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Comparison of biotransformation and efficacy of aminoacetonitrile anthelmintics *in vitro*

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The present *in vitro* study was designed to test and compare anthelmintic activity, hepatotoxicity, and biotransformation of four selected aminoacetonitrile derivatives (AADs): monepantel (MOP, anthelmintic approved for the treatment), AAD-970, AAD-1154, and AAD-1336. Micro-agar larval development test, MTT test of cytotoxicity, and biotransformation study coupled with Ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) technique were used for this purpose. Larvae of two *Haemonchus contortus* strains (drug susceptible and multi-drug resistant) and primary cultures of rat and ovine hepatocytes served as model systems. All AADs (including MOP) exhibited significant larvicidal effect in *H. contortus* susceptible as well as multi-resistant strains, much higher than those of reference anthelmintics thiabendazole and flubendazole. AAD-1154 provides the best results for most tested parameters among all AADs in this study. The cytotoxicity test showed that all AADs can be considered as nontoxic for hepatocytes. In the biotransformation study, Phase I and Phase II metabolites of AADs were identified and schemes of possible metabolic pathways in ovine hepatocytes were proposed. Biotransformation of MOP was much more extensive than biotransformation of other AADs. Based on obtained results, AAD-1154 and AAD-1336 can be considered as promising candidates for further *in vivo* testing. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: monepantel; drug metabolism; micro-agar larval development test; hepatocytes; structure-metabolism relationships

Introduction

The battle against parasitic helminths is a major problem facing the human health and agriculture industry worldwide. Anthelmintics remain the sole accessible means in helminthoses therapy, but the frequent use of similar anthelmintics over many years has led to the development of drug resistance in helminths. This increased drug-resistance, especially characteristic for nematodes, motivated the development of a new anthelmintic class: aminoacetonitrile derivatives (AADs), low molecular mass compounds bearing different aryloxy and aroyl moieties on an aminoacetonitrile core.[1] Based on the initial results obtained with the lead molecule (assigned as AAD-450), more than 700 different AAD molecules were synthetized and their potential anthelmintic activity was assessed. AAD-1566 was selected for the industrial development phase after previous comprehensive testing.[2,3] AAD-1566 was named as monepantel (MOP; N-[(1S)-1-cyano-2-(5-cyano-2-trifluoromethyl-phenoxy)-1-methyl-ethyl]-4-trifluoro methylsulfanyl-benzoamide) and it has been introduced on the market as Zolvix® anthelmintic (Novartis Animal Health) for sheep against nematodes.

MOP is absorbed into the bloodstream of sheep after the oral administration and then quickly biotransformed to MOP-sulfone, which has a similar efficacy as the parent molecule.[4] Although MOP is metabolized to MOP-sulfone in the liver, the large concentrations of both anthelmintically active molecules recovered during the first 48 h post-treatment from the abomasum and small intestine may greatly contribute to the well-established pharmacological activity of MOP against GI nematodes.[5] Many other MOP metabolites are also formed in sheep.[6,7] It is generally known that extensive drug biotransformation increases the risk of drug-drug interactions as well as inter-individual differences in drug pharmacokinetics.[8,9]

MOP and other AADs have a novel mode of action involving a unique, nematode-specific clade of nicotinic acetylcholine receptor (nAChR) subunits.[2,10] AADs act as positive allosteric modulators of this receptor subunit which is forced to open on stimulus, but it cannot close again. This results in a constant uncontrolled flux of ions and finally in a depolarization of muscle cells.[11] This hypercontraction of body wall muscles leads to the paralysis, spasmodic contractions of the anterior portion of the pharynx and ultimately death of adult nematodes.[12] MOP is also effective against nematodes' earlier larval stages (L_1-L_3) and larval susceptibility is not dependent on the drug-sensitivity status of the nematode isolate.[13] The present information about MOP has been reviewed recently.[14]

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Since 2010, MOP has been used in the therapy of nematode infections in sheep mostly with success. However, some cases of failure of MOP therapy have been recently reported.[15,16] In addition, our recent study has revealed that MOP acts as a significant inducer of cytochrome P4503A (CYP3A24), the main biotransformation enzyme, in sheep which can manifest as undesirable interactions of MOP with other drugs.[17] These facts evoked the hypothesis that some other AADs with good anthelmintic activity (at least comparable with MOP) but with different and less extensive biotransformation pathways without undesirable induction effect may exist. The present *in vitro* study was designed to test and compare anthelmintic activity, hepatotoxicity and biotransformation of four selected AADs. In addition to MOP, AAD-970, AAD-1154, and AAD-1336 were included in the study. These AADs were selected according to their promising anthelmintic efficacy *in vitro* and *in vivo*[10] and based on their structural components predicting that their biotransformation will be different to MOP. Larvae of two *Haemonchus contortus* strains (drug sensitive and multi-drug-resistant) and primary cultures of rat and ovine hepatocytes were used as a model systems.

