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# Comparison of albendazole cytotoxicity in terms of metabolite formation in four model systems

Lidia Radko, Maria Minta, Piotr Jedziniak, Sylwia Stypuła-Trębas

Department of Pharmacology and Toxicology,
National Veterinary Research Institute, 24-100 Pulawy, Poland

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**Abstract**

**Introduction:** Albendazole is used to treat endoparasitic diseases in animals and humans. After oral administration, it is quickly oxidised into its pharmacologically active metabolite albendazole sulfoxide and then to sulfone. However, it is not clear which compound is responsible for toxic effects towards mammalian cells. **Material and Methods:** The model systems comprised cultures of isolated rat hepatocytes, two hepatoma cell lines (FaO, HepG2), and non-hepatic Balb/c 3T3 line. Cells were exposed for 24, 48, and 72 h to eight concentrations of albendazole ranging from 0.05 to 100 μg/mL. At all three time points cytotoxic effects were assessed by MTT assay and metabolites in the culture media were determined by LC-MS/MS analysis. **Results:** The effective concentrations $EC_{50\text{-}72h}$ showed that Balb/c 3T3 cells were the most sensitive to albendazole (0.2 ±0.1 μg/mL) followed by FaO (1.0 ±0.4 μg/mL), and HepG2 (6.4 ±0.1 μg/mL). In the case of isolated hepatocytes this value could not be attained up to the highest concentration used. Chemical analysis revealed that the concentrations of albendazole in hepatocytes and HepG2 and FaO culture media gradually decreased with incubation time, while the concentrations of its metabolites increased. The metabolism in isolated hepatocytes was dozens of times greater than in HepG2 and FaO cells. Two metabolites (albendazole sulfoxide, albendazole sulfone) were detected in isolated hepatocytes and HepG2 culture medium, one (albendazole sulfoxide) in FaO culture medium and none in Balb/c 3T3. **Conclusion:** The obtained data indicate that metabolism of albendazole leads to its detoxification. The lower cytotoxic potential of metabolites was confirmed in the independent experiments in this study.

**Keywords:** rat hepatocytes, albendazole, metabolites, cytotoxicity, cell lines.

## Introduction

Albendazole (ABZ) is one of the most important anthelmintics belonging to the large chemical family of benzimidazoles, used in humans and animals. ABZ has also been exhaustively evaluated for use in food-producing animals (15, 27). Its acute toxicity is low and its wide safety margin is due to its greater affinity to parasitic β-tubulin than to mammalian tissues. However, it was classified as "high" priority for detailed risk assessment (5) because some adverse effects (developmental toxicity) were demonstrated in rats (3, 8, 26) and in other animals (10, 12). The most sensitive species are cattle and sheep. The observed differences in species sensitivity are most likely associated with varying degrees of absorption and pharmacokinetics (4, 11, 12, 17, 18). Oral absorption of ABZ in mouse and rat is about 20%–30% and in cattle and sheep 50%, compared to about 1% – 5% in humans (9). After absorption from the gastrointestinal tract this drug is rapidly oxidised to albendazole sulfoxide (ABZ-SO) and subsequently to albendazole sulfone (ABZ-$SO_2$). Albendazole sulfoxide is considered to be the therapeutically active form of the drug. The role of albendazole metabolism in its toxicity for humans was also decribed in the definition of marker residue, where the "sum" of albendazole sulfoxide, albendazole sulfone, and albendazole 2-amino sulfone was expressed as albendazole (15). However, from *in vivo* studies it is difficult to draw conclusions as to which compounds, ABZ or its metabolites, are responsible for toxic effects in treated animals.

The current study is a continuation of our previous research (20) in which cytotoxic potential of albendazole and its two main metabolites was compared

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using three assays in which different biochemical endpoints were assessed (lysosomal activity, proliferation, and membrane integrity).

The aim of this study was to assess cytotoxicity of ABZ in terms of metabolites formation. The same model systems *i.e.* cultures of isolated rat hepatocytes, two hepatoma cell lines (FaO, HepG2), and non-hepatic Balb/c 3T3 line were applied and another assay was used to evaluate influence on mitochondrial activity (MTT).

