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# Albendazole causes stage-dependent developmental toxicity and is deactivated by a mammalian metabolization system in a modified zebrafish embryotoxicity test

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ABSTRACT

The zebrafish embryotoxicity test has previously been combined with an external metabolic activation system (MAS) to assess developmental toxicity of metabolites produced by maternal metabolism. Due to toxicity of MAS the exposure was limited to one early and short period. We have modified the method and included additional testing time points with extended exposure durations. Using the anthelmintic drug albendazole as a model substance, we demonstrated stage-dependent toxic effects at three windows of zebrafish embryo development, *i.e.* 2–3, 12–14 and 24–28 h post fertilization, and showed that MAS, by metabolic deactivation, reduced the toxicity of albendazole at all time points. Chemical analysis confirmed that albendazole was efficiently metabolized by MAS to the corresponding sulfoxide and sulfone, which are non-toxic to zebrafish embryos. To conclude, the modified zebrafish embryotoxicity test with MAS can be expanded for assessment of metabolites at different developmental stages.

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## 1. Introduction

Development is a delicately coordinated process that may be disrupted by exposure to chemical substances. An increasingly popular model organism for assessing developmental toxicity is the zebrafish (*Danio rerio*) embryo, which has the convenience of cell-culture studies (*e.g.* fast, cost-effective assays amendable to high-throughput screening) but contrary to cell cultures it has the advantages of animal studies as it is a complete vertebrate embryo that can be studied regarding numerous relevant endpoints. Zebrafish embryos are transparent and develop externally, which greatly facilitates chemical exposure, manipulation and evaluation of numerous morphological, developmental and behavioral endpoints in the intact living embryo. The zebrafish embryo model has been evaluated regarding its ability to predict the teratogenic potential of chemicals in mammals. Using a 48-h *D. rerio* toxicity/teratogenicity test (*Dar*T), 88% of 41 tested compounds were found to be in agreement with findings from developmental toxicity tests in mammals [1,2]. A similarly high concordance was obtained in two more recent studies where 87% of 31 tested compounds in a 5-day assay [3] and 81% of 27 tested compounds in a 6-day assay [4] were correctly categorized concerning the embryotoxic potential. In contrast, Van den Bulck and colleagues recently reported a concordance of only 60% among 15 tested compounds. Of the misclassified compounds, four were false positives and two were false negatives [5]. The overall concordance in a study may depend on factors such as study design, bioavailability (placenta, chorion, etc.), the physico-chemical properties of the test compounds and the mammalian tests to which the results were compared. Furthermore, kinetic factors such as metabolism of the test compound may in certain cases explain the discordance between species. In the zebrafish embryotoxicity model, the exposure occurs directly via the ambient medium, and consequently the maternal metabolism of the test substance is not taken into account [6]. Endogenous metabolism by the zebrafish embryo itself is not much investigated and may be insignificant in a test situation and/or qualitatively different from that of humans. The zebrafish embryo test may thus underestimate the teratogenic potency of a substance that would be metabolically activated in a pregnant woman. Conversely, the potency may be overestimated

*Abbreviations:* ABZ, albendazole; ABZSO, albendazole sulfoxide; $ABZSO_2$, albendazole sulfone; $ABZSO_2NH_2$, albendazole-2-aminosulfone; dpf, days post fertilization; hpf, hours post fertilization; MAS, metabolic activation system; m*Dar*T, zebrafish embryotoxicity test with MAS; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; β-NF, β-naphtoflavone; DMSO, dimethyl sulfoxide; PB, phenobarbital.

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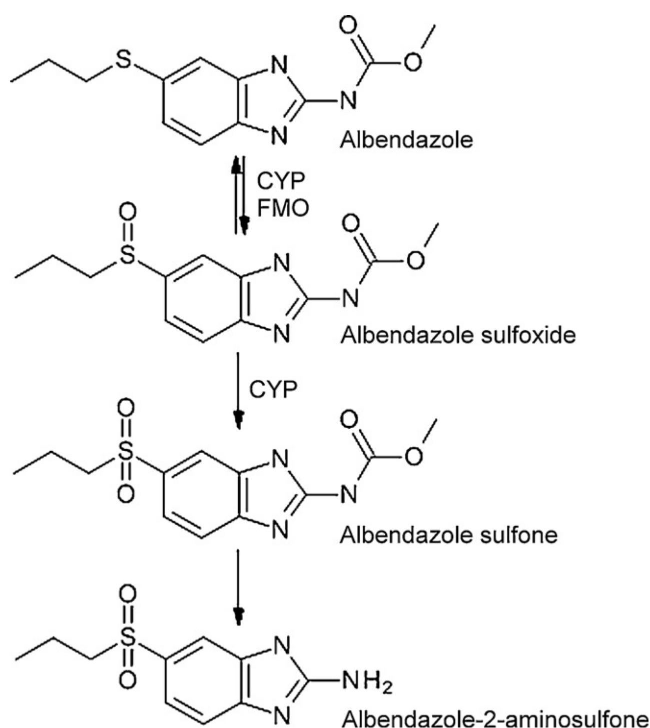


**Fig. 1.** Albendazole (ABZ) and its major metabolites. Albendazole is sequentially metabolized to albendazole sulfoxide (ABZSO), albendazole sulfone ($ABZSO_2$) and albendazole-2-aminosulfone ($ABZSO_2NH_2$) [16]. FMO: flavin-containing monooxygenase, CYP: cytochrome P450 monooxygenase.