Materials and methods

Chemicals and reagents

MOP and other AADs were prepared at the Department of Inorganic and Organic Chemistry (Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Czech Republic) according to Kaminsky *et al.*[10] Starting substituted phenols were firstly converted to appropriate ketones in 89–96% yield followed by Strecker reaction employing potassium cyanide, ammonium chloride, and ammonia solution affording the products in 67, 50, and 91% yields, respectively. The resultant aminoacetonitriles were finally converted to their amides by the reaction with 4-(trifluoromethoxy)benzoyl chloride (82–98% yield). MOP was prepared according to Kaminsky *et al.*[10] with minor modifications.[6] The structure and purity of prepared substances were tested using nuclear magnetic resonance spectrometry and ultrahigh-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS). Liquid sterile-filtered medium Ham F12, Williams medium, bovine serum albumin (BSA), ethylene glycol-bis(β-aminoethyl ether) - *N*, *N*, *N*, *N* - tetraacetic acid (EGTA), 3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and other chemicals (MALDT, UHPLC, MS or analytical grade) were obtained from Sigma-Aldrich (Prague, Czech Republic).

Animals

For experiments, parasite-free Texel lambs (Běleč, Czech Republic) and Wistar rats (MediTox, Konárovice, Czech Republic) were used. Male rats were housed in air-conditioned animal quarters with a 12 h light/dark cycle. Food (a standard rat chow diet) and water were provided *ad libitum*. All experimental procedures were undertaken in accordance with the Czech guidelines for the care and use of farm and experimental animals and were performed under the supervision of Ethical Committee of the Charles University in Prague, Faculty of Pharmacy in Hradec Králové (Protection of Animals from Cruelty Act No. 246/92, Czech Republic).

Obtaining of *Haemonchus contortus* eggs

One susceptible isolate of *H. contortus* – Inbred Susceptible Edinburgh (ISE) and one resistant strain – White River (WR) have been used in this study. The *H. contortus* ISE strain is an anthelminthic-susceptible inbred type of the SE strain,[18] which had been isolated from the field before benzimidazole anthelmintics were introduced to the market. The South African, multi-resistant WR isolate has been isolated from the field, and it has demonstrated resistance to ivermectin (30% efficacy at 0.2 mg/kg) as well as the benzimidazoles, rafoxanide and closantel.[19] Lambs were infected orally with L_3 larvae of *H. contortus*. Each animal obtained a suspension with 5000–6000 L_3 larvae. Lambs had been reared and maintained indoors under conditions designed to minimize the risk of infection with gastrointestinal nematodes. Faecal samples were collected on the day 35 after the inoculation, and nematode eggs were collected by differential sieving through three stacked sieves of 250, 100, and 25 μm mesh, successively. The material retained on a 25 μm mesh sieve was washed with water, sedimented, and floated, followed by washing over 20 μm mesh sieve with water. The flotation was performed by less aggressive modified Sheather's solution instead of saturated sodium chloride. Obtained eggs were subsequently used for the *in vitro* micro-agar larval development test (MALDT).

In vitro micro-agar larval development test (MALDT)

The MALDT test was performed as described by Coles *et al.*[20] in 96-well-plates. Stock drug solutions were prepared by pre-dissolving drugs in DMSO with the subsequent dilution with distilled water (1:4). 10 μL of drug solution was added to each well (or DMSO for control wells). Flubendazole (FLU) and thiabendazole (TBZ) were used as a positive control and their final concentration ranged from 0.00062 to 1.28 $\mu g.mL^{-1}$. The molar concentration of MOP and its derivatives in stock solutions was 0.244 mM. According to this the final concentration (*m/v*) of MOP in wells ranged from 0.00062 to 1.28 $\mu g.mL^{-1}$, concentration of AAD-970 and AAD-1154 from 0.0005 to 1.17 $\mu g.mL^{-1}$ and the final concentration of AAD-1336 from 0.00063 to 1.29 $\mu g.mL^{-1}$. 150 μL of 2% agar at 45 °C was added to each well. After the solidification of the gel, 10 μL of culture medium (1 g yeast extract and 90 mL 0.85% NaCl, autoclave for 20 min; add 3 mL of 10x concentrated Earle's solution per 27 mL of yeast extract) and 10 μL of a suspension diluted 1:1 with amphotericin B (5 μg/mL) (Sigma-Aldrich, Prague, Czech Republic) containing 50–100 eggs were added on the top of agar matrix. To prevent evaporation, the outer wells on each plate were filled with distilled water. The plates were then incubated for 7 days at 27 °C and the test was finished by adding 10 μL of Lugol's solution into each well. Before counting, the whole liquid content of each well was collected and transferred to new 96-well-plates. The numbers of unhatched eggs and L_1–L_3 larvae in each well were counted under an inverted microscope. Two independent tests were performed, both with two replicates for each drug concentration.

