, 32°C, 100 rpm, 1 hpf, Tris-HCl (0.1 M) at pH 7.6, β-Naphthoflavone and Phenobarbital induced rat liver XenoTech microsomes (0.35 mg/ml), NADPH (1 mM) and PP test tubes.

Note:
a Percentage of teratogenic eggs based on surviving eggs.

The following terms were abbreviated: hpf: hours post fertilization; CPA: Cyclophosphamide; MAS: Metabolic Activation System; PB: Phenobarbital; βNF: β-Naphthoflavone; -: No embryo effects; N: Number.

Control groups have ≤ 10% malformed embryos. There were no concentration-dependent lethal and teratogenic effects at 1 or 2 hpf. However, when test groups were incubated 1 hpf, teratogenic and lethal effects were in general stronger than 2 hpf. Therefore, chorion might be more permeable at early stages leading to more severe impairments in fish embryos.
### 4.1.3.6 Vessels

Polypropylene test tubes were used for the mDarT development method since they are thought to limit microsomal protein and lipophilic xenobiotic adhesion onto the test tubes surface. However, glass test tubes are generally used for liver microsomes studies. To verify the impact of the vessel on CPA biotransformation, 20 fish eggs were exposed in glass test tubes with CPA and MAS. Results are displayed in Tab. 4-25.

**Table 4-25 Influence of glass test tubes on CPA biotransformation rate**

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control</th>
<th>MAS control (βNF-PB)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA 28 mM</td>
<td></td>
<td>MAS CPA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.5 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28 mM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>-</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>-</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 90 min, 32°C, 100 rpm, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, β-Naphthoflavone rat liver XenoTech microsomes (0.35 mg/ml), NADPH (1 mM) and glass test tubes.

**Note:** Percentage of teratogenic eggs based on surviving eggs.

The following terms were abbreviated: CPA: Cyclophosphamide; MAS: Metabolic Activation System; PB: Phenobarbital; βNF: β-Naphthoflavone; -: No embryo effects; N: Number. %: percentage.

The experiment is considered to be valid as the number of impaired embryos is ≤ 10% in control groups. The test groups combined with the MAS and CPA showed teratogenic and lethal effects, but it was not concentration-dependent as the greatest effect was observed with one concentration (14 mM). Therefore, glass test tubes did not enhance CPA biotransformation.
4.1.4 Standard protocol

Microsome concentration was previously 0.35 mg/ml as a standard value with MAS. Biotransformation of the test material is usually proportional to the microsome concentration. To verify this assumption, 20 fish eggs were exposed with CPA and a rat liver microsome final concentration of 0.7 mg/ml. Results are displayed in Tab. 4-26.

Table 4-26 Influence of microsomes concentration on CPA biotransformation rate

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative Control CPA 28 mM</th>
<th>MAS Control (βNF-PB)</th>
<th>Test groups CPA Mas CPA 3.5 mM</th>
<th>Test groups CPA Mas CPA 7 mM</th>
<th>Test groups CPA Mas CPA 14 mM</th>
<th>Test groups CPA Mas CPA 28 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>5</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>19</td>
<td>19</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>26</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>65</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 60 min, 32°C, 100 rpm, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, β-Naphthoflavone rat liver XenoTech microsomes (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

**Note:** Percentage of teratogenic eggs based on surviving eggs.

The following terms were abbreviated: **CPA**: Cyclophosphamide; **MAS**: Metabolic Activation System; **PB**: Phenobarbital; **βNF**: β-Naphthoflavone; - : No embryo effects; **N**: Number.

The experiment is considered to be valid as the number of impaired embryos is ≤ 10% in the controls. The test groups were concentration-dependent effects for lethality and teratogenic. Indeed, while the lowest concentration (3.5 mM) displayed little teratogenicity (5%), the highest concentrations, namely 14 and 28 mM showed respectively 90% and 100% teratogenic eggs.

The mDarT standard protocol (see Tab. 4-27) consists therefore of a one hour incubation at 32°C, 100 rpm, with 20 fish embryos 2 hpf, in Tris-HCl.
(0.1 M) at pH 7.6 exposed to a test material, a final concentration of XenoTech rat liver microsomes of 0.7 mg/ml and 1 mM NADPH in polypropylene test tubes.

Table 4-27 Standard protocol incubation conditions

<table>
<thead>
<tr>
<th></th>
<th>mDarT standard protocol conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>32°C</td>
</tr>
<tr>
<td>Time of exposure</td>
<td>1 h</td>
</tr>
<tr>
<td>Start of exposure</td>
<td>2 hpf</td>
</tr>
<tr>
<td>Vessels</td>
<td>Polypropylene test tubes</td>
</tr>
<tr>
<td>Agitation</td>
<td>100 rpm</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.1 M Tris-HCl dissolved in fish medium</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
<tr>
<td>Cofactors</td>
<td>1 mM NADPH</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.7 mg/ml</td>
</tr>
<tr>
<td>Organic solvent</td>
<td>DMSO 0.2 %</td>
</tr>
</tbody>
</table>

4.1.5 Sample preparation for lipophilic conditions

4.1.5.1 Overview

B[a]P solubility is dramatically low in standard test conditions. Poorly soluble compounds tend to give erratic assay results (Di et al., 2006). While the presence of an organic solvent in the aqueous buffer can enhance the solubility of compounds, the assay itself can only tolerate a very small amount of organic solvent. Indeed, a high amount of organic solvent can inhibit CYP enzyme activity and/or affect fish egg development (Chauret et al., 1998). Two sample preparation methods were adapted from the aqueous dilution and cosolvent method as described by Di et al. in 2006 to enhance B[a]P solubility. These two methods may limit precipitation and reduce non-specific binding to plastic-surfaces.
4.1.5.2 Cosolvent method

B[a]P was prepared following the sample preparation described in Annexe A and added to 20 fish eggs. Results are displayed in Table 4-28.

Table 4-28 Influence of the cosolvent method on benzo[a]pyrene biotransformation rate

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control</th>
<th>MAS control (βNF)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B[a]P 50 µM</td>
<td></td>
<td>MAS B[a]P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 µM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 60 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, βNF induced rat liver XenoTech microsomes (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

**Note:**

* Percentage of teratogenic eggs based on surviving eggs. Every control and test group had a DMSO and ACN final concentration of 0.2% and 1% (v/v), respectively.

The following terms were abbreviated: B[a]P: Benzo[a]pyrene; MAS: Metabolic Activation System; βNF: β-Naphthoflavone; -: No embryo effects; DMSO: Dimethyl sulfoxide; ACN: Acetonitrile; N: Number; v/v: volume/volume.

Neither controls nor test groups fulfilled the validation criterion (i.e., 100% coagulated embryos). Therefore, the cosolvent method was not appropriate for mDarT.
4.1.5.3 Aqueous dilution method

B[a]P was prepared following the sample preparation described in Annexe A and added to 20 fish eggs. Results are displayed in Table 4-29.

Table 4-29 Influence of the aqueous dilution method on benzo[a]pyrene biotransformation rate

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative Control</th>
<th>MAS control (βNF)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B[a]P 50 µM</td>
<td></td>
<td>MAS B[a]P 6 µM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Normal developed eggs</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Incubation conditions: 60 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, βNF induced rat liver XenoTech microsomes (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

Note:

* Percentage of teratogenic eggs based on surviving eggs. Every control and test group had a DMSO final concentration of 0.2% (v/v). The following terms were abbreviated: B[a]P: Benzo[a]pyrene; MAS: Metabolic Activation System; βNF: β-Naphthoflavone; -: No embryo effects; DMSO: Dimethyl Sulfoxide; N: Number; v/v: volume/volume.

All controls fulfilled the validation criterion (≤ 10% impaired embryos). However, all test groups exhibited no effect on fish embryos. The aqueous dilution method addition did not improve B[a]P biotransformation.

4.1.5.4 Summary of sample preparation

The cosolvent or aqueous dilution methods did not promote greater lethal or teratogenic effects on fish embryos. Therefore, the standard mDarT protocol was used further with B[a]P.
4.2 Examples of proteratogens tested with mDarT

4.2.1 Cyclophosphamide

4.2.1.1 Controls

As summarized in Table 4-30, the negative control (CPA without MAS) triggered no lethal effects in the fish embryos and only 1 of 60 fish eggs displayed malformations (head) within 3 experiments (2% of surviving eggs) (see Table 4-31). In the 3 MAS control experiments (MAS alone), 3 fish embryos were coagulated (5% of all eggs) and 1 fish embryo displayed malformations of the sacculi/otoliths and the end tail (2% of surviving eggs) (Table 4-32). The total percentage of teratogenic and/or lethal eggs in each individual control group experiment consisting of 20 eggs each (negative or MAS control) was $\leq 10\%$ 48 hpf and, thus, all experiments were considered to be valid.

Table 4-30 Lethal effects of cyclophosphamide with mDarT

<table>
<thead>
<tr>
<th>Effects</th>
<th>Time</th>
<th>Negative control(^a) (CPA)</th>
<th>MAS control(^a) (βNF-PB)</th>
<th>MAS CPA 3.5 mM</th>
<th>MAS CPA 7 mM</th>
<th>MAS CPA 14 mM</th>
<th>MAS CPA 28 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulated eggs</td>
<td>8 hpf</td>
<td>- - -</td>
<td>- 1</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td></td>
<td>24 hpf</td>
<td>- - -</td>
<td>- 2</td>
<td>- - -</td>
<td>1 3 1</td>
<td>1 2 1</td>
<td>6 5 7</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- - -</td>
<td>1 2</td>
<td>- - -</td>
<td>1 3 1</td>
<td>2 2 1</td>
<td>15 18 8</td>
</tr>
</tbody>
</table>

Note.

\(^a\) Three independent experiments values are represented in each group

The following terms were abbreviated: CPA: Cyclophosphamide; MAS: Metabolic Activation System; hpf: hours post fertilization; - : No lethality observed; PB: Phenobarbital; βNF: β-Naphthoflavone.

