UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

katedra biochemických věd

METABOLISMUS ANTHELMINTIK U HELMINTŮ

disertační práce

Hradec Králové, 2008 Mgr. Viktor Cvilink

Práce prezentované v této disertační práci vznikly za finanční podpory Grantové agentury České republiky (grant číslo 524/06/1345), Grantové agentury Univerzity Karlovy (grant číslo 71808/B/2008) a Fondu mobility Univerzity Karlovy.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

Na tomto místě chci poděkovat v první řadě své školitelce doc. RNDr. Lence Skálové, Ph.D., za zaštítění mého studia na katedře biochemických věd, za její milý, přátelský a optimismem naplněný přístup, za vědecké nadšení a plány, toleranci, trpělivost a laskavou všestrannou péči, kterou mi nezištně věnovala. Děkuji.

Děkuji prof. Jiřímu Lamkovi, CSc., za ochotu při zajišťování biologického materiálu a doc. Ing. Barboře Szotákové, Ph.D., za obětavou pomoc při přípravě experimentů.

Speciální poděkování patří Aleně Pakostové, za umění hbitě pomoci v jakékoli situaci, za její lidský postoj a permanentně dobrou náladu.

Děkuji svým kolegům a přátelům z řad bývalých či současných Ph.D. studentů z katedry biochemických věd, Markovi, Heleně, Romaně, Bohunce, Michalovi, Ivě, Yogeetě, Laďce, Veronice, Lucce a Lucce, Pavlíně, Aničce, Adamovi a Hance, stejně tak všem ostatním pracovníkům katedry biochemických věd za vytvoření prostředí, ve kterém jsem se cítil dobře a doma.

Děkuji doc. Ing. Michalu Holčapkovi, Ph.D., za to, že mě představil záhadě jménem hmotnostní spektrometrie.

Děkuji prof. Ristovi Kostiainenovi a assoc. prof. Raimovi A. Ketolovi za otevřené dveře ve Viikki v Helsinkách.

V neposlední řadě děkuji svým rodičům za podporu, kterou mi poskytovali po celou dobu studia, a směju se na malou Alenku, že vstoupila do Říše divů a chytila se prstů mé ruky.

OBSAH

Seznam zkratek .... - 4 -4

1. ÚVOD .... - 5 -5

2. TEORETICKÁ ČÁST .... - 6 -6

2.1. Biotransformace xenobiotik u savců .... - 6 -6
2.1.1. První fáze biotransformace .... - 7 -7
2.1.2. Druhá fáze biotransformace .... - 10 -10

2.2. Biotransformace xenobiotik u helmintů .... - 12 -12

2.3. Helmintózy .... - 13 -13
2.3.1. Haemonchóza .... - 13 -13
2.3.2. Dikroceliόza .... - 13 -13

2.4. Benzimidazolová anthelmintika .... - 14 -14

2.5. Hmotnostní spektrometrie při studiu biotransformace .... - 15 -15
2.5.1. Příprava vzorku .... - 17 -17
2.5.2. Ionizační techniky .... - 18 -18
2.5.3. Hmotnostní analyzátory .... - 21 -21

2.6. Literatura .... - 24 -24

3. CÍLE PRÁCE .... - 28 -28

4. EXPERIMENTÁLNÍ ČÁST .... - 29 -29

4.1. Biotransformace xenobiotik u helmintů .... - 29 -29

4.2. První fáze biotransformace anthelmintik u vlasovky slézové .... - 29 -29

4.3. První a druhá fáze biotransformace anthelmintik u vlasovky slézové .... - 30 -30

4.4. První fáze biotransformace anthelmintik u motolice kopinaté .... - 31 -31

4.5. První a druhá fáze biotransformace anthelmintik u motolice kopinaté .... - 32 -32

5. ZÁVĚR .... - 34 -34

6. CONCLUSION .... - 36 -36

7. PŘÍLOHY .... - 38 -38

7.1. Publikace vztahující se k tématu disertační práce .... - 38 -38

7.2. Seznam všech publikací .... - 122 -122

7.3. Seznam prezentací na vědeckých setkáních .... - 123 -123

Seznam zkratek

ABZ	albendazol
ABZSO	albendazol sulfoxid
$ABZSO_2$	albendazol sulfon
CoA	koenzym A
CYP 450	cytochromy P450
FMO	flavinové monooxygenasy
FLU	flubendazol
FLUR	redukovaný flubendazol
FT-ICR	iontová cyklotronová rezonance s Fourierovou transformací
GSH	glutathion
GST	glutathion S-transferasa
HPLC	vysokoúčinná kapalinová chromatogafie
LLE	extrakce kapalina-kapalina
MALDI	ionizace laserem za použití matrice
MEB	mebendazol
MEBR	redukovaný mebendazol
MS	hmotnostní spektrometrie
MS/MS	tandemová hmotnostní spektrometrie
MS^n	tandemová hmotnostní spektrometrie do vyššího stupně
m/z	poměr hmotnosti a náboje iontu
NADH	nikotinamidadenindinukleotid
NADPH	nikotinamidadenindinukleotidfosfát
NAT	N-acetyltransferasa
PAPS	3´-fosfoadenosin-5´-fosfosulfát
ppm	jedna miliontina celku, tisícina promile
SPE	extrakce na pevné fázi
SPME	mikroextrakce na pevné fázi
SRM	selected reaction monitoring
SULT	sulfotransferasa
TOF	analyzátor doby letu
UGT	uridindifosfátglukuronosyltransferasa
UV	ultrafialový

1. Úvod

Hlavní cestou v boji s helmintickými parazitózami jsou farmakoterapie a farmakoprofylaxe prováděné v ohrožených či napadených chovech prostřednictvím dostupných vhodných anthelmintik. Celosvětově závažnou otázkou při léčbě helmintóz je vývoj rezistence parazitujících červů vůči působení anthelmintik. Mechanismus vzniku rezistence není dosud u většiny anthelmintik objasněn. Jedním z důvodů jsou nedostatečné znalosti o vlastních biotransformačních enzymech helmintů.

Biotransformační enzymy jednotlivých helmintů a jejich role, kterou mohou v detoxikaci anthelmintik hrát, nejsou zatím důkladněji zmapovány a popsány. Jsou to ale právě biotransformační enzymy, které mohou do určité míry chránit organismus parazita před působením anthelmintik (a xenobiotik jako představitelů chemického stresu pro organismus obecně). Schopnost zapojit své vlastní biotransformační enzymy a metabolizovat jimi podané anthelmintické léčivo na neúčinný metabolit může pro parazita v tomto směru představovat výhodný obranný mechanismus.

Krokem k bližší charakterizaci enzymů potenciálně metabolizujících určité anthelmintikum ve vybraném živočišném druhu je určení všech metabolitů, na které je xenobiotikum daným druhem transformováno. Kapalinová chromatografie ve spojení s hmotnostně spektrometrickou detekcí je pro nalezení a identifikaci metabolitů velmi vhodným nástrojem. Díky selektivitě a vysoké citlivosti hmotnostních analyzátorů, a také díky možnosti předseparace látek na koloně chromatografického systému, dosahuje toto spojení vynikajících výsledků i při analýze látek v složitých biologických matricích.

2. Teoretická část

2.1. Biotransformace xenobiotik u savců [1, 2]

Živé organismy během života neustále přicházejí do kontaktu s chemickými látkami z okolního prostředí. Látky mohou do organismu vstoupit a být využity například jako zdroje energie či stavební kameny pro syntézu dalších sloučenin. Na takové je pohlíženo jako na látky tělu vlastní, tzv. eobiotika. Organismus se však setkává i s látkami, které jsou tělu cizí, tzv. xenobiotiky. I xenobiotika, z našeho pohledu nejčastěji léčiva, mohou do organismu vstupovat.

Biotransformace xenobiotik je základním mechanismem pro udržení homeostázy organismů. Probíhá za účasti velkého počtu enzymů, které se vyznačují nízkou substrátovou selektivitou. Syntéza biotransformačních enzymů může sice být stimulována přímo přítomností xenobiotika v organismu (proces enzymové indukce), většinou jsou ale biotransformační enzymy syntetizovány neustále a nezávisle na aktuální expozici organismu xenobiotickému stimulu. Selektivita těchto enzymů je nízká a mohou se účastnit metabolických přeměn celé škály xenobiotických substrátů. Kromě toho mnoho biotransformačních enzymů katalyzuje také reakce endogenních látek, např. steroidních hormonů, vitaminů, bilirubinu, eikosanoidů a žlučových a mastných kyselin.

Biotransformačními enzymy katalyzované reakce xenobiotik se obecně dělí do dvou skupin: reakce první fáze a druhé fáze biotransformace. Jako nultá a třetí fáze metabolismu xenobiotik bývají označovány procesy spojené s transportem xenobiotika do buňky, respektive pozměněného xenobiotika z buňky. První fáze zahrnuje oxidační, redukční a hydrolytické pochody. Obvykle při těchto reakcích dochází k zavedení funkčních skupin do struktury xenobiotika (např. hydroxyskupiny, aminoskupiny, thiolové skupiny, karboxyskupiny) nebo jejich odhalení, „demaskování" (např. dealkylace). Reakce první fáze většinou přinášejí jen malý nárůst hydrofility substrátu. Během reakcí druhé fáze mohou být tyto substráty v místech odhalených či vnesených funkčních skupin konjugovány s endogenními, převážně velmi polárními konjugačními činidly, čímž je dosaženo výrazného zvýšení hydrofility xenobiotického substrátu a usnadnění jeho exkrece z organismu. Biotransformační procesy se odráží v pozměněné struktuře xenobiotika a potažmo tedy i v jeho modifikované biologické aktivitě. Mělo-li xenobiotikum před vstupem do biotransformačních reakcí biologickou aktivitu, mohlo

dojít k jeho deaktivaci nebo detoxikaci. Biotransformační pochody mohou naopak způsobit i aktivaci původně biologicky neaktivní molekuly xenobiotika, popřípadě navození či zvýšení její toxicity. Tyto důsledky jsou závislé na struktuře xenobiotika a mezi jednotlivými typy xenobiotik existují v tomto ohledu velké rozdíly.

2.1.1. První fáze biotransformace [1, 2, 3, 4, 5, 6]

2.1.1.1. Oxidace

2.1.1.1.1. Cytochromy P450

Pro cytochromy P450 je typický univerzální výskyt. Nalézají se prakticky ve všech organismech napříč spektrem. Jsou to klíčové enzymy v metabolismu léčiv a dalších xenobiotik a plní důležité funkce i v eobiotických metabolických drahách (zejména v drahách lipofilních látek, např. žlučových a mastných kyselin, cholesterolu a prostaglandinů). Existuje vysoký počet isoforem (cca 3700 – rok 2007) katalyzujících přeměnu obrovského množství substrátů a taktéž disponujících širokým rejstříkem enzymatických aktivit (kofaktorem je NADPH). Strukturně se jedná o velice rozmanitou skupinu hemoproteinů; pro jejich klasifikaci byla vyvinuta nomenklatura postavená na stupni shody aminokyselinové sekvence mezi jednotlivými isoformami. Isoformy s homologií v sekvenci přesahující 40% jsou řazeny do stejné rodiny (např. CYP3) a isoformy se sekvenční homologií nad 70% do stejné podrodiny (např. CYP3A). Každá individuální isoforma je dále označena číslicí (např. CYP3A4, CYP3A5). U eukaryotních organismů jsou cytochromy P450 vázány v komplexech s NADPH cytochrom P450 reduktasou na membránu endoplasmatického retikula (tzv. mikrosomální cytochromy). S cytochromem P450 souvisí další enzymy, cytochrom b5 a cytochrom b5 reduktasa, které se účastní přenosu elektronů z alternativního kofaktoru NADH na mikrosomální cytochrom P450. Ferredoxin závislé isoformy CYP 450 zapojené do metabolismu eobiotických substrátů je možno nalézt navázané na membránu mitochondrií. Pro funkci cytochromu P450 mají klíčový význam membránové fosfolipidy, zejména fosfatidylcholin, které udržují nativní konformaci enzymu.

Cytochromy P450 jsou obecně známé jako systémy s monooxygenasovou či oxytransferasovou aktivitou. Vůči některým xenobiotikům mohou však vykazovat i

aktivitu reduktasovou. Reduktasovou aktivitu může u některých substrátů za určitých podmínek doprovázet tvorba volných radikálů a mít značný toxikologický význam. Kromě CYP 450 se oxidačních reakcí xenobiotik a eobiotik účastní též další oxidační enzymy. Jako příklady je možno uvést NAD^+ závislé alkoholdehydrogenasu a aldehyddehydrogenasu, dále xanthinoxidasu a monoaminooxidasu. Do metabolismu xenobiotik jsou u člověka zapojeny zejména rodiny CYP1, CYP2 a CYP3, isoformy z ostatních rodin jsou zaměřené na eobiotické substráty. Nejvýznamnějšími jsou CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 a v prenatálním stavu i CYP3A7. Až 50% všech léčiv je u člověka přeměňováno prostřednictvím CYP3A4. Tento fakt je možné vysvětlit široce otevřeným aktivním místem isoformy enzymu a tudíž vysokým stupněm univerzality ve výběru substrátu.

2.1.1.1.2. FMO

Flavinové monooxygenasy (FMO) jsou přítomny v mnoha tkáních, ovšem nejvyšší hladiny FMO byly nalezeny ve tkáni jaterní a plicní. Podobně jako cytochromy P450 jsou lokalizovány na membránách hladkého endoplasmatického retikula. Jako prostetickou skupinu obsahují flavinové monooxygenasy flavinadenindinukleotid. Ke své funkci dále potřebují přísun elektronů (donorem NADPH nebo NADH) a molekulární kyslík. Působením FMO je metabolizována řada léčiv, typicky látky obsahující ve své molekule pouze jedno nukleofilní centrum, např. dusík, síru nebo fosfor. Eobiotika v drtivé většině obsahují nukleofilních center více, proto jsou ze spektra substrátů pro FMO vyjmuty, a tudíž vůči působení FMO chráněny. Substráty FMO bývají často současně též substráty cytochromů P450, produkty reakcí katalyzovaných FMO se však mohou od produktů reakcí katalyzovaných CYP 450 lišit. Mezi reakce katalyzované FMO patří např. N-oxidace sekundárních a terciárních aminů, hydroxylaminů, iminů a hydrazinů, S-oxidace thiolů, disulfidů, thioamidů, thiomočoviny a analogické reakce na fosforu.

Skupina FMO je nadrodinou enzymů zahrnující řadu isoforem majících různou substrátovou specifitu. Členění FMO je analogické členění cytochromů. V současné době je rozlišováno pět isoforem FMO1-FMO5. Hlavní jaterní isoformou u člověka je FMO3.

2.1.1.2. Hydrolýza

Hydrolytické enzymy katalyzují rozklad kovalentních vazeb, které vznikly kondenzací, tj. reakcí v jež vedly ke vzniku produktu za odštěpení molekuly vody. Při hydrolýze substrátu často dochází k odštěpení velké části molekuly substrátu, a tím k rozsáhlé změně jeho struktury. Mezi substráty hydrolytických enzymů patří estery, amidy, hydrazidy, karbamáty, hydroxamové kyseliny a další. Hydrolytické enzymy jsou všudypřítomné a vyskytují se v krevní plasmě, erytrocytech i buňkách tkání. Jako příklady hydrolas mohou být uvedeny arylesterasy, karboxyesterasy, acetylesterasy a cholinesterasa. Zvláštní formou hydrolýzy je hydratace, kdy dochází k zavedení molekuly vody do molekuly substrátu, aniž by současně došlo k jejímu rozkladu.

2.1.1.3. Redukce

Navzdory faktu, že v živých organismech, jako aerobních systémech, jsou upřednostňovány spíše oxidační reakce, rozsáhlá skupina látek v nich může podléhat enzymatické či neenzymatické redukci. Pro řadu léčiv je redukční mechanismus důležitou a významnou cestou biotransformace. Reduktasy jsou enzymy cytosolické i membránově vázané, NADH i NADPH závislé, existují též mitochondriální reduktasy. Pro organismus je významná redukční role střevní mikroflóry, zejména při redukcích některých alkenů (kyselina skořicová) nebo látek obsahujících azo- nebo nitroskupinu. Mezi hlavní enzymové systémy redukující xenobiotika patří reduktasy/dehydrogenasy s krátkým řetězcem (SDR), aldo/keto reduktasy (AKR), reduktasy/dehydrogenasy se středním řetězcem (MDR), nitrochinonreduktasy, cytochromy P450, NADPH-cytochrom P450 reduktasa a NADH-cytochrom b5 reduktasa. Redukční procesy zahrnují redukce násobných vazeb mezi uhlíkovými atomy, deoxygenaci arenoxidů, redukci chinonů a hydroperoxidů. Některé halogenované uhlovodíky, např. halothan, mohou být redukční cestou dehalogenovány (možná je i oxidační cesta, za katalýzu těchto přeměn je pak zodpovědný cytochrom P450). Do redukčních reakcí mohou vstupovat i sloučeniny obsahující dusík (azosloučeniny, N-oxidy, nitrosloučeniny), síru (disulfidy, sulfoxidy) nebo selen, dále organokovové a anorganické sloučeniny. Některé funkční skupiny mohou být jak redukovány, tak oxidovány. Například aldehydické látky mohou být redukovány za vzniku alkoholů, či oxidovány za vzniku příslušné karboxylové kyseliny. Látka typu sulfoxidu může být oxidativně přeměněna na sulfon nebo redukována na sulfid.

2.1.2. Druhá fáze biotransformace [1, 2, 4]

Mezi reakce druhé fáze biotransformace xenobiotik patří glukuronidace, sulfatace, acetylace, methylace a konjugace s glutathionem nebo aminokyselinami. Pro průběh konjugační reakce je nutná energetická aktivace substrátu či kofaktoru (spotřeba energie). Aktivovaný kofaktor (či substrát) pak dále reaguje s vhodnými funkčními skupinami substrátu (či kofaktoru). Vhodné funkční skupiny mohou být přítomny v molekule od začátku nebo být do molekuly vneseny během první fáze biotransformace. Většina reakcí druhé fáze vede k velkému zvýšení hydrofility substrátu a výrazně tak usnadňuje jeho vyloučení z organismu.

2.1.2.1. Glukuronidace

Glukuronidace je hlavní metabolickou cestou druhé fáze biotransformace u člověka. Je katalyzována uridindifosfátglukuronosyltransferasami (UGT) a zahrnuje přenos aktivované α-D-glukuronové kyseliny na substrát. UGT jsou mikrosomální enzymy hojně zastoupené ve tkáních, např. v játrech, ledvinách, střevech a dalších. V buněčném cytosolu dochází k dvoukrokové syntéze aktivované UDP-glukuronové kyseliny, která je pak transportním systémem přenášena do lumen endoplasmatického retikula. Substráty UGT jsou především molekuly obsahující nukleofilní heteroatom, např. alkoholy, fenoly, thioly, aminy, z eobiotických látek především bilirubin, steroidní a thyroidní hormony. Produkty reakcí jsou O-glukuronidy, N-glukuronidy a S-glukuronidy, které se vzájemně liší stabilitou v kyselém prostředí a citlivostí vůči působení ß-glukuronidas. Nomenklatura UGT je obdobná jako u cytochromů P450. U lidí jsou zatím objeveny 2 třídy UGT: UGT1 a UGT2.

2.1.2.2. Sulfatace

Sulfatace je velice běžná konjugační reakce. Enzymy se jí účastnící se nazývají sulfotransferasy (SULT), energeticky aktivovaným kofaktorem je 3´-fosfoadenosin-5´-fosfosulfát (PAPS), jehož siřičitanová skupina je sulfotransferasami přenášena na hydroxylovou skupinu substrátu. Sulfotransferasy jsou cytosolické enzymy, vyznačují se relativně vysokou afinitou k substrátu, avšak mají nižší reakční kapacitu, a to z důvodu nízké pohotovostní endogenní koncentrace PAPS. Nejčastějšími substráty jsou látky obsahující hydroxylovou skupinu: alkoholy, fenoly. Z endogenních substrátů

jsou to například steroidní a thyroidní hormony. Sulfataci mohou podstupovat i aminy. Vznikající organické sulfáty jsou hydrofilnější než původní látky a velmi snadno se vylučují z organismu. Nomenklatura SULT je opět založena na stupni shody v aminokyselinové sekvenci a rozřazuje SULT do tří rodin: SULT1, SULT2 a SULT4.

2.1.2.3. Konjugace s glutathionem

Reakce s glutathionem jsou katalyzovány glutathion S-transferasami (GST). GST jsou převážně cytosolické enzymy, které využívají jako kofaktor tripeptid glutathion (γ-L-glutamyl-L-cysteinyl-glycin, GSH). Většina konjugačních reakcí probíhá za účasti GST, avšak GSH obvykle bývá v buňkách přítomný v tak vysoké koncentraci, že může docházet i k neenzymatické konjugaci substrátů. Konjugaci s glutathionem podléhají hlavně sloučeniny elektrofilního charakteru, např. látky halogensubstituované, nitrosubstituované nebo sulfosubstituované, případně heterocyklické sloučeniny. Nomenklatura a třídění GST do skupin znovu stojí na míře podobnosti aminokyselinové sekvence.

Konjugace s glutathionem též hraje důležitou roli při biosyntéze leukotrienů. Tyto reakce jsou katalyzovány mikrosomálními glutathion S-transferasami.

2.1.2.4. Konjugace s aminokyselinami

Do konjugací s aminokyselinami vstupují převážně xenobiotika či eobiotika obsahující karboxylovou skupinu. Samotné konjugaci předchází aktivace xenobiotického substrátu reakcí s acetylkoenzymem A za působení mitochondriální acyl-CoA syntetasy. V druhé fázi je acyl aktivovaného karboxyl nesoucího xenobiotika přenesen na aminoskupinu aminokyseliny. Tato reakce je katalyzována N-acyltransferasou. Mezi aminokyseliny zapojující se do konjugačních reakcí patří glycin, taurin nebo glutamin. Konkrétní zúčastněná aminokyselina závisí na druhu xenobiotika podstupujícího konjugaci. Těmi jsou, jak už bylo zmíněno, různé alifatické, aromatické či heterocyklické karboxylové kyseliny. Typická je pro nesteroidní antiflogistika. Z eobiotik konjugaci s aminokyselinami podstupují hlavně žlučové kyseliny.

2.1.2.5. Acetylace

Koenzymem acetylačních reakcí je podobně jako v případě konjugace s aminokyselinami acetylkoenzym A. Enzymy katalyzující acetylace se nazývají N-acetyltransferasy (NAT). Jsou to cytosolické enzymy katalyzující přenos acetylové skupiny. V první fázi dochází k acetylaci enzymu a posléze přenosu acetylu do molekuly substrátu. Nejčastějšími substráty jsou látky obsahující aminoskupinu, např. aromatické aminy nebo látky s hydrazinovou skupinou. Látky s alifatickou aminoskupinou nejsou NAT běžně metabolizovány. U člověka byly nalezeny 2 druhy N-acetyltransferas: NAT1 a NAT2. Zatímco NAT1 je přítomna ve většině tkání, NAT2 byla nalezena pouze v jaterní a nepatrně ve střevní tkáni. U NAT2 byl nalezen výrazný genetický polymorfismus (vyskytují se tzv. rychlí a pomalí acetylátoři).

2.1.2.6. Methylace

Je poměrně zvláštní konjugační reakcí, ve které dochází ke zvýšení lipofility xenobiotika, navíc se methylací obvykle maskují hydrofilní funkční skupiny vnesené do struktury látky během první fáze biotransformace. Methylace katalyzují mikrosomální i cytosolické enzymy nazývané methyltransferasy. Kofaktorem reakcí je S-adenosylmethionin. Substráty se rozdělují podle místa konečného přenosu methylskupiny. Ta může být přenesena na atom kyslíku (O-methyltransferasy přeměňující fenoly, katecholaminy, dopamin), na atom dusíku (N-methyltransferasy metabolizující nikotin, různé aminy xeno- i eobiotického původu) nebo na atom síry (S-methyltransferasy methylující thioly, merkaptopurin). Detekována byla i methylace přímo na atomu uhlíku xenobiotika (u benzo[*a*]pyrenů), jedná se však o vzácnou metabolickou přeměnu. Pro methyltransferasy je opět typický genetický polymorfismus.