Preparation of primary culture of hepatocytes

Rats or lambs were stunned and exsanguinated in agreement with Czech slaughtering rules for farm animals. After removal of the liver from the abdominal cavity, the liver was flushed through the main veins with Euro Collins solution (15 mM KH_2PO_4, 42.5 mM K_2HPO_4, 15 mM KCl, 10 mM $NaHCO_3$ and 0.2 M glucose). The hepatocytes were obtained from the liver by two-step collagenase method.[21,22] Briefly, left lobe (ovine liver) or whole liver (rat liver) were perfused with salt solution (0.14 M NaCl, 5.0 mM KCl, 0.8 mM $MgSO_4$) in Na^+/K^+ phosphate buffer (0.2 mM, pH 7.4) containing a calcium binding component (0.4 mM EGTA). Consequently, the liver was perfused

with phosphate buffer containing calcium chloride (1.46 mM) and collagenase (30 mg/100 mL) at 37 °C. The collagenase perfusion proceeded for 5–10 min depending on the rate of digestion. After perfusion, hepatocytes were released into the phosphate buffer containing calcium chloride and BSA. The obtained suspension was filtered through a nylon mesh and centrifuged at 40 g for 5 min at 4 °C. The pellet was re-suspended in chilled buffer and the washing procedure was twice repeated. Finally, suspensions containing 1 million viable (75–80%) cells in 1 mL of culture medium ISOM (1:1 mixture of Ham F12 and Williams' E) with 5% fetal bovine serum were placed into 60 mm plastic dishes pre-coated with collagen or into 96-well plates. Cultures were maintained without the substrate for 4 h at 37 °C in a humid atmosphere of air and 5% CO_2.

Test of hepatocytes viability

The mitochondria of living cells are able to reduce yellow MTT to purple formazan. Tested compounds (AADs) were pre-dissolved in DMSO to obtain stock solutions with the concentration of 1, 2, 5, 10, 20, 50, and 100 mM. Stock solutions were diluted 1000 times with an appropriate cell culture medium to obtain the final concentration of 1, 2, 5, 10, 20, 50, and 100 μM. The final concentration of DMSO in all samples was 0.1%. Rat hepatocytes (prepared from liver from one or two animals) in 96-well plates were exposed for 24 h to culture medium with AADs. Control cells were exposed to the culture medium with 0.1% DMSO. Each AADs concentration was tested in 12 parallels. After the exposure, 25 μL of MTT solution (3 mg of MTT in 1 mL of phosphate buffer saline, pH 7.4) was added to each well. The final MTT concentration in all wells was 1.8 mM. Plates were incubated at 37 °C for an additional 2 h, then the medium was removed and the formed formazan was dissolved in 50 μl of 0.08 M HCl in isopropanol by 30 min shaking. The absorbance in each well was quantified by measuring at 570 nm with the background correction at 690 nm using UV spectrophotometer (Tecan Infinite M200, Mannedorf, Switzerland). Three independent experiments were performed.

Incubation of ovine hepatocytes with AADs and solid-phase extraction (SPE)

Mixed suspension of hepatocytes (prepared from two lambs) were seeded in 60-mm-dishes pre-coated with collagen. After the attachment of hepatocytes, the medium was replaced with fresh serum-free one with AADs (10 μM in 3 mL of fresh medium). AADs were pre-dissolved in DMSO. The final concentration of DMSO in the medium did not exceed 0.1%. Each AADs was tested in triplicates. After 24 h, the hepatocytes were scraped and homogenized. Samples were centrifuged at 3000 x *g* for 5 min. The supernatant was extracted using the SPE method. The sample was loaded onto the Phenomenex Strata X (3 mL, 60 mg, 33 Wm; Phenomenex, Torrance, CA, USA) extraction cartridge.[6] Samples were evaporated to dryness using vacuum concentrator. Dry samples were quantitatively reconstituted in the mixture of acetonitrile–water (30:70, *v/v*) by sonication. The solutions were mixed using a vortex for 5 min. One microliter of reconstituted samples was injected into the UHPLC-MS system.

Ultra high-performance liquid chromatography - mass spectrometry conditions

UHPLC-MS/MS analysis of samples was measured in the negative-ion electrospray ionization (ESI) mode on a hybrid quadrupole – time-of-flight mass analyzer (micrOTOF-Q, Bruker Daltonics, Bremen, Germany). UHPLC was performed on an Agilent 1290 Infinity liquid chromatography (Agilent Technologies, Waldbronn, Germany) using Zorbax Eclipse C18 column 150x2.1 mm, 1.8 μm (Agilent Technologies, Waldbronn, Germany), temperature 25 °C, flow rate 0.4 mL/min and the injection volume 1 μL. The mobile phase consisted of 0.5 mM ammonium acetate adjusted to pH 4.0 (A) and acetonitrile (B). The linear gradient was used (Table 1). The QqTOF mass spectrometer with the average resolving power higher than 13 000 (full width at half maximum definition) was used with the following setting of tuning parameters: capillary voltage 4.5 kV, drying temperature 220 °C, the flow rate and pressure of nitrogen were 8 L/min and 1.3 bar, respectively. The external calibration was performed with sodium formate clusters before individual measurements. ESI mass spectra were recorded in the range of *m/z* 501000 in the negative-ion mode. The isolation width *m/z* 4 and the collision energy 25 eV (found as optimal energy for the fragmentation of studied metabolite ions) using argon as the collision gas were used for MS/MS experiments.

Statistical analysis

All calculations were done using Microsoft Excel and GraphPad Prism 5.04. Data analysis was performed using Student t-test (GraphPad Prism Software, La Jolla, CA, USA). All values were expressed as mean ± SD. A probability of $p < 0.05$ was considered statistically significant.

Results

The larvicidal efficacy, hepatotoxicity and biotransformation of AAD-970, AAD-1154 and AAD-1336 were tested and compared to MOP. For this purpose, *in vitro* micro-agar larval development test, test of cytotoxicity in rat hepatocytes and biotransformation study in primary culture of ovine hepatocytes using UHPLC-MS/MS technique were applied.