## Material and Methods

**Standards and reagents.** Analytical standards of albendazole (ABZ, CAS 54965-21-8), albendazole sulfoxide (ABZ-SO, CAS 54029-12-8), albendazole sulfone (ABZ-$SO_2$, CAS 75184-71-3), albendazole sulfoxide-D3 (ABZ-SO-D3, CAS 1448346-38-0) and albendazole-2-aminosulfone-(propyl-3,3,3) hydrochloride (ABZ-NH-$SO_2$, CAS 1435902-07-0) were purchased from Sigma-Aldrich, Poland. Dimethyl sulfoxide (DMSO), foetal bovine serum (FBS), bovine calf serum (BCS), 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), trypsin-EDTA, insulin, hydrocortisone, and antibiotic solution (10,000 U/mL of penicillin, 10 mg/mL of streptomycin) were purchased from Sigma-Aldrich (Poland). All other chemicals were purchased from commercial suppliers and were of the highest available purity.

**Isolation of hepatocytes and culture conditions**. Wistar albino rats were housed under standard laboratory conditions of lighting (12 h dark/12 h light), temperature (22 ±2°C), and relative humidity (50% – 60%) with free access to commercial feed (Altromin, Spezialfutter, Germany) and tap water. The cells were isolated from the liver of males weighing 250–350 g. The two-step collagenase perfusion technique was used, as described previously (25). Hepatocytes were cultured in a humidified incubator at 37°C, in an atmosphere of 5% $CO_2$, using William's E medium supplemented with 10% foetal bovine serum, 1 μM of insulin, 1 μM of hydrocortisone, 1% of glutamine, and 1% of antibiotics. The cells were seeded on 96-well plates coated with fibronectin (Corning BioCoat, USA) at density of $5 \times 10^5$ cells/well in 100 μL of medium and incubated until attached. After 4–5 h the medium was replaced with fresh medium containing the studied drugs.

**Cell lines and culture conditions.** HepG2 cell line was purchased from the American Type Culture Collection (ATCC HB-8065). These cells were cultured in Minimum Essential Medium Eagle (MEME, Sigma, USA). FaO cell line was purchased from the European Collection of Cell Cultures (ECACC 89042701). These cells were cultured in F12 nutrient mixture (Kaighn's modification) (GIBCO, UK). Balb/c 3T3 clone A31 cell line (gift from the Department of Swine Diseases of the National Veterinary Research Institute in Pulawy, Poland) was cultured in Dulbecco's Modified Eagle's Medium (DMEM), (GIBCO, UK). The media were supplemented with 10% BCS (Balb/c 3T3), 10% FBS (HepG2, FaO), 1% L-glutamine, 1% antibiotic solution. The cells were maintained in 75 $cm^2$ cell culture flasks (NUNC) in humidified incubator at 37°C, in an atmosphere of 5% $CO_2$. The medium was refreshed every two or three days and the cells were trypsinised by 0.25% trypsin–0.02% EDTA after reaching 70%–80% confluence. Single cell suspensions were prepared and adjusted to a density of $2 \times 10^5$ cell/mL (HepG2, FaO) and $1 \times 10^5$ cell/mL for 24 h, 48 h exposition or $5 \times 10^4$ cell/mL for 72 h exposition (Balb/c 3T3). The cell suspension was transferred to 96-well plates (100 μL/well) and incubated for 24 h before the exposure to the studied drugs.

**Compound preparation and exposure.** ABZ and its metabolites were dissolved in DMSO. The final concentration of DMSO was 0.1%. The same final concentration of the solvent was used in the corresponding control. The medium used for test solutions and in control preparation did not contain serum and antibiotics. All drug solutions in medium were freshly prepared and protected from light. Drugs were tested in eight concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 μg/mL (ABZ) and 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 μg/mL (ABZ-SO and ABZ-$SO_2$). Each concentration was tested in six replicates (cell lines) and in four independent experiments (isolated rat hepatocytes). Cytotoxicity was assessed after 24, 48, and 72 h of exposure and media from ABZ cultures were collected for evaluation towards the presence of metabolites. The medium was not changed during the incubation time.