2.2. Biotransformace xenobiotik u helmintů

Biotransformačním procesům xenobiotik u helmintů bylo v porovnání s biotransformačními pochody u savců věnováno mnohem méně pozornosti. Našimi pracemi jsme se snažili přispět k prohloubení znalostí této problematiky. Současný stav vědění na tomto poli jsme shrnuli v přehledném článku, který je přílohou této disertační práce.

2.3. Helmintózy [7, 8, 9]

Helmintózy, nemoci způsobené parazitujícími červy, představují jednu z nejrozšířenějších příčin ohrožení zdravotního stavu hospodářských, domácích i volně žijících zvířat. Chovatelům hospodářských zvířat působí tyto nemoci značné ekonomické ztráty, samotným zvířatům pak zhoršení zdravotního stavu, které vede v mnoha případech k předčasnému úhynu.

Jako modelové parazitující druhy pro studium metabolismu vybraných benzimidazolových léčiv byly pro účely našich experimentů vybrány vlasovka slézová (*Haemonchus contortus*, Trichostrongylidae, Nematoda) a motolice kopinatá (*Dicrocoelium dendriticum*, Dicrocoeliidae, Trematoda).

2.3.1. Haemonchóza

Vlasovka slézová (*Haemonchus contortus*) je častý a relativně velmi patogenní parazit domácích i volně žijících přežvýkavců (hlavně ovcí, koz). Je to oblý červ, gonochorista, délky zhruba 10-20 mm (samci) a 20-30 mm (samice). U zvířat žije ve slézu, přichycen ústním otvorem k mukóze žaludeční stěny. Infekce vlasovkami, jako krev sajícími parazity, se projevuje anémií a hypoproteinémií. Nakažená zvířata jsou slabá, apatická, ztrácí na váze. I relativně malý počet jedinců (v řádu několika desítek až set) může zapříčinit doprovodné krvácení do žaludku z lézí způsobených přichycením parazita na žaludeční stěnu (cca 50 ml denně). Pokud je hostitel vystaven působení velkého počtu jedinců parazita během krátké doby, může se vyskytnout tzv. hyperakutní forma haemonchózy. Hostitel pak umírá zhruba za 7 dní kvůli masivním ztrátám krve (až 600 ml denně, přispívá též krvácení do žaludku z lézí). U chronické haemonchózy, která může trvat několik měsíců, nebývá anémie tak razantně vyjádřena z důvodu kompenzace zvýšením krvetvorby.

2.3.2. Dikrocelióza

Dikrocelióza, helmintóza způsobená motolicí kopinatou, je v současnosti považována za celosvětově významné, avšak dosud méně prozkoumané onemocnění domácích i volně žijících zvířat a též relativně vzácnou chorobu člověka. Jedná se o jednu z šesti nejčastěji se vyskytujících helmintóz hospodářských přežvýkavců, ale postihuje také volně žijící zvířata. Výskyt helmintózy je celosvětový. Motolice kopinatá je plochý červ s průhledným tělem dosahujícím délky 5 až 15 mm a šířky 1,5 až 3 mm. Parazituje zejména ve žlučových cestách, žlučovém měchýři a ve vývodu pankreatu

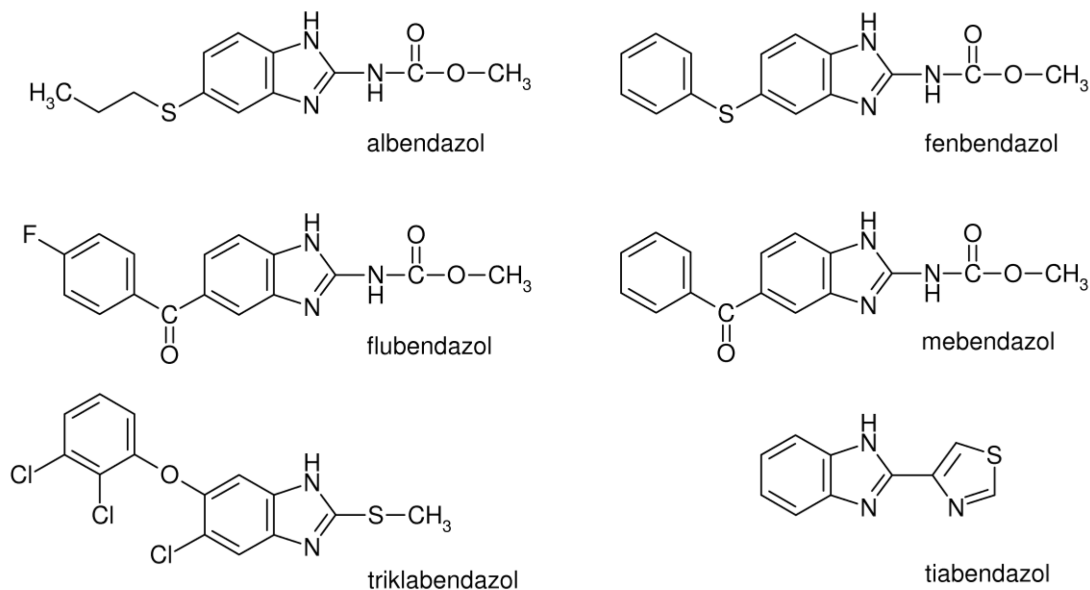
svého hostitele. U definitivního hostitele působí zánět žlučovodů, který postupně přechází na chronickou formu s typickými znaky, kterými jsou rozšíření duktů, hyperplazie, proliferace a deskvamace mukózy.

2.4. Benzimidazolová anthelmintika [10, 11, 12, 13, 14, 15, 16]

Mezi léčivy používanými k terapii helmintóz, tj. anthelmintiky, hrají významnou roli benzimidazolová anthelmintika. Benzimidazolová anthelmintika patří mezi léčiva ve veterinární i humánní medicíně celosvětově hojně využívaná. Spektrum účinku je poměrně široké, liší se u konkrétních zástupců. U většiny derivátů zahrnuje tasemnice i hlístice, některé širokospektré deriváty navíc působí i na motolice, či vykazují navíc antimykotickou aktivitu. Většina benzimidazolových anthelmintik působí nejen proti dospělým helmintům, ale i proti vývojovým stádiím parazitů, některá z nich mají toxický účinek i na jejich vajíčka.

Ve veterinární medicíně hrají benzimidazolová anthelmintika velmi významnou roli jak v prevenci, tak i v terapii parazitárních infekcí a jsou podávána mnoha zvířecím druhům. K nejčastěji používaným patří albendazol, fenbendazol, flubendazol, mebendazol, triklabendazol a tiabendazol (Obrázek 1). Anthelmintická aktivita léčiv je závislá na době udržení terapeutických koncentrací v tělních tekutinách a tkáních. U polygastrických zvířat a dalších býložravců (např. skot, ovce, kůň) lze benzimidazoly podávat jednorázově, u monogastrických zvířat (např. pes, kočka, prase, drůbež) je většinou nutné opakované podání. K typickým indikacím patří nematodózy gastrointestinálního traktu, plic, příp. i dalších orgánů a tkání. Albendazol, triklabendazol a fenbendazol vykazují antitrematodní aktivity a používají se při léčbě fasciolózy a dikroceliózy. Anticestodní aktivitu mají albendazol, fenbendazol, mebendazol a flubendazol. Mechanismus účinku benzimidazolových anthelmintik spočívá v selektivní vazbě na β-podjednotku tubulinu parazitů. Touto vazbou je zabráněno polymerizaci monomerů a vzniku mikrotubulů, které tvoří strukturní jednotky mnoha buněčných organel a které jsou nezbytné pro řadu buněčných pochodů včetně mitózy. Po jejich podání bylo u parazitů pozorováno poškození struktury a funkce tegumentu, hypodermis, svalových vrstev a střeva. Také docházelo k blokování vzniku gamet. Dále může být působením benzimidazolových anthelmintik narušen energetický metabolismus a pozorováno snížení příjmu glukózy, což někdy bývá uváděno jako další mechanismus účinku.

Benzimidazolová anthelmintika vykazují pouze mírné nežádoucí účinky na organismy hostitelů. Toto specifické působení, selektivní toxicita benzimidazolových anthelmintik, je důsledkem mnohem nižší reverzibility jejich vazby na tubulin parazita oproti tubulinu hostitele. Rezistence na léčbu, která se u některých kmenů vyskytuje, má často podstatu v bodové mutaci genu pro β-tubulin vedoucí k syntéze proteinu s odlišnými vlastnostmi.



Obrázek 1. Chemické struktury vybraných benzimidazolových anthelmintik

2.5. Hmotnostní spektrometrie při studiu biotransformace [4, 17, 18, 19, 20, 21, 22, 23, 24, 25]

Hmotnostní spektrometrie se ukázala jako užitečná technika pro identifikaci strukturně rozmanitých látek. Její online spojení s HPLC separací představuje efektivní a citlivou techniku s dostatečnou robustností. Kombinace HPLC s tandemovou hmotnostní spektrometrií (HPLC-MS/MS) se stala jednou z hlavních technik pro identifikaci neznámých látek. Určení totožnosti neznámé látky ale zůstává náročným úkolem. Data totiž často neposkytují dostatečně jednoznačnou možnost interpretace, nezřídka jsou nejasná a málo zřejmá, a interpretace takových dat je potenciálně problematickým místem.

Při studiu metabolismu látek a identifikaci jejich metabolitů je ovšem parentní látka vždy známa a obyčejně bývá dostatek prostoru studovat její fragmentační chování,

případně fragmentační chování synteticky připravených a teoreticky očekávaných metabolitů. Každá metabolická změna v molekule látky s sebou nese určitou změnu v molekulové hmotnosti vzniklého metabolitu, adekvátní počtu atomů vnesených či odtržených od struktury původního, nezměněného léčiva. Srovnání fragmentačního chování neznámého metabolitu a parentní látky (popřípadě synteticky připraveného standardu metabolitu) lze poté *per analogiam* využít při identifikaci, místa a druhu metabolické změny.

Primární snahou při studiu metabolismu xenobiotika je zachycení všech přítomných vytvořených metabolitů z parentní látky a jejich strukturní charakterizace. Dalším případným krokem může být jejich kvantifikace. K naplnění těchto úkolů je často využíváno spojení kapalinové chromatografie a hmotnostní spektrometrie. Obvyklým postupem při získávání dat je porovnání výsledků tzv. „full scan" analýz slepých vzorků a skutečných vzorků získaných při *in vitro*, *in vivo* či *ex vivo* experimentech. Toto porovnání by manuálně bylo nesmírně časově náročné, a proto se provádí s využitím speciálních počítačových programů. Software porovná chromatogramy jednotlivých vzorků a označí všechny ionty, které jsou přítomné ve skutečných vzorcích a chybí ve vzorcích slepých. Program také na základě rozdílu molekulových hmotností právě zkoumaného iontu a iontu parentní sloučeniny navrhne pro analyzovaný ion možnou metabolickou změnu, a to podle tabulky předpokládaných metabolických modifikací, která je součástí softwaru a je uživatelem editovatelná. Vybrané ionty lze podrobit dalším analýzám, které mohou přinést bližší informace o zkoumaném jedinci a případně potvrdit identitu metabolitu. Právě taková data dovoluje získat tandemová hmotnostní spektrometrie. Během tandemové hmotnostní analýzy je v jednom experimentu uskutečněna analýza vybraného iontu do druhého nebo vyššího stupně. Zahrnuje řadu typů skenů, mezi které řadíme skeny produktových iontů, skeny prekurzorových iontů a skeny neutrálních ztrát.

Pomocí hmotnostního spektrometru lze nejprve vybraný ion izolovat a poté podrobit fragmentaci v tzv. kolizní cele (u některých typů analyzátorů probíhá izolace a fragmentace iontu v jednom kompartmentu). Kolizní cela je naplněna inertním plynem, často argonem nebo heliem. Dochází v ní ke srážkám zkoumaného iontu s molekulami inertního plynu za štěpení chemických vazeb a vzniku fragmentů zkoumaného iontu. Ty jsou zaznamenány jako spektrum produktových iontů. Takto získaná spektra produktových iontů obsahují pouze ionty vzniklé štěpením daného vybraného prekurzoru a lze z nich usuzovat na strukturní charakter prekurzoru. V případě MS^n

analýz lze vybraný produktový ion znovu izolovat a podrobit jej dalšímu koliznímu štěpení, které přinese dodatečné strukturní informace týkající se již jen iontu analyzovaného v posledním stupni. Fragmentovat ionty lze i přímo v iontovém zdroji, zde však odpadá možnost výběru prekurzorového iontu. V případě, že je zvolen takový průběh analýzy, ve kterém dochází k výběru iontu (krok 1), jeho fragmentaci (krok 2) a zaznamenání výsledného spektra (krok 3), nazýváme takový sken skenem produktových iontů a získáváme jím produktové spektrum. V případě skenu prekurzorových iontů je nastavení výběru iontu prováděno až v kroku 3 (vybírán je požadovaný produktový ion). Zaznamenávány pak jsou všechny prekurzorové ionty, které svou disociací poskytují právě vybraný produktový ion. Výsledkem je spektrum prekurzorových iontů. Při skenu neutrálních ztrát jsou vybírány sledované ionty jak v kroku 1, tak v kroku 3, a to takovým způsobem, že rozdíl v *m/z* hodnotách (poměr hmotnost iontu vs. náboj iontu) pro oba ionty vybrané v krocích 1 a 3 zůstává konstantní (z iontu se odštěpuje charakteristická neutrální skupina). Například se ověřuje, zda ve vybraném intervalu hodnot *m/z* dochází k neutrální ztrátě 80 u (ztráta typická pro sulfáty), tedy zda ion o hodnotě *m/z* 380 poskytuje produktový ion o hodnotě *m/z* 300. Následuje sken, který toto zjišťuje pro hodnotu 381, hledá se produktový ion *m/z* 301, poté 382 a 302 atd. Velikost neutrální ztráty, pro kterou má spektrometr skenovat, je samozřejmě volitelná. Některé přístroje díky své vysoké přesnosti měření umožňují stanovit *m/z* iontů s přesností na několik desetinných míst, tj. běžně s přesností pod 5 ppm (analyzátory doby letu). Analyzátory využívající Fourierovu transformaci jsou schopny dosáhnout i hodnot menších než 1 ppm. Díky znalosti přesné molekulové hmotnosti je možné zjistit elementální složení analyzované látky. Znalost elementálního složení může výrazně napomoci při strukturní identifikaci analytu a bývá tím faktorem, který ze skupiny návrhů možných struktur vybere tu jednu správnou, eventuálně potvrdí či vyvrátí hypotézu metabolické modifikace v dané části molekuly. Důležitou roli při měřeních s přesným určením hmoty hraje správná kalibrace hmotnostní stupnice. Ideálně se při měřeních využívá interní kalibrace, kdy se během analýzy vzorku souběžně sprejuje kalibrant, nejlépe pomocí další sprejující kapiláry.

2.5.1. Příprava vzorku [17, 18, 20, 26, 27,28]

Ačkoli jsou HPLC-MS(/MS) analýzy velmi citlivé a selektivní, jistá příprava vzorků, obzvláště pokud se jedná o práci s komplexními biologickými matricemi, bývá výhodná a je důležitým krokem při zisku platných analytických dat. Důkladným a

pečlivým zpracováním vzorku lze nejen docílit zlepšené citlivosti, ale lze i předejít zanesení chromatografické kolony či dalších částí chromatografického systému balastními látkami přítomnými v biologické matrici (soli, proteiny, jiné makromolekuly). Nejběžnější metody dnes používané zahrnují homogenizaci vzorku, eventuální vysrážení proteinů a centrifugaci, případně filtraci nebo úpravu pH, které jdou ruku v ruce s následnou extrakcí kapalina-kapalina (LLE), extrakcí na pevných fázích (SPE) nebo mikroextrakcí na pevné fázi (SPME) Cílem je co možná nejefektivnější extrakce analytu z matrice. Při srážení proteinů je nutné pamatovat na fakt, že eventuální použití kyseliny za tímto účelem může mít za následek např. hydrolýzu některých konjugátů a zkreslit tak výsledek analýzy. Elegantně se lze tomuto vyhnout použitím organických rozpouštědel v precipitačním kroku. Taktéž při SPE je třeba vhodnou volbou sorbentu zajistit, aby nedošlo k úniku látek zájmu. Výše zmíněné techniky patří mezi zavedené a dlouhodobě užívané. V poslední době je snaha o automatizaci a urychlení přípravy vzorku.

2.5.2. Ionizační techniky [4, 17,19, 20, 21,22, 23, 29]

Tato kapitola se omezuje pouze na stručné představení ionizačních technik využívaných při studiu metabolismu léčiv.

Rané ionizační techniky (moving belt, particle-beam, termosprej), ač nebyly vždy snadné a praktické na použití a trpěly nízkou robustností a citlivostí, jistě našly ve specifických aplikacích své uplatnění. Úlohou ionizační techniky je převést molekuly analytu do stavu, který je vhodný pro zavedení do hmotnostního analyzátoru, tj. zionizovat neutrální molekuly analytu, případně odpařit kapalné nosné médium. Intenzivní vývoj hmotnostně spektrometrických aplikací, hlavně ve spojení s kapalinovou chromatografií, byl zapříčiněn až nástupem citlivých a robustních ionizačních technik za atmosferického tlaku (API). Jako API jsou souhrnně označovány všechny ionizační techniky, při kterých jsou ionty tvořeny bez nutnosti přítomnosti vakua. V HPLC-MS metabolomických aplikacích nacházejí uplatnění zejména ionizace elektrosprejem (ESI) a chemická ionizace za atmosferického tlaku (APCI). Velmi blízká APCI je fotoionizace za atmosferického tlaku (APPI). Ionizace laserem za použití matrice (MALDI) nachází významné využití převážně při analýze biopolymerů.

2.5.2.1. Ionizace elektrosprejem

Elektrosprej (ESI) je v současnosti nejběžněji používanou API ionizační technikou. ESI lze úspěšně kombinovat s různými typy analyzátorů, je využíván při analýze polárních až středně polárních látek, látek termálně nestabilních a látek s vysokou molekulovou hmotností. Jedná se o „měkkou“, velice šetrnou ionizační techniku: minimum fragmentací během procesu ionizace zaručuje, že vazby v molekule zůstanou neporušené a nedojde degradaci molekuly (labilních vazeb konjugátů). Pro elektrosprej je charakteristický vznik vícenásobně nabitých iontů analytu. Vícenásobně nabitý ion je detekován na nové hodnotě *m/z*, dojde k posunu na stupnici *m/z* směrem dolů. Tímto způsobem je umožněna analýza látek o vysoké molekulové hmotnosti, které by nesoucí jediný náboj přesahovaly svou hodnotou *m/z* dynamický rozsah přístroje. Elektrosprej je technikou volby pro online spojení kapalinové chromatografie a hmotnostního spektrometru. Analyt je v proudu mobilní fáze přiváděn do kovové kapiláry v iontovém zdroji. Na kapiláru je vloženo vysoké napětí (několik kV), které je zodpovědné za vznik nabitých částic z molekul analytu. Za pomoci zmlžujícího a sušícího plynu je z proudu kapaliny vytvářen proud miniaturních analyt nesoucích kapek, které dále podstupují tzv. „coulombické štěpení“ (rozpad kapky nesoucí nabité molekuly na menší kapky) a „vypařování iontů“, které vedou k převedení nabitých částic analytu z kapaliny do stavu izolovaných nabitých molekul v plynné fázi. U ionizace elektrosprejem, stejně jako u ostatních „měkkých“ ionizačních technik, je častý výskyt adduktů analyzovaných molekul s ionty sodnými, draselnými či amonnými (v závislosti na druhu mobilní fáze použité při předcházející chromatografické separaci). Vyskytnout se mohou též addukty s neutrálními molekulami rozpouštědel (např. methanol, acetonitril, voda). Pro podporu ionizace analytu je možné přidávat k eluentu v nízkých koncentracích vhodný modifikátor, při záznamu kladných iontů obvykle kyselinu octovou či mravenčí, případně při záznamu záporných iontů hydroxid amonný. Nevhodné jsou přísady jakýchkoli netěkavých sloučenin (soli, netěkavé pufry v mobilní fázi), které ve vyšších koncentracích mohou výrazně potlačit signál analytu. Miniaturizovanou variantou elektrospreje je nanoelektrosprej, který vyniká svou citlivostí a též lepší tolerancí k obsahu solí ve vzorku, ztrácí ovšem co se týče robustnosti.

2.5.2.2. Chemická ionizace za atmosferického tlaku, fotoionizace za atmosferického tlaku

Lepší toleranci k obsahu solí v mobilní fázi než elektrosprej má též chemická ionizace za atmosferického tlaku (APCI). Tato měkká ionizační technika je vhodná pro látky jak nepolární, tak polární, obzvlástě opět ve spojení s HPLC-MS (APCI pojme až 2 ml eluentu za minutu). Tzv. koronárním výbojem (napětí na koronární jehle v řádu několika kV) dojde k ionizaci molekul zmlžené mobilní fáze a posléze ionizované molekuly mobilní fáze ion molekulárními reakcemi ionizují molekuly analytu (v tento moment již v plynné fázi). Molekuly mobilní fáze a analytu jsou během procesu ionizace zahřívány na relativně vysoké teploty (cca 500°C). Proto se tato technika nehodí pro ionizaci termolabilních látek jako jsou např. glukuronidy, sulfáty a N-oxidy. Obdobou APCI je fotoionizace za atmosferického tlaku (APPI), jen místo koronárního výboje se pro ionizaci využívá ultrafialového záření. Je vhodná i pro ionizaci látek nepolárního charakteru.

2.5.2.3. Ionizace laserem za účasti matrice

Ionizace laserem za účasti matrice (MALDI) je měkká ionizační technika a používá se k ionizaci různých druhů biopolymerů, nejčastěji proteinů a peptidů, případně oligonukleotidů nebo sacharidů. Časté je spojení MALDI s analyzátorem doby letu (TOF). Desorpce a ionizace analytu je v MALDI dosaženo jediným laserovým pulsem. Analyzovaný vzorek je připraven smísením s vhodnou matricí, vybranou tak, aby dovedla absorbovat záření o vlnové délce daného laseru, odpařen a poté vystaven krátkému laserovému pulsu, jehož energii matrice absorbuje a je ionizována. Matrice předá část své absorbované energie molekulám analytu: dojde k ionizaci a desorpci molekul analytu. Jako matrice slouží nejčastěji hydroxysubstituované aromatické kyseliny, které silně absorbují v UV oblasti (použití dusíkových UV laserů) a zároveň díky svému kyselému charakteru podporují ionizaci analytu jako donor protonů. Typické MALDI spektrum obvykle obsahuje převážně jednou nabité ionty (protonované molekuly analytu), mohou se vyskytnout dvojnásobně nabité, zřídka i vícenabité.

2.5.3. Hmotnostní analyzátory [4, 17, 19, 20, 21, 23]

Aby se minimalizovala možnost nechtěných kolizí molekul analytu s dalšími molekulami, pracují všechny hmotnostní analyzátory za vysokého vakua. Jakákoli srážka by mohla způsobit fragmentaci analytu, tvorbu adduktů či shluků, eventuelně deionizaci analytu, a negativně by ovlivnila hmotnostní spektrum. Proto jsou hmotnostní spektrometry evakuovány, obvykle na 10^{-2} – 10^{-5} Pa, v závislosti na přístroji. Evakuace probíhá na několika úrovních. V první linii mechanická vakuová pumpa poskytuje hrubé vakuum cca 0,1 Pa. V druhé linii se využívají difuzní nebo turbomolekulární pumpy, které již dosáhnou vysokého vakua. U přístrojů typu FT-ICR, majících ještě vyšší nároky na vakuum, dochází k nasazení vysoce výkonných kryogenních pump. V této kapitole jsou krátce zmíněny vybrané hmotnostní analyzátory a jednoduše vysvětlen princip jejich fungování.