for a substance that would undergo maternal metabolic deactivation in humans. Busquet et al. recently presented a zebrafish embryotoxicity model, called m*Dar*T, in which they incorporated an external mammalian metabolic activation system (MAS) that would mimic maternal metabolism [7]. The MAS was based on liver microsomes from rats and contains various xenobiotica-metabolizing flavin-containing monooxygenases (FMO) and cytochrome P450 (CYP) monooxygenases. The authors demonstrated metabolic activation of the pro-teratogenic compounds ethanol, cyclophosphamide and acetaminophen in the m*Dar*T assay [7,8]. Zebrafish embryos were exposed together in a vial containing the test substance and MAS for 1 h starting at 2 h post fertilization (hpf). The short exposure was chosen to avoid the toxicity of MAS itself. A major drawback of such a limited exposure window is that susceptible developmental processes that occur during other windows of development will not be considered, resulting in false negative results. One of Wilson's six principles of teratology stresses that susceptibility to developmental toxicants varies with the developmental stage at the time of exposure [9], and thus it would be a great advantage to enable use of the metabolizing system at several time points during development.

Albendazole (ABZ) is a benzimidazole methylcarbamate, which is used as an anthelmintic drug to control gastrointestinal parasites in humans and domestic animals. Administration of ABZ during gestation has been shown to cause embryotoxic effects in cattle, rat, rabbit and sheep [10]. Observed effects include increase of resorptions, decreased fetal weight and increase of teratogenic effects, such as vascular, craniofacial, skeletal and external malformations [11–15].

The metabolism of ABZ is similar in humans, rat, mice, cattle and sheep [10]. In mammals, ABZ undergoes a rapid and extensive first-pass oxidation to the pharmacologically active metabolite albendazole sulfoxide (ABZSO), which is sequentially metabolized to the inactive albendazole sulfone ($ABZSO_2$) and albendazole-2-aminosulfone ($ABZSO_2NH_2$) [16] (see Fig. 1). The formation of ABZSO has been shown to depend on both the CYP and the FMO system in the studied species [17–19]. CYP2C6, CYP3A1/2 and CYP2A1 are suggested to be involved in sulfoxidation of ABZ in rat liver microsomes [18].

There is conflicting evidence of the embryotoxic potential of ABZ. Due to the rapid metabolism, the unmetabolized form of ABZ has either not been identified or only found at low concentrations in plasma after treatment of animals or humans. Thus, the *in vivo* pharmacological and embryotoxic action of ABZ in mammals are generally assumed to be related to ABZSO [10]. ABZSO, but no other ABZ metabolite, produced the embryotoxic effects related to ABZ treatment in rats [20–22]. The association between developmental toxicity and metabolite disposition has been studied in rats 12 h following oral administration of the ABZ-forming pro-drug netobimin on gestation day 10 [13]. The authors found a significant correlation between rate of malformed embryos and concentrations of ABZ and ABZSO, but not $ABZSO_2$, in embryonic tissue. The lowest teratogenic dose tested resulted in ABZSO concentrations of ~1.5 μg/mL in maternal plasma and ~1.5 μg/g in embryo tissue at the termination of the experiment. Although the corresponding ABZ concentrations were substantially lower (~0.06 μg/g in embryo tissue) it could not be excluded that ABZ contributed to the observed embryotoxicity. However, co-administration of ABZ and an inhibitor of oxidative metabolism from day 8–15 of gestation nearly eliminated the toxic effects of ABZ in rat [20], suggesting that the metabolite rather than the parent compound is the cause of developmental toxicity. In contrast to the conclusions from the above mentioned *in vivo* studies, there are results from *in vitro* models suggesting that ABZ is a more potent toxicant than ABZSO, *e.g.* regarding inhibition of cell proliferation in micromass cell cultures of rat embryo midbrain and limb bud cells [23] and in a human hepatoma cell line lacking ABZ-metabolizing capacity [24]. The *in vitro* studies are in accordance with results from our laboratory, which show that only ABZ and not the metabolites ABZSO, $ABZSO_2$ and $ABZSO_2NH_2$ is embryotoxic in zebrafish embryos after continuous exposure from 2 hpf [25]. The reason for the discrepancy between the mammalian *in vivo* studies on the one hand and the zebrafish studies together with the *in vitro* studies on the other hand remains to be clarified. Nevertheless, it is clear that only ABZ, and not its mammalian metabolites, shows toxicity in zebrafish embryos and ABZ may thus be a useful model substance to study effects of metabolic transformation by the external mammalian metabolization system utilized in the m*Dar*T method.

The aim of the current work was to modify and optimize the m*Dar*T conditions to enable exposure of zebrafish embryos together with a mammalian metabolizing system for extended durations and at several developmental stages, *i.e.* at 2–3, 12–14 and 24–28 hpf. To improve the possibility to detect late and more subtle effects, we included additional endpoints of developmental toxicity and assessed these at several time points up to six days post fertilization. The exposure was also changed from grouped exposure to individual exposure in a microplate format, which renders each embryo a replicate. The modified m*Dar*T method was evaluated using ABZ as a model substance which, according to expectations, was efficiently metabolized and showed reduced toxicity in zebrafish embryos when tested together with the metabolization system. Formation of metabolites was confirmed by chemical analysis of the exposure medium.

## 2. Material and methods

### 2.1. Materials

Albendazole (ABZ, CAS# 54965-21-8, purity 99.0%) was obtained from Dr. Ehrensdorfer GmbH, Germany. Dimethyl sulfoxide (DMSO) and reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from

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