4.2.1.2 Lethal and/or teratogenic effects of metabolic activated cyclophosphamide in zebrafish D. rerio embryos

The lethal and/or teratogenic effects observed 48 hpf, which were induced in fish embryos incubated with both CPA and rat liver microsomes, are summarized in Tables 4-30, 4-31 and 4-32.
Table 4-30 summarizes the lethal effects observed in fish eggs incubated with different concentrations of metabolized CPA. There were no or 5 coagulated eggs of 60 fish eggs at 3.5, 7.0 and 14.0 mM CPA, respectively. However, in all 3 single experiments a significant increase of coagulated fish eggs was observed 48 hpf at the highest concentration 28 mM (41 of 60 fish eggs). In this group a strong increase in the incidence of coagulated eggs was observed between 24 and 48 hpf, respectively.

Table 4-31 Overview of lethal and teratogenic effects of cyclophosphamide with mDarT

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MAS control&lt;sup&gt;(βNF-PB)&lt;/sup&gt;</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA 28 mM</td>
<td>CPA 3.5 mM</td>
<td>CPA 7 mM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>59</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td>% Teratogenic eggs&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (±3)</td>
<td>2 (±3)</td>
<td>7 (±3)</td>
</tr>
<tr>
<td>% Lethal eggs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>5 (±5)</td>
<td>-</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 60 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, βNF & PB induced rat liver microsomes from XenoTech (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

**Note.**

<sup>a</sup> Three independent experiments values are represented in each group

<sup>b</sup> Mean percentage ± SD of teratogenic eggs based on surviving eggs (3 experiments)

<sup>c</sup> Mean percentage ± SD of lethal eggs based on all eggs (3 experiments)

**At p < 0.01, there was a significant difference with the other groups

The following terms were abbreviated: **CPA:** Cyclophosphamide; **MAS:** Metabolic Activation System; **PB:** Phenobarbital; **βNF:** β-Naphthoflavone; -: No embryo effects; Σ: Sum; N: Number.
In the test groups containing both MAS and CPA, the percentage of teratogenic eggs (teratogenic eggs/surviving eggs) clearly increased with increasing CPA concentration from 7% (3.5 mM CPA) to 100% (28 mM CPA) reaching significance at the two highest CPA concentrations 14 and 28 mM ($p < 0.01$) as summarized in Table 4-31. The absolute number of fish eggs displaying teratogenic effects increased from 4 (3.5 mM CPA) to 34 (14 mM CPA). At the highest concentration (28 mM CPA) the lethal effects were predominant i.e., 41 from 60 fish eggs were coagulated. Thus, the absolute number of teratogenic fish eggs in the highest concentration group dropped, however, all 19 surviving fish embryos of this group displayed malformations (100% teratogenic eggs).

The absolute and relative incidences of the different CPA-induced teratogenic endpoints in zebrafish are summarized in Table 4-32 for the different control and test groups. With the exception of rachischisis all possible malformations listed in Table 3-2 were observed in fish embryos treated with metabolized CPA. However, some malformations, such as effects on the head occurred more frequently than others. Teratogenic effects were considered as “fingerprint-endpoints” if the following criteria were fulfilled: (i) concentration-response relationship and (ii) the endpoint must be observed in $\geq 50\%$ of all teratogenic fish eggs in the test groups. As 67 fish eggs exposed to CPA displayed one or more teratogenic effects, a fingerprint-endpoint must be observed in at least 33 fish eggs within the three experiments (Tab. 4-32).
Table 4-32 Overview of teratogenic effects of cyclophosphamide metabolite(s) in zebrafish *D. rerio* embryos

<table>
<thead>
<tr>
<th>Malformations</th>
<th>Time</th>
<th>Control groups</th>
<th>Test groups</th>
<th>(\Sigma_t)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative control (CPA)</td>
<td>MAS CPA</td>
<td>MAS CPA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAS control (βNF-PB)</td>
<td>3.5 mM</td>
<td>7 mM</td>
</tr>
<tr>
<td>Head</td>
<td>24 hpf</td>
<td>-</td>
<td>2/58</td>
<td>3/60</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>1/60 (2%) (^a)</td>
<td>-</td>
<td>2/60 (3%)</td>
</tr>
<tr>
<td>Tail</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>End tail</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sacculi/Otoliths</td>
<td>48 hpf</td>
<td>-</td>
<td>1/57 (2%)</td>
<td>2/60 (3%)</td>
</tr>
<tr>
<td>Heart</td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spinal cord structure</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>24 hpf</td>
<td>-</td>
<td>1/58</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rachischisis</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4-32 to be continued

<table>
<thead>
<tr>
<th>Malformations</th>
<th>Time</th>
<th>Negative control (CPA)</th>
<th>MAS control (βNF-PB)</th>
<th>MAS CPA 3.5 mM</th>
<th>MAS CPA 7 mM</th>
<th>MAS CPA 14 mM</th>
<th>MAS CPA 28 mM</th>
<th>Σ₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deformity of yolk</td>
<td>24 hpf</td>
<td>-</td>
<td>1/58</td>
<td>1/60</td>
<td>1/55</td>
<td>2/56</td>
<td>7/42</td>
<td>20/67</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3/55 (5%)</td>
<td>8/55 (15%)</td>
<td>9/19 (47%)</td>
<td></td>
</tr>
<tr>
<td>Growth retardation</td>
<td>24 hpf</td>
<td>-</td>
<td>1/58</td>
<td>1/60</td>
<td>7/55</td>
<td>4/56</td>
<td>39/42</td>
<td>20/67</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>1/60 (2%)</td>
<td>7/55 (13%)</td>
<td>2/55 (4%)</td>
<td>10/19 (53%)</td>
<td></td>
</tr>
<tr>
<td>Sum of teratogenic eggs</td>
<td>1/60</td>
<td>1/57</td>
<td>4/60</td>
<td>10/55</td>
<td>34/55</td>
<td>19/19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note.**

One teratogenic egg can display multiple malformations

a Number of eggs displaying this endpoint/number of surviving eggs (%)

b Number of eggs displaying this endpoint / total number of teratogenic eggs in the test groups.

c Major fingerprints (> 50%) are represented in bold

d Sum of the teratogenic eggs per group / number of surviving eggs after 48 hpf

The following terms were abbreviated: **CPA**: Cyclophosphamide; **MAS**: Metabolic Activation System; **hpf**: hours post fertilization; **PB**: Phenobarbital; **βNF**: β-Naphthoflavone; - : No embryo effects; Σ₁: Sum of the teratogenic eggs per endpoint out of all teratogenic eggs in the 4 test groups after 48 hpf.
Following these criteria, malformations of the head (51/67) (Fig. 4-2 A, B and D), the sacculi/otoliths (46/67) (Fig. 4-2 A, B, C and D), and the spinal cord structure (38/67) (Fig. 4-2 C and D), are defined as fingerprint malformations of CPA in fish embryos (Fig. 4-2). Other malformations such as effects on the end tail (Fig. 4-2 C) or scoliosis (Fig. 4-2 B and C) were also observed, however, they didn't fulfill the criteria for fingerprint endpoints.

Figure 4-2 Inverted microscope image of fish embryos exposed to cyclophosphamide and microsomes

A. 24 hpf, 3.5 mM CPA. B. 24 hpf, 7 mM CPA. C. 48 hpf, 14 mM CPA. D. 48 hpf, 28 mM CPA. Abbreviations: E: Eye; ET: End Tail; H: Head; hpf: hours post fertilization; PO: Pericardial Oedema; S: Scoliosis; SC: Spinal Cord; S/O: Sacculi/Otoliths; YSO: Yolk Sac Oedema; *: See Glossary.
Figure 4-3 illustrates CPA-induced teratogenic effects depending on the concentration of CPA and on the anatomic region within the fish embryo. Figures 4-3 A, 4-3 B and 4-3 C display frontal, axial and ventral malformations, respectively. Figure 4-3 D presents growth retardation, i.e., the developmental stages of the test groups were compared with the control groups.

**Figure 4-3** Summary of individual morphologic malformations in zebrafish *D. rerio* embryos exposed to cyclophosphamide and microsomes 48 hpf.

Effects on the frontal zone (A), on the axial zone (B) and on the ventral zone (C) are differentiated. (D) CPA-induced growth retardation. The standard deviation for each parameter is the result of 3 independent experiments.
A clear concentration-response was obtained for all malformations observed at the frontal (Fig. 4-3A), axial (Fig. 4-3B) and ventral (Fig. 4-3C) zones (see also Table 4-32), i.e., the incidence of malformations increased with increasing concentrations of CPA. In contrast, a clear incidence of growth retardation (Fig. 4-3D) was only observed in the test group with the highest CPA concentration.

Taken together, a clear concentration-response was observed for both lethal and teratogenic effects in *D. rerio* fish eggs exposed to CPA and a mammalian metabolic activation system. Significant increases (p < 0.01) in teratogenic effects compared to the controls were observed in test groups exposed to 14 and 28 mM CPA. Moreover, a significant increase (p < 0.01) in lethality was observed in the highest concentration group (28 mM CPA). Three fingerprint malformations (effects on the head, sacculi/otoliths and spinal cord structure) were identified in the fish embryos.

Based on these three studies, a clear concentration-response relationship was observed with CPA and MAS, regarding both teratogenic and lethal effects.
4.2.2 Ethanol

4.2.2.1 Concentration range finding

A concentration range finding with multiple concentrations of ethanol was investigated with 20 fish embryos per group (see Tab. 4-33).

Table 4-33 Influence of ethanol on zebrafish *D. rerio* development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Internal control</th>
<th>Ethanol groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl 0.1 M</td>
<td>EtOH 42.5 mM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

**Incubation conditions**: 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, and PP test tubes.

**Note:**
The following terms were abbreviated: EtOH: Ethanol; -: No embryo effects; N: Number.

The internal control fulfilled the validation criterion (≤ 10% impaired embryos). At 340 mM, ethanol was highly lethal (80% coagulated eggs). Therefore, a final ethanol concentration of up to 170 mM will be combined with MAS and fish eggs.

Microsomes are able to oxidize ethanol via CYPs isoforms 2E1, 1A2 and 3A4 (Das and Vasudevan, 2007). However, CYP2E1 has the highest specificity to oxidize ethanol (Guengerich, 2003),(Jain and Lyer, 2004). Isoniazid induced rat liver microsomes have the highest content of CYP2E1. Therefore, these microsomes will be combined with ethanol.
4.2.2.2 Controls

As shown in Tables 4-34 and 4-35, only 1 coagulated fish egg (2% of all fish eggs) and 4 fish eggs displaying teratogenic effects (7% of surviving embryos) were obtained in 3 negative control experiments each consisting of 20 fish embryos exposed to 170 mM ethanol without MAS. In the 3 MAS control experiments (MAS alone), no teratogenic effects were observed, but 4 of 60 fish eggs were coagulated (7% of all fish eggs). However, for each control group and for the 3 experiments, the acceptance criterion of ≤ 10% impaired embryos after 48 hpf was satisfied and, thus, the experiments were considered to be valid.