2.5.3.1. Kvadrupólový analyzátor, trojitý kvadrupólový analyzátor

Kvadrupólový analyzátor je jeden z nejběžnějších hmotnostních analyzátorů. Bývá stolních rozměrů, schopen rychlého skenování, cenově dostupný, rozlišení se pohybuje v řádu několika jednotek tisíc. Skládá se ze čtyř rovnoběžně umístěných elektrod, nejlépe hyperbolického průřezu, na které je vloženo stejnosměrné napětí a vysokofrekvenční střídavé napětí (během cyklu mají vždy dvě protilehlé elektrody kladný potenciál, zbylé dvě záporný, na všechny je vloženo vysokofrekvenční střídavé napětí). Po opuštění iontového zdroje jsou před vstupem do kvadrupólu ionty urychleny elektrickým polem. Během průletu kvadrupólem jsou pomocí na elektrody vkládaných napětí jednotlivé ionty filtrovány podle svých hodnot *m/z* tak, že vždy je umožněn úspěšný průlet celou délkou kvadrupólu jen iontům o jedné hodnotě *m/z*. Použitá napětí totiž způsobují oscilaci letících iontů a právě pouze ionty se stabilní oscilací mohou dosáhnout detektoru, ostatní ionty s nestabilní oscilací kolidují s elektrodami kvadrupólu. Plynulou změnou hodnot napětí lze postupně umožnit dosažení detektoru iontům o všech hodnotách *m/z*. V praxi se častěji vyskytuje trojitý kvadrupól (tři jednoduché kvadrupóly v sérii), který umožňuje provádět některé výhodné typy skenů (např. sken produktových iontů, sken prekurzorových iontů, sken neutrálních ztrát, SRM skenování…). Prostřední kvadrupól je přitom použit jako kolizní cela, která je naplněna kolizním plynem. Analyzátory typu trojitého kvadrupólu jsou díky vysoké citlivosti a selektivitě v SRM módu vhodně využívány pro kvantifikaci látek.

2.5.3.2. Kvadrupólová iontová past

Analyzátory typu iontových pastí umožňují detailně studovat fragmentační mechanismy molekul. V principu se jedná o trojrozměrný trojitý kvadrupól. Analyzátor se skládá ze dvou koncových elektrod hyperbolického tvaru a jedné kruhové elektrody s hyperbolickým průřezem umístěné mezi dvěma koncovými. Na elektrody je vkládáno stejnosměrné a vysokofrekvenční střídavé napětí, vytváří se trojrozměrné pole, které umožňuje zadržení iontů o určitých vybraných hodnotách *m/z* (mající za daných podmínek stabilní oscilaci), zatímco ostatní ionty díky svým nestabilním trajektoriím kolidují s elektrodami. Do iontové pasti vstupují ionty o různých hodnotách *m/z*, které jsou posléze podle hodnot *m/z*, zvyšováním amplitudy vysokofrekvenčního napětí vkládaného na kruhovou elektrodu, vypuzovány na detektor. Ionty o vybrané *m/z* mohou být v pasti zachyceny, jejich oscilace utlumeny tlumícím plynem (nejčastěji helium) a „zakoncentrovány" ve středu iontové pasti. Zachycené ionty pak mohou být podrobeny fragmentaci, a to do druhého (MS/MS) či vyššího stupně (MS^n). V praxi bývají obvykle detekovány ionty do cca MS^5.

Lineární iontová past je typ iontové pasti, kde jsou ionty zachytávány uvnitř lineárního kvadrupólu vhodným nastavením hodnot napětí v jeho koncových částech. Výhodou lineární iontové pasti je její vyšší iontová kapacita a dynamický rozsah.

2.5.3.3. Magnetický analyzátor, magnetický analyzátor s dvojí fokusací

Magnetický analyzátor rozlišuje ionty v magnetickém poli podle jejich kinetické energie a náboje. Po opuštění iontového zdroje je ion urychlen elektrickým polem a směřuje do magnetického analyzátoru. Jelikož nese náboj, dochází v magnetickém poli k zakřivení jeho dráhy letu. Míra zakřivení závisí na kinetické energii daného iontu, dále na náboji iontu a síle magnetického pole. Ionty s nižší kinetickou energií, tedy ionty s nižší hodnotou *m/z*, mají vyšší míru zakřivení trajektorie (působící odstředivá síla je menší než u iontů s vyšší hodnotou *m/z*). Zařazení elektrostatického analyzátoru za magnetický dalo vzniknout magnetickému analyzátoru s dvojí fokusací. V elektrostatickém analyzátoru dochází, poté co ionty opustí magnetický analyzátor, k zakřivení drah iontů podle hodnot jejich kinetických energií, čímž „se zaostří" ionty o stejné kinetické energii na stejnou úroveň a docílí se tak zvýšeného rozlišení až v řádech několika desítek tisíc. Existuje několik variant uspořádání magnetického a elektrostatického analyzátoru: liší se pořadím zapojených analyzátorů a propojovacími

úhly mezi analyzátory. Magnetický sektorový analyzátor s dvojí fokusací je poměrně rozměrné zařízení, avšak lze na něm dosáhnout měření s vysokou přesností a vysokým rozlišením. V současné době jsou však přístroje tohoto typu vytlačovány buď přístroji typu TOF, případně FT-ICR.

2.5.3.4. Analyzátor doby letu

Svou stavbou a principem je analyzátor doby letu (TOF) nejjednodušším hmotnostním analyzátorem. Jelikož se jedná o pulzní analyzátor, je výhodné a časté jeho spojení s MALDI. Ionty desorbované ze vzorku jsou urychleny napěťovým pulsem, čímž získávají přibližně stejnou kinetickou energii, a letí dále již nijak neurychlovány směrem k detektoru. Čas, za který daný ion urazí vzdálenost odpovídající délce letové trubice a dopadne na detektor, slouží k určení *m/z* daného iontu. Vychází se přitom z faktu, že ionty s nižší hodnotou *m/z* (platí zmíněný předpoklad stejné kinetické energie udělené všem iontům) dorazí na detektor za kratší čas než ionty s vyšší hodnotou *m/z*. Výhodou analyzátorů doby letu je prakticky neomezený dynamický rozsah a schopnost velice rychle skenovat, která je limitována jen dobou, za jakou „nejtěžší“ z analyzovaných iontů dosáhne detektoru. Další výhodou analyzátorů doby letu je schopnost měření s přesným určením hmoty (s přesností cca 5 ppm), což může významně napomoci při získání sumárního vzorce při identifikaci neznámé sloučeniny.

Reflektron (též reflektor, iontové zrcadlo) je prvek sloužící ke zvýšení rozlišení TOF analyzátoru. Proud iontů je po průniku do reflektronu vystaven působení elektrického pole, které mění trajektorii iontů a vrací ionty zpět směrem, kterým přiletěly. V iontovém zrcadle dochází ke kompenzaci drobných rozdílů mezi kinetickými energiemi udělenými jednotlivým iontům o stejné hodnotě *m/z* během urychlení. Ionty s vyššími kinetickými energiemi proletí do reflektronu hlouběji; urazí tak delší vzdálenost než ionty o stejné *m/z*, ale s nižší kinetickou energií. Tímto způsobem dochází ke srovnání délky drah, které ionty se stejnou hodnotou *m/z* urazí, a razantnímu zvýšení rozlišení. Rozlišení může být ještě dále zvýšeno, například použitím techniky opožděné extrakce iontů. V současnosti dosahují v praxi TOF přístroje rozlišení v řádu desítek tisíc.

2.5.3.5. Iontová cyklotronová rezonance s Fourierovou transformací

Cyklotronové hmotnostní spektrometry s Fourierovou transformací (FT-ICR) jsou v současné době přístroje velmi nákladné a provozně náročné, avšak nabízející

ultravysoké rozlišení (v řádu statisíců) a nejvyšší přesnost určení hmoty (pod 1 ppm). Ionty z iontového zdroje kolmo vstupují do silného magnetického pole detekční cely, jehož vlivem se začnou s určitou frekvencí, která závisí na *m/z* iontu, pohybovat po cyklické trajektorii (opět kolmo ve vztahu ke směru svého původního pohybu). Excitací iontů vysokofrekvenčním napětím se zvětší poloměr jejich cyklického pohybu, ionty se přiblíží destičkám detekčního systému a dojde k indukci proudu v detekčním systému cely. Tento proud je zaznamenáván a pomocí Fourierovy transformace převeden na frekvenční spektrum, ze kterého se získá spektrum hmotnostní.

2.6. Literatura

[1] Gibson, G.G., Skett, P.: Introduction to drug metabolism. 3rd edition. (2001) Nelson Thornes Publishers, Cheltenham.

[2] Parkinson, A.: Biotransformation of Xenobiotics. In: Klaassen, C.D. (Ed): Casarett & Doull's Toxicology, The Basic Science of Poisons. 6th edition. (2003) McGraw-Hill Professional.

[3] Anzenbacherová, E., Anzenbacher, P.: Cytochromes P450 and metabolism of xenobiotics. (1999) *Bulletin of Czech Society for Biochemistry and Molecular Biology*, 27, 4-33.

[4] Rossi, D.T., Sinz, M.W.: Mass Spectrometry in Drug Discovery. (2002) Marcel Dekker, New York.

[5] Lewis, D.F.V.: Guide to cytochromes P450 structure and function. 1st edition. (2001) Taylor and Francis, London.

[6] Testa, B., Caldwell, J.: Metabolism of drugs and other xenobiotics. (1995) Academic Press, London.

[7] Jíra, J.: Lékařská helmintologie. (1998) Galén, Praha.

[8] http://parasitology.informatik.uni-wuerzburg.de/login/n/h/2229.html.

[9] Otranto, D., Traversa, D.: Dicrocoeliosis of ruminants: a little known fluke disease. (2003) *Trends Parasitol.* 19:12-15.

[10] Lamka, J., Ducháček, L.: Anthelmintika. In: Veterinární léčiva pro posluchače farmacie. (1998) Karolinum, Praha, 67-84.

[11] Lacey, E.: The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. (1988) *Int J Parasitol.* 18:885-936.

[12] Hanser, E., Mehlhorn, H., Hoeben, D., Vlaminck, K.: In vitro studies on the effects of flubendazole against Toxocara canis and Ascaris suum. (2003) *Parasitol Res.* 89:63-74.

[13] Walker, S. M., McKinstry, B., Boray, J. C., Brennan, G. P., Trudgett, A., Hoey, E. M., Fletcher, H., Fairweather, I.: Response of two isolates of Fasciola hepatica to treatment with triclabendazole in vivo and in vitro. (2004) *Parasitol Res.* 94:427-438.

[14] McConville, M., Brennan, G. P., McCoy, M., Castillo, R., Hernandez-Campos, A., Ibarra, F., Fairweather, I.: Immature triclabendazole-resistant Fasciola hepatica: tegumental responses to in vitro treatment with the sulphoxide metabolite of the experimental fasciolicide compound alpha. (2007) *Parasitol Res.* 100:365-377.

[15] Petersen, M. B., Friis, C.: Glucose uptake in Oesophagostomum dentatum and the effect of oxfendazole. (1998) *Vet Parasitol.* 80:159-166.

[16] Sangster, N.: Pharmacology of anthelmintic resistance. (1996) *Parasitology.* 113 Suppl:S201-216.

[17] Clarke, N. J., Rindgen, D., Korfmacher, W. A., Cox, K. A.: Systematic LC/MS metabolite identification in drug discovery. (2001) *Anal Chem.* 73:430A-439A.

[18] Kostiainen, R., Kotiaho, T., Kuuranne, T., Auriola, S.: Liquid chromatography/ atmospheric pressure ionization-mass spectrometry in drug metabolism studies. (2003) *J Mass Spectrom.* 38:357-372.

[19] Holčapek, M.: Hmotnostní spektrometrie v organické analýze, přednášky k předmětu.

[20] Holčapek, M., Kolářová, L., Nobilis, M.: High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. (2008) *Anal Bioanal Chem.* 391:59-78.

[21] Gross, J.H.: Mass Spectrometry: A Textbook. (2004) Springer-Verlag, Berlín.

[22] Dettmer, K., Aronov, P. A., Hammock, B. D.: Mass spectrometry-based metabolomics. (2007) *Mass Spectrom Rev.* 26:51-78.

[23] Korfmacher W.A.: Mass spectrometry in drug metabolism studies. (2005) CRC Press, Boca Raton.

[24] Prakash, C., Shaffer, C. L., Nedderman, A.: Analytical strategies for identifying drug metabolites. (2007) *Mass Spectrom Rev.* 26:340-369.

[25] Oliveira, E. J., Watson, D. G.: Liquid chromatography-mass spectrometry in the study of the metabolism of drugs and other xenobiotics. (2000) *Biomed Chromatogr.* 14:351-372.

[26] Bolden, R. D., Hoke, S. H., 2nd, Eichhold, T. H., McCauley-Myers, D. L., Wehmeyer, K. R.: Semi-automated liquid--liquid back-extraction in a 96-well format to decrease sample preparation time for the determination of dextromethorphan and dextrorphan in human plasma. (2002) *J Chromatogr B Analyt Technol Biomed Life Sci.* 772:1-10.

[27] Chen, Y., Guo, Z., Wang, X., Qiu, C.: Sample preparation. (2008) *J Chromatogr A.* 1184:191-219.

[28] Ulrich, S.: Solid-phase microextraction in biomedical analysis. (2000) *J Chromatogr A.* 902:167-194.

[29] Kaufmann, R.: Matrix-assisted laser desorption ionization (MALDI) mass spectrometry: a novel analytical tool in molecular biology and biotechnology. (1995) *J Biotechnol.* 41:155-175.

3. Cíle práce

Hlavním cílem naší práce bylo přinést nové informace a poznatky týkající se metabolismu benzimidazolových anthelmintik u parazitujících červů vlasovky slézové a motolice kopinaté, přispět tak ke snahám zefektivnit anthelmintickou léčbu a porozumět mechanismům rezistence vůči anthelmintikům obecně.

Dílčími cíli bylo:

1) Zkoumat první fázi biotransformace flubendazolu *in vitro* a *ex vivo* u vlasovky slézové (*Haemonchus contortus*), zabývat se též stereospecifitou případně detekovaných pochodů

2) Identifikovat metabolity první i druhé fáze biotransformace albendazolu a flubendazolu u vlasovky slézové (*Haemonchus contortus*) s využitím hmotnostně spektrometrické detekce

3) Zkoumat první fázi biotransformace albendazolu a albendazol sulfoxidu *in vitro* a *ex vivo* u motolice kopinaté (*Dicrocoelium dendriticum*), zabývat se též stereospecifitou případně detekovaných pochodů

4) Identifikovat metabolity první i druhé fáze biotransformace albendazolu, flubendazolu a mebendazolu u motolice kopinaté (*Dicrocoelium dendriticum*) s využitím hmotnostně spektrometrické detekce

4. Experimentální část

4.1. Biotransformace xenobiotik u helmintů

Cvilink, V., Lamka, J., Skálová, L. - Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths (submitted)

Cílem práce bylo shrnout současný stav znalostí na poli biotransformačních enzymů u helmintů, účast těchto enzymů v metabolických přeměnách xenobiotických substrátů, dále zhodnotit možnou úlohu těchto biotransformačních enzymových systémů při vzniku a vývoji rezistence vůči působení anthelmintických léčiv. Tato práce je první prací tohoto typu týkající se biotransformačních systémů parazitických červů. Bližší informace lze nalézt v práci samotné v sekci Přílohy.

4.2. První fáze biotransformace anthelmintik u vlasovky slézové (Haemonchus contortus)

Cvilink, V., Kubíček, V., Nobilis, M., Křížová, V., Szotáková, B., Lamka, J., Várady, M., Kuběnová, M., Novotná, R., Gavelová, M., Skálová, L. (2008). Biotransformation of flubendazole and selected model xenobiotics in *Haemonchus contortus*. Vet Parasitol. 151:242-248.

V tomto projektu bylo cílem zkoumat první fázi biotransformace anthelminticky účinného benzimidazolového léčiva flubendazolu parazitickým červem vlasovkou slézovou (*Haemonchus contortus*) s důrazem na redukční enzymy parazita. Za účelem studia biotransformace byli izolováni jedinci *H. contortus* ze slézu hostitele, v tomto případě byla hostitelem ovce domácí. Část izolovaných helmintů byla použita na přípravu subcelulárních frakcí pro *in vitro* experimenty, část byla užita pro *ex vivo* inkubace. Získané vzorky byly po extrakci analyzovány pomocí HPLC s UV detekcí. První fáze biotransformace flubendazolu byla hodnocena také z pohledu stereospecifity. Reduktasová aktivita biotransformačních enzymů *H. contortus* vůči karbonylové skupině xenobiotika byla dále testována pomocí dalších modelových substrátů nesoucích karbonylovou skupinu: metyraponu, acenaphthenolu, D,L-glyceraldehydu,

daunorubicinu a oracinu, které jsou poměrně specifickými substráty pro karbonylreduktasy člověka.

V provedených experimentech byla detekována významná reduktasová aktivita biotransformačních enzymů *H. contortus* vůči flubendazolu. Jak v *in vitro*, tak v *ex vivo* pokusech došlo k redukci karbonylové skupiny flubendazolu za vzniku metabolitu – redukovaného flubendazolu (FLUR). Tato aktivita byla *in vitro* striktně NADPH závislá. Redukční pochod neprobíhal byl-li jako koenzym použit NADH. Redukcí karbonylové skupiny flubendazolu vznikala chirální molekula redukovaného flubendazolu, byla nalezena významná stereospecifita enzymové aktivity pozorovaná jako preferenční tvorba (-)-FLUR. Poměr (-)-FLUR k (+)-FLUR činil 9:1. Reduktasová aktivita enzymů vlasovky slézové byla potvrzena schopností redukovat karbonylovou skupinu testovaných modelových substrátů. *In vitro* experimenty ukázaly, že enzymy obsažené v cytosolické frakci homogenátu parazita účinně redukovaly všechny modelové substráty, enzymy mitochondriální frakce však *in vitro* redukovaly pouze daunorubicin.

4.3. První a druhá fáze biotransformace anthelmintik u vlasovky slézové (Haemonchus contortus)

Cvilink, V., Skálová, L., Szotáková, B., Lamka, J., Kostiainen, R., Ketola, R. A. (2008). LC-MS-MS identification of albendazole and flubendazole metabolites formed *ex vivo* by *Haemonchus contortus*. Anal Bioanal Chem. 391:337-343.

Úkolem v této práci bylo nalézt a identifikovat všechny metabolity první a druhé fáze biotransformace anthelmintik albendazolu a flubendazolu parazitickým helmintem vlasovkou slézovou (*Haemonchus contortus*). Po získání jedinců parazita byly provedeny *ex vivo* inkubace parazitů s vybranými léčivy. Pro nalezení metabolitů bylo po extrakci na pevné fázi analyzováno jak živné médium z inkubací, tak homogenát těl inkubovaných parazitů. K určení metabolického profilu jsme využili hmotnostně spektrometrickou detekci (trojitý kvadrupól).

V *ex vivo* pokusech byly nalezeny metabolity první i druhé fáze biotransformace. Identifikace metabolitů proběhla na základě shody retenčních časů daného metabolitu a jeho standardu při LC separaci (byl-li k dispozici) a na základě kombinace informací získaných z příslušných full scan spekter a produktových spekter

iontů. Z reakcí první fáze byla u albendazolu (*m/z* 266) zaznamenána S-oxidace, za tvorby albendazol sulfoxidu (*m/z* 282). Další oxidace sulfoxidu na sulfon detekována nebyla. Flubendazol (*m/z* 314) byl biotransformován na redukovaný flubendazol (*m/z* 316). Nalezena byla i významná konjugační aktivita enzymů parazita. V případě albendazolu byly na základě retenčních časů identifikovány dva různé konjugáty (*m/z* 428) s hexosou (glukosou), patrně lišící se od sebe místem navázání glukosy na aglykon. Tvorba glukosových konjugátů *ex vivo* nepřímo ukazuje na přítomnost jak glukosyltransferas, tak pro konjugační reakci nutného kofaktoru v parazitickém organismu. Albendazol sulfoxid do konjugačních reakcí nevstupoval, nebyly zaznamenány žádné ionty, které by na toto ukazovaly. Konjugaci s glukosou naopak ve velké míře podléhal redukovaný flubendazol (tvorba O-konjugátu, *m/z* 478) a flubendazol (konjugát *m/z* 476). Při MS/MS experimentech byla pro glukosid redukovaného flubendazolu typická ztráta 180 u. Tato neutrální ztráta je charakteristická pro benzylglukosidy či acylglukosidy. Toto posloužilo k identifikaci místa konjugace. Detekovaný O-glukosid redukovaného flubendazolu, jako benzylglukosid, tuto ztrátu velmi ochotně vykazoval. U konjugátů albendazolu i flubendazolu docházelo při MS/MS experimentech ke ztrátě 162 u, což napovídalo, že hexosová jednotka byla na aglykon vázána jiným způsobem než přes kyslík. Z důvodu lability glykosidické vazby však nebylo možné ani přibližně určit místo metabolické modifikace. Tvorba konjugátů s hexosou byla potvrzena skeny neutrálních ztrát, kdy u všech konjugátů docházelo k zmíněným charakteristickým neutrálním ztrátám (162 u a 180 u).

4.4. První fáze biotransformace anthelmintik u motolice kopinaté (Dicrocoelium dendriticum)

Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L. (2008). Phase I biotransformation of albendazole in lancet fluke (*Dicrocoelium dendriticum*). Res Vet Sci. (*in press*)

Cílem tohoto projektu bylo studovat první fázi biotransformace benzimidazolového anthelmintika albendazolu (ABZ) a jeho metabolitu albendazol sulfoxidu (ABZSO) parazitickým červem motolicí kopinatou (*Dicrocoelium*

dendriticum). První fáze biotransformace albendazolu byla hodnocena i po stránce stereospecifity. Jako zdroj motolice kopinaté byl použit přirozeně infikovaný hostitel, ovce muflon (*Ovis musimon*). Část získaných dospělých jedinců *D. dendriticum* byla *ex vivo* inkubována s příslušným anthelmintikem, část byla použita pro přípravu subcelulárních frakcí, které byly následně využity pro *in vitro* experimenty. Analýza vzorků byla po příslušné extrakci analytů prováděna pomocí HPLC s fluorimetrickou detekcí.

In vitro pokusy ukázaly striktně NADPH dependentní sulfoxidaci albendazolu, a to jak v mikrosomální, tak v mitochondriální frakci. Detekována byla také dvoustupňová oxidace albendazolu, dávající vznik albendazol sulfonu. Tento proces byl však pozorován jen v mitochondriální subcelulární frakci. Přesto jde o první důkaz, že parazitický červ je schopen deaktivovat albendazol sulfonací na albendazol sulfon (albendazol sulfoxid je sám anthelminticky účinný). Cytosolická frakce nevykazovala vůči albendazolu žádnou oxidační aktivitu. Byl-li jako substrát použit albendazol sulfoxid, byla detekována jeho oxidace na albendazol sulfon, opět ale pouze v mitochondriální subcelulární frakci. Sulforedukce albendazol sulfoxidu nebyla pozorována v žádné z frakcí. V *ex vivo* experimentech byl jediným detekovaným metabolitem albendazol sulfoxid, a to v živném médiu z inkubace i homogenátu inkubovaných těl parazitů. Sulfoxidace ani sulforedukce albendazol sulfoxidu nebyla pozorována. Albendazol sulfoxid ve své molekule obsahuje chirální centrum. Chirální analýza enantiomerů *in vitro* tvořeného albendazol sulfoxidu ukázala na nepříliš vysokou specifitu tvorby enantiomerů: poměr (+)-ABZSO : (-)-ABZSO byl 55:45. *Ex vivo* výsledky ukázaly převahu tvroby (-)-ABZSO. Tento posun oproti *in vitro* výsledkům mohl být zapříčiněn zvýšenou vazbou (+)-ABZSO na makromolekuly parazita.

4.5. První a druhá fáze biotransformace anthelmintik u motolice kopinaté (Dicrocoelium dendriticum)

Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L. - LC-MS identification of benzimidazole anthelminthics metabolites formed *ex vivo* by *Dicrocoelium dendriticum* (submitted)

V řešeném projektu bylo cílem testovat metabolismus benzimidazolových anthelmintik albendazolu (ABZ), flubendazolu (FLU) a mebendazolu (MEB) u motolice kopinaté (*Dicrocoelium dendriticum*) v podmínkách *ex vivo* a *in vitro*. Dále pomocí spojení kapalinové chromatografie a hmotnostní spektrometrie zkoumat první a druhou fázi biotransformace, identifikovat metabolity, popsat biotransformační cesty vybraných anthelmintik a charakterizovat hlavní biotransformační enzymy parazita. Získaní jedinci parazita byli vystaveni působení anthelmintika při *ex vivo* inkubacích v živném médiu. Po extrakci na pevné fázi byly analyzovány homogenát těl inkubovaných parazitů i živné médium z inkubací.