**Cytotoxicity assessment.** Cytotoxicity was assessed using the MTT assay (19). The assay is based on the reduction of tetrazolium salt MTT in live cells to dark formazan product inside the cell *via* mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH)-dependent dehydrogenases. The amount of generated formazan is assumed to be directly proportional to the cell density and is colorimetrically quantified.

The stock solution of MTT was dissolved in phosphate buffered saline (5 mg/mL) and sterilised by filtration through a 0.22 μm Millipore filter. Then working solution (0.05 μg MTT/mL PBS) was prepared and 100 μL was added to every well of the microplate. Cultures were allowed to incubate for further 4 h at 37°C in 5% $CO_2$ humidified atmosphere. After this time, the MTT solution was removed and the intracellular formazan crystals were dissolved in 100 μL of DMSO. The plate was shaken for 15 min at room temperature and transferred to a microplate reader (Multiscan RC Labsystems, USA) to measure the absorbance at 570 nm, using blank as a reference. The mean optical density (OD) was used to calculate the percentage of cell survival for each concentration of the tested drug.

**Determination of ABZ and its metabolites in the culture media.** After 24, 48, and 72 h incubation, media from cultures were collected for chemical analysis. ABZ, ABZ-SO, ABZ-$SO_2$, and ABZ-NH-$SO_2$ were determined using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The culture medium (100 µL) was diluted with 350 µL of 0.01 M ammonium acetate (pH 5.0) and 50 µL of internal standard solution (ABZ-SO-D3) and injected onto chromatography column (Kinetex, 50 × 2.1 mm, 1.3 µm particle diameter, Phenomenex, USA). Applied mobile phase consisted of acetonitrile and 0.01 M ammonium acetate (pH 5.0) and was pumped into the gradient mode flow 0.4 mL/min. The column oven temperature was 40°C.

Mass spectrometry analysis was performed using electrospray (ESI, positive ionisation) in the multiple reaction monitoring (MRM) mode. For each analyte, two fragmentation reactions were monitored, whereas one was monitored for internal standards (ABZ m/z = 266.1→234.1; 266.1→191.0, ABZ-SO m/z = 282.0→240.0; 282.0→208.8, ABZ-SO2 m/z = 298.0→266.1; 298.0→159.1 and ABZ-NH-SO2 m/z = 240.0→198.1; 240.0→133.1, internal standard ABZ-SO-D3 m/z = 301.1→159.0). Limits of detection (LOD) for all analytes were 0.01 µg/mL.

Standard calibration curves were prepared by the injection of mixed standard solutions on five concentration levels. The ratios of peak areas of standards and internal standard were plotted *versus* concentration expressed as „µg/mL". The equations and regression coefficients were calculated for the curves and were used for calculation of analytes concentrations. Such calibration curves were prepared with each series of samples. The concentrations of analytes were calculated as a difference between tested samples and the control sample containing albendazole.

**Data analysis.** The values representing effective concentration ($EC_{50}$) at three time points (24, 48, and 72 h) were calculated according to the Hill's equation (GraphPad Prism version 5.0 for Windows, GraphPad Software, USA) and expressed as standard error of the mean (±SEM). Statistical comparisons among $EC_{50}$ results were performed by analysis of variance (ANOVA) followed by Tukey test. Differences were considered as statistically significant at $P \le 0.05$. The results of LC-MS/MS analysis were expressed as the mean standard of deviation (±SD) of three (cell lines) or four (isolated rat hepatocytes) independent experiments. Differences ($P \le 0.05$) between mean values at corresponding time point were analysed by Student's *t*-test.

## Results

Qualitative and quantitative analysis of albendazole and its metabolites in culture media showed that ABZ was metabolised efficiently by rat hepatocytes, less by human (HepG2) and rat (FaO) hepatoma cells, but not in Balb/c 3T3 cells.

In HepG2 culture media a dose-dependent increase in parent compound was observed at 24 h time point. Then (at 48 and 72 h), a gradual decrease in ABZ in medium was observed up to the concentration of 10 µg/mL (Fig. 1A). Exposure to the higher concentrations used in this study, *i.e.* 50 and 100 µg/mL, resulted in both higher concentration of ABZ (Fig. 1 A) and its metabolites (Fig. 2 A, D).