Table 4-34 Lethal effects of ethanol with mDarT

<table>
<thead>
<tr>
<th>Effects</th>
<th>Time</th>
<th>Negative control* (EtOH)</th>
<th>MAS control (INH)</th>
<th>MAS EtOH 42.5 mM</th>
<th>MAS EtOH 85 mM</th>
<th>MAS EtOH 170 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulated Eggs</td>
<td>8 hpf</td>
<td>1 - -</td>
<td>1 - -</td>
<td>1 - -</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td></td>
<td>24 hpf</td>
<td>1 - -</td>
<td>1 1 2</td>
<td>1 2 1</td>
<td>- 1 1</td>
<td>- 10 2</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>1 - -</td>
<td>1 1 2</td>
<td>2 2 1</td>
<td>- 1 2</td>
<td>9 11 2</td>
</tr>
</tbody>
</table>

Incubation conditions: 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, Isoniazid induced rat liver microsomes from XenoTech (0.7 mg/ml), NADPH (1 mM), 2 hpf, and PP test tubes.

Note.

* Three independent experiments values are represented in each group

The following terms were abbreviated: EtOH: Ethanol; MAS: Metabolic Activation System; hpf: hours post fertilization; INH: Isoniazid; - : No lethality observed.

4.2.2.3 Lethal and/or teratogenic effects of metabolic activated ethanol in zebrafish D. rerio embryos

Lethal and/or teratogenic effects observed 48 hpf in fish embryos incubated with both ethanol and rat liver microsomes, are summarized in Tables 4-34, 4-35 and 4-36.
Table 4-34 summarizes the lethal effects observed in fish eggs incubated with different concentrations of metabolized ethanol. Only 5 and 3 coagulated eggs out of 60 eggs were observed in the test groups exposed to 42.5 and 85 mM ethanol, respectively. However, a significant increase in coagulated fish embryos (22 of 60 fish eggs) was observed after 48 hpf at the highest concentration of 170 mM ethanol.

Table 4-35 Overview of lethal and teratogenic effects of ethanol with mDarT

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control</th>
<th>MAS control (INH)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td></td>
<td></td>
<td>MAS EtOH</td>
</tr>
<tr>
<td>170 mM</td>
<td></td>
<td></td>
<td>42.5 mM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>- 2 2</td>
<td>2 2 2</td>
<td>5 2 7</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>1 - -</td>
<td>2 2 1</td>
<td>- 1 2</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>19 18 18</td>
<td>16 16 17</td>
<td>15 17 11</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>4 - -</td>
<td>6 14</td>
<td>17</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>1 4 5</td>
<td>5 3</td>
<td>22</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>55 56 49</td>
<td>49 43</td>
<td>21</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>7 (±6)</td>
<td>11 (±0)</td>
<td>25 (±14)*</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>2 (±3)</td>
<td>7 (±3)</td>
<td>5 (±5)</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, Isoniazid induced rat liver microsomes from XenoTech (0.7 mg/ml), NADPH (1 mM), 2 hpf, and PP test tubes.

**Note.**

* Three independent experiments values are represented in each group
* Mean percentage ± SD of teratogenic eggs based on surviving eggs (3 experiments)
* Mean percentage ± SD of lethal eggs based on all eggs (3 experiments)
* At p < 0.05, there was a significant difference with the other groups

The following terms were abbreviated: EtOH: Ethanol; MAS: Metabolic Activation System; INH: Isoniazid; -: No embryo effects; Σ: Sum; N: Number.
In the test groups, the incidence of eggs displaying malformations (teratogenic eggs/surviving eggs) increased with increasing ethanol concentrations from 11% (42.5 mM ethanol) to 45% (170 mM) reaching significance at the two highest ethanol concentrations 85 and 170 mM (p < 0.05) as summarized in Table 4-35. The absolute number of fish eggs displaying teratogenic effects were 6, 14 and 17 at 42.5, 85, and 170 mM ethanol, respectively, i.e., the absolute number of teratogenic eggs was comparable for the two highest ethanol concentrations. However, the incidence of teratogenicity (teratogenic eggs/surviving eggs) increased with increasing ethanol concentrations, as the lethality was much higher at 170 mM ethanol (22 coagulated eggs) compared to 85 mM ethanol (3 coagulated eggs).

The absolute and relative incidences of the different ethanol-induced teratogenic endpoints in zebrafish are summarized in Table 4-36. With the exception of rachischisis all possible malformations listed in Table 3-2 have been observed in fish embryos treated with metabolized ethanol. However, some malformations, such as effects on the sacculi/otoliths, occurred more frequently than others. According to the criteria mentioned in section 4.2.1.2, as 37 fish eggs exposed to ethanol displayed one or more teratogenic effects, a fingerprint-endpoint must be observed in at least 18 fish eggs within the 3 experiments.
Table 4-36 Overview of teratogenic effects of ethanol metabolite(s) in the zebrafish *D. rerio*

<table>
<thead>
<tr>
<th>Malformations</th>
<th>Time</th>
<th>Control groups</th>
<th>Test groups</th>
<th>Σ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative control (EtOH)</td>
<td>MAS control (INH)</td>
<td>MAS EtOH 42.5 mM</td>
<td>MAS EtOH 85 mM</td>
</tr>
<tr>
<td>Head</td>
<td>24 hpf</td>
<td>1/59</td>
<td>-</td>
<td>4/56</td>
<td>9/58</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>3/55 (5%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7/57 (12%)</td>
</tr>
<tr>
<td>Tail</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>1/58</td>
<td>-</td>
</tr>
<tr>
<td>End tail</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>2/56</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>1/58 (2%)</td>
<td>-</td>
<td>3/55 (5%)</td>
<td>2/57 (4%)</td>
</tr>
<tr>
<td>Sacculi/Otoliths</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>4/56</td>
<td>7/58</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>2/58 (3%)</td>
<td>-</td>
<td>6/55 (11%)</td>
<td>13/57 (23%)</td>
</tr>
<tr>
<td>Heart</td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>4/55 (7%)</td>
<td>5/57 (9%)</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>24 hpf</td>
<td>1/59</td>
<td>-</td>
<td>6/56</td>
<td>5/58</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>1/58 (2%)</td>
<td>-</td>
<td>3/55 (5%)</td>
<td>8/57 (14%)</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>4/55 (7%)</td>
<td>10/57 (18%)</td>
</tr>
<tr>
<td>Rachischisis</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4-36 to be continued

<table>
<thead>
<tr>
<th>Malformations</th>
<th>Time</th>
<th>Negative control (EtOH)</th>
<th>MAS control (INH)</th>
<th>MAS EtOH 42.5 mM</th>
<th>MAS EtOH 85 mM</th>
<th>MAS EtOH 170 mM</th>
<th>Σt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deformity of Yolk</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4/58</td>
<td>3/48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>3/55 (5%)</td>
<td>4/57 (7%)</td>
<td>6/38 (16%)</td>
<td>13/37</td>
</tr>
<tr>
<td>Growth Retardation</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>2/56</td>
<td>5/58</td>
<td>7/48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>4/55 (7%)</td>
<td>4/57 (7%)</td>
<td>6/38 (16%)</td>
<td>14/37</td>
</tr>
<tr>
<td><strong>Sum of teratogenic eggs</strong></td>
<td></td>
<td>4/58</td>
<td>-/55</td>
<td>6/55</td>
<td>14/57</td>
<td>17/38</td>
<td></td>
</tr>
</tbody>
</table>

**Note.**

- One teratogenic egg can display multiple malformations
- Number of eggs displaying this endpoint/number of surviving eggs (%)
- Number of eggs displaying this endpoint / total number of teratogenic eggs in the test groups.
- Major fingerprints malformations (> 50%) are represented in bold
- Sum of the teratogenic eggs per group / number of surviving eggs after 48 hpf

The following terms were abbreviated: EtOH: Ethanol; INH: Isoniazid; MAS: Metabolic Activation System; hpf: hours post fertilization; -: No embryo effects; Σt: Sum of the teratogenic eggs per endpoint out of all teratogenic eggs in the 3 test groups after 48 hpf.
According to the above-mentioned definition of fingerprint malformations, the major endpoints were the effects on the sacculi/otoliths (29/37) (Fig. 4-4 A, B and C), the spinal cord structure (26/37) (Fig. 4-4 A, B and C), and the head (19/37) (Fig. 4-4 A and C) as well as scoliosis (19/37) (Fig. 4-4 B and C) (≥ 50% of all teratogenic fish eggs in the test groups).

Figure 4-4 Inverted microscope image of fish embryos exposed to ethanol and microsomes.

A. 42.5 mM EtOH, 24 hpf. B. 85 mM EtOH, 48 hpf. C. 170 mM EtOH, 48 hpf. The following terms are abbreviated: SC: Spinal Cord; H: Head; Ht: Heart; PO: Pericardial Oedema; S/O: Sacculi/Otoliths; YS: Yolk sac; YSO: Yolk Sac Oedema.
In Figure 4-5, the teratogenic effects of ethanol are presented depending on the anatomic region within the fish embryo. Figures 4-5 A, 4-5 B and 4-5 C display frontal, axial and ventral malformations, respectively. Figure 4-5 D summarizes growth retardation.

A clear concentration-response was obtained for all teratogenic effects at the frontal (Fig. 4-5 A) and ventral (Fig. 4-5 C) zones. In addition, a concentration-dependent retardation of fish embryo development was observed in 4 (42.5 mM and 85 mM) and 6 fish eggs (170 mM) (Fig. 4-5 D). The incidence of most malformations of the axial region increased with increasing concentrations of ethanol. However, incidences of end tail defects (Fig. 4-5 B) were not concentration dependent and again no rachischisis was observed (Fig.4-5 B).
Figure 4-5 Summary of individual morphologic malformations in zebrafish *D. rerio* embryos exposed to ethanol and microsomes 48 hpf.