Výsledky ukázaly, že enzymy *D. dendriticum* byly schopné katalyzovat oxidační i redukční reakce první fáze, stejně jako konjugační reakce druhé fáze biotransformace. Albendazol byl oxidován na albendazol sulfoxid (*m/z* 282), což byl také jediný detekovaný ABZ metabolit. Karbonylová skupina flubendazolu byla redukována za vzniku redukovaného flubendazolu (*m/z* 316). Detekovány byly dva monomethylkonjugáty redukovaného flubendazolu (*m/z* 330). Díky výskytu produktových iontů na *m/z* 174 a *m/z* 146 bylo na základě MS^n analýz určeno, že se jedná v prvním případě o methylaci na benzimidazolovém jádře a v druhém na postranním řetězci flubendazolu. Pomocí MS^n analýzy byla též detekována tvorba deoxydimethylflubendazolu (*m/z* 328). Mebendazol byl enzymy motolice redukován za vzniku redukovaného mebendazolu (*m/z* 298). Jediný detekovaný metabolit druhé fáze biotransformace mebendazolu byl nalezen na *m/z* 326 a po sérii MS^n experimentů určen jako dimethylderivát redukovaného mebendazolu. Účast parentních látek albendazolu, flubendazolu, mebendazolu ani ABZSO jako metabolitu první fáze nebyla v konjugačních reakcích detekována.

Zajímavým výsledkem, do jisté míry překvapivým, však bylo, že biotransformační enzymy motolice kopinaté vykazovaly překvapivou selektivitu ve výběru substrátů a též nečekanou specifitu ve tvorbě produktů. Ač je flubendazol v podstatě fluorsubstituovaný mebendazol, nedocházelo k monomethylaci derivátu mebendazolu, pozorován byl pouze dimethylderivát; albendazol nebyl methylován vůbec. Detekované methylované deriváty ukázaly, že *Dicrocoelium dendriticum* pravděpodobně disponuje enzymovými systémy schopnými methylace i syntézy kofaktoru methylačních procesů, S-adenosylmethioninu.

5. Závěr

Terapie anthelmintiky je v současné době hlavní metodou v boji s parazitickými červy. Znalost a pochopení detoxikačních systémů, kterými tyto organismy disponují a které jsou jimi využívány k obraně proti působení xenobiotik, jsou důležité pro porozumění pochodům ovlivňujícím hladinu léčiva v těle parazita a rozhodujícím tak o výsledku anthelmintické léčby. Nedosažení efektivní koncentrace léčiva v organismu parazita nevyhnutelně vede ke snížení účinnosti léčby, může vést k indukci přítomných biotransformačních enzymů parazita a vyústit ve vznik rezistence vůči podávanému léčivu. Biotransformační enzymy helmintů jsou však doposud jen málo prostudovány a často je nezřejmé, zda je jimi daný druh vůbec vybaven a zda je dovede v tomto ohledu využít.

V předkládané práci bylo naším cílem popsat biotransformační cesty vybraných anthelmintik ve vybraných druzích helmintů, identifikovat vytvářené metabolity a charakterizovat biotransformační enzymy zkoumaných parazitů. Pozornost jsme zaměřili na dva druhy helmintů, vlasovku slézovou (*Haemonchus contortus*) a motolici kopinatou (*Dicrocoelium dendriticum*) a jejich schopnost biotransformovat benzimidazolová anthelmintika albendazol, flubendazol a mebendazol.

Výsledky experimentů jednoznačně ukázaly, že oba druhy helmintů disponují enzymatickými systémy schopnými zasáhnout do metabolismu léčiv. Prostřednictvím online spojení kapalinové chromatografie a hmotnostně spektrometrické detekce jsme zkoumali první a druhou fázi biotransformace benzimidazolových anthelmintik. Enzymy studovaných parazitických druhů vykazovaly oxidasovou i reduktasovou aktivitu: byly schopny katalyzovat sulfoxidaci albendazolu na albendazol sulfoxid a albendazol sulfon a redukovat karbonylovou skupinu flubendazolu a mebendazolu. U obou druhů jsme na základě detekce konjugátů parentních léčiv i produktů první fáze jejich biotransformace nalezli aktivitu enzymů druhé fáze biotransformace. Zatímco u druhu *Haemonchus contortus* je jednalo o konjugaci albendazolu, flubendazolu a redukovaného flubendazolu s hexosou (předpokládáme glukosou), druh *Dicrocoelium dendriticum* biotransformoval molekuly flubendazolu, mebendazolu a redukovaného flubendazolu methylmodifikací na příslušné monomethyl- a dimethylderiváty. Žádný z nalezených procesů druhé fáze biotransformace xenobiotik u helmintů nebyl do této doby publikován. Metabolické změny ve struktuře podávaného léčiva významně

ovlivňují jeho účinnost je z hlediska zkoumaného parazita považovat za detoxikační, obranné a xenobiotikum neutralizující. Selektivity biotransformačních enzymů druhé fáze se navíc mezidruhově velmi lišily, což bylo jen potvrzením, že poznatky načerpané u jednoho druhu nelze spolehlivě přenášet na druh jiný.

Hlubší a podrobnější znalost metabolismu anthelmintik v cílových druzích helmintů a také znalost rozdílů mezi biotransformačními enzymy hostitele a parazita mohou pomoci zefektivnit anthelmintickou léčbu, vést ke zmírnění dopadů helmintorezistence a být cenné v potenciálním vývoji nových druhově specifických léčiv.

6. Conclusion

Anthelminthic drugs are presently the principal method for the control of helminth diseases. Knowledge of detoxification mechanisms that helminths possess is important for understanding the processes that affect the drug concentration within a helminth organism and may be crucial in the treatment efficacy. When the drug concentration in a target organism does not reach the therapeutic level, it results in a decreased efficacy of pharmacotherapy which may further lead to the induction of helminth biotransformation enzymes and eventually issue in resistance development to the administered drug.

Present knowledge of helminth biotransformation enzymes is insufficient. Often it is not known whether a given individual helminth species possesses such enzymes or whether it is capable of employing them in treatment evasion.

In the presented thesis, the objective was to investigate the biotransformation pathways of selected anthelminthics in model helminth species, identify the formed metabolites and characterize biotransformation enzymes of the studied parasites. Our research was focused on two species, the barber pole worm (*Haemonchus contortus*) and the lancet fluke (*Dicrocoelium dendriticum*) and their ability to metabolize selected benzimidazole anthelminthics albendazole, flubendazole and mebendazole.

The results showed unambiguously that both helminth species have enzymatic systems capable of engaging in the xenobiotic metabolism. By means of liquid chromatography in conjunction with mass spectrometry, the investigation of phase I and phase II biotransformation in selected helminths showed that the studied parasitic species possessed an ability to oxidize and reduce given anthelminthic substrates. Sulfoxidation of albendazole to albendazole sulfoxide and albendazole sulfone and reduction of flubendazole and mebendazole carbonyl groups occurred. Formation of second phase biotransformation products indicated presence of conjugation enzymes in examined helminths. Glucose conjugates of albendazole, flubendazole and reduced flubendazole were found in experiments with *H. contortus*. Methylmodification of flubendazole, mebendazole and reduced flubendazole was observed in experiments with *D. dendriticum*. None of the identified conjugation reactions of xenobiotics in helminths has been published until now. Such metabolic modifications of drugs can substantially affect their anthelminthic action and can be considered a detoxication and defense

mechanisms. Substantial interspecies differences in the second phase enzyme selectivities were observed. This finding confirmed the fact that data valid for one species cannot be easily extrapolated to another.

Deeper and more detailed knowledge of anthelminthic biotransformation in helminths, and also the knowledge of differences in host and parasite biotransformation enzymes, can contribute to render the anthelminthic treatment more effective, can help to cushion the impact of helminth resistance development and have implicatons for the design of new anthelminthic drugs.

7. Přílohy

7.1. Publikace vztahující se k tématu disertační práce

I. Cvilink, V., Lamka, J., Skálová, L. - Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths. Drug Metab Rev. (*submitted*)

II. Cvilink, V., Kubíček, V., Nobilis, M., Křížová, V., Szotáková, B., Lamka, J., Várady, M., Kuběnová, M., Novotná, R., Gavelová, M., Skálová, L. (2008). Biotransformation of flubendazole and selected model xenobiotics in *Haemonchus contortus*. Vet Parasitol. 151:242-248.

III. Cvilink, V., Skálová, L., Szotáková, B., Lamka, J., Kostiainen, R., Ketola, R. A. (2008). LC-MS-MS identification of albendazole and flubendazole metabolites formed *ex vivo* by *Haemonchus contortus*. Anal Bioanal Chem. 391:337-343.

IV. Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L. (2008). Phase I biotransformation of albendazole in lancet fluke (*Dicrocoelium dendriticum*). Res Vet Sci. (*in press*)

V. Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L. - LC-MS identification of benzimidazole anthelminthics metabolites formed *ex vivo* by *Dicrocoelium dendriticum* (*submitted*)

I.

Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths

Cvilink, V., Lamka, J., Skálová, L.

submitted

Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths

V. Cvilink*, J. Lamka and L. Skálová

Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, Hradec Králové, Czech Republic

Abstract

Anthelminthics remain the only accessible means in the struggle against helminth parasites which cause significant morbidity and mortality in man and farm animals. The treatment of helminthic infections has become problematic because of frequent drug resistance of helminth parasites. The development of drug resistance can be facilitated by the action of xenobiotic metabolizing enzymes (XME). In all organisms, XME serve as an efficient defense against the potential negative action of xenobiotics. The activities of XME determine both desired and undesired effects of drugs and the knowledge of drug metabolism is necessary for safe and effective pharmacotherapy. While human and mammalian XME have been intensively studied for many years, XME of helminth parasites have undergone relatively little investigation so far. However, many types of XME including oxidases, reductases, hydrolases, transferases and transporters have been described in several helminth species. XME of helminth parasites may protect these organisms from toxic effects of anthelminthics. In case of certain anthelminthics, metabolic deactivation was reported in helminth larvae and/or adults. Moreover, if a helminth is in the repeated contact with an anthelminthic, it defends itself against the chemical stress by the induction of biotransformation enzymes or transporters. This induction can represent an advantageous defense strategy of the parasites and may facilitate the drug resistance development.

Key words: drug metabolism, helminth parasites, biotransformation enzymes, resistance, xenobiotics, anthelminthic treatment

1. Introduction

1.1. Position of helminths in parasitology and possibilities of their control

The coexistence of millions of species in nature (unicellular organisms, algae, fungi, plants, animals) has resulted from the course of their evolution in development of several types of mutual relationships: predator-prey, phoresis, mutualism, commensalism and parasitism. A parasitic relationship, common in both plant and animal kingdoms, is characterized by an association of two organisms of different species. The parasite, living on or within the host, is metabolically dependent on the host. Parasites generally do harm to a host, but they also play an important role in species evolution (Hendrix, 2006).

Disorders caused by parasites affect and trouble man and animals all over the world. Seven out of the 10 most serious human diseases belong to parasitoses and eight of them are spread by human parasites (World Health Organisation, WHO, www.who.int/whosis/whostat/2007). Human parasitosis is frequent especially in tropical and subtropical territories. Approximatelly 1.3 million people die of a parasitosis each year and 3.8 million people are simultaneously infected (Molyneux, 2006; Hotez, 2007). Thirty-two out of 81 serious animal diseases (cattle, sheep, goat, equine, avian, swine, bee and lagomorph) listed by World Organisation for Animal Health (OIE) are directly caused or supported by parasites (www.oie.int./eng/maladies/en_classification2008). Numerous parasitoses are also zoonoses; this means the transmission of the causative agent from an infected animal to man. Human or animal parasites live either within the host body (endoparasites) or on the host surface (ectoparasites). The causative agents are classified in regnum Animalia in two subregna Metazoa and Protozoa (Mehlhorn, 2001). The first one contains the especially parasitically important Platyhelminths of three classes (primitive and true tapeworms, flukes), Nematoda (roundworms) and Arthropoda of two classes (ticks and mites, insects). The subregnum Protozoa contains parasitically significant subphylum Mastigophora and phyla Apicomplexa, Microspora and Ciliophora.

A number of helminth species (Platyhelminths and Nematoda) affect man and/or animals causing a disease termed helminthosis. Man is affected by a helminthosis

directly (as a host of a helminth) or indirectly as a breeder of parasitized farm and other animals. Helminthic impacts are of a definitive (death of the human or animal host, culling of livestock) or a temporary character (losses in general yield and especially in the production of farm animals, decreased immunity to other infectious diseases). Human and veterinary medicine fight with helminthic infections over the world. Several approaches have been historically used, such as regulations of vectors in populations, zootechnical strategies, breeding of animals for parasitic resistance and treatment with drugs of antiparasitic effect. The last mentioned possibility is the most practiced approach in the last four decades in the form of pharmacotherapy and pharmacoprophylaxis. However, the situation is complicated by the fact that the number of available anthelminthic agents is relatively low, they are long term used and their efficacy decreases. Helminths have developed mechanisms that protect them against the toxic effects of anthelminthics and the resistance of many helminths to common antiparasitic drugs has become a worldwide problem. There are a number of drug resistance mechanisms in helminths, some of them have been well recognized and understood, but many others remain to be elucidated. Recently it has been pointed out that the drug resistance development can be partly related to systems of enzymes and transporters which protect an organism from the potential negative action of xenobiotics (Kerboeuf, 2003; Robinson, 2004).

1.2. Introduction to xenobiotic metabolizing enzymes

Many xenobiotic compounds, e.g., environmental contaminants, drugs or food additives, permanently enter animal bodies. In an organism, most of these xenobiotics are metabolized by unique enzymes into more polar metabolites that are easier to excrete. Xenobiotic metabolism is considered to occur in three phases. Oxidation, reduction or hydrolysis of drugs represent phase I of metabolism. In this step, reactive and hydrophilic groups are inserted or uncovered in the structures of xenobiotics. In phase II, xenobiotics or their phase I metabolites can undergo conjugation reactions with endogenous compounds. Glutathione, glucuronic acid, amino acids and sulphates represent the main conjugation agents. The active transport of substrates, metabolites or conjugates through membranes mediated by special protein transporters is now considered to be the third phase of xenobiotic metabolism.

The most important enzymes involved in phase I biotransformation are cytochromes P450 (CYPs), heme-proteins ubiquitous in living organisms. Thousands of different CYPs have been identified and classified into several families and subfamilies on the basis of the sequence homology of the genes. CYPs commonly catalyze monooxygenation of substrates, but they are also able to act as peroxidases or reductases. In addition to xenobiotic biotransformation, CYPs play a very important role in endogenous metabolism of steroids, fatty acids and prostaglandins. Flavin-containing monooxygenases (FMO) mediate the oxidation of a large number of xenobiotics, preferring substrates with nucleophilic nitrogen or sulphur atoms. The FMO gene family is much smaller than the CYP family, and the FMO physiological functions are poorly understood. In spite of this fact, FMO activities are important in biotransformation of many xenobiotics (Krueger, 2005). Peroxidases, monoamine oxidase and xanthine oxidase represent other enzymes which can catalyze the oxidation of xenobiotics (Uetrecht, 2007). Alcohols, aldehydes and ketones are often metabolized via the action of reductases/dehydrogenases. These enzymes are classified into three protein classes: medium chain dehydrogenases (MDR), short chain dehydrogenases (SDR) and aldo-keto reductases (AKR) (e.g., Jez, 2001). The substrates of reductases/dehydrogenases are not only xenobiotics but also a number of endogenous substances. Reductive biotransformation is less frequent than the oxidative one, but in case of ketones, aldehydes, quinones, nitro compounds, N-oxides or S-oxides the reduction can represent the main metabolic pathway. Many xenobiotics, such as esters, amides and epoxides, undergo hydrolytic biotransformation. Several hydrolases (e.g., acetylcholine esterases, peptidases), the primary function of which lies in endogenous metabolism, can participate in hydrolysis of these xenobiotics (Testa, 2003, 2007).

Most of the metabolites formed in phase I and appropriate functional groups containing xenobiotics are conjugated with certain endogenous compounds. Conjugation usually introduces hydrophilic ionizable functional groups to the molecule of a xenobiotic thus making it more polar and facilitating renal excretion. Glucuronidation is the most common conjugation pathway in mammals. The enzymes involved are UDP-glucuronosyl transferases (UGT) of two gene families. Typical UGT substrates are xenobiotic or eobiotic alcohols, phenols or carboxylic acids. Electrophilic compounds are mostly conjugated with glutathione through the action of glutathione S-transferases (GST). These ubiquitous conjugation enzymes, found in all organisms which were tested for their presence, protect biomolecules against harmful

electrophiles. Carboxylic acids or amines serve as substrates for conjugation with amino acids. Aromatic amines or hydroxylamines can also undergo acetylation by N-acetyltransferases (NAT). Sulfation mediated by sulfotransferases (SULT) represents another important type of conjugation reaction (Uetrecht, 2007).

As hydrophilic conjugates or metabolites are not able to pass through a lipid membrane they must be transported by means of specific transport proteins. ATP-binding cassette (ABC) transporters play a key role in the efflux of xenobiotic as well as eobiotic compounds. Some of these transporters, e.g., P-glycoprotein (ABCB1) or multi-drug resistance proteins (ABCC1-6), are important for drug resistance of cancer cells or insect (Ouellette, 2003).

All three systems: phase I enzymes, conjugation enzymes and transporters serve for detoxication of potentially harmful xenobiotics and their activities are crucial for the effect and/or efficacy of drugs and other xenobiotics.

Generally, the parent substance and the metabolite or conjugate differ in both physico-chemical properties and pharmacodynamic effect as well as in toxicity and pharmacokinetic behaviour (Testa, 2007). The occurrence and activity of xenobiotic metabolizing enzymes (XME) determines the way and extent in which a drug or a xenobiotic is metabolized. Hence the XME fundamentally affect the biological effects (both desired and undesired) of an administered drug. Thus in the case of anthelminthics, the activity of XME of both the helminth parasite and the host substantially affects the efficacy of pharmacotherapy of a helminthosis.

If an organism is in increased contact with xenobiotics, it may defend itself against the chemical stress by increasing the activity (expression) of XME (e.g., Testa, 1995; Nebbia, 2001). This induction of XME can have significant pharmacological and toxicological consequences. In case of helminthosis treatment, with increased activity of host biotransformation enzymes (e.g., farm animals) the plasmatic levels of anthelminthics are decreased, which can result in failure of pharmacotherapy. This decrease in plasmatic levels of active substances increases the parasites' chances of survival after the application of a drug. It is well known that just the contact of parasites with low doses of anthelminthics results in the activation of parasite defense mechanisms and a subsequent reduction of drug sensitivity of the parasites (Geerts, 2000). The induction of host XME can thus indirectly contribute to the development of the parasite's drug resistance.

The biotransformation enzymes and transporters may to a certain extent protect the parasites from toxic effects of anthelminthics and the induction of XME can represent an advantageous defense strategy (Robinson, 2004). With respect to this fact, metabolism of drugs in helminth parasites and helminth XME deserve more attention and a better understanding.

2. Xenobiotic metabolizing enzymes in helminths

2.1. Phase I enzymes

In phase I of metabolism, drugs or other xenobiotics undergo oxidative, reductive or hydrolytic transformations.

2.1.1. Oxidases

The oxidation of numerous xenobiotics in bacteria, fungi, plants, insects and vertebrates is mediated by cytochromes P450 (CYPs). Whilst CYP monooxygenase activities are found widely distributed in the nature, attempts to detect them in helminths were unsuccessful for many years, and the helminths were considered exceptional organisms lacking CYP (Precious, 1989a; Barrett, 1997). The view has changed with the progress in genetic analyses which provided the evidence that CYP genes exist in the genome of *Caenorhabditis elegans* (*C. elegans*, extensively used model roundworm without parasitic importance). The *C. elegans* genome contains over 80 CYP encoding genes, which are included in the *CYP35* family (Menzel, 2001; Lindblom, 2006), and a NADPH-CYP reductase gene (www.brenda-enzymes.info). The expression of several *CYP35* genes significantly increased when *C. elegans* adults had been exposed to known CYP inducers, e.g., beta-naphtoflavone, clofibrate, fluoranthene, atrazine, phthalate or heavy metals (Menzel, 2001; Menzel, 2005; Reichert, 2005; Roh, 2006, 2007). In spite of this fact, very little is known about involvement of corresponding CYP35 enzymes in biotransformation of xenobiotics (Menzel, 2005). *CYP*-like gene has also been found in mitochondrial genome of the ectoparasitic mosquito *Romanomermis culicivorax* (Azevedo, 1993). In present, the genome sequencing of other helminths of high public importance, such as *Schistosoma*

mansoni (*S. mansoni,* fluke, parasite of man and other vertebrates), *Trichinella spiralis* (*T. spiralis*, roundworm, parasite of carnivores and man)*, Onchocerca volvulus* (*O. volvulus,* roundworm, parasite of man) and *Ascaris suum (A. suum,* roundworm, parasite of pig and man) is almost completed and a new information on helminth CYP genes may be expected soon (http://www.sanger.ac.uk/Projects/Helminths).

In addition to looking for CYP genes, the effort has been directed to the detection of CYP activities and proteins. Kotze (1997) found typical CYP monooxygenase activities, epoxidation of aldrin and deethylation of ethoxycoumarin in microsomes isolated from L1 and L3 larvae of *Haemonchus contortus (H. contortus,* roundworm of ruminants, important model for experimental parasitology). These activities were NADPH dependent, inhibited by carbon monoxide and piperonyl butoxide (CYP inhibitors) and induced by exposure to phenobarbital (typical CYP2B inducer). While larvae possessed significant aldrin epoxidase as well as 7-ethoxycoumarin deethylase activities, microsomes from adult *H. contortus* showed only a very low level of aldrin epoxidation activity, 10000-fold less intensive than that reported for rat liver microsomes (Kotze, 1997). Oxidative metabolism of model CYP substrates, aldrin and ethoxycoumarin, has been examined also in *H. contortus* adults *ex vivo*. Intact adults possessed only a low aldrin epoxidase activity (Kotze, 2000). It remains to be proved if this aldrin epoxidase activity is really an activity of *H. contortus* CYP. The CYP-mediated oxidative biotransformation pathways in adult parasites may be expected to be of lower importance compared to the oxidative biotransformation pathways in free living larval stages due to the reduced oxygen concentration in the intestinal environment (Kotze, 2006).

However, in *S. mansoni* adults high CYP-like activities were observed. A variety of CYP substrates (aminopyrine, ethylmorphine, benzphetamine, N-nitrosyldimethylamine, aniline, ethoxyresorufin and pentoxyresorufin) were used to identify CYP enzyme activites in adults extract (10000 g supernatant of homogenates). All of the aforementioned specific activities were detected (with the exception of ethoxyresorufin-O-deethylase and aniline hydroxylase) and were comparable with those in rat liver microsomes. In the extract from other *Schistosoma haematobium* adults, only aminopyrine N-dealkylase activity was detected. In both species, different anti – rat CYP antibodies were used to detect the presence of CYPs. AntiCYP2E1 and antiCYP2B reacted with *Schistosoma* proteins, while no immunoreactivity was observed with anti-CYP1A, CYP4A, CYP3A (Saeed, 2002)

Kerboeuf et al. (1995) demonstrated very low activities of aminopyrine N-dealkylase in L1 and L2 larvae of *Heligmosomoides polygyrus (H. polygyrus,* roundworm of experimental use without parasitic importance). This activity was inhibited by benzylimidazole, a CYP inhibitor, which indicated the participation of CYP in this reaction. In other experiments by Kotze et al. (2006), the toxicity of rotenone in adults and larvae of *H. contortus* and larvae of *Trichostrongylus colubriformis (T. colubriformis,* roundworm, parasite of ruminants and horse) was increased in the presence of piperonyl butoxide. In mammals and insect, the detoxification of rotenone is catalyzed by CYP. A CYP inhibitor piperonyl butoxide possessed no toxicity to helminths when used alone. Synergism of rotenone and piperonyl butoxide toxicity is in line with the assumption that CYP takes part in rotenone detoxification in helminths (Kotze, 2006).

Although CYPs act mainly as monooxygenases (i.e., enzymes transferring one oxygen atom from molecular oxygen to a substrate), they also possess additional catalytic activities (e.g., oxytransferase or reductase activities). The oxytransferase activity (the transfer of oxygen from one substrate to another substrate) was found in *H. contortus* adults and larvae, utilizing cumene hydroperoxide for oxidative deethylation of ethoxycoumarin (Kotze, 1999). The larval oxytransferase activity was inhibited by typical CYP inhibitors (metyrapone, n-octylamine, chloramphenicol, piperonyl butoxide) and unaffected by the peroxidase inhibitor salicylhydroxamic acid, while the activity in adults was inhibited by both CYP and peroxidase inhibitors.