Exposure of FaO cells to ABZ resulted in a dose-dependent increase in the concentration of parent compound at 24 h followed by its gradual decrease at 48 and 72 h at all concentrations used (Fig. 1 C). In contrast to HepG2 culture, only ABZ-SO was detected in the FaO culture media and its amount was slightly greater than the corresponding values in HepG2 cells (Fig. 2 A, C).

In the case of isolated hepatocyte culture exposed to ABZ, the amount of ABZ in the medium was lower (*t*-test, $P \le 0.05$) at corresponding time points and concentrations used than in the media of both hepatoma cultures (Fig. 1 A, B, C). At the same time both established metabolites increased with time and dose (Fig. 2 B, E). ABZ-SO was the main metabolite and its amount was dozens of times greater (*t*-test, $P \le 0.05$) than in hepatoma lines. Maximal ABZ-SO concentration (1.5 µg/mL) in hepatocyte cultures was revealed after the highest dose of 100 µg/mL (Fig. 2B) *vs*. 0.02 and 0.03 µg/mL in HepG2 and FaO culture media, respectively (Fig. 2 A, C).

No metabolites (>LOD) were found in the culture medium of Balb/c 3T3 cells. However, when compared with the value at 24 h, the amount of ABZ slightly decreased with increasing time of exposure (Fig. 1 D). Albendazole 2-amino sulfone was not detected in any case.

The observed cytotoxic effects were dose- and time-dependent but in isolated hepatocytes the effect was minimal. The effective concentrations, calculated from the dose-response curves, reflecting a decrease in cell viability by 50%, are shown in Table 1. Comparison of the most representative value *i.e.* $EC_{50\text{-}72h}$, revealed that ABZ was much more toxic than ABZ-SO and ABZ-$SO_2$. The $EC_{50\text{-}72h}$ values were as follows:

for ABZ – 0.2 µg/mL (Balb/c 3T3), 1.0 µg/mL (FaO), and 6.4 µg/mL (HepG2).

for ABZ-SO – 14.0 µg/mL (Balb/c 3T3) and 55.5 µg/mL (HepG2).

for ABZ-$SO_2$ – 37.8 µg/mL (Balb/c 3T3) and 69.5 µg/mL (FaO).

The obtained results revealed that Balb/c 3T3 cells were the most sensitive to ABZ, followed by FaO and HepG2. Balb/c 3T3 cells were also the most sensitive to ABZ-SO, followed by HepG2, and the highest sensitivity to ABZ-$SO_2$ was in the case of Balb/c 3T3 cells, followed by FaO lines. In none of the isolated hepatocytes could this value be attained up to the highest (100 µg/mL) concentration used.