Effects on the frontal zone (A), on the axial zone (B) and on the ventral zone (C). (D). Ethanol-induced growth retardation. The standard deviation for each parameter is the result of 3 independent experiments.

Based on these 3 studies, a clear increase of teratogenic incidences was observed with increasing concentrations of metabolic activated ethanol, reaching significance (p < 0.05) at 85 and 170 mM ethanol, respectively. In addition, lethality was significantly increased (p < 0.05) at the highest test material concentration (170 mM ethanol). Moreover, four characteristic malformations (effects on the sacculi/otoliths, cordal structure and head as well as scoliosis) were identified in fish embryos exposed to metabolically activated ethanol.
4.2.3 Benzo[a]pyrene

4.2.3.1 Concentration range finding

A concentration range finding study with multiple concentrations of B[a]P alone was investigated with 20 fish embryos per group (see Tab. 4-37).

Table 4-37 Influence of benzo[a]pyrene on zebrafish *D. rerio* development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Internal control</th>
<th>Benzo[a]pyrene groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl 0.1 M</td>
<td>B[a]P 6 µM</td>
</tr>
<tr>
<td></td>
<td>0.2% DMSO (v/v)</td>
<td></td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

*Incubation conditions:* 60 min, 100 rpm, 32°C, Tris-HCl (0.1 M) at pH 7.6, 2 hpf, and PP test tubes.

*Note:* The following terms were abbreviated: B[a]P: Benzo[a]pyrene; DMSO: Dimethyl Sulfoxide; -: No embryo effects; N: Number; v/v: volume/volume.

The internal control and all the B[a]P groups fulfilled the validation criterion (≤10% impaired embryos). Therefore B[a]P concentration was combined with mDarT up to 50 µM.
4.2.3.2 Selection of microsomes

4.2.3.2.1 βNF & PB induced rat liver microsomes

20 Fish eggs were incubated with different concentrations of B[a]P and β-Naphthoflavone & Phenobarbital induced rat liver microsomes that cover a broad range of CYP activities (see Tab. 1-2). Results are displayed in Tab. 4-38.

Table 4-38 Influence of βNF-PB induced rat liver microsomes and benzo[a]pyrene on zebrafish *D. rerio* development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control</th>
<th>MAS control (βNF-PB)</th>
<th>Positive control</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B[a]P 50 µM</td>
<td>MAS CPA 28 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>1</td>
<td>-</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>2</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>19</td>
<td>18</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>5</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>-</td>
<td>10</td>
<td>65</td>
<td>20</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 60 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, βNF-PB induced rat liver XenoTech microsomes (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

**Note:**

* Percentage of teratogenic eggs based on surviving eggs.

Every control and test group had a DMSO final concentration of 0.2% (v/v). The following terms were abbreviated: B[a]P: Benzo[a]pyrene; CPA: Cyclophosphamide; MAS: Metabolic Activation System; PB: Phenobarbital; βNF: β-Naphthoflavone; -: No embryo effects; N: Number; v/v: volume/volume.

The negative and the MAS control groups fulfilled the validation criterion (≤ 10% impaired embryos) and the positive control showed that MAS was active (100% teratogenic eggs).
When B[a]P was incubated with MAS some defects on fish embryos were observed. Low concentrations caused 4 coagulated eggs (20%) at 6 µM and 2 teratogenic eggs (11%) and 1 lethal egg (5%) at 12 µM. However, for both high concentrations (25 µM and 50 µM), no differences from the negative and MAS control groups were observed.

There was no concentration dependent response between B[a]P-concentration and no significant difference was observed between the test groups and the controls. In conclusion, the βNF-PB induced rat liver microsomes were not appropriate for B[a]P biotransformation.
4.2.3.2.2 βNF induced rat liver microsomes

B[a]P can undergo monooxygenation catalyzed by the microsomal CYP isoforms 1A1 and 1B1, to yield a series of arene oxides, which can be hydrated by mEHs to yield the corresponding toxic metabolites B[a]P-7,8-dihydrodiol-9,10-epoxide (Jain and Lyer, 2004) (see Fig. 1-26).

20 Fish eggs were incubated with B[a]P and β-Naphthoflavone induced rat liver microsomes that contain high concentrations of CYP1A amongst others (see Tab. 1-2). Results are displayed in Tab. 4-39.

Table 4-39 Influence of βNF induced rat liver microsomes and benzo[a]pyrene on zebrafish D. rerio development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control</th>
<th>MAS control (βNF)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>% Teratogenic eggs(^a)</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>-</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

Incubation conditions: 60 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, βNF induced rat liver XenoTech microsomes (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

Note:
\(^a\) Percentage of teratogenic eggs based on surviving eggs.
Every control and test group had a DMSO final concentration of 0.2% (v/v). The following terms were abbreviated: B[a]P: Benzo[a]pyrene; MAS: Metabolic Activation System; βNF: β-Naphthoflavone; -: No embryo effects; N: Number; v/v: volume/volume.

Negative and MAS control groups fulfilled the validation criterion of ≤ 10% impaired embryos. In the test groups, defects (teratogenic and lethal) were
detected for the first three concentrations (6, 12 and 25 µM). However, at 50 µM, no effect was observed.

B[a]P’s high lipophilicity (LogK_{OW} = 6) may limit its solubilisation in Tris-HCl buffer and decrease the biotransformation potential with microsomes at B[a]P concentration above 25 µM. To overcome B[a]P insolubility, new sample preparation methods were investigated as described in the section 4.1.5.

### 4.2.3.3 Controls

As shown in Tables 4-40 and 4-41, there was 1 coagulated fish embryo (2% of all fish eggs) in 3 negative control experiments each consisting of 20 fish embryos exposed to 50 µM B[a]P without MAS. In the 3 MAS control experiments (MAS alone) there was 1 teratogenic fish egg. For each control group and for the 3 experiments, the acceptance criterion of ≤ 10% impaired embryos 48 hpf was satisfied and thus the experiment were considered to be valid.

### 4.2.3.4 Lethal and/or teratogenic effects of metabolic activated benzo[a]pyrene in zebrafish D. rerio embryos

Lethal and/or teratogenic effects observed 48 hpf in fish embryos incubated with both B[a]P and induced rat liver microsomes are summarized in Tables 4-40, 4-41 and 4-42.

**Table 4-40 Lethal effects benzo[a]pyrene with mDarT**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulated eggs*</td>
<td>8 hpf</td>
<td>- - 1</td>
<td>- - -</td>
<td>2 1 1</td>
<td>2 - 2</td>
<td>1 1 -</td>
<td>- - -</td>
</tr>
<tr>
<td></td>
<td>24 hpf</td>
<td>- - 1</td>
<td>- - -</td>
<td>2 3 3</td>
<td>4 1 2</td>
<td>4 6 2</td>
<td>- 2 1</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- - 1</td>
<td>- - -</td>
<td>2 3 3</td>
<td>5 1 2</td>
<td>4 6 2</td>
<td>- 2 1</td>
</tr>
</tbody>
</table>

*Note.

* Three independent experiments values are represented in each group
The following terms were abbreviated: **B[a]P**: Benzo[a]pyrene; **MAS**: Metabolic Activation System; **hpf**: hours post fertilization; - : No lethality observed.

Table 4-41 summarizes the lethal effects observed in fish eggs incubated with different concentrations of metabolized B[a]P. Lethality increased with the time for the first 3 concentrations from 8 hpf to 24 hpf due to early severe impairments leading to coagulation. However, a significant decrease in coagulated fish embryos (3 out of 60 fish eggs) was observed for the highest concentration (50 µM B[a]P). Lethality was not significantly different between test and control groups.

**Table 4-41 Overview of lethal and teratogenic effects of benzo[a]pyrene with mDarT**

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control</th>
<th>MAS control (βNF)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Teratogenic eggs</td>
<td>- - -</td>
<td>- - 1</td>
<td>2 1</td>
</tr>
<tr>
<td>N Lethal egg</td>
<td>- - 1</td>
<td>- - -</td>
<td>2 3 3</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20 20 19</td>
<td>20 19</td>
<td>16 16 13 14 18 16</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>1</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>59 59</td>
<td>45 48</td>
<td>37</td>
</tr>
<tr>
<td>% Teratogenic eggs</td>
<td>-</td>
<td>2 (±3)</td>
<td>14 (±9)</td>
</tr>
<tr>
<td>% Lethal eggs</td>
<td>2 (±3)</td>
<td>-</td>
<td>13 (±3)</td>
</tr>
</tbody>
</table>

**Incubation conditions**: 60 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, βNF induced rat liver XenoTech microsomes (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

- **a** Three independent experiments values are represented in each group
- **b** Mean percentage ± SD of teratogenic eggs based on surviving eggs (3 experiments)
- **c** Mean percentage ± SD of lethal eggs based on all eggs (3 experiments)
- **At p < 0.05, there was a significant difference with the other groups**

Every control and test group had a DMSO final concentration of 0.2% (v/v).
The following terms were abbreviated: B[a]P: Benzo[a]pyrene; MAS: Metabolic Activation System; βNF: β-Naphthoflavone; -: No embryo effects; Σ: Sum; N: Number; v/v: volume/volume.

As summarized in Tab. 4-41, at the lowest concentration (6 µM) 7 teratogenic eggs were detected (14% of surviving embryos). At 12 µM, 4 fish embryos (8% of surviving embryos) displayed abnormalities. There was a significant increase (p < 0.05) in teratogenic eggs at 25 µM with 11 fish eggs exhibiting multiple malformations (23% of surviving embryos), including growth retardation (see Fig. 4-6 A) or head malformations (see Fig. 4-6 C). The number of impaired eggs for the highest concentration (50 µM) dropped with 6 fish embryos (11% of surviving embryos) showing defects, mainly on the tail (see Fig. 4-6 B and D). In spite of teratogenic clear effects in fish embryos exposed to different concentrations, there was no concentration-dependent effect.