In vitro micro-agar larval development test

Results of MALDT are presented in Table 2 as LC_{50} and LC_{99} values, which are defined as the anthelmintic concentration where the hatching and development of eggs to the L_3 stage is inhibited by 50% and 99%, respectively. The data were analyzed using a logistic regression model to determine LC_{50} and LC_{99}.[23] LC_{50} gives information on the resistance of the average worm in the population, and LC_{99} shows which proportion of the population is the most resistant. Statistically significant ($p \leq 0.05$) differences in LC_{50} or LC_{99} values of MOP and other compounds were analyzed using Student t-test. The degree of anthelmintic resistance was expressed as the resistance factor (RF), calculated as the LC_{50} or LC_{99} value for the resistant isolate (WR) divided by the respective value for the susceptible isolate (ISE).

Table 1. Linear gradient used for UHPLC-MS

Time (min)	A %	B %
0	70	30
11	5	95
12	5	95
13	70	30

Table 2. Lethal concentrations (LC; ng.mL^{-1}; mean ± SD) of MOP/AADs/FLU/TBZ for resistant (WR) and susceptible (ISE) isolates of *H. contortus* obtained by *in vitro* micro-agar larval development tests and resistance factors (RF). Data was obtained from two independent experiments performed in duplicates. Triangles indicate significant differences ($p \leq 0.05$) among $LC_{50/99}$ of MOP and other compounds (▲ increase, ▼ decrease)

Drug	WR		ISE			
	LC_{50}	LC_{99}	LC_{50}	LC_{99}	RF_{50}[a]	RF_{99}[b]
MOP	4.6 ± 0.3	8.1 ± 1.5	3.7 ± 0.1	9.0 ± 1.0	1.24	0.90
AAD-970	5.4 ± 0.0▲	25.6 ± 14.7	5.6 ± 0.7▲	24.5 ± 16.6	0.96	1.05
AAD-1154	4.2 ± 0.1▼	5.0 ± 0.2▼	4.2 ± 0.1▲	5.1 ± 0.1▼	1.00	0.98
AAD-1336	5.3 ± 0.0▲	6.5 ± 0.1	5.1 ± 0.2▲	6.3 ± 0.3▼	1.02	1.02
FLU	12.0 ± 0.3▲	57.6 ± 13.4▲	6.7 ± 0.2▲	16.5 ± 2.1▲	1.79	3.48
TBZ	16.1 ± 3.3▲	429.1 ± 151.1▲	7.4 ± 1.4▲	18.0 ± 6.5▲	2.17	23.80

[a] LC_{50} for WR divided by LC_{50} for ISE
[b] LC_{99} for WR divided by LC_{99} for ISE

Obtained results showed a significant effect of all ADDs against *H. contortus* larvae. All AADs were significantly more effective in larval development inhibition in comparison to reference anthelmintics TBZ and FLU. In ISE strain, MOP had the lowest value of LC_{50} from all AADs, but LC_{99} values for AAD-1154 and AAD-1336 were significantly lower than those for MOP. In WR strain, AAD-1154 was more effective than MOP, as its values of LC_{50} and LC_{99} were lower than those of MOP.

Comparing WR and ISE strains, the LC_{50} as well as LC_{99} values of AADs did not differed significantly, contrary to LC_{50} and LC_{99} values of TBZ. It corresponds with values of resistance factor (RF) that express differences of anthelmintic efficacies between WR and ISE strains. While TBZ had significantly decreased efficacy on development of *H. contortus* larvae of WR strain than ISE strain ($RF_{99} = 23.8$), all AADs were similarly effective in both strains as resistant factors around 1 were obtained ($RF_{50} = 0.96$-1.24; $RF_{99} = 0.90$-1.05). FLU, which was used as the second reference anthelmintic, exhibited relatively low values of resistant factors ($RF_{99} = 3.48$)

Effect of AADs on the viability of isolated hepatocytes

The primary culture of isolated rat hepatocytes was used to compare the potential cytotoxicity of AADs (Figure 1). Hepatocytes were exposed to AADs for 24 h and then the viability of hepatocytes was assayed using MTT test. While AAD-1336 was mildly hepatotoxic at the concentration up to 50 μM, MOP and AAD-1154 decreased viability of hepatocytes only at the highest concentration (100 μM).

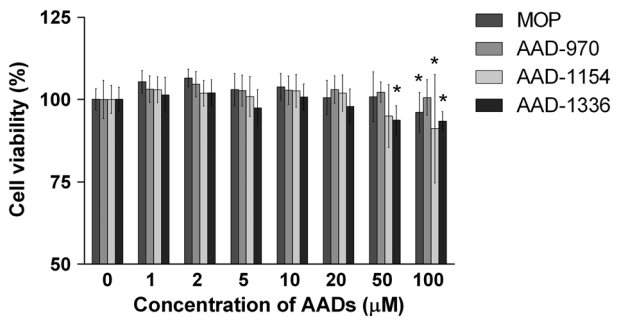

Figure 1. The effect of MOP and its derivatives at concentration 1-100 μM on the viability of isolated hepatocytes. The exposure lasted 24 h. The data are expressed as percentage of the control cells (cell exposed to vehicle 100%) ± SD ($n = 3$). The viability of control cells represents 100%. The asterisk indicates a significant difference from the control ($p < 0.05$)

Exposure of hepatocytes to AAD-970 had no effect on the cell viability.