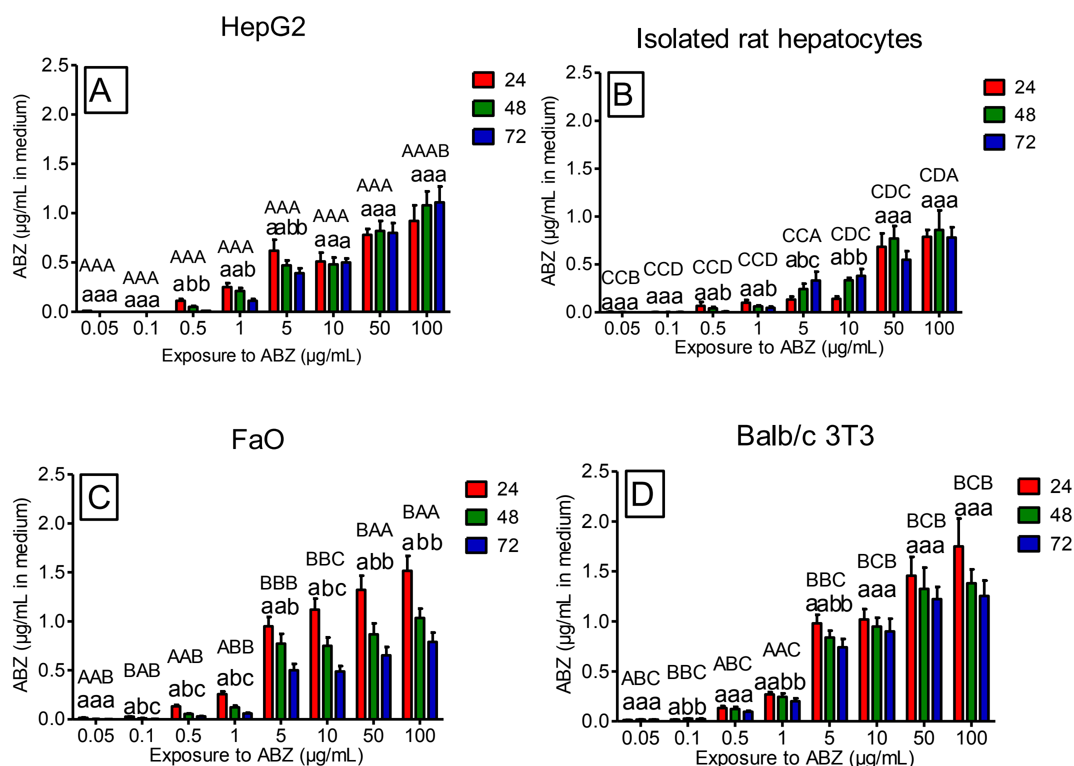


**Fig. 1.** Amounts (µg/mL) of albendazole in extracellular media from HepG2, hepatocytes, FaO, and Balb/c 3T3 cell cultures incubated for 24, 48, and 72 h with 8 concentrations of the drug. The values are expressed as means ± SD (n = 3) for cell lines or (n = 4) for isolated rat hepatocytes. The different capital letters (A–D) indicate significant differences (P ≤ 0.05) among cell models at the corresponding concentration and time of exposure for respective drugs. The different small letters (a–c) within the same concentration indicate significant difference (P ≤ 0.05) among times of exposure for respective drugs

**Table 1.** The mean (±SEM) value of $EC_{50}$ (µg/mL) for albendazole and its two metabolites evaluated by MTT assay at three time points, in three cell lines (n = 3), and in isolated rat hepatocytes (n = 4)

| Cell cultures | Exposure time | Albendazole, ABZ (0.05–100 µg/mL) | Albendazole sulfoxide, ABZ-SO (0.78–100 µg/mL) | Albendazole sulfone, $ABZ-SO_2$ (0.78–100 µg/mL) |
|---|---|---|---|---|
| HepG2 | 24 h | 37.5 ±2.7$^{a}$ | ne | 43.1 ±1.1$^{a}$ |
| | 48 h | 26.4 ±2.9$^{a}$ | ne | 37.9 ±3.8$^{a}$ |
| | 72 h | 6.4 ±0.9$^{a}$ | 55.5 ±5.2$^{a}$ | ne |
| FaO | 24 h | 22.5 ±4.5$^{b}$ | ne | 78.8 ±1.5$^{b}$ |
| | 48 h | 8.8 ±2.0$^{b}$ | ne | 70.2 ±5.1$^{b}$ |
| | 72 h | 1.0 ±0.4$^{b}$ | ne | 69.5 ±1.8$^{a}$ |
| Balb/c 3T3 | 24 h | 0.5 ±0.1$^{c}$ | 67.6 ±5.1 | 54.0 ±2.1$^{c}$ |
| | 48 h | 0.3 ±0.05$^{c}$ | 22.4 ±1.5 | 47.7 ±1.4$^{c}$ |
| | 72 h | 0.2 ±0.1$^{c}$ | 14.0 ±1.1$^{b}$ | 37.8 ±1.4$^{b}$ |
| Isolated rat hepatocytes | 24 h | ne | ne | ne |
| | 48 h | ne | ne | ne |
| | 72 h | ne | ne | ne |

The different letters (a–c) within columns indicate significant differences (P ≤ 0.05) between cell lines at the corresponding time of exposure; ne – no effect ($EC_{50}$ could not be attained at the highest concentration (100 µg/mL) tested)