Peroxidases represent an additional group of enzymes that may be able to oxidize xenobiotics in helminths (Kotze, 1999) as several peroxidases catalyze the oxidation of xenobiotics in mammals (Testa, 1995, 2007). The involvement of peroxidases in detoxification of xenobiotics in helminths has already been entertained in a review by Barrett (1997). More detailed information on participation of these primarily antioxidant enzymes in xenobiotic detoxification in helminths is not available yet. Helminth peroxidases have been mostly studied only from the view of their antioxidant function. The system which protects the helminths against oxidative stress is relatively well understood. Helminth presence in host organism leads to the activation of immune system and to an increase of the free radical production. It was well documented that the concentration of reactive oxygen species correlated with parasites expulsion. On the other hand, parasites possess a number of enzymes which protect them from the host produced oxidants. The enzymes involved are superoxide dismutase

(SOD), catalase, glutathione peroxidase, xanthin oxidase, cytochrome c peroxidase and peroxiredoxins (www.brenda-enzymes.info; Kotze, 2001). The said enzymes are able to detoxify the reactive oxygen species produced during own parasite metabolism as well as oxidants produced by the host immune system. Superoxide dismutase and peroxiredoxins are probably the major hydrogen peroxide detoxifying enzymes in helminths (Dzik, 2006). The protective role of catalase, peroxiredoxins and glutathione peroxidase in defense of adult and larval *H. contortus* against hydrogen peroxide has been demonstrated (Kotze, 2001; Bagnall, 2004). Ferredoxin NADPH oxidoreductase protected *S. mansoni* adults against cumene hydroperoxide and superoxide generating herbicide methyl viologen (Girardini, 2002b). Since some of the antioxidant enzymes (e.g., peroxidases, xanthin oxidase) also oxidize xenobiotics in vertebrates (Testa, 1995, 2007), the participation of these enzymes in biotransformation of xenobiotics in helminths can be considered.

The oxidation of xenobiotics in helminths could be mediated also by flavin-containing monooxygenases (FMO). In vertebrates, the FMO represent important biotransformation enzymes catalyzing oxidation of a heteroatom bearing nucleophilic xenobiotics. FMO gene family is conserved and ancient, with representatives present in almost all phyla examined so far. The genome of *C. elegans* contains five predicted genes encoding putative homologs of mammalian FMOs. One of them was classified as *FMO15*, but the function of the corresponding protein remains unknown (Petalcorin, 2005; www.brenda-enzymes.info).

Taking together, the present information on participation of helminth oxidases in biotransformation of xenobiotics is still insufficient and unclear. Only the expanding proteomic studies may finally answer the question about occurrence, function and importance of oxidative biotransformation enzymes in helminths.

2.1.2. Reductases and hydrolases

In contrast to oxidases, reductases and hydrolases have been considered important biotransformation enzymes of xenobiotics in helminths for many years (Precious, 1989a; Barrett, 1997).

Reductive metabolism represents the main metabolic pathway of carbonyl group bearing compounds. Homogenates of *Hymenolepis diminuta (H. diminuta,* tapeworm,

parasite of rat and mouse) reduced effectively several aldehydes (acetaldehyde, glyceraldehyde, p-nitrobenzaldehyde). NADPH was the preferred cofactor in this reduction. On the other hand, no reductive activities were found toward xenobiotic ketones in this helminth species (Munir, 1985).

The reduction of a carbonyl bearing compound was studied in subcellular fractions of adult *H. contortus* (Cvilink, 2008a). For the evaluation of activities of carbonyl reducing enzymes several model substrates were employed: D,L-glyceraldehyde and daunorubicin (substrates of human AKR1A enzymes and carbonyl reductase), acenaphthenol (a substrate of AKR1C subfamily), metyrapone (a substrate of AKR1C, AKR1A and carbonyl reductase in cytosol and 11β-hydroxysteroid dehydrogenase in microsomes) and oracin (a substrate of almost all carbonyl reducing enzymes tested). In *H. contortus*, all model substrates were metabolized and the specific activities of carbonyl reducing enzymes were similar to the activities found in farm animal species (Szotakova, 2004). As the reduction of a carbonyl group mainly represents a deactivation pathway that protects organisms against the toxic effect of reactive aldehydes and ketones, the significant activity of carbonyl reducing enzymes may help *H. contortus* to counteract the effect of anthelminthics or other carbonyl group bearing xenobiotics (Cvilink, 2008a).

In spite of this fact, the information on the carbonyl group reductases in helminths is negligible: one can expect the existence of many carbonyl reducing enzymes of different families and subfamilies as at least 68 genes encoding a short chain dehydrogenases/reductases were detected in *C. elegans* genome (Lindblom, 2006).

Reductive transformation is an important metabolic pathway for azo- and nitro-compounds. This type of xenobiotic metabolism has been detected also in several species of parasites. *Ascaris lumbricoides (A. lumbricoides,* roundworm, parasite of man) and *Moniezia expansa (M. expansa,* tapeworm, parasite of ruminants) were capable of reducing aromatic nitro compounds, azobenzene, azo-dyes and sulphoxides (Precious, 1989b). Homogenates of *Hymenolepis diminuta (H. diminuta,* tapeworm, parasite of rat and mice) reduced effectively azo-compounds but no reductive activities toward nitro-compounds were observed (Munir, 1985).

Xenobiotic esters, amides and cyclic compounds can be hydrolyzed during phase I biotransformation. A wide range of hydrolases have been found in helminths (Barrett, 1997). Parasites seem well equipped with estrases, including phosphatases

capable of hydrolyzing nitrophenylphosphates and organophosphates. *A. lumbricoides* and *M. expansa* possess esterases capable of hydrolyzing nitrophenyl, p-aminobenzoyl, naphthyl and methylumbelliferyl esters and arylamides. In addition, the activities of arylsulphatases and deacetylases were detected in these parasites. Homogenates of *H. diminuta* hydrolyzed a number of organic phosphates, sulphates and esters (Munir, 1985).

Conjugates of xenobiotics, formed in phase II of xenobiotic metabolism, may undergo a cleavage by the action of hydrolases. A range of α- and β- glucosidases, galactosidases and mannosidases was discovered in *H. diminuta.* N-deacetylase activity was observed in *A. lumbricoides, H. diminuta* and *M. expansa* (Munir, 1985). Cysteine conjugate β-lyase was found in *H. polygyrus, M. expansa* and *Necator americanus (N. americanus,* roundworm, parasite of man) (Adcock, 1999).

2.2. Phase II enzymes

Among the established phase II reactions in mammals and plants belong, e.g., glucuronidation, sulfation, acetylation, methylation, glucosidation, glutathione conjugation and conjugation with amino acids.

As distinct from mammals, to our knowledge, only a couple of papers dealing with phase II detoxification enzymes in helminths have been published, except for the glutathione S-transferase family. While the helminth glutathione S-transferases are the enzymes of a great interest, the rest of phase II enzymes seem to be neglected and overlooked by researchers.

2.2.1. Glutathione S-transferases

The superfamily of glutathione S-transferases (GST) comprises omnipresent, multi-substrate, both cytosolic and membrane bound microsomal enzymes involved in the biotransformation of endogenous and xenobiotic compounds, including drugs and environmental pollutants, mostly by catalysing the conjugation of electrophilic substrates to glutathione. These dimeric proteins (either homo- or heterodimers) also neutralize and detoxify the substrates by peroxidase activity or passive binding (Salinas, 1999).

The GST activity is also involved in tissue protection against oxidative damage caused by formation of reactive peroxides (hydroperoxides of phospholipids, fatty acids and DNA) (Hayes, 1995b), originating either from the parasite endogenous metabolism or from the host's immune system. Furthermore, GSTs take part in the non-catalytical intracellular transport of hydrophobic substances and also play a role as a determinant of cellular pathways of stress response (Wilce, 1994; Salinas, 1999).

Organisms express multiple forms of GSTs, as a likely consequence of their diverse roles (Wilce, 1994). Initially, the individual GSTs classification was based on biochemical and immunological properties (Mannervik, 1985) but due to wide and often overlapping substrate specificities, the classes are assigned by the direct primary amino acid sequence analysis. Up to now, mammalian Alpha (basic), Mu (near neutral), Pi (acidic), Sigma, Theta, Kappa, Zeta, and Omega GST classes have been established (Mannervik, 1985; Pemble 1996; Board, 1997; Tew, 1999; Board, 2000; Edwards, 2000; Hoque, 2007). Also a "MAPEG" class, a separate mammalian class of membrane associated GST activity exhibiting proteins, is distinguished (Jakobsson, 1999, 2000). Non-mammalian GSTs have been much less well characterized. Plants appear to express the plant specific Phi and Tau classes of GST (Edwards, 2000). Expression of a Beta class GST in bacteria (Vuilleumier, 1997), and a Delta class GST in insect (Zhou, 1997) has been reported.

All GST classes are involved in conjugation of 1-chloro-2,4-dinitrobenzene (CDNB), except for the Theta class enzymes which exhibit high levels of activity with 1,2-epoxy-3-(4 nitrophenoxy)propane (EPNP) (Dowd, 1997). 1,2-dichloro-4-nitrobenzene (DCNB) is a preferential substrate of the Mu class GST isoenzyme (Lopez, 1994).

2.2.1.1 Structure and localization of GSTs

GSTs have been detected in many kinds of helminth organisms, including roundworms, flukes and tapeworms. Among helminth species, the GSTs differ in substrate specificities, catalytic activities, immunogenicity and exhibit, in general, a low level of sequence similarity to both the helminth and the mammalian classes of GSTs. Although helminths do express GSTs that resemble those of mammals there can be found specific regions of structural difference in the helminth GSTs (Sheehan, 2001). Clearly, enzyme activities vary in accordance with a helminth class. Highest GST

activities were detected in tapeworms, and the activity appears to be higher in parasites of the gut than in blood or tissue-dwelling parasites. This fact may be a reflection of the cytoplasmic tegument of tapeworms, making them more susceptible to the xenobiotic exposition. Relatively low GST activities were found in roundworms (Barrett, 1997). Since GSTs are thought to act as protective detoxification enzymes, their occurrence in tissues which come into contact with xenobiotics or which are crucial for reproduction would be anticipated. However, the localization of GSTs shows a surprising interspecies variability in species investigated.

Five forms of GSTs isolated from *Fasciola hepatica (F. hepatica,* fluke, parasite of grazing vertebrates) had a catalytic activity with a number of hydrophobic ligands and secondary lipid peroxidation products (Brophy, 1990c). The level of GSTs in *F. hepatica* was approximately 4% of the total soluble protein (Brophy, 1990c). At least seven isoforms were purified from *F. hepatica* extracts by Panaccio (1992) who found that *F. hepatica* GSTs can be aligned to the mammalian Mu class. This is in agreement with Rossjohn and associates (1997) who found that *F. hepatica* contains at least seven GST isoforms, four of which had been cloned (Fh-1, Fh-7, Fh-47, and Fh-51). All four cloned isoforms belong to the Mu class and share greater than 71% sequence identity (Rossjohn, 1997). In *F. hepatica* the GST presence was associated with the lamellae of the intestinal epithelium (Panaccio, 1992). In addition to that, Wijffels et al. (1992) reported a GST activity in the tegument, muscular tissues and parenchymal cells. This is consistent with the investigation conducted by Creaney et al. (1995) who localized *Fh*-GST1 to the parenchyma of *F. hepatica* adults and demonstrated the presence of other GSTs in the gut and subtegumental tissues of *F. hepatica*. A correlation between the level of GST activity and development of resistance in *F. hepatica* was reported (Miller, 1994). Surprisingly, a decrease in GST activity was observed (in liver flukes which had survived the exposure to salicylanilide anthelminthics) and associated with an increase in drug resistance.

The GSTs isolated from *S. mansoni* have been reported as both monomeric and dimeric proteins (O'Leary, 1988; Taylor, 1988). Six GST isoenzymes have been identified in *S. mansoni* (O'Leary, 1988, 1992). Two major groups of *S. mansoni* GST subunits, *Sm*28 and *Sm*26, have been shown to belong to the Alpha and Mu classes, respectively (Brophy, 1994b). The highest activity observed toward the traditional GST substrate CDNB was with the GST *Sm*28 (Taylor, 1988). An Omega class GST, detected in all life stages of *S. mansoni*, was found by Girardini and colleagues

(Girardini, 2002a) and showed a very low activity toward CDNB. Interestingly, the investigated Omega class GST displayed a significant glutathione-dependent dehydroascorbate reductase and thiol transferase enzymatic activities which might occur due to a cysteine residue located in the GST active site, instead of in eukaryotic cells usual serine and tyrosine. They also observed a higher expression of the Omega *Sm*-GST in adults rather than in free-living developmental life stages. Its involvement in the survival of the parasite within the host has been proposed (Vande Waa, 1993; Girardini, 2002a). *Sm*-26 and *Sm*-28 GSTs were reported to be found in the tegument and subtegumental tissue of *Schistosoma* adults (Trottein, 1992). Immuno-electron microscope studies of *S. mansoni* showed the *Sm*-GST presence in both tegument and excretory epithelial cells. It was absent from the caecal epithelium and the flame cells (Taylor, 1988). *Sm*-26GST and *Sm*-28GST were localized in parenchymal cells of adults (Holy, 1989; Porchet, 1994) and were absent in the tegument, muscles, digestive tract, neural mass, vitelline glands and mature gametes which were not immunoreactive (Porchet, 1994).

A three-dimensional structure for the *S. japonicum* (parasite related to *S. mansoni*) GST has been determined (Lim, 1994) and the enzyme has been found to be closely related to the Mu class of mammalian GSTs. This finding is in agreement with McTigue et al. who reported an overall structural similarity of *Sj*-GST with the rat Mu class GSTs (McTigue, 1995). The structural resemblance of *S. japonicum* GST to the structure of Fh-47 GST from *F. hepatica*, which belongs to the Mu class, has also been observed (Rossjohn, 1997). Brophy (1994b) reported that the *Sj*-28GST family belongs to the Alpha, and the *Sj*-26GST family to the Mu class. The *Sm*-28GST and *Sj*-28GST have a high sequence similarity (75%), as do their respective *Sm*-26 and *Sj*-26 GST analogues (over 80%).

A. lumbricoides and *A. suum* (roundworm, parasite of pig and man) are frequent and dangerous parasites in tropical and subtropical areas. The recombinant *A. suum* GST displayed a comparatively low level of sequence similarity to the human liver Alpha (29%), pig lung Pi (37%), mouse liver Pi (34%) and human liver Mu class GSTs (28%). A three-dimensional structural model of *As*-GST showed a topological resemblance to mammalian Pi class GSTs (Liebau, 1997). In *A. suum*, the GST enzyme activity has been localized to the intestinal epithelium (Douch, 1978; Liebau, 1997), indicating the GST participation in metabolism of substances imported from the outer environment or secreted to it. GST enzymatic activity was also found in intestinal and

muscle tissue of *A. suum* (Douch, 1978). A glutathione transferase has been purified from *Ascaridia galli (A. galli,* roundworm of poultry) (Meyer, 1996). According to the analysis, the enzyme subunit exhibited structural identity to the Sigma class GSTs as the N-terminal sequence and tryptic peptide sequences were similar to other nematode Sigma class GSTs. The purified enzyme showed high activity and specificity in the GSH-dependent isomerization of prostaglandin H to prostaglandin E (Meyer, 1996).

Much research on *Onchocerca volvulus* (*O. volvulus*, roundworm, parasite of man) GST has been conducted by Liebau and colleagues. Three different GSTs have been identified in *O. volvulus*: *Ov*-GST1, *Ov*-GST2, and *Ov*-GST3 (Liebau, 1994b; Liebau, 1994c; Liebau, 2000; Krause, 2001). *Ov*-GST3 was found to be readily induced in response to oxidative stress, providing a defense against both intracellular metabolism derived and host immune system derived reactive oxygen species (Brophy, 2000; Kampkotter, 2003). The intensity of the up-regulation is dependent on the nature of oxidative stress (Liebau, 2000). As of the structural analysis, it was possible to find an *Ov*-GST1 relation to the Sigma class (Sommer, 2001, 2003). However, its low level of sequence similarity to the rat Pi class GST has also been reported (Liebau, 1994a). *Ov*-GST1 is a secretory GST located in the outer zone of the hypodermis, directly at the parasite-host interface. It displays a GSH-dependent prostaglandin D synthase activity, thus showing a potential to participate in modulation of immune cell functions (Perbandt, 2005). Structurally, *Ov*-GST1 is a glycoprotein which is active as a non-glycosylated dimer (Liebau, 1994c). A mass spectrometric study revealed that an *Ov*-GST1 carries high-mannose type oligosaccharides on at least four glycosylation sites (Sommer, 2001). *Ov*-GST2, the major cytosolic GST in *O. volvulus*, seems to be topologically related to the mammalian Pi class, sharing 42% of sequence identity with the human Pi class GSTs (Hoppner, 2004). The *Ov*-GST3 belongs to the Omega class (Kampkotter, 2003; Hoppner, 2004; Perbandt, 2005). The *Ov*-GST3 analysis revealed a low sequence identity with *Ov*-GST1 (14%) and *Ov*-GST2 (21%) (Liebau, 2000), but demonstrated a significant resemblance to GST domain containing stress response proteins (Kodym, 1999). In *O. volvulus*, the *Ov*-GST1 seems to be located at the parasite-host interface, associated with the outer membrane of the hypodermis (Liebau, 1994c) while *Ov*-GST2 was observed all over the hypodermal cytoplasm and in uterine epithelial cells (Liebau, 1996; Wildenburg, 1998). This corresponds with their respective functions in the organism since *Ov*-GST1 is a secretory GST and Ov-GST2 acts as an intracellular “housekeeping” enzyme. The *Ov*-GST1 was also detected in L1,

L2 and L3 larval stages (Wildenburg, 1998). Salinas et al. reported the GST localization in the seminal receptacle and spermatozoa of *Onchocerca* adults (Salinas, 1994).

Four acidic GST forms were isolated from *H. diminuta* by Brophy and associates (1990a). More than 95% of the GST activity with CDNB was detected in the tapeworm cytosol compared to the microsomes. No activity with cumene hydroperoxide, a mammalian alpha class GST substrate, and no activity with the Alpha/Pi class substrate ethacrynic acid was detected (Brophy, 1990a).

Brophy et al. also resolved four forms of GSH transferase from *M. expansa*. Three isolated GSTs showed no relationship to any of the GST classes, but the N-terminal of the most abundant GST exhibited a mixture of characteristics of the Alpha and Mu GST classes (Brophy, 1989b).

GSTs of *Taenia solium* (tapeworm, parasite of man and pig) were investigated by Vibanco-Perez et al. (1999, 2002). The analysis of the N-terminal showed that both *Ts*-GST subunits are related to mammalian Mu class. The further analysis revealed the presence of typical features, including the Mu loop, similar to several previously reported Mu class GSTs (Vibanco-Perez, 1999, 2002). A *Ts*-GST purified from *T. solium* adults displayed significant CDNB, hexa-2,4-dienal and trans-non-2-enal catalytic activities (Plancarte, 2004). The *T. solium* GSTs from interhost stages (protoscoleces) exhibited much lower activities with the model substrates and the lipid peroxidation products (Nava, 2007).

A 28kDa cytosolic GST transferase was isolated, purified and cloned by Hong et al. (2000) from *Paragonimus westermani (P. westermani,* fluke, parasite of carnivores, pig and birds). A high activity with CDNB, a relatively low activity with ethacrynic acid and reactive carbonyls and no activity with epoxy-3-(p-nitrophenoxy)-propane was observed. Biochemical and molecular studies suggested that *Pw*-28GST was classifiable to the Sigma class of GSTs (Hong, 2000), the sequence similarity between *Pw*-28GST and the Sigma class GSTs in the major catalytic domains was as high as 58%. In contrast, bacterially generated recombinant 26kDa *Pw*-GST exhibited an activity toward universal and Mu class specific substrates (Kim, 2007) and was found to be a Mu class GST member.

Comparison of the cDNAs derived from *Wuchereria bancrofti* and *Brugia malayi* GSTs (roundworms, parasites of man, monkeys, carnivores) revealed an enormously high sequence identity (97%) (Rathaur, 2003). Lacking the extra helix of the Alpha class and the loop characteristic of the Mu class GSTs, *Wb*-GST and *Bp*-GST

were found to be topologically related to the Pi-class GSTs. Furthermore, ethacrynic acid, a typical Pi class substrate, was preferentially conjugated by the *Wb*-GST (Rathaur, 2003).

GST activity has been detected in *Echinococcus granulosus (E. granulosus,* tapeworm, parasite of carnivores) interhost stages by Morello et al. (1982). A protein fraction migrating as a M_r band was isolated from the *E. granulosus* protoscoleces and its N-terminal amino acid sequence most closely resembled the Mu class GSTs (Fernandez, 1994). A GST cloned from *E. multilocularis* (tapeworm, parasite related to *E. granulosus*) protoscoleces was found to be related to the Mu class GSTs and possessed significant activities towards CDNB and lipid peroxidation products (Liebau, 1996). A recombinant *Eg*-GST showed striking sequence similarity to *Em*-GST (99%). The sequence similarity of *Eg*-GST with other Mu class GSTs was up to 48% and with the 26kDa trematode GSTs it was about 40% (Fernandez, 2000).

A recombinant *Clonorchis sinensis (C. sinensis,* fluke, parasite of vertebrates including man) *Cs*-GST (28kDa) had enzymatic activity and was grouped into the Sigma class (Hong, 2002) and a recombinant *Cs*-GST (26kDa) was classified as a Mu class GST (Hong, 2001). The GST tissue localization in *C. sinensis* was examined by Kang et al. using immunoelectron microscopy and immunohistochemical staining (Kang, 2001). *Cs*-28GST was detected in several locations, including the tegument, parenchyma, intra-uterine eggs and sperm tails, with major abundance in the cells of subtegumental parenchyma (Kang, 2001).

In *Brugia* spp. the GSTs were predominantly associated with reproductive organs of the adults which in females signalized a particular susceptibility of embryos to oxidative stress (Rao, 2000).

The recombinant *H. contortus* GST protein (*Hc*-GST1) was investigated (van Rossum, 2004) and displayed about 70% similarity to a predicted GST (Hc-GST-E) demonstrated in the excretory-secretory products of *H. contortus* by Yatsuda et al. (2003). It was also suggested that the *Hc*-GST1 and *Hc*-GST-E may form a new class of roundworm GSTs. Some of GSTs predicted from the *C. elegans* proteome would also belong to this class (van Rossum, 2004).

A *C. elegans* GST cDNA sequence found by Weston et al. shared about 28-40% sequence similarity with the rat Pi class GST subunits (Weston, 1989). Campbell et al. predicted over 30 GST isoforms in the *C. elegans* proteome and speculated that the variety of recently discovered GSTs would require a new class nomenclature

(Campbell, 2001). Campbell and colleagues successfully cloned a *H. polygyrus bakeri* GST. It shared approximately 45-57% sequence identity with the GSTs predicted from the roundworms *C. elegans* and *H. contortus*, all of which could be aligned to the new class of helminth specific GSTs. Using *H. polygyrus bakeri* GST antisera, antigenic cross-reactivity between the recombinant *H. p. bakeri*, *H. contortus* and *C. elegans* GSTs was observed and supported this theory (Campbell, 2001). A relationship between GSTs from *H. contortus* and *C. elegans* was reported (van Rossum, 2001b). Van Rossum et al. (2001a) isolated 12 glutathione S-transferases from *C. elegans* and employed mass spectrometry for their identification. Three GSTs could be classified as members of the Alpha, one of the Pi and one of the Sigma GST class. Seven of the GST proteins isolated, according to the classification of Campbell (2001), fell into the newly proposed roundworm specific GST class. Searching the databases, Lindblom and Dodd (2006) found 48 predicted genes that coded for GSTs in *C. elegans* genome, 46 of which have documented mRNAs and one has a documented phenotype (Lindblom, 2006).

2.2.1.2. Role of GSTs

As it was previously pointed out, the substantial role of GSTs lies in protective detoxification of potentially harmful substances, including reactive oxygen species and products of lipid peroxidation. On the other hand, additional endogenous roles may be attributed to a number of GSTs in helminth organisms.