Biotransformation of AADs in ovine hepatocytes

The biotransformation of MOP and AADs was studied in the primary culture of ovine hepatocytes. After 24 h incubation of hepatocytes with AADs and SPE extraction, metabolites of AADs were identified using UHPLC-ESI-MS/MS technique. All measurements were carried out in the negative-ion mode due to a better sensitivity. The mass accuracy of *m/z* determination for all $[M\text{-}H]^-$ ions was better than 3 ppm in accordance with the typical requirements for high mass accuracy measurements. Structures of possible metabolites were predicted using the MetabolitePredict software (Bruker Daltonics, Bremen, Germany) and the knowledge of fragmentation behavior associated with metabolic reactions.[24] The signal-to-noise ratio (S/N) was higher than five for all metabolites. Metabolic pathways of AAD-970, AAD-1154 and AAD-1336 are shown in Figure 2. The relative abundances of AADs metabolites in ovine hepatocytes are given in Figure 3. Only MOP is capable of S-oxidation, because it contains sulfur for this biotransformation step. The hydroxylation occurred in all compounds, but the number and position of hydroxylated metabolites differed among AADs. The hydrolysis of nitrile to amide group was identified only in AAD-970. The hydroxyl group of metabolites formed during Phase I biotransformation is often the site of Phase II biotransformation. From Phase II metabolites, glucuronides, sulfates and acetyl cysteine conjugates were found. Acetyl cysteine conjugates were detected only in MOP,[5] while all AADs formed sulfates and glucuronides. Number of conjugates formed and position of binding of conjugation agents differed among AADs. Relative abundance of metabolites of individual AADs shows that the biotransformation of MOP was much more extensive than biotransformation of other AADs tested. Sulfone represented the dominant MOP metabolite, while glucuronides were the major metabolites of AAD-970 and AAD-1154. In AAD-1336, arene hydroxylation was the main biotransformation step.

Discussion

Gastro-intestinal nematodes cause a significant disease in grazing sheep worldwide, with important economic repercussions to the sheep industry.[25] Nowadays, the situation has become more problematic by increasing the occurrence of drug-resistant helminths.[26,27] The treatment of helminthoses is mainly based on the administration of anthelmintic drugs from the following