The absolute and relative incidences of the different B[a]P-induced teratogenic endpoints are summarized in Table 4-42. With the exception of rachischisis all possible malformations listed in Table 3-2 were observed in fish embryos treated with metabolized B[a]P. According to the criteria mentioned in section 4.2.1.2, as 8 fish eggs exposed to B[a]P displayed one or more teratogenic effects, a fingerprint-endpoint must be observed in at least 14 fish eggs within the 3 experiments.
Table 4-42 Overview of teratogenic effects of benzo[a]pyrene metabolite(s) on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>Malformations</th>
<th>Time</th>
<th>Control groups</th>
<th>Test groups</th>
<th>( \Sigma_t )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative Control (B[a]P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAS Control ((\beta) NF)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>24 hpf</td>
<td>- 2/60</td>
<td>4/52 2/53</td>
<td>4/48 1/57</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- -</td>
<td>1/52 (2%)</td>
<td>1/52 (2%)</td>
</tr>
<tr>
<td>Tail</td>
<td>24 hpf</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- -</td>
<td>1/52 (2%)</td>
<td>- -</td>
</tr>
<tr>
<td>End tail</td>
<td>24 hpf</td>
<td>- -</td>
<td>- 1/53</td>
<td>1/48 -</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- -</td>
<td>3/52 (6%)</td>
<td>1/52 (2%)</td>
</tr>
<tr>
<td>Sacculi/Otoliths</td>
<td>24 hpf</td>
<td>- -</td>
<td>3/52 2/53</td>
<td>4/48 -</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- -</td>
<td>3/52 (6%)</td>
<td>1/52 (2%)</td>
</tr>
<tr>
<td>Heart</td>
<td>48 hpf</td>
<td>- 1/60 (2%)</td>
<td>1/52 (2%)</td>
<td>1/52 (2%)</td>
</tr>
<tr>
<td>Spinal Cord Structure</td>
<td>24 hpf</td>
<td>- -</td>
<td>2/52 2/53</td>
<td>3/48 -</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- -</td>
<td>4/52 (8%)</td>
<td>1/52 (2%)</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>24 hpf</td>
<td>- -</td>
<td>1/52 -</td>
<td>1/48 -</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- -</td>
<td>3/52 (6%)</td>
<td>1/52 (2%)</td>
</tr>
<tr>
<td>Rachischisis</td>
<td>24 hpf</td>
<td>- -</td>
<td>- - -</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>- -</td>
<td>- - -</td>
<td>- -</td>
</tr>
</tbody>
</table>
Table 4-32 to be continued

<table>
<thead>
<tr>
<th>Malformations</th>
<th>Time</th>
<th>Negative Control (B[a]P)</th>
<th>MAS Control (βNF)</th>
<th>MAS B[a]P 6 µM</th>
<th>MAS B[a]P 12 µM</th>
<th>MAS B[a]P 25 µM</th>
<th>MAS B[a]P 50 µM</th>
<th>Σt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deformity of Yolk</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/53</td>
<td>-</td>
<td>6/28</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>2/52 (4%)</td>
<td>-</td>
<td>3/48 (6%)</td>
<td>1/57 (2%)</td>
<td></td>
</tr>
<tr>
<td>Growth Retardation</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>1/52</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/48 (2%)</td>
<td>-</td>
<td>1/28</td>
</tr>
<tr>
<td><strong>Sum of teratogenic eggs</strong>c</td>
<td></td>
<td>-/59</td>
<td>1/60</td>
<td>7/52</td>
<td>4/52</td>
<td>11/48</td>
<td>6/57</td>
<td></td>
</tr>
</tbody>
</table>

Note.

One teratogenic egg can display multiple malformations

a Number of eggs displaying this endpoint / number of surviving eggs (%)

b Sum of the teratogenic eggs per group / number of teratogenic eggs after 48 hpf

c Sum of the teratogenic eggs per group / number of surviving eggs after 48 hpf

The following terms were abbreviated: B[a]P: Benzo[a]pyrene; MAS: Metabolic Activation System; hpf: hours post fertilization; βNF: β-Naphthoflavone; -: No embryo effects; Σt: Sum of the teratogenic eggs per endpoint out of all teratogenic eggs in the 4 test groups after 48 hpf.

No malformation fulfilled the “fingerprint” criteria. Therefore no characteristic teratogenic endpoint was detected for B[a]P in fish embryos.
Figure 4-6 Inverted microscope image of fish embryos exposed to benzo[a]pyrene and microsomes.

In Figure 4-7, the teratogenic effects are presented depending on the B[a]P concentrations and the anatomic region within the fish embryo. Figures 4-7 A, 4-7 B and 4-7 C display frontal, axial and ventral malformations, respectively. Figure 4-7 D summarizes growth retardation.

There was no concentration-response for any of the teratogenic effects at the frontal (Fig. 4-7 A) axial (Fig. 4-7 B) or ventral (Fig. 4-7 C) zones. There was no effect on growth retardation (Fig. 4-7 D).

**Figure 4-7** Summary of individual morphologic malformations in zebrafish *D. rerio* embryos exposed to benzo[a]pyrene and microsomes 48 hpf.

Effects on the frontal zone (A), on the axial zone (B) and on the ventral zone (C) are differentiated. (D). Benzo[a]pyrene-induced growth retardation. The standard deviation for each parameter is the result of 3 independent experiments.
Based on these 3 studies, there was no concentration-response of B[a]P in the mDarT, with a drop of lethal and teratogenic effect at 50 µM. However, the incidence of malformations in fish embryos combined with B[a]P and MAS reached significance (p < 0.05) at 25 µM. In spite of the teratogenic effects observed, no clear fingerprint was identified with B[a]P metabolites on zebrafish *D. rerio* embryos.
4.2.4 Thalidomide

4.2.4.1 Concentration range finding

A concentration range finding study with multiple concentrations of thalidomide (Thal) alone was investigated with 20 fish embryos per group (see Tab. 4-43).

Table 4-43 Influence of thalidomide on zebrafish *D. rerio* development

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Internal control</th>
<th>Thalidomide groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl 0.1 M</td>
<td>Thal 100 µM</td>
</tr>
<tr>
<td></td>
<td>0.2% DMSO (v/v)</td>
<td></td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>% Normal eggs</td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

**Incubation conditions:** 60 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, and PP test tubes.

**Note:**
The following terms were abbreviated: Thal: Thalidomide; DMSO: Dimethyl Sulfoxide; -: No embryo effects; N: Number; v/v: volume/volume.

The internal control and thalidomide groups fulfilled the validation criterion (≤ 10% impaired embryos). Therefore, Thal concentration up to 400 µM was combined with MAS.

4.2.4.2 Overview

Although much careful work has been done in an attempt to understand the mechanism of action of thalidomide, the reason for the chemical and species specificity remain unclear. In *in vivo* studies, rats are least sensitive to thalidomide whereas rabbits display concordant limb malformations to
humans (see Chapter 1.5.4.3.). Therefore, rabbit liver microsomes were used to be combined with the fish embryos.

4.2.4.3 Controls

As shown in Tables 4-44 and 4-45, no coagulated or teratogenic egg were observed in the 3 negative control experiments each consisting of 20 fish embryos exposed to 400 µM Thal without MAS. In the 3 MAS control experiments, 1 teratogenic effect (heart malformation) was observed and 2 out of 60 fish eggs were coagulated (3% of all fish eggs). However, for each control group and for the 3 experiments, the acceptance criterion of \( \leq 10\% \) impaired embryos 48 hpf was satisfied and, thus, the experiments were considered to be valid.

4.2.4.4 Lethal and/or teratogenic effects of metabolic activated thalidomide in D. rerio embryos

Lethal and/or teratogenic effects observed 48 hpf in fish embryos incubated with both thalidomide and rabbit liver microsomes, are summarized in Tables 4-44, 4-45 and 4-46.

Table 4-44 Lethal effects of thalidomide with mDarT

<table>
<thead>
<tr>
<th>Effects</th>
<th>Time</th>
<th>Negative control(^a) (Thal)</th>
<th>MAS control (Rabbit)</th>
<th>MAS Thal 100 µM</th>
<th>MAS Thal 200 µM</th>
<th>MAS Thal 400 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulated Eggs</td>
<td>8 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Note.

\(^a\) Three independent experiments values are represented in each group

The following terms were abbreviated: Thal: Thalidomide; MAS: Metabolic Activation System; hpf: hours post fertilization; - : No lethality observed.
Table 4-44 summarizes the lethal effects observed in fish eggs incubated with different concentrations of metabolized thalidomide. Lethality was only observed in the highest concentration (400 µM). However, lethality was not significantly different between test and control groups.

Table 4-45 Overview of lethal and teratogenic effects of thalidomide with mDarT

<table>
<thead>
<tr>
<th>48 hpf</th>
<th>Negative control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MAS control (Rabbit)</th>
<th>Test groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thal 400µM</td>
<td>MAS Thal 100 µM</td>
<td>MAS Thal 200 µM</td>
</tr>
<tr>
<td>N Teratogenic eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Lethal eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N Normal eggs</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Σ Teratogenic eggs</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Σ Lethal eggs</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Σ Normal eggs</td>
<td>60</td>
<td>57</td>
<td>59</td>
</tr>
<tr>
<td>% Teratogenic eggs&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>2 (±3)</td>
<td>2 (±3)</td>
</tr>
<tr>
<td>% Lethal eggs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>3 (±3)</td>
<td>-</td>
</tr>
</tbody>
</table>

Incubation conditions: 60 min, 32°C, Tris-HCl (0.1 M), 2 hpf at pH 7.6, non-induced rabbit liver XenoTech microsomes (0.7 mg/ml), NADPH (1 mM) and PP test tubes.

Note.

<sup>a</sup> Three independent experiments values are represented in each group

<sup>b</sup> Mean percentage ± SD of teratogenic eggs based on surviving eggs (3 experiments)

<sup>c</sup> Mean percentage ± SD of lethal eggs based on all eggs (3 experiments)

*At p < 0.05, there was a significant difference with the other groups

Every control and test group had a DMSO final concentration of 0.2% (v/v)

The following terms were abbreviated: DMSO: Dimethyl Sulfoxide; Thal: Thalidomide; MAS: Metabolic Activation System; -: No embryo effects; Σ: Sum; N: Number, v/v: volume/ volume.

In the test groups, the incidence of eggs displaying malformations (teratogenic eggs/surviving eggs) increased with increasing Thal concentrations.
from 2% (100 and 200 µM thalidomide- see Fig. 4-8 A) to 16% (400 µM) reaching significance at the highest Thal concentration (p < 0.05) (Table 4-45).