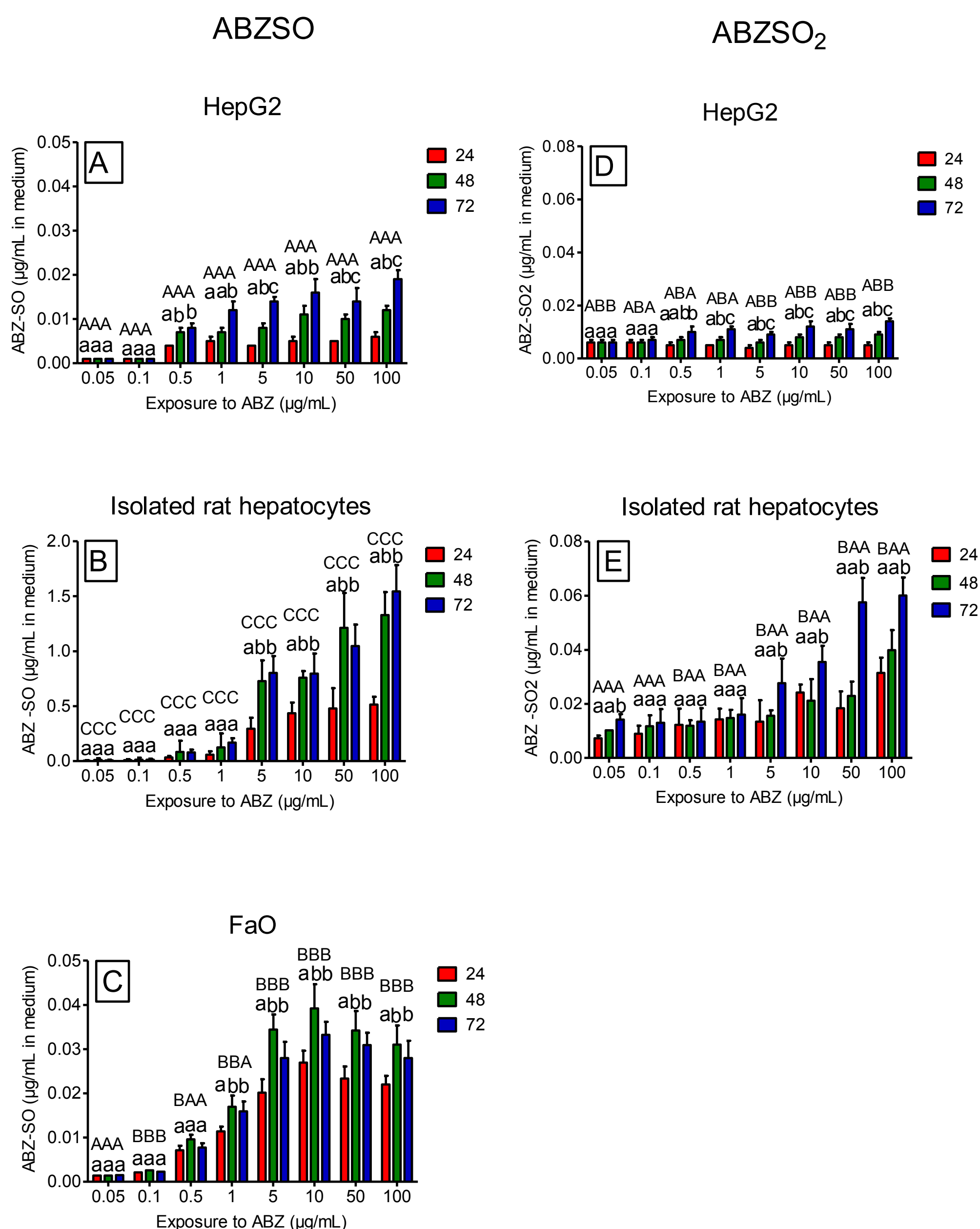


**Fig. 2.** Metabolic profile of ABZ in hepatoma (HepG2, FaO) cell lines and in isolated rat hepatocytes. Note: two metabolites were detected in HepG2 (A, D) and hepatocytes (B, E) culture media; one – in FaO (C) cultures; no metabolite was detected in Balb/c 3T3 cultures. The values are expressed as means ± SD (n = 3) for cell lines or (n = 4) for isolated rat hepatocytes. The different capital letters (A–D) indicate significant differences ($P \leq 0.05$) among cell models at the corresponding concentration and time of exposure for respective drugs. The different small letters (a–c) within the same concentration indicate significant difference ($P \leq 0.05$) among times of exposure for respective drugs