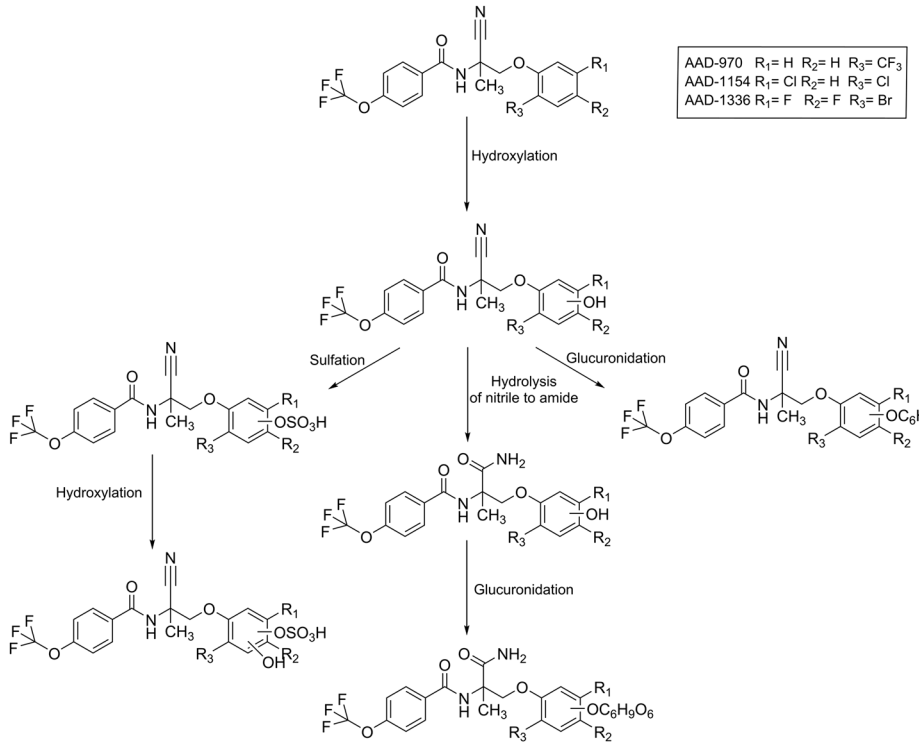

Figure 2. Scheme of metabolic pathways of AADs *in vitro* in ovine hepatocytes

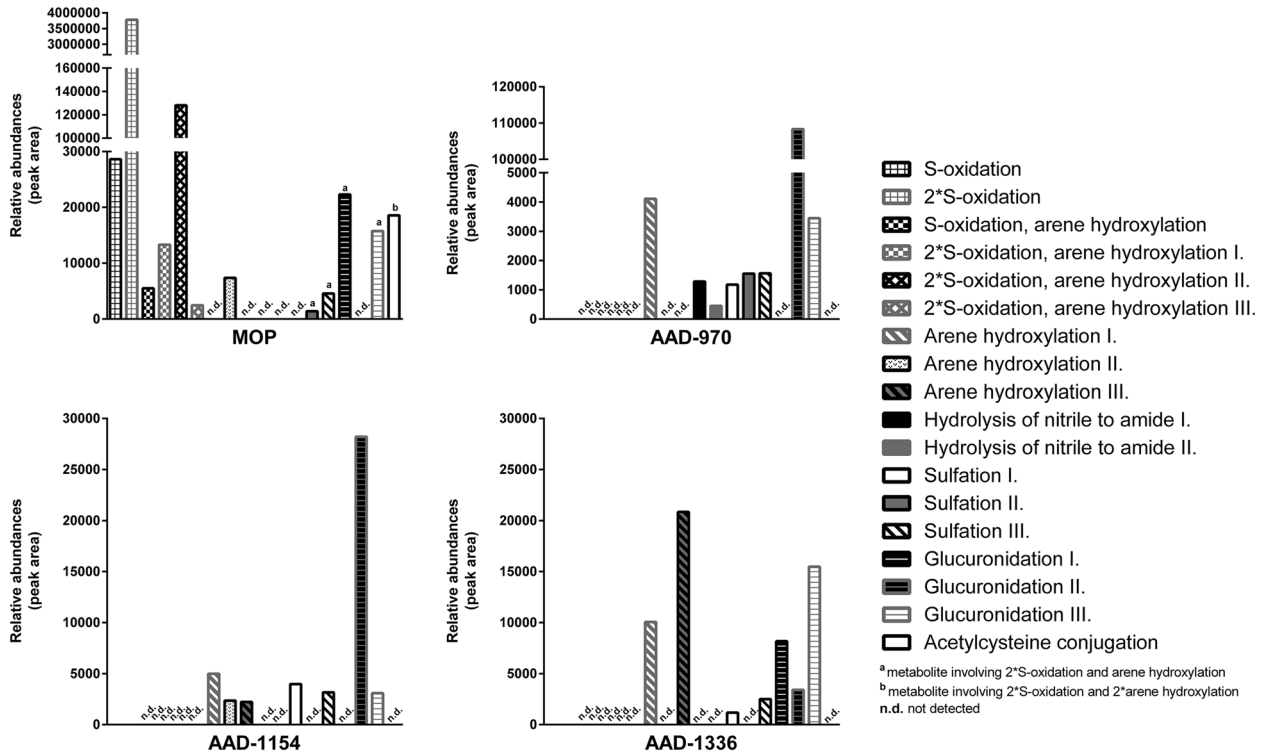

Figure 3. Relative abundances (peak area) of MOP and other AADs metabolites in ovine hepatocytes

groups: benzimidazoles, imidazothiazoles, macrocyclic lactones and AADs. Contrary to other groups, only AADs are sufficiently effective against multi-resistant nematodes.[14] In spite of this fact, only one representative of AADs, MOP, has been approved for veterinary practice which limits the choice for veterinarians. Moreover, MOP is extensively biotransformed in sheep and induces CYPs activities in ovine liver[6,7,17] which increase a risk of drug-drug interactions and inter-individual differences in pharmacokinetics.[8,9] For that reasons, the finding of other AAD with good anthelmintic activity in multi-resistant strains but without extensive biotransformation in sheep could be beneficial. In present project, anthelmintic efficacy, hepatotoxicity and biotransformation of three promising

AADs were tested and the obtained results were compared with those for MOP.

The larval development test showed that all AADs (including MOP) exhibited a significant larvicidal effect in *H. contortus* susceptible ISE strain as well as in multi-resistant WR strain. The efficacy of all AADs was higher than the efficacy of reference anthelmintics TBZ and FLU, especially in multi-resistant WR strain. Due to widespread anthelmintic resistance, this high sensitivity of *H. contortus* resistant strain to AADs is a big advantage for successful therapy. AAD-1154 was even better than MOP in almost all tested parameters among AADs in this study.

The primary culture of isolated rat hepatocytes was used for the cytotoxicity test of AADs. Rat hepatocytes were chosen as obtaining them is simpler, cheaper and their attachment better for the viability test than ovine ones. AADs were tested at the concentration range 1–100 μM. Our results showed that all AADs can be considered as nontoxic for hepatocytes, as certain hepatotoxicity occurs only at the concentration over 50 μM, which is much higher than the highest concentration achievable in the organism during the recommended dosage. This information could also be useful for the potential use of AADs in anti-cancer therapy, as several AADs (including MOP and AAD-1336) exhibited promising antiproliferative effect in several cancer cell lines.[28]

The biotransformation of AADs was studied in primary culture of ovine hepatocytes after excluding the hepatotoxic effect of AADs. In biotransformation study, the ovine hepatocytes are more appropriate model system than rat ones as sheep is a target species and considerable inter-species differences in drug biotransformation are probable Moreover, the metabolic pathway of MOP in ovine hepatocytes and in sheep *in vivo* was described in our previous study.[6,7] However, the biotransformation of other AADs has not been studied till now. Our study in ovine hepatocytes revealed that all AADs underwent arene hydroxylation in Phase I. Unlike MOP, AAD-1336 and AAD-1154 were hydroxylated on two or three different positions, respectively. Only MOP can undergo two-step S-oxidation, because other AADs do not have a sulfur in their structures. AAD-970 is also transformed via the hydrolysis of nitrile to amide. This unusual biotransformation step[29] was previously described for MOP in sheep *in vivo*.[7] Present *in vitro* study proved that this reaction was catalyzed by hepatic enzymes and it required certain structural features, because AAD-1154 and AAD-1336 were not subject to this hydrolysis although they also have a nitrile group.