The absolute and relative incidences of the different thalidomide-induced teratogenic endpoints in zebrafish are summarized in Table 4-46. With the exception of scoliosis and rachischisis all possible malformations listed in Table 3-2 were observed in fish embryos treated with metabolized Thal. However, some malformations, such as effects on the head, occurred more frequently than others. According to the criteria mentioned in section 4.2.1.2, as 11 fish eggs exposed to Thal displayed 1 or more teratogenic effects, a fingerprint-endpoint must be observed in at least 6 fish eggs within the 3 experiments (Tab. 4-46).
Table 4-46 Overview of teratogenic effects of thalidomide metabolite(s) on zebrafish *D. rerio* embryonic development

<table>
<thead>
<tr>
<th>Malformations</th>
<th>Time</th>
<th>Control groups</th>
<th>Test groups</th>
<th>( \Sigma_t )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative control (Thal)</td>
<td>MAS control (Rabbit)</td>
<td>MAS Thal 100 µM</td>
</tr>
<tr>
<td>Head</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tail</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>End tail</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sacculi/Otoliths</td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heart</td>
<td>48 hpf</td>
<td>-</td>
<td>1/59 (2%)</td>
<td>-</td>
</tr>
<tr>
<td>Spinal Cord Structure</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rachischisis</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 4-46 to be continued

<table>
<thead>
<tr>
<th>Malformations</th>
<th>Time</th>
<th>Negative control (Thal)</th>
<th>MAS control (Rabbit)</th>
<th>MAS Thal 100 µM</th>
<th>MAS Thal 200 µM</th>
<th>MAS Thal 400 µM</th>
<th>Σt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deformity of Yolk</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/60</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>1/60 (2%)</td>
<td>1/60 (2%)</td>
<td>2/57 (4%)</td>
<td>4/11</td>
</tr>
<tr>
<td>Growth Retardation</td>
<td>24 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 hpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Sum of teratogenic eggs</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-/60</td>
<td>1/59</td>
<td>1/60</td>
<td>1/60</td>
<td>9/57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note.**

One teratogenic egg can display multiple malformations

<sup>a</sup> Number of eggs displaying this endpoint / number of surviving eggs (%)

<sup>b</sup> Number of eggs displaying this endpoint / total number of teratogenic eggs in the test groups.

<sup>c</sup> Major malformations (> 50%) represented in bold

<sup>d</sup> Sum of the teratogenic eggs per group / number of surviving eggs after 48 hpf

The following terms were abbreviated: **Thal**: Thalidomide; **MAS**: Metabolic Activation System; **hpf**: hours post fertilization; -: No embryo effects; Σt: Sum of the teratogenic eggs per endpoint out of all teratogenic eggs in the 3 test groups after 48 hpf.
According to the above-mentioned definition of fingerprint malformations, the major endpoints are shown in Figure 4-8. Effects on the head (6/11), sacculi/otoliths (6/11) (Fig. 4-8 B, C and D), (Fig. 4-8 C), and the heart (19/37) (Fig. 4-8 B, C and D) were predominant thalidomide-induced malformations in *D. rerio* embryos (≥ 50% of all teratogenic fish eggs in the test groups).

**Figure 4-8** Inverted microscope image of fish embryos exposed to thalidomide and microsomes

A. 24 hpf, 200 µM Thal. B. 48 hpf, 200 µM Thal. C. 24 hpf, 400 µM Thal. D. 48 hpf, 400 µM Thal. The following terms are abbreviated: H: Head; Ht: Heart; S/O: Sacculi/Otoliths; T: Tail; YS: Yolk sac.
In Figure 4-9, the teratogenic effects are presented depending on the thalidomide concentrations and the anatomic region within the fish embryo. Figures 4-9 A, 4-9 B and 4-9 C display frontal, axial and ventral malformations, respectively. A concentration-response was observed for the major endpoints on the frontal (Fig. 4-9 A) and ventral (Fig. 4-9 C) zones. The malformations (when present) on the axial region were independent of thalidomide concentration.

Figure 4-9 Summary of individual morphologic malformations in zebrafish *D. rerio* embryos exposed to thalidomide and microsomes 48 hpf. Effects on the frontal zone (A), on the axial zone (B) and on the ventral zone (C) are differentiated. The standard deviation for each parameter is the result of 3 independent experiments. Abbreviations: Thal: Thalidomide; MAS: Metabolic Activation System.
Based on these 3 studies, an increase in teratogenic incidences was observed with increasing concentrations of metabolic activated thalidomide, reaching significance ($p < 0.05$) at 400 µM. However, lethality was not significantly different between the control and test groups. Nevertheless, 3 characteristic malformations (effects on the head, sacculi/otoliths, and heart) were identified in fish embryos exposed to metabolically activated thalidomide.
5 Discussion

We have demonstrated the successful combination of the fish embryo assay, used for screening for embryotoxic/teratogenic effects, with a mammalian metabolic activation system (i.e., rat liver microsomes). The efficiency of this advanced in vitro screening model was tested using 4 mammalian proteratogens, namely, cyclophosphamide, ethanol, benzo[a]pyrene and thalidomide, as references. It was shown that the exposure of fish eggs to each proteratogen alone didn’t result in any significant teratogenic effect. Only the presence of the mammalian metabolic activation system triggered the formation of teratogenic and lethal effects in the exposed fish embryos.

With the exemption of rachischisis all teratogenic endpoints listed in Table 3-2 were observed in fish embryos exposed to metabolically activated CPA. The incidences of lethality and most teratogenic effects were clearly concentration-dependent and significantly increased (p < 0.01) in test groups exposed to 14 and 28 mM CPA. Moreover, a significant increase (p < 0.01) in lethality was observed in the highest concentration group (28 mM CPA). The major malformations, i.e., the teratogenic endpoints with the highest incidences, comprised effects on the head, the sacculi/otoliths and the spinal cord structure. These effects were defined as fingerprint-endpoints in fish embryos according to the definition provided in section 4.2.1.2. Like many other antineoplastic agents, CPA has been reported to elicit a full spectrum of developmental toxicities in mammals, including mice (Hackenberg and Von Kreybig, 1965), rats (Murphy, 1962) and rabbits (Gerlinger and Clavert, 1964). Defects included growth retardation (reduced fetal body weight) and effects on the limb, digit, palate and jaw when administered intraperitoneally during organogenesis at dose levels of 2 to 50 mg/kg/day. In humans, a specific syndrome of defects was identified, including congenital malformations of the digit, palate, ears, face, skin and in some cases the skull (Schardein and Macina, 2007). Thus, some of the major effects observed in fish embryos exposed to metabolically activated CPA are similar to characteristic anomalies observed in animals and humans.

As with CPA, exposure to ethanol without an external metabolic activation system was insufficient to induce any significant developmental malformations in fish embryos up to a concentration of 170 mM. Only the concomitant use of ethanol and microsomes provoked lethality and teratogenicity starting at the lowest concentration (42.5 mM). As demonstrated
by other groups, ethanol is able to induce malformations in fish embryos without a metabolic activation system and, thus, acting as a direct teratogen in this test system (Reimers et al., 2004; Reimers et al., 2006). However, these teratogenic effects were observed only at higher ethanol concentrations (200 mM) and/or increased incubation times. In fish eggs exposed to ethanol and microsomes, a clear increase in teratogenic incidences was observed with increasing concentrations of metabolic activated ethanol, reaching significance ($p < 0.05$) at 85 and 170 mM ethanol, respectively. In addition, lethality was significantly increased ($p < 0.05$) at the highest test material concentration (170 mM ethanol). Again, all malformations with the exception of rachischisis, were observed in fish embryos with different incidences. Based on the definition of fingerprint-endpoints, effects on the spinal cord structure, the sacculi/otoliths, the head as well as scoliosis were the major malformations observed. It is well known that ethanol is teratogenic in animals and humans. *In vivo* studies in several species (e.g., mice, rats, rabbits, guinea pigs) clearly demonstrated potent developmental toxicity, including retarded fetal growth, increased mortality and malformations. In humans, the most typical abnormalities are associated with the central nervous system, the craniofacial development, the cardiac system and the skeleton (Schardein and Macina, 2007). Again, major ethanol-induced effects observed in the fish embryos are similar to effects observed in ethanol exposed mammals and especially in humans. The malformations of the head and the spinal cord observed in fish embryos correspond to the craniofacial and skeletal abnormalities in humans.

As for the previous compounds, benzo[a]pyrene alone did not provoke significant lethal or teratogenic effect. The incidence of malformations on fish embryos combined with benzo[a]pyrene and MAS reached significance ($p < 0.05$) at 25 µM. However, there was no difference between the controls and the test groups in lethality. Moreover, there was no concentration-response due to a drop of lethal and teratogenic effects at the highest concentration (50 µM benzo[a]pyrene). Insolubility may explain those results. Indeed, if a heterogenous suspension is formed within the test tube, the compound quickly precipitates of solution as large particles and may not be able to interact with metabolizing enzymes in the liver microsomes, leading to no or little biotransformation. Furthermore, its high lipophilicity ($\log K_{\text{OW}} = 6$) may contribute to non-specific binding with microsomes, chorion and plasticware which may limit the benzo[a]pyrene free part in the suspension and at last reduce its biotransformation (Di et al., 2006). Again, high lipophilicity of benzo[a]pyrene could inhibit enzyme activity by denaturing microsomes vesicles.
3D structure (N. Hewitt, personal communication). Specific malformations on the fish eggs based on the definition of fingerprint-endpoints were not detected even though there were pronounced teratogenic effects. In mammals, benzo[a]pyrene is known to be teratogenic with mice being the most sensitive species. Reported defects include, exencephaly, thoraco- and gastroschisis, phocomelia and oedema (Shepard and Lemire, 2004). Benzo[a]pyrene-induced effects detected in the fish embryos were not specific and could not be compared with mammals.