The antioxidant activities of GSTs have been intensively studied. One of the GSTs isolated from *M. expansa* by Brophy et al. (1989b) had the ability to conjugate alka-2,4-dienals and especially alk-2-enals, well-known secondary products of lipid peroxidation (Esterbauer, 1982). As the levels of other lipid peroxidation protective enzymes (superoxide dismutase, catalase and glutathione peroxidase) are low in *M. expansa* (Paul, 1980), the *Me*-GSTs may represent an ultimate component of the protection system against toxic electrophilic and oxidative products.

The purified ES GST pool from *N. americanus* was able to metabolize one group of potential peroxidation products (cumene hydroperoxide and linoleic acid peroxides) (Brophy, 1995) whereas the GST pool from *H. polygyrus* could neutralize model and lipid peroxides via peroxidase activity, but failed to show the activity with

carbonyl breakdown products of lipid peroxides (*trans*-2-nonenal or *trans*, *trans*-2, 4-decadienal) via glutathione transferase activity (Brophy, 1994a). The GST pool isolated from *P. westermani* was able to conjugate the aldehyde products of lipid peroxidation such as t-butyl hydroperoxide, *trans*-2-nonenal and *trans, trans*-decaenal (Hong, 2000).

A number of helminth GSTs may be involved in prostaglandin synthesis and thus provide the parasite with a powerful tool able to effectively counteract and suppress the host immune system response. Located at the parasite-host interface, as a component of helminth excretory-secretory products, GST prostaglandin synthetase/isomerase activity may help the helminth to survive within the host organism. Meyer et al. (1996) purified a prostaglandin isomerase activity possessing GST from *A. galli*. The enzyme showed a high and specific activity in glutathione dependent PGH to PGE isomerization. Sommer et al. (2003) isolated an *O. volvulus* GST, *Ov*-GST1, located in the outer hypodermal lamellae and in parts of the cuticle, which exhibited prostaglandin D2 synthase activity. Both mentioned parasite GSTs could be classed as Sigma class GST, and the prostaglandin production could then possibly be attributed to the Sigma class H-site unique structure, which has a wide and open cleft, distinct from other GST classes (Sommer, 2001).

The important protective and housekeeping roles of GSTs lie in the binding of potentially toxic (or non-toxic) substrates, their intracellular transport and their deactivation by conjugation with glutathione. The impact of these GSTs activities on anthelminthics and other xenobiotics is discussed in Section 3.

2.2.1.3. Induction and inhibition of GSTs

There are many reports clearly showing the fact that the GST activity, including helminth GST activity, can be easily modulated by exposure to xenobiotics. The outcome of such an exposure depends on the nature of the modulating agent and may lead to a decrease in enzyme activity or to an increased enzyme activity characterized by increased protein level and typically accompanied by an increased steady state level of mRNA (Vande Waa, 1993).

The GST expression can be easily induced by treatment with a number of chemicals including known or suspected carcinogens, drugs and oxidative stress metabolites (Hayes, 1995a). A frequently used typical inducer phenobarbital induced GST activity

in *H. diminuta* adults (Brophy, 1990a), protoscoleces (developmental stage) of *E. granulosus* (Morello, 1982) and adults of *S. mansoni* (Vande Waa, 1993). 3-methylcholanthrene and butylated hydroxyanisole were investigated by Vande Waa et al. (Vande Waa, 1993) and both compounds significantly induced *Sm*-GST activity against CDNB. An *Ov*-GST3 from *O. volvulus* represents an oxidative stress sensitive GST which is considerably induced in presence of oxidative stress-related compounds (Liebau, 2000; Perbandt, 2005).

Gupta et al. (2005) reported that GST of adult female *Setaria cervi* (*S. cervi*, roundworm, parasite of ruminants) could be induced without difficulty by *in vitro* exposure to diethylcarbamazine, butylated hydroxyanisole and phenobarbital. The exposure of *S. cervi* microfilariae to butylated hydroxyanisole also yielded an increase in GST activities. Conversely, a dramatic decrease in GST activity was observed after exposure of *S. cervi* microfilariae to diethylcarbamazine. Moreover, an extra electrophoretic band of GST activity was detected in extracts of diethylcarbamazine and butylated hydroxyanisole treated adult worms, suggesting the presence of a chemical stress responsive GST (Gupta, 2005).

The inhibition of helminth GST enzyme activity was reported in *A. suum* by Liebau et al. (1997). The recombinant *As*-GST interacted with natural breakdown products of haem, bile acids and a variety of model inhibitors (triphenyltin chloride, bromosulphophthalein, cibacron blue) as well as with mammalian GST inhibitors and several anthelminthic compounds. The anthelminthics bithionol and hexachlorophen inhibited the GST activity significantly while the benzimidazole anthelminthic albendazole displayed a low potential to inhibit r*As*-GST (Liebau, 1997). After the treatment of *E. granulosus* infected mice with mebendazole, Feng et al. (1995) reported approximately 30% inhibition in the *Ec*-GST activity. The capability of inhibiting the GST activity by anthelminthics was further demonstrated in *F. hepatica* (Brophy, 1990c) and *N. americanus* (Asojo, 2007). Inhibition of the *P. westermani* GST activity by bromosulfophthalein, cibacron blue and albendazole (Hong, 2000), and inhibition of activities of recombinant *A. suum* and recombinant *O. volvulus* GSTs by medicinal plant extracts (Fakae, 2000) have also been reported. As demonstrated by Brophy et al. (2000), glutathione conjugates can act as effective inhibitors of the GST activity and even exhibit a slight selectivity to *O. volvulus* GST in comparison with the human Pi class GSTs.

However, although a variety of GST inhibitors is available, there is no specific helminth GST inhibitor reported so far (Liebau, 1997), the advantage of which could be taken to the selective treatment of a parasitosis.

2.2.2. Other conjugation enzymes

In addition to GSTs, other transferases (e.g., UDP-glucuronosyltransferases, N-acetyltransferases, methyltransferases, sulfotransferases) can catalyze the conjugation reactions of xenobiotics and eobiotics in mammals.

The presence of conjugation enzymes in helminths is often predicted in compliance with the sequenced genome of *C. elegans* (Lindblom, 2006). However, the mRNAs and phenotypes of such enzymes are documented sporadically. Certain examined helminth species clearly exhibited various conjugation activities but the reported reactions were mostly not associated with metabolism of xenobiotic compounds and rather compounds of physiological character.

Isaac et al. (1990) demonstrated the ability of *Brugia pahangi (B. pahangi,* roundworm, parasite of domestic carnivores and man) to N-acetylate several biogenic amines such as serotonine, octopamine and dopamine. The enzymatic reaction required the addition of acetyl-CoA. The same investigators detected another N-acetyltransferase activity in *A. galli* (Isaac, 1991; Muimo, 1993), thus they showed the potential of possible acetylation of xenobiotic substrates in the helminths investigated. A cytosolic enzyme which in the presence of acetyl-CoA catalysed the acetylation of diamines (putrescine, cadaverine, 1,3-diaminopropane and 1,6-diaminohexane) was purified from *A. suum* (Wittich, 1989). Wittich and Walter (1990) then confirmed the N-acetylase activity toward putrescine also in *O. volvulus*. Acetylation of naturally occurring biogenic amines has also been described in *F. hepatica* (Aisien, 1992, 1993) and in *C. elegans* (Abo-Dalo, 2004).

O'Hanlon et al. (1987, 1991a, 1991b) investigated the metabolism of ecdysteroids, moulting hormones, in *A. suum* and *Paraascaris equorum* (roundworm, parasite of horse) and reported the formation of 20-hydroxyecdysone-25-glucoside conjugate and putative ecdysone-22-phosphate. Pica-Mattoccia et *al*. (2006) reported a sulphotransferase-like activity involved in activation of the antitrematode oxamniquine in *S. mansoni*. The activity was competitively inhibited by beta-estradiol and quercetin, common sulfotransferase substrates. However, the exact nature of the enzyme was not

established (Pica-Mattoccia, 2006). Sulfation of important structural protein components was observed in *C. elegans* (Kim, 2005). A tyrosylproteinsulfotransferase, which is responsible for this modification, was detected in the hypodermis of *C. elegans* and was shown to be crucial for the cuticle formation (Kim, 2005). On the other hand, no sulfotransferase activity was detected in *H. diminuta*: neither helminths nor helminth extracts were able to sulfate various substrates (Raines, 1988).

Taking together, the current state of research in this field does not provide any proof that any of the aforementioned conjugation enzymes, including GSTs, are actually involved in the metabolism of xenobiotic compounds. A further and targeted investigation on this issue is required in order to clarify the roles of the occurring enzyme activities in xenobiotic metabolism in helminths.

2.3. Transporters

Transport of xenobiotics is now considered the phase III of biotransformation. Transporters, membrane-bound proteins transporting compounds across membranes, represent an important tool in xenobiotics detoxification. Two main types of transporters are recognized: transporters importing xenobiotics into the cells and transporters exporting xenobiotics or their metabolites out of the cells. The second type of transporters has been investigated much more because of its importance in drug resistance. ATP-binding cassette (ABC) transporters are the major family of exporting transporters. ABC transporters are omnipresent proteins, found in all cells of all species from microbes to man. Their structure consists of four core domains, two transmembrane domains and two nucleotide-binding domains. Transport clearly involves major conformational changes and conventional enzyme-like mechanism. The transport cycle is initiated by the interaction with a substrate, followed by ATP binding which induces conformational changes leading to the transfer of the substrate. After the ATP hydrolysis, the former state of a transporter is regenerated (Higgins, 2003).

In metabolism of xenobiotics, ABC transporters mediate an active efflux of both lipophilic xenobiotics, which passed through plasmatic membranes, and hydrophilic metabolites and conjugates, which were formed by the action of biotransformation enzymes. Comprehensive information on the role of ABC transporters in veterinary drug research and parasitic resistance was brought in a review by Alvarez and associates

(2006). ABC transporters have been found in a number of helminth species. In *C. elegans* genome, a large family of 60 genes encoding ABC transporter-like proteins was identified (Lindblom, 2006). The research on drug transport has been mainly focused on P-glycoprotein (Pgp, ABCB1), the best known ABC transporter and the major efflux pump involved in xenobiotic metabolism. The first Pgp described in helminths was identified in *C. elegans* (Lincke, 1992). Thereafter, the Pgp genes were found in several flukes (e.g., *S. mansoni*, *F. hepatica*) as well as roundworms (e.g., *H. contortus, Onchocerca* spp.) (Kerboeuf, 2003). The use of specific monoclonal antibodies allowed the localization of Pgp proteins in helminth tissue. In *H. contortus* Pgp was predominantly detected along the intestinal tract of the worms, with the most intense presence in the pharynx and anterior intestine (Smith, 2002). Detailed information on Pgp and other ABC transporters in helminths is available in a review by Kerboeuf and associates (2003) and Alvarez and associates (2006).

3. Metabolism of anthelminthics

To the best of our knowledge, there are a handful of documented reports where a xenobiotic compound is clearly metabolized by helminth XME and the metabolite is then identified as a biotransformation product. The published papers deal with metabolism of anthelmintically active agents, which is understandable, because XME catalyzed deactivation or/and activation substantially affects the fate of an administered anthelminthic. There are a number of anthelminthic drugs used for treatment of helminthosis but only a few of them have been studied from this point of view. Moreover, most researchers have studied only the phase I of the anthelminthic metabolism or the interaction of anthelminthics with one selected enzyme (e.g., GST). Therefore, the knowledge of a large proportion of metabolic pathways of anthelminthic drugs in helminths still remains incomplete.

Phase I biotransformation of benzimidazole anthelminthics albendazole (ABZ) and/or triclabendazole (TCBZ) was studied in several helminth species. The oxidation of both drugs was detected in *F. hepatica, M. expansa, A. suum, H. contortus* and *Dicrocoelium dendriticum (D. dendriticum,* fluke, parasite of ruminants) (Solana, 2001; Mottier, 2004; Robinson, 2004; Alvarez, 2005; Cvilink, 2008b, 2008c). Although all the above mentioned helminths possess the ability to oxidize ABZ and/or TCBZ, their

metabolic activities and localization of helminth oxidases significantly differ among helminth species.

In *D. dendriticum* ABZ sulphoxidation occurred in mitochondrial and microsomal subcellular fractions, while no sulphoxidation was detected in cytosol (Cvilink, 2008c). In the related species *F. hepatica* the sulphoxidation of ABZ in both microsomes and cytosol occurred (Solana, 2001). Microsomal sulphoxidation of TCBZ was described in *F. hepatica* (Mottier, 2004; Alvarez, 2005).

Ex vivo, when living *D. dendriticum* adults were incubated with ABZ, time- and concentration-dependent formation of ABZ sulphoxide (ABZSO) and efflux of ABZSO into the incubation medium was observed. The extent of ABZSO formation was as low as only 2.5% of the initial ABZ concentration were metabolized during the incubation (Cvilink, 2008c). Since ABZSO itself is an anthelmintically active compound, any extent of ABZSO formation would not decrease the ABZ efficacy.

As ABZ is a prochiral substrate, chiral discrimination in its biotransformation can be assumed. In *D. dendriticum*, a slight but significant predomination of (+)-ABZ.SO was observed *in vitro* (Cvilink, 2008c) while *in vitro* ABZ sulphoxidation was not stereospecific in *F. hepatica, M. expansa* and *A. suum* (Solana, 2001). On the other hand, a pronounced stereospecificity in ABZ oxidation was described in mammalian microsomes. (-)-ABZSO was preferentially formed in rat and deer species, while (+)-ABZSO predominated in sheep, cattle, pig and mouflon (Delatour, 1990, 1991a, 1991b; Velik, 2005). In mammals, the formation of (-)-ABZSO is catalyzed by cytochrome P450 (particularly P4501A), whereas (+)-ABZSO formation depends on the activity of flavin containing monooxygenases (Delatour, 1991a, 1991b; Moroni, 1995). It is likely that oxidases of a different structure, less stereospecific, with a more open entry to the binding place, participate in the ABZ sulphoxidation in helminths.

Chiral discrimination may also occur in binding and transport of ABZSO enantiomers. Solana *et al*. (2002) described an enantioselective binding of ABZSO to cytosolic proteins of *F. hepatica, M. expansa* and *A. suum*. An active efflux of anthelminthics in these helminth species was also reported (Mottier, 2006a). On the other hand, the ABZSO transfer into *F. hepatica* was not enantioselective, as the ratio of ABZSO enantiomers found in helminth bodies corresponded to the ABZSO ratio detected in plasma and bile of the hosts (Alvarez, 2000).

The second step of ABZ oxidation, sulphonation, was observed in *D. dendriticum* mitochondrial fractions (Cvilink, 2008c). This was the first piece of evidence that helminth enzymes are able to deactivate ABZ via the formation of the biologically inactive ABZ sulphone ($ABZSO_2$). However, no ABZ sulphonation was detected in *ex vivo* experiments and the rate of the *in vitro* sulphonation was so low that any significant protective role of this reaction cannot be assumed. Similarly, the formation of $ABZSO_2$ was not observed in *ex vivo* experiments with *H. contortus* (Cvilink, 2008b). In the case of TCBZ, very intensive sulphonation was reported in *F. hepatica* (Robinson, 2004). The parasite was able to effectively convert TCBZ sulphoxide to the inactive TCBZ sulphone and in this way *F. hepatica* might significantly decrease the concentration of the toxic compound in its body. The reduction of ABZSO back to ABZ occurred in *M. expansa* (Solana, 2001) but neither in *F. hepatica* nor in *D. dendriticum* (Robinson, 2004; Cvilink, 2008c).

Phase II of ABZ metabolism has been studied only in *H. contortus ex vivo* (Cvilink, 2008b). Using mass spectrometry, two glucose conjugates of ABZ were detected in incubation medium samples as well as in roundworm bodies samples and tentatively assigned as N-glucosides. Although ABZSO was formed in the said experiments too, no glucose conjugation of this metabolite was detected.

Biotransformation of the benzimidazole anthelminthic thiabendazole was studied in *Trichostrongylus colubriformis (T. colubriformis,* roundworm, parasite of ruminants). In *ex vivo* incubations with thiabendazole, no metabolite or conjugate was detected. The conjugation of hydroxythiabendazole (probably with sulfate) was detected when hydroxythiabendazole was used as a substrate (Sangster, 1986).

Alvinerie and associates (2001) revealed the formation of one phase I metabolite of the antiparasitic drug moxidectin formed in *H. contortus* homogenate incubations. The formation of this metabolite was inhibited by carbon monoxide which indicates the participation of cytochromes P450 in this reaction. On the other hand, *H. contortus* was not able to metabolize the anthelminthic closantel, neither *in vitro* nor *ex vivo* (Rothwell, 1997).

A. suum metabolized the anthelminthic drug mebendazole via carbonyl reduction and carbamate hydrolysis *in vitro* (Kohler, 1981). Metabolism of the benzimidazole drug flubendazole (FLU) was investigated in *H. contortus*. Cvilink et *al*. (2008a) studied the phase I biotransformation of FLU *in vitro* as well as *ex vivo*. The results showed that cytosolic NADPH-dependent enzymes of *H. contortus* metabolized FLU

via reduction of the carbonyl group. The reduction of FLU was stereospecific, the ratio of (-) : (+) enantiomers of reduced FLU formed was 9 : 1. Reduced flubendazole was the only phase I metabolite found. In mammals and birds, two phase I metabolites of FLU (reduced FLU and hydrolyzed FLU) arise (www.emea.europa.eu/pdfs/vet/mrls/Flubendazole.pdf). Reduction of flubendazole to the reduced flubendazole was also detected in *ex vivo* incubation with *M. benedeni* (Moreno, 2004).

A mass spectrometry study on phase I and II metabolism of FLU in *H. contortus ex vivo* has proved the formation of reduced FLU and revealed the formation of glucose conjugates of both parent drug FLU and reduced FLU. FLU was conjugated with glucose forming a putative FLU-N-glucoside while O-glucoside was formed from the reduced FLU (Cvilink, 2008b).

The glucosidation of anthelminthics is interesting as glucose conjugation, a common metabolic pathway in plants, occurs rarely in the animal kingdom. Focusing on helminths, only O'Hanlon and associates (1987, 1991a, 1991b) reported glucose conjugation in metabolism of endogenous ecdysteroids in *A. suum* and *P. equorum*. The anthelminthic metabolism via glucose conjugation requires further examination as nil is known about glucosyltransferases involved in this reaction in helminths.

The inverse situation is in the case of glutathione S-transferases (GSTs): The present knowledge of helminth GSTs is relatively large but the evidence of their action in anthelminthic metabolism is still lacking.

Doubtless, helminth GSTs appear to have a potential to neutralize exogenous toxic compounds including anthelminthics via binding and/or conjugation with glutathione. GSTs are active non-covalent binding proteins for a range of lipophilic ligands (Mannervik, 1988). The binding of an anthelminthic drug to GST proteins may result in an inactivation of the pharmacologically active substance (Brophy, 1995). In *A. suum*, the GSTs showed the potential to bind anthelminthics such as ABZ, bithionol and hexachlorophen (Liebau, 1997). Tapeworm GSTs can bind to many anthelminthics (Brophy, 1990b) and the high levels of GSTs in tapeworms could lead to reduced concentrations of the anthelminthic agent (Milhon, 1997). Praziquantel was able to bind to the dimer interface groove of a schistosomal GST which resulted in steric inhibition of *Sj*-GST catalytic and transport activities for large ligands (McTigue, 1995). In contrast, Milhon et al. (1997) observed that praziquantel did not competitively inhibit

recombinant *Sj*-GST (McTigue, 1995). The GST activity in *P. westermani* was also not affected by praziquantel even at high concentrations (Hong, 2000).

The main GSTs isolated from *M. expansa*, *H. diminuta, S. mansoni* and *F. hepatica* were able to bind to several commercially available anthelminthics; in spite of this fact, the experiments with purified GSTs did not prove any conjugation of anthelminthic drugs with glutathione (Brophy, 1989a, 1989b, 1990b; Walker, 1993). On the other hand, Lo and colleagues (Lo, 2007) showed that extracts from *S. mansoni* efficiently conjugated an array of synthetically prepared electrophilic compounds with glutathione. It has been demonstrated that some of the glutathione conjugates formed served as substrates for further conjugation thus giving rise to bisglutathionyl conjugates (Lo, 2007). The results of this study indicate the ability of helminth GSTs to catalyze conjugation of certain compounds, but common anthelminthics appear not to be the proper substrates. O'Leary et al. (1991) investigated the GST catalyzed biotransformation of the anthelminthic dichlorvos in *S. mansoni*. They surprisingly detected the O-demethylation of dichlorvos and the formation of demethylated dichlorvos and S-methylglutathione. The presence of both glutathione and dichlorvos in the reaction mixture was essential as no metabolites were detected while one of the components was omitted from the reaction mixture (O'Leary, 1991).

The detection of atypical metabolites indicates that the biotransformation of anthelminthics in helminths is hardly predictable and may substantially differ from that in mammals. Moreover, significant inter-species differences in anthelminthic metabolism have been found among helminth parasites. These differences may be relevant to the understanding of drug efficacy against different kinds of helminths. For this purpose much more comprehensive information on biotransformation and transport of anthelminthics is necessary.

The knowledge of transport mechanisms of anthelminthics is essential as the action of the anthelminthics depends on the ability of the active drug to reach its specific receptor within the target parasite. Passive drug transfer through external helminth surfaces is the predominant entry mechanism for most widely used anthelminthics. Despite the structural differences in the external surfaces of helminth parasites, the mechanism of drug entrance depends mainly on the lipophilicity of the anthelminthic agent (Mottier, 2006a; Alvarez, 2007). In the efflux of anthelminthics out of the parasite body, the participation of helminth ABC transporters is assumed (Alvarez, 2006). A direct proof is not available but many indirect evidences support this

hypothesis: e.g., the interaction of many anthelminthics with several mammalian transporters, the presence of transporter genes in helminth genomes, inhibition studies. More information can be found in an excellent review by Alvarez et al. (2006).

4. Role of helminth XME in drug resistance development

The resistance of helminths to common anthelminthics represents a worldwide problem. Most probably, there are many mechanisms of drug resistance in helminths, some of which have been elucidated but a lot remains unclear. One can distinguish two types of resistance mechanisms: pharmacokinetic-mediated mechanisms and pharmacodynamic-mediated mechanisms. The first type includes processes such as a decreased drug uptake, an accelerated drug efflux and an increased drug inactivation. By these means the concentration of the active drug within parasite cells is decreased, a lower number of drug molecules can bind to target macromolecules and the drug effect is reduced. These unwanted processes can be overcome using higher doses of anthelminthics. The pharmacodynamic-mediated mechanisms may involve an increase in the amount of target macromolecules or changes in their structures that both cause the reduction in drug efficacy. If an anthelminthic is not able to bind to altered target molecules, a higher dose of a drug does not lead to a higher efficacy (Alvarez, 2005). The individual mechanisms are often combined which makes the solving of the resistance problem more difficult. Present knowledge of resistance mechanisms in helminths is summarized and discussed in several reviews (e.g., Wolstenholme, 2004; Gilleard, 2006; Brennan, 2007).

Pharmacokinetic-mediated drug resistance is based on an increase in activities of drug transporters or/and biotransformation enzymes. In stable resistant strains, the resistance might be caused by changes in genes for XME or in corresponding control (regulatory) segments. In sensitive strains, the helminth XME may represent a tool contributing to the development of resistance as the active efflux of a drug together with its deactivation via biotransformation diminishes drug efficacy and may allow some worms to survive the anthelminthic therapy. Moreover, contact of a helminth with a given drug may lead to the induction (increased expression or protein stabilization) of helminth XME and acceleration of drug deactivation can furthermore facilitate the survival of some helminth individuals. Repetitive outliving of these “less-sensitive”

helminths causes a gradual selection and may end up in development of a resistant strain.

The induction of selected biotransformation enzymes by xenobiotics has been intensively studied in *C. elegans*. The potential of the environmental pollutants (e.g., phthalates, polychlorobiphenyls, heavy metals) to significantly increase the expression of several roundworm genes including CYPs (CYP35A) and GSTs has been described (Roh, 2006, 2007; Menzel, 2007). Also, the well-known CYP1A inducers such as beta-naphthoflavone or lansoprazol strongly induced CYP35A2 (Menzel, 2001). The induction of CYP35A might be mediated via the aryl hydrocarbon receptor, the xenobiotic sensor regulating the expression of certain biotransformation enzymes and transporters. This receptor controls CYP1A expression in many animal species and it was also found in *C. elegans* (Hahn, 2002). Using *C. elegans* whole genome DNA microarray, the effects of beta-naphthoflavone, fluoranthene, atrazine, clofibrate and diethylstilbestrol on the roundworm gene expression were investigated. The analysis clearly showed an induction of 203 genes belonging to different families, e.g., CYPs, GSTs, UDP-glucuronosyltransferases, carboxylesterases (Reichert, 2005).