## Discussion

One of the most frequently cited limitations of *in vitro* tests for assessing health effects are qualitative and quantitative deficiencies in the biotransformation of test chemicals, in comparison with the situation found *in vivo* (7). Knowledge of metabolism is of great importance in the area of drug safety because biotransformation can lead either to detoxification or to formation of more toxic metabolites (2, 16). Since the liver is the main target organ in systemic toxicity, and since it plays a major role in the metabolism of many compounds, the liver-derived cell models are among the most frequently used in the *in vitro* studies. Currently the most used model systems are hepatoma cell lines. Although some studies attribute sufficient metabolic activities to these cells (23, 24), they are in general considered to lack substantial liver-specific functions and hepatic enzyme activity (6, 7, 13, 14, 21, 28, 29).

This study evaluated the cytotoxic effects of ABZ towards cells in terms of the role of formation of

metabolites. ABZ was found to be metabolised by isolated rat hepatocytes and human (HepG2) and rat (FaO) hepatoma lines but not in the Balb/c 3T3 cell line. However, the metabolic profile varied in terms of quality and quantity. Two metabolites (ABZ-SO and ABZ-$SO_2$) were detected in isolated hepatocytes and HepG2 culture media, and one (ABZ-SO) in FaO culture medium. The indicated metabolism in isolated hepatocytes was dozens of times greater than in HepG2 and FaO cells (Fig. 2).

As expected, the obtained data indicate that metabolism of ABZ leads to its detoxification. ABZ was highly toxic to non-metabolising Balb/c 3T3 cells ($EC_{50\text{-}72h}$ values at three time points were lower than 1 µg/mL). FaO cells were more sensitive than HepG2 with $EC_{50\text{-}72h}$ of 1 and 6.4 µg/mL, respectively. In the case of isolated hepatocytes, at any time point of assessment, $EC_{50}$ value could not be attained up to the highest concentration used, *i.e.* 100 µg/mL. Comparative evaluation of cytotoxicity of individual metabolites separately confirmed that ABZ-SO and ABZ-$SO_2$ are much less toxic than ABZ (Table 1).

To some extent our results are in line with the literature data which are rather scanty. Previously, it was demonstrated that SK-HEP-1 cells were much more susceptible to cytotoxic action of ABZ than HepG2 cells (22). According to the authors the fact that ABZ was more toxic than metabolites resulted from the incapacity of SK-HEP-1 cells to convert ABZ to its less toxic derivatives. Similar situation took place in our study in the case of Balb/c 3T3 cells. More recently, Baliharova *et al.* (2) investigated the effects of ABZ in rat hepatocytes and HepG2 cells. No effects were noted in rat hepatocytes up to the concentration of 50 µM (13.25 µg/mL). In contrast, a significant decrease in HepG2 cell viability (up to 30% compared to control) was detected after 48 and 72 h incubation with 5 and 50 µM (1.325 and 13.25 µg/mL) of ABZ (1).

The obtained data indicate that metabolism of albendazole leads to its detoxification. The lower cytotoxic potential of metabolites was confirmed in the independent experiments in this study.

**Conflict of Interests Statement:** The authors declare that there is no conflict of interests regarding the publication of this article.

**Financial Disclosure Statement:** The study, titled "Comparison of cytotoxicity and metabolism of selected veterinary medicines in the culture hepatoma cells (cell lines: FaO and HepG2), and primary culture of rat hepatocytes", was conducted within the statutory activity of the National Veterinary Research Institute in Pulawy, Poland.

**Animal Rights Statement:** The experiment was approved by the Local Ethics Committee (University of Life Sciences in Lublin, Poland).

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