Exposure to thalidomide without an external metabolic activation system did not significantly induce malformations in fish embryos up to a concentration of 400 µM. Only the concomitant use of thalidomide and rabbit liver microsomes provoked little but significant teratogenicity (p < 0.05) at the highest concentration tested. However, lethality was not affected at any concentration. Based on the definition of fingerprint-endpoints, effects on the sacculi/otoliths, the head as well as heart were the major malformations observed in the fish embryos. In mammals, thalidomide teratogenicity is species specific. In vivo studies in the most sensitive species (e.g., rabbits and monkeys) clearly demonstrated potent developmental toxicity, including retarded foetal growth and limb malformations. In humans, the most typical abnormalities are associated with the limbs (e.g., phocomelia), the central nervous system, the face, the ears, the cardiac system and the abdominal and visceral organs (Brent and Holmes, 1988). Major thalidomide-induced effects observed in the fish embryos are similar to effects observed in thalidomide exposed in humans but not in animals. Malformations of the head, the sacculi/otoliths and the heart observed in fish embryos correspond to the face, the ears and the cardiac system in humans. However, no concordant limb reduction defects were detected in fish embryos which are considered to be the most likely to occur when associated with thalidomide exposure in humans (Brent and Holmes, 1988). Furthermore, there is yet no agreement that (i) limb reduction defects would actually be present or detectable in fish embryos and (ii) what can be considered as a limb reduction defects in fish embryos. Furthermore, thalidomide effectiveness in humans produces severe limb reduction defects during a very narrow period of early organogenesis from the 22nd to the 36th day post conception whereas fish embryos exposure time with MAS covers the blastula period. Thus, fish embryos could be further incubated during organogenesis (75% Epiboly stadium) with MAS and thalidomide to investigate any teratogenic effects. Besides the exposure time, the activation system (i.e., rabbit liver microsomes) might not be suitable to provoke limb reduction defects.
Based on these results, hydrophilic compounds (cyclophosphamide and ethanol) appeared to be intensively metabolized with the MAS inducing severe abnormalities on fish embryos while the more lipophilic test materials (benzo[a]pyrene and thalidomide) didn’t provoked pronounced lethal and teratogenic effects: (i) Non-specific binding to microsomes, chorion and plasticware may reduce the free part of the test material in the test tubes and (ii) non-homogenous solution as well as (iii) hydrophobic effects are likely to limit lipophilic compounds biotransformation. Further input should be done on the standard test conditions for sample preparation.

Numerous advantages make this model an excellent in vitro alternative method to screen for teratogenic effects; (i) short time of the assay based on the short embryonic development (48 hpf, from the oviposition to the end of the test) (ii) the huge number of fish eggs daily available to perform screening studies and (iii) the ease to applying this technique in new facilities.

However, despite these advantages some limitations of the system have to be taken into account: (i) The chorion membrane enclosing the fish embryo might limit the uptake of very lipophilic substances or substances with a high molecular weight making it difficult to detect their proteratogenic potential (ii) Due to the toxicity of the microsomes to the fish embryos the contact time with the test material is limited to 1 hour, i.e., the fish embryos are exposed to the test item only during a small part of the developmental period. Although the exposure time covers the most sensitive phase of fish developmental period (as demonstrated with CPA, ethanol), some proteratogens, which are active at a later developmental stage, might be missed. (iii) The CYP P450 activity of liver microsomes depends on the inducing compound, with which the rats were treated. In general, the use of microsomes exhibiting a broad range of CYP activities is preferred (e.g., by using Phenobarbital/β-naphthoflavone-induced microsomes). However, proteratogens activated by a specific CYP P450 isoenzyme (e.g., CYP 2E1) might exhibit lower activity or might be even missed when using generally induced microsomes. Therefore, the application of different liver microsomes from rats treated with different inducers should be considered depending on the test material.
6 Conclusion

For the first time it has been described that an exogenous mammalian activation system (i.e., rat and rabbit liver microsomes) was successfully incorporated with zebrafish *D. rerio* embryos for the detection of teratogenic effects. Based on the results of cyclophosphamide, ethanol, benzo[a]pyrene and thalidomide, it could be shown that only the combination of the fish embryos with the metabolic activation system was able to detect the developmental toxicity of these proteratogenic compounds. For cyclophosphamide, ethanol and thalidomide a concentration-response relationship was shown and the qualitative nature of the malformations was similar between fish embryos and humans. Benzo[a]pyrene was demonstrated to be significantly teratogenic in fish embryos in spite of a lack of concentration-response and specific teratogenic fingerprints.
7  Outlook

In the future, a series of experiments should be performed to prove that the proteratogen metabolites of CPA, ethanol, B[a]P are active (if commercially available) within the fish eggs and compare them with the results obtained with the parent compound and MAS. Moreover, other known proteratogens if possible referring to a class of chemical or being a specific CYP family substrate should be tested in order to establish a database. Furthermore, microsome activity should be further demonstrated using (i) CYP inhibitors, or (ii) test material detoxified by the MAS (e.g., Cytochalasin D). Other efforts should be made (i) in testing others liver subcellular fractions (e.g., S9-mix, human microsomes) (ii) in the evaluation of this fish egg teratogenic tool, as the emergence of genomic and proteomic methods for zebrafish are now available. Lastly, a clear strategy must be established for the screening of new compounds. Metabolic stability assessed during xenobiotic development process or quantitative structure-activity relationship (QSAR) may give a clue to the CYP family involved in its metabolism. This can first orientate the choice of the appropriate animal induced liver microsomes. However, if no information are available, the test material might be incubated with induced liver microsomes covering a broad range of CYP activities. If the results were not satisfactory, it should be further refined using induced liver microsomes representing specific major human isoforms involved in the biotransformation of xenobiotics, namely CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. Furthermore, a positive control (e.g., CPA, ethanol) should always be incorporated in the test system to assess MAS activity when screening new compounds.
8 Summary

The assessment of teratogenic effects of chemicals is generally performed using in vivo teratogenicity assays e.g., in rats or rabbits. Following the 3R principles, the development of alternative methods is encouraged to reduce the number of animal tests. From this perspective, we have developed an in vitro assay (mDarT) using the zebrafish Danio rerio embryo teratogenicity assay (DarT) combined with an exogenous mammalian metabolic activation system (MAS), able to biotransform proteratogenic compounds. Cyclophosphamide, ethanol, benzo[a]pyrene and thalidomide were used as test materials to assess the efficiency of this assay. Briefly, the zebrafish embryos were co-cultured at 2 hpf (hours post fertilization) with the test material at varying concentrations, mammalian liver microsomes from different species and NADPH for 60 min at 32°C under moderate agitation in Tris buffer. The negative control (test material alone) and the MAS control (MAS alone) were incubated in parallel. For each test group, 20 eggs were used for statistical robustness. Afterwards fish embryos were transferred individually into 24-well plates filled with fish medium for 48 hours at 26°C with a 12 hour-light cycle. Teratogenicity was scored after 24 and 48 hpf using morphological endpoints. The test was considered to be valid if a minimum of 90% of fish eggs developed normally for the two controls (test material alone and MAS alone). For each test material, the experiment was repeated three times with the controls satisfying the validation criteria (≤ 10% impaired embryos). Indeed, no significant teratogenic effects were observed compared to controls in fish embryos exposed to the proteratogens alone (i.e., without metabolic activation) or the MAS alone. In contrast, the four test materials induced significant abnormalities in fish embryos when co-incubated with animal liver microsomes. For cyclophosphamide, ethanol and thalidomide a concentration-response relationship was shown and the qualitative nature of the malformations was similar between fish embryos and humans. Benzo[a]pyrene was demonstrated to be significantly teratogenic in fish embryos in spite of no concentration-response and unspecific teratogenic fingerprints. We conclude that the application of animal liver microsomes will improve and refine the DarT as a predictive and valuable alternative method to screen teratogenic substances.
Abbreviations

ACN: Acetonitrile
ADME: Absorption, Distribution, Metabolism and Excretion
ANOVA: Analysis of Variance
AT: Acetyl Transferase
Aro: Aroclor1254
B[a]P: Benzo[a]pyrene
BCA: Bicinchoninic Acid
BMELV: Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz
βNF: β-Naphthoflavone
BSA: Bovin Serum Albumin
CPA: Cyclophosphamide
CYP P450 or CYP: Cytochrome P450
DarT: Zebrafish D. rerio Teratogenic assay
DEX: Dexamethasone
DIN: Deutsch Industrie Norm
DMEM: Dulbecco's modified eagle medium
DMSO: Dimethyl sulfoxide
DOC: Dynamic organ culturing
E: Eye
ECVAM: European Center for the Validation of Alternative Methods
EDTA: Ethylenediaminetetraacetic acid
EHs: Epoxide Hydrolases
ENL: Erythema Nodosum Leprosum
ET: End tail
EtOH: Ethanol
ER: Endoplasmic Reticulum
EST: Embryonic stem cell test
FAS: Fetal Alcohol Syndrome
FET: Fish embryo toxicity test
FETAX: Frog Embryo Teratogenic Assay: Xenopus
FM: Fish Medium
FMOs: Flavin Monooxygenases
GST: Glutathione S-transferase
GT: Glutathione
H: Head
Ht: Heart
hpf: hours post fertilization
ICCVAM: Interagency coordinating committee on the validation of alternatives methods
INH: Isoniazid or Isonicotinyl Hydrazine
IVT: In Vitro Technologies
MAS: metabolic activation system
mDarT: metabolic Zebrafish *D. rerio* Teratogenic assay
mEHs: microsomal Epoxide Hydrolases
MM: Micromass test
NADPH: nicotinamide adenine dinucleotide phosphate reduced
da.: no data
NoT: No Tail detached
NRS: NADPH Regenerating System
OECD: Organization for Economic Cooperation and Development
PAHs: Polycyclic Aromatic Hydrocarbons
PAPS: 3’-Phosphoadenosine 5’-Phosphosulfate
PIC: Protein Inhibitor Cocktail
PB: Phenobarbital
PO: Pericardial Oedema
PP: Polypropylene
QMS: Quality Management System
REACH: Registration, Evaluation, and Authorization of Chemicals
ROS: Reactive Oxygen Species
rpm: rotation per minute
RT: Room temperature
S: Scoliosis
SC: Spinal Cord
SD: Standard deviation
sEH: Soluble epoxide hydrolase
S/O: Sacculi/otoliths
SM: Somites
STs: Sulfotransferases
T: Tail
Thal: Thalidomide
UDP-GA: α-D-UDP-glucuronic acid
UGT: UDP-glucuronosyltransferase
v/v: volume/volume
WEC: Whole embryo culture
YS: Yolk sac
**Abbreviations**

**YSE**: Yolk sac extension  
**YSO**: yolk sac oedema  
**ZEBET**: Die Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch
heart structure in the zebrafish *D. rerio*. At 48hpf, in fish embryos, the normal looping process places the ventricle and atrium side by side, so that the two chambers largely overlap each other in lateral view (Antkiewicz et al., 2005).

sacculi/otoliths. This is the primitive ear structure in the zebrafish *D. rerio* embryo. The zebrafish possess two otoliths per sacculi. The otoliths are dark round bones within the sacculi (or vesicle). The otoliths malformations included one to multiple otoliths per sacculi. The sacculi malformations included abnormally shaped vesicles (Reimers et al., 2004).

somites. Undifferenciated mesodermal component of an early trunk or tail segment or metamere, derived from paraxial mesoderm; forms the myotome, the sclerotome and perhaps the dermatome (Kimmel et al., 1995).

yolk. Nutrient store for embryonic development in the form of semicrystalline phospholipoprotein and contained within yolk granules (Kimmel et al., 1995)

yolk sac. Giant syncitial uncleaved cells containing the yolk; underlies the blastodisc early, and becomes enveloped by the blastoderm during epiboly (Kimmel et al., 1995)

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Mushiroda, T., Douya, R., Takahara, E., and Nagata, O. (2000). The Involvement of Flavin-Containing Monooxygenase but not CYP3A4 in Metabolism of Itpride Hydrochloride, a Gastroprokinetic Agent: Comparison with Cisapride and Mosapride Citrate. Drug Metab. Disp. 28, 1231-1237.