Although these results indicate that the induction of XME as a response to chemical stress may represent an important defense strategy, unequivocal examples of XME induction in helminths are rare. Similarly, only insufficient evidence on a direct association between XME and drug resistance in helminths is available.

The metabolism of triclabendazole (TCBZ) and TCBZ sulphoxide (TCBZSO) was investigated in TCBZ-resistant and in TCBZ-susceptible strains of *Fasciola hepatica.* A significantly faster oxidation of TCBZ and a greater conversion of TCBZSO to the inactive TCBZ-sulphone were found in the resistant flukes (compared with the susceptible individuals) (Robinson, 2004; Alvarez, 2005). The activity of TCBZ and TCBZSO oxidases could be on the grounds of an increased expression of the enzymes or changes in enzyme structures. Anyway, the increased deactivation of TCBZ via sulphonation represents an efficient tool for the flukes to survive the attack of TCBZ (Brennan, 2007).

Resistance–induced changes in TCBZ transport in *F. hepatica* have been studied by Mottier et al. (2006b). The concentration of TCBZ and its main metabolite TCBZSO was significantly lower within the TCBZ-resistant flukes than within TCBZ-susceptible flukes. The performed inhibition study indicated that this decrease was caused by the enhanced activity of P-glycoprotein (ABCB1) transporter in the resistant flukes. An

altered efflux mechanism may account for the TCBZ resistance development in *F. hepatica*.

Data from Xu et al. (1998) suggested that P-glycoprotein may play a role in ivermectin resistance in *Haemonchus contortus.* The gene structures were altered and expression of P-glycoprotein mRNA was higher in the ivermectin-resistant than susceptible strains of *H.contortus*. Similarly, genetic polymorphism in P-glycoprotein gene was detected in moxidectin-resistant *H.contortus* strain (Blackhall, 1998).

The abovementioned results demonstrate the importance of the enhanced drug efflux in helminth drug resistance development. On the other hand, helminths are probably not able to restrict the drug entry into their bodies; for most of the anthelminthics the main way of drug transfer into the body is represented by the passive drug transfer (Mottier, 2006a; Alvarez, 2007).

Taking together, drug-efflux transporters and biotransformation enzymes seem to be crucial mechanisms in helminth resistance development. Further studies and knowledge enhancement in this field are needful.

5. Conclusion

The anthelminthic drugs are presently the principal method for the control of helminth parasites. Knowledge of the detoxification mechanisms that helminths possess is very important for understanding the processes that affect the drug concentration within the helminth parasites and may decrease the efficacy of pharmacotherapy.

Nevertheless, present information on the anthelminthic metabolism and XME in helminths is insufficient. Moreover, the available data indicate that the structures, presence, expression and activities of XME in helminths are distinct from most of the animal XME, and that anthelminthics undergo different metabolization in helminths and in mammals. Therefore it is not possible to employ the data obtained in mammals either for the estimation of anthelminthic metabolism or for the prediction of helminth XME properties. The helminth inter-species differences in XME and anthelminthic metabolism occur and this fact limits the generalization and the extrapolation of data obtained in one helminth species to other helminths.

In conclusion, the XME and anthelminthic metabolism in helminths should deserve more attention. More detailed information on anthelminthic metabolism in helminths can contribute to rendering the pharmacotherapy more effective. More data on the XME differences between parasites and their hosts might be valuable for the design of new potential anthelminthics. Lastly, deeper knowledge of inter-species differences among helminth species might help to clarify the diverse drug efficacies in individual helminth parasites.

Abo-Dalo, B., Ndjonka, D., Pinnen, F., Liebau, E., Luersen, K. (2004). A novel member of the GCN5-related N-acetyltransferase superfamily from Caenorhabditis elegans preferentially catalyses the N-acetylation of thialysine [S-(2-aminoethyl)-L-cysteine]. *Biochem J.* 384:129-137.

Adcock, H. J., Brophy, P. M., Teesdale-Spittle, P. H., Buckberry, L. D. (1999). Cysteine conjugate beta-lyase activity in three species of parasitic helminth. *Int J Parasitol.* 29:543-548.

Aisien, S. O., Walter, R. D. (1992). Polyamine N-acetyltransferase from Fasciola hepatica. *Mol Biochem Parasitol.* 51:65-72.

Aisien, S. O., Walter, R. D. (1993). Biogenic-amine acetylation: an additional function of the N-acetyltransferase from Fasciola hepatica. *Biochem J.* 291 (Pt 3):733-737.

Alvarez, A. I., Merino, G., Molina, A. J., Pulido, M. M., McKellar, Q. A., Prieto, J. G. (2006). Role of ABC transporters in veterinary drug research and parasite resistance. *Curr Drug Deliv.* 3:199-206.

Alvarez, L. I., Imperiale, F. A., Sanchez, S. F., Murno, G. A., Lanusse, C. E. (2000). Uptake of albendazole and albendazole sulphoxide by Haemonchus contortus and Fasciola hepatica in sheep. *Vet Parasitol.* 94:75-89.

Alvarez, L. I., Mottier, M. L., Lanusse, C. E. (2007). Drug transfer into target helminth parasites. *Trends Parasitol.* 23:97-104.

Alvarez, L. I., Solana, H. D., Mottier, M. L., Virkel, G. L., Fairweather, I., Lanusse, C. E. (2005). Altered drug influx/efflux and enhanced metabolic activity in triclabendazole-resistant liver flukes. *Parasitology.* 131:501-510.

Alvinerie, M., Dupuy, J., Eeckhoutte, C., Sutra, J. F., Kerboeuf, D. (2001). In vitro metabolism of moxidectin in Haemonchus contortus adult stages. *Parasitol Res.* 87:702-704.

Asojo, O. A., Homma, K., Sedlacek, M., Ngamelue, M., Goud, G. N., Zhan, B., Deumic, V., Asojo, O., Hotez, P. J. (2007). X-ray structures of Na-GST-1 and Na-GST-2 two glutathione S-transferase from the human hookworm Necator americanus. *BMC Struct Biol.* 7:42.

Azevedo, J. L., Hyman, B. C. (1993). Molecular characterization of lengthy mitochondrial DNA duplications from the parasitic nematode Romanomermis culicivorax. *Genetics.* 133:933-942.

Bagnall, N. H., Kotze, A. C. (2004). cDNA cloning and expression patterns of a peroxiredoxin, a catalase and a glutathione peroxidase from Haemonchus contortus. *Parasitol Res.* 94:283-289.

Barrett, J. (1997). Helminth detoxification mechanisms. *J Helminthol.* 71:85-89.

Blackhall, W. J., Liu, H. Y., Xu, M., Prichard, R. K., Beech, R. N. (1998). Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of Haemonchus contortus. *Mol Biochem Parasitol.* 95:193-201.

Board, P. G., Baker, R. T., Chelvanayagam, G., Jermiin, L. S. (1997). Zeta, a novel class of glutathione transferases in a range of species from plants to humans. *Biochem J.* 328 (Pt 3):929-935.

Board, P. G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermiin, L. S., Schulte, G. K., Danley, D. E., Hoth, L. R., Griffor, M. C., Kamath, A. V., Rosner, M. H., Chrunyk, B. A., Perregaux, D. E., Gabel, C. A., Geoghegan, K. F., Pandit, J. (2000). Identification, characterization, and crystal structure of the Omega class glutathione transferases. *J Biol Chem.* 275:24798-24806.

Brennan, G. P., Fairweather, I., Trudgett, A., Hoey, E., McCoy, McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G., Brophy, P. M. (2007). Understanding triclabendazole resistance. *Exp Mol Pathol.* 82:104-109.

Brophy, P. M., Barrett, J. (1990a). Blocking factors and the isolation of glutathione transferases from Hymenolepis diminuta (Cestoda: Cyclophyllidea). *Parasitology.* 100 Pt 1:137-141.

Brophy, P. M., Barrett, J. (1990b). Glutathione transferase in helminths. *Parasitology.* 100 Pt 2:345-349.

Brophy, P. M., Bensmith, A., Brown, A., Behnke, J. M., Pritchard, D. I. (1994a). Glutathione S-Transferases from the Gastrointestinal Nematode Heligmosomoides-Polygyrus and Mammalian Liver Compared. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology.* 109:585-592.

Brophy, P. M., Campbell, A. M., van Eldik, A. J., Teesdale-Spittle, P. H., Liebau, E., Wang, M. F. (2000). Beta-carbonyl substituted glutathione conjugates as inhibitors Of O. volvulus GST2. *Bioorg Med Chem Lett.* 10:979-981.

Brophy, P. M., Crowley, P., Barrett, J. (1990c). Detoxification reactions of Fasciola hepatica cytosolic glutathione transferases. *Mol Biochem Parasitol.* 39:155-161.

Brophy, P. M., Papadopoulos, A., Touraki, M., Coles, B., Korting, W., Barrett, J. (1989a). Purification of Cytosolic Glutathione Transferases from Schistocephalus-Solidus (Plerocercoid) - Interaction with Anthelmintics and Products of Lipid-Peroxidation. *Molecular and Biochemical Parasitology.* 36:187-196.

Brophy, P. M., Patterson, L. H., Brown, A., Pritchard, D. I. (1995). Glutathione S-transferase (GST) expression in the human hookworm Necator americanus: potential roles for excretory-secretory forms of GST. *Acta Trop.* 59:259-263.

Brophy, P. M., Pritchard, D. I. (1994b). Parasitic helminth glutathione S-transferases: an update on their potential as targets for immuno- and chemotherapy. *Exp Parasitol.* 79:89-96.

Brophy, P. M., Southan, C., Barrett, J. (1989b). Glutathione transferases in the tapeworm Moniezia expansa. *Biochem J.* 262:939-946.

Campbell, A. M., Teesdale-Spittle, P. H., Barrett, J., Liebau, E., Jefferies, J. R., Brophy, P. M. (2001). A common class of nematode glutathione S-transferase (GST) revealed by the theoretical proteome of the model organism Caenorhabditis elegans. *Comp Biochem Physiol B Biochem Mol Biol.* 128:701-708.

Creaney, J., Wijffels, G. L., Sexton, J. L., Sandeman, R. M., Spithill, T. W., Parsons, J. C. (1995). Fasciola hepatica: localisation of glutathione S-transferase isoenzymes in adult and juvenile liver fluke. *Exp Parasitol.* 81:106-116.

Cvilink, V., Kubicek, V., Nobilis, M., Krizova, V., Szotakova, B., Lamka, J., Varady, M., Kubenova, M., Novotna, R., Gavelova, M., Skalova, L. (2008a). Biotransformation of flubendazole and selected model xenobiotics in Haemonchus contortus. *Vet Parasitol.* 151:242-248.

Cvilink, V., Skalova, L., Szotakova, B., Lamka, J., Kostiainen, R., Ketola, R. A. (2008b). LC-MS-MS identification of albendazole and flubendazole metabolites formed ex vivo by Haemonchus contortus. *Anal Bioanal Chem.* 391:337-343.

Cvilink, V., Szotakova, B., Krizova, V., Lamka, J., Skalova, L. (2008c). Phase I biotransformation of albendazole in lancet fluke (Dicrocoeliumdendriticum). *Res Vet Sci.*

Delatour, P., Benoit, E., Besse, S., Boukraa, A. (1991a). Comparative enantioselectivity in the sulphoxidation of albendazole in man, dogs and rats. *Xenobiotica.* 21:217-221.

Delatour, P., Benoit, E., Caude, M., Tambute, A. (1990). Species differences in the generation of the chiral sulfoxide metabolite of albendazole in sheep and rats. *Chirality.* 2:156-160.

Delatour, P., Garnier, F., Benoit, E., Caude, I. (1991b). Chiral behaviour of the metabolite albendazole sulphoxide in sheep, goats and cattle. *Res Vet Sci.* 50:134-138.

Douch, P. G., Buchanan, L. L. (1978). Glutathione conjugation of some xenobiotics by Ascaris suum and Moniezia expansa. *Xenobiotica.* 8:171-176.

Dowd, C. A., Buckley, C. M., Sheehan, D. (1997). Glutathione S-transferases from the white-rot fungus, Phanerochaete chrysosporium. *Biochem J.* 324 (Pt 1):243-248.

Dzik, J. M. (2006). Molecules released by helminth parasites involved in host colonization. *Acta Biochim Pol.* 53:33-64.

Edwards, R., Dixon, D. P., Walbot, V. (2000). Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. *Trends Plant Sci.* 5:193-198.

Esterbauer, H., Cheeseman, K. H., Dianzani, M. U., Poli, G., Slater, T. F. (1982). Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP-Fe2+ in rat liver microsomes. *Biochem J.* 208:129-140.

Fakae, B. B., Campbell, A. M., Barrett, J., Scott, I. M., Teesdale-Spittle, P. H., Liebau, E., Brophy, P. M. (2000). Inhibition of glutathione S-transferases (GSTs) from parasitic nematodes by extracts from traditional Nigerian medicinal plants. *Phytother Res.* 14:630-634.

Feng, J. J., Guo, H. F., Yao, M. Y., Xiao, S. H. (1995). Effects of mebendazole, albendazole, and praziquantel on glutathione S-transferase and superoxide dismutase of Echinococcus granulosus cyst wall harbored in mice. *Zhongguo Yao Li Xue Bao.* 16:297-300.

Fernandez, C., Hormaeche, C. E. (1994). Isolation and biochemical characterisation of a glutathione S-transferase from Echinococcus granulosus protoscoleces. *Int J Parasitol.* 24:1063-1066.

Fernandez, V., Chalar, C., Martinez, C., Musto, H., Zaha, A., Fernandez, C. (2000). Echinococcus granulosus: molecular cloning and phylogenetic analysis of an inducible glutathione S-transferase. *Exp Parasitol.* 96:190-194.

Geerts, S., Gryseels, B. (2000). Drug resistance in human helminths: current situation and lessons from livestock. *Clin Microbiol Rev.* 13:207-222.

Gilleard, J. S. (2006). Understanding anthelmintic resistance: the need for genomics and genetics. *Int J Parasitol.* 36:1227-1239.

Girardini, J., Amirante, A., Zemzoumi, K., Serra, E. (2002a). Characterization of an omega-class glutathione S-transferase from Schistosoma mansoni with glutaredoxin-like dehydroascorbate reductase and thiol transferase activities. *Eur J Biochem.* 269:5512-5521.

Girardini, J. E., Dissous, C., Serra, E. (2002b). Schistosoma mansoni ferredoxin NADP(H) oxidoreductase and its role in detoxification. *Mol Biochem Parasitol.* 124:37-45.

Gupta, S., Rathaur, S. (2005). Filarial glutathione S-transferase: its induction by xenobiotics and potential as drug target. *Acta Biochim Pol.* 52:493-500.

Hahn, M. E. (2002). Aryl hydrocarbon receptors: diversity and evolution. *Chem Biol Interact.* 141:131-160.

Hayes, J. D., Pulford, D. J. (1995a). The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol.* 30:445-600.

Hayes, J. D., Strange, R. C. (1995b). Potential contribution of the glutathione S-transferase supergene family to resistance to oxidative stress. *Free Radic Res.* 22:193-207.

Hendrix, C. M., Robinson, E. (2006). *Diagnostic parasitology for veterinary technicians.* St. Louise: Mosby Elsevier.

Higgins, C. F., Linton, K. J. (2003). ABC transporters, an introduction and overview. In: I. B. Holland (ed.), *ABC proteins* (pp. 317-335). London: Academic Press.

Holy, J. M., O'Leary, K. A., Oaks, J. A., Tracy, J. W. (1989). Immunocytochemical localization of the major glutathione S-transferases in adult Schistosoma mansoni. *J Parasitol.* 75:181-190.

Hong, S. J., Kang, S. Y., Chung, Y. B., Chung, M. H., Oh, Y. J., Kang, I., Bahk, Y. Y., Kong, Y., Cho, S. Y. (2000). Paragonimus westermani: a cytosolic glutathione S-transferase of a sigma-class in adult stage. *Exp Parasitol.* 94:180-189.

Hong, S. J., Lee, J. Y., Lee, D. H., Sohn, W. M., Cho, S. Y. (2001). Molecular cloning and characterization of a mu-class glutathione S-transferase from Clonorchis sinensis. *Mol Biochem Parasitol.* 115:69-75.

Hong, S. J., Yu, J. R., Kang, S. Y. (2002). Ultrastructural localization of 28 kDa glutathione S-transferase in adult Clonorchis sinensis. *Korean J Parasitol.* 40:173-176.

Hoppner, J., Perbandt, M., Betzel, C., Walter, R. D., Liebau, E. (2004). Crystallization of the major cytosolic glutathione S-transferase from Onchocerca volvulus. *Acta Crystallogr D Biol Crystallogr.* 60:1496-1497.

Hoque, E., Pflugmacher, S., Fritscher, J., Wolf, M. (2007). Induction of glutathione S-transferase in biofilms and germinating spores of Mucor hiemalis strain EH5 from cold sulfidic spring waters. *Appl Environ Microbiol.* 73:2697-2707.

Hotez, P., Raff, S., Fenwick, A., Richards, F., Jr., Molyneux, D. H. (2007). Recent progress in integrated neglected tropical disease control. *Trends Parasitol.* 23:511-514.

Isaac, R. E., Eaves, L., Muimo, R., Lamango, N. (1991). N-acetylation of biogenic amines in Ascaridia galli. *Parasitology.* 102 Pt 3:445-450.

Isaac, R. E., Muimo, R., Macgregor, A. N. (1990). N-Acetylation of Serotonin, Octopamine and Dopamine by Adult Brugia-Pahangi. *Molecular and Biochemical Parasitology.* 43:193-198.

Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., Persson, B. (1999). Common structural features of MAPEG -- a widespread superfamily of

membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein Sci.* 8:689-692.

Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., Persson, B. (2000). Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). A widespread protein superfamily. *Am J Respir Crit Care Med.* 161:S20-24.

Jefferies, J. R., Campbell, A. M., van Rossum, A. J., Barrett, J., Brophy, P. M. (2001). Proteomic analysis of Fasciola hepatica excretory-secretory products. *Proteomics.* 1:1128-1132.

Jez, J. M., Penning, T. M. (2001). The aldo-keto reductase (AKR) superfamily: an update. *Chem Biol Interact.* 130-132:499-525.

Kampkotter, A., Volkmann, T. E., de Castro, S. H., Leiers, B., Klotz, L. O., Johnson, T. E., Link, C. D., Henkle-Duhrsen, K. (2003). Functional analysis of the glutathione S-transferase 3 from Onchocerca volvulus (Ov-GST-3): a parasite GST confers increased resistance to oxidative stress in Caenorhabditis elegans. *J Mol Biol.* 325:25-37.

Kang, S. Y., Ahn, I. Y., Park, C. Y., Chung, Y. B., Hong, S. T., Kong, Y., Cho, S. Y., Hong, S. J. (2001). Clonorchis sinensis: molecular cloning and characterization of 28-kDa glutathione S-transferase. *Exp Parasitol.* 97:186-195.

Kerboeuf, D., Blackhall, W., Kaminsky, R., von Samson-Himmelstjerna, G. (2003). P-glycoprotein in helminths: function and perspectives for anthelmintic treatment and reversal of resistance. *Int J Antimicrob Agents.* 22:332-346.

Kerboeuf, D., Soubieux, D., Guilluy, R., Brazier, J. L., Riviere, J. L. (1995). In vivo metabolism of aminopyrine by the larvae of the helminth Heligmosomoides polygyrus. *Parasitol Res.* 81:302-304.

Kim, T. H., Hwang, S. B., Jeong, P. Y., Lee, J., Cho, J. W. (2005). Requirement of tyrosylprotein sulfotransferase-A for proper cuticle formation in the nematode C. elegans. *FEBS Lett.* 579:53-58.

Kim, T. Y., Lee, J. Y., Kim, T. I., Moon, K. H., Kang, S. Y., Hong, S. J. (2007). Molecular cloning and enzymatic characterization of a class mu glutathione S-transferase of Paragonimus westermani. *Parasitol Res.* 101:1225-1231.

Kodym, R., Calkins, P., Story, M. (1999). The cloning and characterization of a new stress response protein. A mammalian member of a family of theta class glutathione s-transferase-like proteins. *J Biol Chem.* 274:5131-5137.

Kohler, P., Bachmann, R. (1981). Intestinal tubulin as possible target for the chemotherapeutic action of mebendazole in parasitic nematodes. *Mol Biochem Parasitol.* 4:325-336.

Kotze, A. C. (1997). Cytochrome P450 monooxygenase activity in Haemonchus contortus (Nematoda). *Int J Parasitol.* 27:33-40.

Kotze, A. C. (1999). Peroxide-supported in-vitro cytochrome P450 activities in Haemonchus contortus. *Int J Parasitol.* 29:389-396.

Kotze, A. C. (2000). Oxidase activities in macrocyclic-resistant and -susceptible Haemonchus contortus. *J Parasitol.* 86:873-876.

Kotze, A. C., Dobson, R. J., Chandler, D. (2006). Synergism of rotenone by piperonyl butoxide in Haemonchus contortus and Trichostrongylus colubriformis in vitro: potential for drug-synergism through inhibition of nematode oxidative detoxification pathways. *Vet Parasitol.* 136:275-282.

Kotze, A. C., McClure, S. J. (2001). Haemonchus contortus utilises catalase in defence against exogenous hydrogen peroxide in vitro. *Int J Parasitol.* 31:1563-1571.

Krause, S., Sommer, A., Fischer, P., Brophy, P. M., Walter, R. D., Liebau, E. (2001). Gene structure of the extracellular glutathione S-transferase from Onchocerca volvulus and its overexpression and promoter analysis in transgenic Caenorhabditis elegans. *Mol Biochem Parasitol.* 117:145-154.

Krueger, S. K., Williams, D. E. (2005). Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacol Ther.* 106:357-387.

Liebau, E., Eckelt, V. H., Wildenburg, G., Teesdale-Spittle, P., Brophy, P. M., Walter, R. D., Henkle-Duhrsen, K. (1997). Structural and functional analysis of a glutathione S-transferase from Ascaris suum. *Biochem J.* 324 (Pt 2):659-666.

Liebau, E., Eschbach, M. L., Tawe, W., Sommer, A., Fischer, P., Walter, R. D., Henkle-Duhrsen, K. (2000). Identification of a stress-responsive Onchocerca volvulus glutathione S-transferase (Ov-GST-3) by RT-PCR differential display. *Mol Biochem Parasitol.* 109:101-110.

Liebau, E., Walter, R. D., Henkle-Duhrsen, K. (1994a). Isolation, sequence and expression of an Onchocerca volvulus glutathione S-transferase cDNA. *Mol Biochem Parasitol.* 63:305-309.

Liebau, E., Walter, R. D., Henkle-Duhrsen, K. (1994b). Onchocerca volvulus: isolation and sequence of a second glutathione S-transferase cDNA. *Exp Parasitol.* 79:68-71.

Liebau, E., Wildenburg, G., Brophy, P. M., Walter, R. D., Henkle-Duhrsen, K. (1996). Biochemical analysis, gene structure and localization of the 24 kDa glutathione S-transferase from Onchocerca volvulus. *Mol Biochem Parasitol.* 80:27-39.

Liebau, E., Wildenburg, G., Walter, R. D., Henkle-Duhrsen, K. (1994c). A novel type of glutathione S-transferase in Onchocerca volvulus. *Infect Immun.* 62:4762-4767.

Lim, K., Ho, J. X., Keeling, K., Gilliland, G. L., Ji, X., Ruker, F., Carter, D. C. (1994). Three-dimensional structure of Schistosoma japonicum glutathione S-transferase fused with a six-amino acid conserved neutralizing epitope of gp41 from HIV. *Protein Sci.* 3:2233-2244.

Lincke, C. R., The, I., van Groenigen, M., Borst, P. (1992). The P-glycoprotein gene family of Caenorhabditis elegans. Cloning and characterization of genomic and complementary DNA sequences. *J Mol Biol.* 228:701-711.