In Phase II, all AADs underwent sulfation in two positions. All AADs were also conjugated with UDP-glucuronic acid, but the number and positions of glucuronides differed among individual AADs. MOP formed only one glucuronide, two different glucuronides arose from AAD-970 and AAD-1154. Three glucuronides including two diastereoisomers were detected in the case of AAD-1336. Only MOP was biotransformed via conjugation with glutathione leading to acetylcysteine conjugate unlike the other AADs. The presence of the strong electron-withdrawing nitrile substituent of an arene ring maybe the reason for glutathione S-transferases affinity only to MOP.[30]

When relative abundances of AADs metabolites were compared, large differences were found. Overall, MOP biotransformation was much more extensive than biotransformation of all other AADs. In MOP biotransformation, S-oxidation absolutely predominated. This finding is in agreement with *in vivo* results.[4,7] In case of other AADs, the amount of metabolites formed in ovine hepatocytes was substantially lower than for MOP. Glucuronides were the major metabolites of AAD-970 and AAD-1154, while hydroxylated metabolite was the main metabolite of AAD-1336.

In conclusion, this is the first report describing the comparison of larvicidal efficacy, hepatotoxicity and biotransformation pathways of MOP and three other AADs *in vitro*. Based on obtained results, we consider AAD-1154 and AAD-1336 as potential candidates for further *in vivo* testing.

Acknowledgements

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References

[1] P. Ducray, N. Gauvry, F. Pautrat, T. Goebel, J. Fruechtel, Y. Desaules, S. S. Weber, J. Bouvier, T. Wagner, O. Froelich, R. Kaminsky. Discovery of aminoacetonitrile derivatives, a new class of synthetic anthelmintic compound. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2935.

[2] R. Kaminsky, L. Rufener. Monepantel: From discovery to mode of action, in *Parasitic Helminths – Targets, Screens, Drugs and Vaccines*. (Eds: C.R. Caffrey, P.M. Selzer), Wiley-Blackwell, Weinheim, Germany, **2012**, pp. 233–249.

[3] B. Good. Anthelmintic resistance – a potential challenge for sheep procedures? in *Technical Updates on Sheep Production*. (Eds: M.G. Diskin, M.P. McHugh), Teagasc, Agriculture and Food Development Authority, Carlow, Ireland, **2012**, pp. 72–75.

[4] D. Karadzovska, W. Seewald, A. Browning, M. Smal, J. Bouvier, J. M. Giraudel. Pharmacokinetics of monepantel and its sulfone metabolite, monepantel sulfone, after intravenous and oral administration in sheep. *J. Vet. Pharmacol. Ther.* **2009**, *32*, 359.

[5] A. Lifschitz, M. Ballent, G. Virkel, J. Sallovitz, C. Lanusse. Accumulation of monepantel and its sulphone derivate in tissues of nematode location in sheep: Pharmacokinetic support to its excellent nematodicidal activity. *Vet. Parasitol.* **2014**, *203*, 120.

[6] L. Stuchlíková, R. Jirásko, I. Vokřál, J. Lamka, M. Špulák, M. Holčapek, B. Szotáková, H. Bártíková, M. Pour, L. Skálová. Investigation of the metabolism of monepantel in ovine hepatocytes by UHPLC/MS/MS. *Anal. Bioanal. Chem.* **2013**, *405*, 1705.

[7] L. Stuchlíková, R. Jirásko, I. Vokřál, M. Valát, J. Lamka, B. Szotáková, M. Holčapek, L. Skálová. Metabolic pathways of anthelmintic drug monepantel in sheep and in its parasites (*Haemonchus contortus*). *Drug Test. Anal.* **2014**, *6*, 1055.

[8] B. Testa, S.D. Krämer. Consequences of drug metabolism, in *The Biochemistry of Drug Metabolism: Principles, Redox Reactions, Hydrolyses*. (Eds: M.V. Kisakűrek, T. Kolitzus), VHCA, Zűrich, Switzerland, Wiley-Blackwell, Weinheim, Germany, **2008**, pp. 24–32.

[9] B. Testa. Xenobiotic metabolism: The global view, in *The Metabolism of Drugs and Other Xenobiotics*. (Eds. B. Testa, B. Testa), Academic Press, Lausanne, Switzerland, **1995**, pp. 5–23.

[10] R. Kaminsky, P. Ducray, M. Jung, R. Clover, L. Rufener, J. Bouvier, S. S. Weber, A. Wenger, S. Wieland-Berghausen, T. Goebel, N. Gauvry, F. Pautrat, T. Skripsky, O. Froelich, C. Komoin-Oka, B. Westlund, A. Sluder, P. Mäser. A new class of anthelmintics effective against drug-resistant nematodes. *Nature*. **2008**, *452*, 176.