Bibliography


14 Annex A

a) Cyclophosphamide

The protocol for CPA drug biotransformation with mDarT is displayed in Table 14-1. CPA stock solution was dissolved in Tris-buffer and stored on ice.

Table 14-1 Protocol for CPA with mDarT

<table>
<thead>
<tr>
<th></th>
<th>MAS 28 mM</th>
<th>CPA 3.5 mM</th>
<th>MAS 7 mM</th>
<th>MAS 14 mM</th>
<th>MAS 28 mM</th>
</tr>
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<tr>
<td>Tris-HCl (0.1 M)</td>
<td>910</td>
<td>380</td>
<td>810</td>
<td>710</td>
<td>510</td>
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<tr>
<td>Microsomes (20 mg/ml)</td>
<td>70</td>
<td>-</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>CPA (20 mg/ml)</td>
<td>-</td>
<td>800</td>
<td>100</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>Fish eggs</td>
<td>820</td>
<td>820</td>
<td>820</td>
<td>820</td>
<td>820</td>
</tr>
<tr>
<td>NADPH (10 mM)</td>
<td>200</td>
<td>-</td>
<td>200</td>
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<tr>
<td>Final Volume</td>
<td>2000 µl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CPA: Cyclophosphamide; MAS: Metabolic Activation System; -: none.
b) Ethanol

The protocol for ethanol drug biotransformation with mDarT is shown in Table 14-2. Ethanol stock solution was stored at room temperature (RT).

<table>
<thead>
<tr>
<th></th>
<th>MAS</th>
<th>EtOH 170 mM</th>
<th>MAS EtOH 42.5 mM</th>
<th>MAS EtOH 85 mM</th>
<th>MAS EtOH 170 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (0.1 M)</td>
<td>910</td>
<td>1160</td>
<td>905</td>
<td>900</td>
<td>890</td>
</tr>
<tr>
<td>Microsomes (20 mg/ml)</td>
<td>70</td>
<td>-</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>EtOH</td>
<td>-</td>
<td>20</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Fish eggs</td>
<td>820</td>
<td>820</td>
<td>820</td>
<td>820</td>
<td>820</td>
</tr>
<tr>
<td>NADPH (10 mM)</td>
<td>200</td>
<td>-</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Final Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2000 µl</td>
</tr>
</tbody>
</table>

Abbreviations: EtOH: Ethanol; MAS: Metabolic Activation System; -: none
c) Benzo[a]pyrene

The protocol for B[a]P drug biotransformation with mDarT is summarized in Table 14-3. Four B[a]P stock solutions, respectively 3, 6, 12 and 25 mM, were dissolved in DMSO under dim light in brown glasses and stored at RT.

Table 14-3 Protocol with B[a]P and mDarT

<table>
<thead>
<tr>
<th>Tris-HCl (0.1 M)</th>
<th>MAS</th>
<th>B[a]P 50 µM</th>
<th>MAS B[a]P 6 µM</th>
<th>MAS B[a]P 12 µM</th>
<th>MAS B[a]P 25 µM</th>
<th>MAS B[a]P 50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>906</td>
<td>1176</td>
<td>906</td>
<td>906</td>
<td>906</td>
<td>906</td>
</tr>
<tr>
<td>Microsomes (20 mg/ml)</td>
<td>70</td>
<td>-</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Fish eggs</td>
<td>820</td>
<td>820</td>
<td>820</td>
<td>820</td>
<td>820</td>
<td>820</td>
</tr>
<tr>
<td>DMSO</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B[a]P</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NADPH (10 mM)</td>
<td>200</td>
<td>-</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Final Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2000 µl</td>
</tr>
</tbody>
</table>

Abbreviations: B[a]P: Benzo[a]pyrene; DMSO: Dimethyl Sulfoxide; MAS: Metabolic Activation System; -: none.
c-1) Aqueous dilution method

In the aqueous dilution method, as described in Fig. 14-1, an aqueous dilution step (Premix) was performed before addition of test material to the MAS (step D).

**Figure 14-1 Aqueous dilution method protocol**

A: PP test tubes at RT with microsomes diluted in Tris-HCl buffer (0.1 M). B: B[a]P was weighted under dim light in brown glasses at RT and dissolved in DMSO. C: PP vial at RT. D: PP test tubes were incubated at 32°C; the reaction was started by adding NADPH. Fish eggs and NADPH were previously diluted in Tris-HCl buffer (0.1 M). The following terms were abbreviated: B[a]P: Benzo[a]pyrene; DMSO: Dimethyl Sulfoxide; M: Microsomes; PP: polypropylene; RT: room temperature; (v/v): volume/volume; VT: Total volume.
c-2) Cosolvent method

As described in Fig. 14-2, the test material is being added directly to the microsomal proteins from an organic media (i.e., DMSO and Acetonitrille).

**Figure 14-2 Cosolvent method protocol**

**A:** B[a]P was weighted under dim light in brown glasses at RT and dissolved in DMSO. **B:** PP test tubes at RT with microsomes diluted in Tris-HCl buffer (0.1 M). **C:** Brown glasses were filled with 160 µl ACN at RT. B[a]P was transferred under dim light. **D:** PP vial at RT. **E:** PP test tubes were incubated at 32°C; the reaction was started by adding NADPH. Fish eggs and NADPH were previously diluted in Tris-HCl buffer (0.1 M). The following terms were abbreviated: **ACN:** Acetonitrille; **B[a]P:** Benzo[a]pyrene; **DMSO:** Dimethyl Sulfoxide; **M:** Microsomes; **PP:** polypropylene; **RT:** room temperature; (v/v): volume/volume; **VT:** Total volume.
d) Thalidomide

In Table 14-4, the protocol for Thal drug biotransformation with the mDarT is displayed. Three stock solutions of Thal respectively 5, 10 and 20 mM were dissolved in DMSO in brown glasses and stored at RT.

**Table 14-4 Protocol of Thal with mDarT**

<table>
<thead>
<tr>
<th></th>
<th>MAS 400 µM</th>
<th>MAS 100 µM</th>
<th>MAS 200 µM</th>
<th>MAS 400 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (0.1 M)</td>
<td>906</td>
<td>1176</td>
<td>906</td>
<td>906</td>
</tr>
<tr>
<td>Microsomes (20 mg/ml)</td>
<td>70</td>
<td>-</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Fish eggs</td>
<td>820</td>
<td>820</td>
<td>820</td>
<td>820</td>
</tr>
<tr>
<td>DMSO</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thal</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NADPH (10 mM)</td>
<td>200</td>
<td>-</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Final Volume</td>
<td></td>
<td></td>
<td></td>
<td>2000 µl</td>
</tr>
</tbody>
</table>

Abbreviations: B[a]P: Benzo[a]pyrene; DMSO: Dimethyl Sulfoxide; MAS: Metabolic Activation System; -: none.
15 Annex B

PUBLICATIONS

ABSTRACTS
German Society of Pharmacology and Toxicology (DGPT) 2005, Mainz:
Use of primary rat and human hepatocytes for the analysis of cytochrome P450
induction and inhibition
*Naunyn-Schmiedeberg's Archives of Pharmacology*. p371, Suppl. 1, R94

German Society of Pharmacology and Toxicology (DGPT) 2007, Mainz:
Busquet F, von Landenberg F, Mueller SO, Nagel R, and Broschard TH
Development of a screening assay for teratogenicity: combination of DarT with a
mammalian metabolic activation system
*Naunyn-Schmiedeberg's Archives of Pharmacology*. p375, Suppl. 1, 98

Altex 2007, Linz:
Busquet F, Nagel R, Mueller SO, von Landenberg F, and Broschard TH
Development of a screening assay for teratogenicity: combination of DarT with
mammalian metabolic activation system
*ALTEx*. p191, 24; 3/07

Eurotox 2007, Amsterdam:
Busquet F, Nagel R, von Landenberg F, and Broschard TH
Teratogenicity of Benzo[a]pyrene in the fish embryo assay (DarT) combined with
mammalian metabolic activation system
*Toxicology Letters*, pS75, 172, Suppl. 1, 10/07

PAPER
Toxicological Sciences:
Busquet F, von Landenberg F, Nagel R, Mueller SO, Huebler N, and Broschard TH
Development of a new screening assay to identify proteratogenic compounds usir
Zebrafish *Danio rerio* embryo combined with an exogenous mammalian metabol
activation system (mDarT)
Advance Access published March 28, 2008

AWARD

Hessian research award for animal welfare 2007 for the investigation and developme
of a method to improve animal welfare in science:
Broschard TH and Busquet F
Development of a new screening assay to identify proteratogenic compounds usir
Zebrafish Danio rerio embryo combined with an exogenous mammalian metabol
activation system (mDarT).
Erklärung

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Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zwecke der Promotion vorgelegt.

Ich bestätige, daß ich die Promotionsordnung der Fakultät Forst-, Geo- und Hydrowissenschaften der TU Dresden anerkenne.

Darmstadt, 22/04/08 François Dominique BUSQUET