[11] C. Epe, R. Kaminsky. New advancement in anthelmintic drugs in veterinary medicine. *Trends Parasitol.* **2013**, *29*, 129.

[12] L. Rufener, P. Mäser, I. Roditi, R. Kaminsky. *Haemonchus contortus* acetylcholine receptors of the DEG-3 subfamily and their role in sensitivity to monepantel. *PLoS Pathog.* **2009**, *5*, e1000380.

[13] L. Lecová, L. Stuchlíková, J. Lamka, M. Špulák, M. Várady, L. Skálová. Efficacy of monepantel against lower development stages of a multi-resistant and susceptible *Haemonchus contortus* isolates: An *in vitro* study. *Helmintologia*. **2013**, *50*, 91.

[14] L. Lecová, L. Stuchlíková, L. Prchal, *L. Skálová*. Monepantel: The most studied new anthelmintic drug of recent years. *Parasitology* **2014**, *141*, 1686.
[15] I. Scott, W. E. Pomroy, P. R. Kenyon, G. Smith, B. Adlington, A. Moss. Lack of efficacy of monepantel against *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. *Vet. Parasitol.* **2013**, *198*, 166.
[16] S. Love. First confirmed case of resistance to new drench in Australia-what does it mean? Available at: http://www.wormboss.com.au/news/articles/drench-resistance/first-confirmed-case-of-resistance-to-new-drench-in-australiawhat-does-it-mean.php [29 July 2014].
[17] L. Stuchlíková, P. Matoušková, H. Bártíková, I. Vokřál, J. Lamka, T. Štolcová, H. Pětníková, V. Kubíček, B. Szotáková, L. Skálová. Monepantel induced hepatic cytochromes P450 in sheep *in vivo* and *in vitro*. *Chem. Biol. Interact.* **2015**, *227*, 63.
[18] M. H. Roos, M. Otsen, R. Hoekstra, J. G. Veenstra, J. A. Lenstra. Genetic analysis of inbreeding of two strains of the parasitic nematode *Haemonchus contortus*. *Int. J. Parasitol.* **2004**, *34*, 109.
[19] J. A. Van Wyk, F. S. Malan. Resistance of field strains of *Haemonchus contortus* to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. *Vet. Rec.* **1988**, *123*, 226.
[20] G. C. Coles, F. Jackson, W. E. Pomroy, R. K. Prichard, G. Von Samson-Himmelstjerna, A. Silvestre, M. A. Taylor, J. Vercruysse. The detection of anthelmintic resistance in nematodes of veterinary importance. *Vet. Parasitol.* **2006**, *136*, 167.
[21] V. Baliharová, J. Velík, M. Savlík, B. Szotáková, J. Lamka, L. Tahotná, L. Skálová. The effects of fenbendazole, flubendazole and mebendazole on activities of hepatic cytochromes P450 in pig. *J. Vet. Pharmacol. Ther.* **2004**, *27*, 85.
[22] M.N. Berry, A.M. Ecwards, G.J. Barritt. Laboratory techniques in biochemistry and molecular biology, in *Isolated Hepatocytes Preparation, Properties and Applications*. (Eds: R.H. Burdow, P.H. vanKnippenberg), Elsevier Science, Amsterdam, **1991**, pp. 15–35.
[23] R. J. Dobson, D. A. Griffiths, A. D. Donald, P. J. Waller. A genetic model describing the evolution of levamisole resistance in *Trichostrongylus colubriformis*, a nematode parasite of sheep. *J. Appl. Math.* **1987**, *4*, 279.
[24] M. Holčapek, L. Kolářová, M. Nobilis. High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. *Anal. Bioanal. Chem.* **2008**, *391*, 59.
[25] G. C. Coles. The future of veterinary parasitology. *Vet. Parasitol.* **2001**, *98*, 31.
[26] H. A. Shalaby. Anthelmintics Resistance; How to Overcome it? *Iran. J. Parasitol.* **2013**, *8*, 18.
[27] I. Sutherland, I. Scott. Anthelmintic resistance, in *Gastrointestinal Nematodes of Sheep and Cattle. Biology and Control*. (Eds: I. Sutherland, I. Scott), Wiley-Blackwell, Oxford, United Kingdom, **2010**, pp. 117–124.
[28] D.L. Morris. New south Innovations Pty Limited. *Kinase inhibitors for the treatment of cancer*. World Patent No. WO2013/138863 A1, **2013**.
[29] B. Testa, J.M. Mayer. Isocyanates and Nitriles, in *Hydrolysis in Drug and Prodrug Metabolism, Chemistry, Biochemistry and Enzymology*. (Eds: T. Kolitzus, P.L. Bounds). VHCA, Zűrich, Switzerland, Wiley-VCH, Weinheim, Germany, **2003**, pp. 702–706.
[30] A. Parkinson. Glutathione conjugation, in *Biotransformation of Xenobiotics*. (Eds: C.D. Klaassen),The McGraw-Hill Companies, Andover, KS, USA, **2001**, pp. 213–218.