Lindblom, T. H., Dodd, A. K. (2006). Xenobiotic detoxification in the nematode Caenorhabditis elegans. *J Exp Zoolog A Comp Exp Biol.* 305:720-730.

Lo, W. J., Chiou, Y. C., Hsu, Y. T., Lam, W. S., Chang, M. Y., Jao, S. C., Li, W. S. (2007). Enzymatic and nonenzymatic synthesis of glutathione conjugates: application to the understanding of a parasite's defense system and alternative to the discovery of potent glutathione S-transferase inhibitors. *Bioconjug Chem.* 18:109-120.

Lopez, M. F., Patton, W. F., Sawlivich, W. B., Erdjument-Bromage, H., Barry, P., Gmyrek, K., Hines, T., Tempst, P., Skea, W. M. (1994). A glutathione S-transferase (GST) isozyme from broccoli with significant sequence homology to the mammalian theta-class of GSTs. *Biochim Biophys Acta.* 1205:29-38.

Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M., Jornvall, H. (1985). Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. *Proc Natl Acad Sci U S A.* 82:7202-7206.

Mannervik, B., Danielson, U. H. (1988). Glutathione transferases--structure and catalytic activity. *CRC Crit Rev Biochem.* 23:283-337.
McTigue, M. A., Williams, D. R., Tainer, J. A. (1995). Crystal structures of a schistosomal drug and vaccine target: glutathione S-transferase from Schistosoma japonica and its complex with the leading antischistosomal drug praziquantel. *J Mol Biol.* 246:21-27.
Mehlhorn, H. (2001). *Encyclopedic Reference of Parasitology*. Berlin: Springer.
Menzel, R., Bogaert, T., Achazi, R. (2001). A systematic gene expression screen of Caenorhabditis elegans cytochrome P450 genes reveals CYP35 as strongly xenobiotic inducible. *Arch Biochem Biophys.* 395:158-168.
Menzel, R., Rodel, M., Kulas, J., Steinberg, C. E. (2005). CYP35: xenobiotically induced gene expression in the nematode Caenorhabditis elegans. *Arch Biochem Biophys.* 438:93-102.
Menzel, R., Yeo, H. L., Rienau, S., Li, S., Steinberg, C. E., Sturzenbaum, S. R. (2007). Cytochrome P450s and short-chain dehydrogenases mediate the toxicogenomic response of PCB52 in the nematode Caenorhabditis elegans. *J Mol Biol.* 370:1-13.
Meyer, D. J., Muimo, R., Thomas, M., Coates, D., Isaac, R. E. (1996). Purification and characterization of prostaglandin-H E-isomerase, a sigma-class glutathione S-transferase, from Ascaridia galli. *Biochem J.* 313 (Pt 1):223-227.
Milhon, J. L., Thiboldeaux, R. L., Glowac, K., Tracy, J. W. (1997). Schistosoma japonicum GSH S-transferase Sj26 is not the molecular target of praziquantel action. *Experimental Parasitology.* 87:268-274.
Miller, C. M., Howell, M. J., Boray, J. C. (1994). Glutathione S-transferases as markers of salicylanilide resistance in isolates of Fasciola hepatica. *Int J Parasitol.* 24:533-542.
Molyneux, D. H. (2006). Control of human parasitic diseases: Context and overview. *Adv Parasitol.* 61:1-45.
Morello, A., Repetto, Y., Atias, A. (1982). Characterization of glutathione S-transferase activity in Echinococcus granulosus. *Comp Biochem Physiol B.* 72:449-452.
Moreno, L., Alvarez, L., Mottier, L., Virkel, G., Bruni, S. S., Lanusse, C. (2004). Integrated pharmacological assessment of flubendazole potential for use in sheep: disposition kinetics, liver metabolism and parasite diffusion ability. *J Vet Pharmacol Ther.* 27:299-308.
Moroni, P., Buronfosse, T., Longin-Sauvageon, C., Delatour, P., Benoit, E. (1995). Chiral sulfoxidation of albendazole by the flavin adenine dinucleotide-containing and cytochrome P450-dependent monooxygenases from rat liver microsomes. *Drug Metab Dispos.* 23:160-165.
Mottier, L., Alvarez, L., Ceballos, L., Lanusse, C. (2006a). Drug transport mechanisms in helminth parasites: passive diffusion of benzimidazole anthelmintics. *Exp Parasitol.* 113:49-57.
Mottier, L., Alvarez, L., Fairweather, I., Lanusse, C. (2006b). Resistance-induced changes in triclabendazole transport in Fasciola hepatica: ivermectin reversal effect. *J Parasitol.* 92:1355-1360.
Mottier, L., Virkel, G., Solana, H., Alvarez, L., Salles, J., Lanusse, C. (2004). Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidized metabolites into Fasciola hepatica. *Xenobiotica.* 34:1043-1057.
Muimo, R., Isaac, R. E. (1993). Properties of an Arylalkylamine N-Acetyltransferase from the Nematode, Ascaridia-Galli. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology.* 106:969-976.

Munir, W. A., Barrett, J. (1985). The metabolism of xenobiotic compounds by Hymenolepis diminuta (Cestoda: Cyclophyllidea). *Parasitology.* 91 (Pt 1):145-156.

Nava, G., Robert, L., Plancarte, A. (2007). Characterization of Taenia solium cysticerci microsomal glutathione S-transferase activity. *Parasitol Res.* 101:1373-1381.

Nebbia, C. (2001). Biotransformation enzymes as determinants of xenobiotic toxicity in domestic animals. *Vet J.* 161:238-252.

O'Hanlon, G. M., Cleator, M., Mercer, J. G., Howells, R. E., Rees, H. H. (1991a). Metabolism and fate of ecdysteroids in the nematodes Ascaris suum and Parascaris equorum. *Mol Biochem Parasitol.* 47:179-187.

O'Hanlon, G. M., Howarth, O. W., Rees, H. H. (1987). Identification of ecdysone 25-O-beta-D-glucopyranoside as a new metabolite of ecdysone in the nematode Parascaris equorum. *Biochem J.* 248:305-307.

O'Hanlon, G. M., Mercer, J. G., Rees, H. H. (1991b). Evidence for the release of 20-hydroxyecdysone 25-glucoside by adult female Parascaris equorum (Nematoda) in vitro. *Parasitol Res.* 77:271-272.

O'Leary, K. A., Hathaway, K. M., Tracy, J. W. (1992). Schistosoma mansoni: single-step purification and characterization of glutathione S-transferase isoenzyme 4. *Exp Parasitol.* 75:47-55.

O'Leary, K. A., Tracy, J. W. (1988). Purification of three cytosolic glutathione S-transferases from adult Schistosoma mansoni. *Arch Biochem Biophys.* 264:1-12.

O'Leary, K. A., Tracy, J. W. (1991). Schistosoma mansoni: glutathione S-transferase-catalyzed detoxication of dichlorvos. *Exp Parasitol.* 72:355-361.

Ouellette, M., Legare, D. (2003). Drug resistance mediated by ABC transporters in parasites of human. In: I. B. Holland (ed.), *ABC proteins* (pp. 317-335). London: Academic Press.

Panaccio, M., Wilson, L. R., Crameri, S. L., Wijffels, G. L., Spithill, T. W. (1992). Molecular characterization of cDNA sequences encoding glutathione S-transferases of Fasciola hepatica. *Exp Parasitol.* 74:232-237.

Paul, J. M., Barrett, J. (1980). Peroxide Metabolism in the Cestodes Hymenolepis-Diminuta and Moniezia-Expansa. *International Journal for Parasitology.* 10:121-124.

Pemble, S. E., Wardle, A. F., Taylor, J. B. (1996). Glutathione S-transferase class Kappa: characterization by the cloning of rat mitochondrial GST and identification of a human homologue. *Biochem J.* 319 (Pt 3):749-754.

Perbandt, M., Hoppner, J., Betzel, C., Walter, R. D., Liebau, E. (2005). Structure of the major cytosolic glutathione S-transferase from the parasitic nematode Onchocerca volvulus. *J Biol Chem.* 280:12630-12636.

Petalcorin, M. I., Joshua, G. W., Agapow, P. M., Dolphin, C. T. (2005). The fmo genes of Caenorhabditis elegans and C. briggsae: characterisation, gene expression and comparative genomic analysis. *Gene.* 346:83-96.

Pica-Mattoccia, L., Carlini, D., Guidi, A., Cimica, V., Vigorosi, F., Cioli, D. (2006). The schistosome enzyme that activates oxamniquine has the characteristics of a sulfotransferase. *Mem Inst Oswaldo Cruz.* 101 Suppl 1:307-312.

Plancarte, A., Rendon, J. L., Landa, A. (2004). Purification, characterization and kinetic properties of the Taenia solium glutathione S-transferase isoform 26.5 kDa. *Parasitol Res.* 93:137-144.

Porchet, E., McNair, A., Caron, A., Kusnierz, J. P., Zemzoumi, K., Capron, A. (1994). Tissue expression of the Schistosoma mansoni 28 kDa glutathione S-transferase. *Parasitology.* 109 (Pt 5):565-572.

Precious, W. Y., Barrett, J. (1989a). The possible absence of cytochrome P-450 linked xenobiotic metabolism in helminths. *Biochim Biophys Acta.* 992:215-222.

Precious, W. Y., Barrett, J. (1989b). Xenobiotic metabolism in helminths. *Parasitol Today.* 5:156-160.

Raines, P. S., Barrett, J. (1988). Hymenolepis-Diminuta - Lack of Sulfate Activation and Sulfotransferase Activity. *Experimental Parasitology.* 65:209-213.

Rao, U. R., Salinas, G., Mehta, K., Klei, T. R. (2000). Identification and localization of glutathione S-transferase as a potential target enzyme in Brugia species. *Parasitol Res.* 86:908-915.

Rathaur, S., Fischer, P., Domagalski, M., Walter, R. D., Liebau, E. (2003). Brugia malayi and Wuchereria bancrofti: gene comparison and recombinant expression of pi-class related glutathione S-transferases. *Exp Parasitol.* 103:177-181.

Reichert, K., Menzel, R. (2005). Expression profiling of five different xenobiotics using a Caenorhabditis elegans whole genome microarray. *Chemosphere.* 61:229-237.

Robinson, M. W., Lawson, J., Trudgett, A., Hoey, E. M., Fairweather, I. (2004). The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant Fasciola hepatica. *Parasitol Res.* 92:205-210.

Roh, J. Y., Jung, I. H., Lee, J. Y., Choi, J. (2007). Toxic effects of di(2-ethylhexyl)phthalate on mortality, growth, reproduction and stress-related gene expression in the soil nematode Caenorhabditis elegans. *Toxicology.* 237:126-133.

Roh, J. Y., Lee, J., Choi, J. (2006). Assessment of stress-related gene expression in the heavy metal-exposed nematode Caenorhabditis elegans: a potential biomarker for metal-induced toxicity monitoring and environmental risk assessment. *Environ Toxicol Chem.* 25:2946-2956.

Rossjohn, J., Feil, S. C., Wilce, M. C., Sexton, J. L., Spithill, T. W., Parker, M. W. (1997). Crystallization, structural determination and analysis of a novel parasite vaccine candidate: Fasciola hepatica glutathione S-transferase. *J Mol Biol.* 273:857-872.

Rothwell, J., Sangster, N. (1997). Haemonchus contortus: the uptake and metabolism of closantel. *Int J Parasitol.* 27:313-319.

Saeed, H. M., Mostafa, M. H., O'Connor, P. J., Rafferty, J. A., Doenhoff, M. J. (2002). Evidence for the presence of active cytochrome P450 systems in Schistosoma mansoni and Schistosoma haematobium adult worms. *FEBS Lett.* 519:205-209.

Salinas, A. E., Wong, M. G. (1999). Glutathione S-transferases--a review. *Curr Med Chem.* 6:279-309.

Salinas, G., Braun, G., Taylor, D. W. (1994). Molecular characterisation and localisation of an Onchocerca volvulus pi-class glutathione S-transferase. *Mol Biochem Parasitol.* 66:1-9.

Sangster, N. C., Prichard, R. K. (1986). Thiabendazole uptake, metabolism and excretion in thiabendazole resistant and susceptible Trichostrongylus colubriformis. *J Parasitol.* 72:798-800.

Sheehan, D., Meade, G., Foley, V. M., Dowd, C. A. (2001). Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J.* 360:1-16.

Smith, J. M., Prichard, R. K. (2002). Localization of p-glycoprotein mRNA in the tissues of Haemonchus contortus adult worms and its relative abundance in drug-selected and susceptible strains. *J Parasitol.* 88:612-620.

Solana, H. D., Rodriguez, J. A., Lanusse, C. E. (2001). Comparative metabolism of albendazole and albendazole sulphoxide by different helminth parasites. *Parasitol Res.* 87:275-280.

Solana, H. D., Sallovitz, J. M., Lanusse, C. E., Rodriguez, J. A. (2002). Enantioselective binding of albendazole sulphoxide to cytosolic proteins from helminth parasites. *Methods Find Exp Clin Pharmacol.* 24:7-13.

Sommer, A., Nimtz, M., Conradt, H. S., Brattig, N., Boettcher, K., Fischer, P., Walter, R. D., Liebau, E. (2001). Structural analysis and antibody response to the extracellular glutathione S-transferases from Onchocerca volvulus. *Infect Immun.* 69:7718-7728.

Sommer, A., Rickert, R., Fischer, P., Steinhart, H., Walter, R. D., Liebau, E. (2003). A dominant role for extracellular glutathione S-transferase from Onchocerca volvulus is the production of prostaglandin D2. *Infect Immun.* 71:3603-3606.

Szotakova, B., Baliharova, V., Lamka, J., Nozinova, E., Wsol, V., Velik, J., Machala, M., Neca, J., Soucek, P., Susova, S., Skalova, L. (2004). Comparison of in vitro activities of biotransformation enzymes in pig, cattle, goat and sheep. *Res Vet Sci.* 76:43-51.

Taylor, J. B., Vidal, A., Torpier, G., Meyer, D. J., Roitsch, C., Balloul, J. M., Southan, C., Sondermeyer, P., Pemble, S., Lecocq, J. P., et al. (1988). The glutathione transferase activity and tissue distribution of a cloned Mr28K protective antigen of Schistosoma mansoni. *Embo J.* 7:465-472.

Testa, B. (1995). *The metabolism of drugs and other xenobiotics: Biochemistry of redox reactions*. London: Academic Press.

Testa, B., Mayer, J.A. (2003). *Hydrolysis in drug and prodrug metabolism*. Weinheim, Zürich: VHCA & WILEY-VCH.

Testa, B., van de Waterbeemd, H. (2007). *ADME-Tox Approaches*. In Triggle, D., Taylor, J. (eds.), *Comprehensive Medicinal Chemistry* II, vol.5, Philadelphia: Elsevier

Tew, K. D., Ronai, Z. (1999). GST function in drug and stress response. *Drug Resist Updat.* 2:143-147.

Trottein, F., Godin, C., Pierce, R. J., Sellin, B., Taylor, M. G., Gorillot, I., Silva, M. S., Lecocq, J. P., Capron, A. (1992). Inter-species variation of schistosome 28-kDa glutathione S-transferases. *Mol Biochem Parasitol.* 54:63-72.

Uetrecht, J. P., Trager, W. (2007). *Drug Metabolism: Chemical and Enzymatic Aspects*. New York: Informa Healthcare.

van Rossum, A. J., Brophy, P. M., Tait, A., Barrett, J., Jefferies, J. R. (2001a). Proteomic identification of glutathione S-transferases from the model nematode Caenorhabditis elegans. *Proteomics.* 1:1463-1468.

van Rossum, A. J., Jefferies, J., Young, C. J., Barrett, J., Tait, A., Brophy, P. M. (2001b). Glutathione S-transferase (GST) functional genomics: role of Caenorhabditis elegans in investigating GST expression in parasitic nematodes. *Chemico-Biological Interactions.* 133:274-277.

van Rossum, A. J., Jefferies, J. R., Rijsewijk, F. A., LaCourse, E. J., Teesdale-Spittle, P., Barrett, J., Tait, A., Brophy, P. M. (2004). Binding of hematin by a new class of glutathione transferase from the blood-feeding parasitic nematode Haemonchus contortus. *Infect Immun.* 72:2780-2790.

Vande Waa, E. A., Campbell, C. K., O'Leary, K. A., Tracy, J. W. (1993). Induction of Schistosoma mansoni glutathione S-transferase by xenobiotics. *Arch Biochem Biophys.* 303:15-21.

Velik, J., Baliharova, V., Skalova, L., Szotakova, B., Wsol, V., Lamka, J. (2005). Liver microsomal biotransformation of albendazole in deer, cattle, sheep and pig and some related wild breeds. *J Vet Pharmacol Ther.* 28:377-384.

Vibanco-Perez, N., Jimenez, L., Mendoza-Hernandez, G., Landa, A. (2002). Characterization of a recombinant mu-class glutathione S-transferase from Taenia solium. *Parasitol Res.* 88:398-404.

Vibanco-Perez, N., Jimenez, L., Merchant, M. T., Landa, A. (1999). Characterization of glutathione S-transferase of Taenia solium. *J Parasitol.* 85:448-453.

Vuilleumier, S. (1997). Bacterial glutathione S-transferases: what are they good for? *J Bacteriol.* 179:1431-1441.

Walker, J., Crowley, P., Moreman, A. D., Barrett, J. (1993). Biochemical-Properties of Cloned Glutathione S-Transferases from Schistosoma-Mansoni and Schistosoma-Japonicum. *Molecular and Biochemical Parasitology.* 61:255-264.

Weston, K., Yochem, J., Greenwald, I. (1989). A Caenorhabditis elegans cDNA that encodes a product resembling the rat glutathione S-transferase P subunit. *Nucleic Acids Res.* 17:2138.

Wijffels, G. L., Sexton, J. L., Salvatore, L., Pettitt, J. M., Humphris, D. C., Panaccio, M., Spithill, T. W. (1992). Primary sequence heterogeneity and tissue expression of glutathione S-transferases of Fasciola hepatica. *Exp Parasitol.* 74:87-99.

Wilce, M. C., Parker, M. W. (1994). Structure and function of glutathione S-transferases. *Biochim Biophys Acta.* 1205:1-18.

Wildenburg, G., Liebau, E., Henkle-Duhrsen, K. (1998). Onchocerca volvulus: ultrastructural localization of two glutathione S-transferases. *Exp Parasitol.* 88:34-42.

Wittich, R. M., Walter, R. D. (1989). A novel type of putrescine (diamine)-acetylating enzyme from the nematode Ascaris suum. *Biochem J.* 260:265-269.

Wittich, R. M., Walter, R. D. (1990). Putrescine N-acetyltransferase in Onchocerca volvulus and Ascaris suum, an enzyme which is involved in polyamine degradation and release of N-acetylputrescine. *Mol Biochem Parasitol.* 38:13-17.

Wolstenholme, A. J., Fairweather, I., Prichard, R., von Samson-Himmelstjerna, G., Sangster, N. C. (2004). Drug resistance in veterinary helminths. *Trends Parasitol.* 20:469-476.

Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R., Prichard, R. (1998). Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. *Mol Biochem Parasitol.* 91:327-335.

Yatsuda, A. P., Krijgsveld, J., Cornelissen, A. W., Heck, A. J., de Vries, E. (2003). Comprehensive analysis of the secreted proteins of the parasite Haemonchus contortus reveals extensive sequence variation and differential immune recognition. *J Biol Chem.* 278:16941-16951.

Zhou, Z. H., Syvanen, M. (1997). A complex glutathione transferase gene family in the housefly Musca domestica. *Mol Gen Genet.* 256:187-194.

II.

Biotransformation of flubendazole and selected model xenobiotics in *Haemonchus contortus*

Cvilink, V., Kubíček, V., Nobilis, M., Křížová, V., Szotáková, B., Lamka, J., Várady, M., Kuběnová, M., Novotná, R., Gavelová, M., Skálová, L.

Vet Parasitol (2008) 151:242-248

ELSEVIER

Available online at www.sciencedirect.com
ScienceDirect

Veterinary Parasitology 151 (2008) 242–248

veterinary parasitology

www.elsevier.com/locate/vetpar

Biotransformation of flubendazole and selected model xenobiotics in *Haemonchus contortus*

V. Cvilink [a], V. Kubíček [a], M. Nobilis [a], V. Křížová [a], B. Szotáková [a], J. Lamka [a], M. Várady [b], M. Kuběnová [a], R. Novotná [a], M. Gavelová [a], L. Skálová [a,*]

[a] *Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, Hradec Králové, CZ-500 05, Czech Republic*

[b] *Parasitological Institute, Slovak Academy of Sciences, Hlinkova 3, Košice, Slovakia*

Received 10 May 2007; received in revised form 12 October 2007; accepted 17 October 2007

Abstract

Haemonchus contortus is one of the most pathogenic parasites of small ruminants (e.g., sheep and goat). The treatment of haemonchosis is complicated because of frequent resistance of *H. contortus* to common anthelmintics. The development of resistance can be facilitated by the action of drug metabolizing enzymes of parasites that can deactivate anthelmintics and thus protect parasites against the toxic effect of the drug. The aim of this project was to investigate the Phase I biotransformation of benzimidazole anthelmintic flubendazole in *H. contortus* and to determine the biotransformation of other model xenobiotics. For this purpose, *in vitro* (subcellular fractions of *H. contortus* homogenate) as well as *ex vivo* (live nematodes cultivated in flasks with medium) experiments were used. The results showed that cytosolic NADPH-dependent enzymes of *H. contortus* metabolize flubendazole via reduction of its carbonyl group. The apparent kinetic parameters of this reaction were determined ($V'_{max} = 39.8 \pm 2.1$ nM min^{-1}, $K'_m = 1.5 \pm 0.3$ μM). The reduction of flubendazole in *H. contortus* is stereospecific, the ratio of (−):(+) enantiomers of reduced flubendazole formed was 90:10. Reduced flubendazole was the only Phase I metabolite found. Effective reduction of other xenobiotics with carbonyl group (metyrapon, daunorubicin, and oracin) was also found. Significant activity of carbonyl-reducing enzymes may be important for *H. contortus* to survive the attacks of anthelmintics or other xenobiotics with carbonyl group.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Drug metabolism; Anthelmintics; Helminthoses; Carbonyl-reducing enzymes; Stereospecificity

1. Introduction

Biotransformation enzymes in all organisms serve as the efficient defense against potential negative action of xenobiotics. These xenobiotics (drugs, food additives, and environmental contaminants) undergo biotransformation in organisms to achieve more easy excretion of xenobiotics via their metabolites. The parent compound and its metabolites generally differ in both physical–chemical properties and biological activity, as well as in pharmacokinetic behavior. Thus, the activity of biotransformation enzymes substantially affects both the desired and undesired effects of any drug administered or of other xenobiotics.

The biotransformation enzymes of parasitic helminths may to a certain extent protect these organisms against

* Corresponding author at: Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, Hradec Králové, CZ-500 05, Czech Republic. Tel.: +420 495067322; fax: +420 495067168.

E-mail address: lenka.skalova@faf.cuni.cz (L. Skálová).

0304-4017/\$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2007.10.010

toxic effects of anthelmintics, and the ability to inactivate anthelmintics via biotransformation can represent an advantageous defense strategy of the parasites (Robinson et al., 2004; Kotze et al., 2006). The biotransformation of benzimidazole anthelmintics by parasitic worms has been relatively little investigated so far. Oxidation of albendazole and triclabendazole in *Fasciola hepatica*, *Haemonchus contortus*, *Moniezia expansa*, and *Ascaris suum* was observed (Solana et al., 2001; Robinson et al., 2004; Mottier et al., 2004). Although, all these helminths have the ability to oxidize albendazole or triclabendazole, their metabolic activities significantly differ among helminthic species. Information about reduction or hydrolysis of anthelmintics in worms is almost nil, although the parasitic helminths possess a number of hydrolases, including the *O*- and *N*-deacetylases, and reductases of aldehydes and ketones, azoreductases, nitroreductases (Barrett, 1997, 1998). Only sulforeduction of albendazole sulfoxide was described in *F. hepatica*, *M. expansa*, and *A. suum* (Solana et al., 2001). The present knowledge about reductases or hydrolases in parasites is insufficient, although their participation in biotransformation of some veterinary drugs can be assumed. From among the currently used anthelmintics, flubendazole and mebendazole are metabolised by reduction and hydrolysis in target animal species (http://www.emea.europa.eu/htms/vet/mrls/a-zmrl.htm#, Moreno et al., 2004).

The present study was designed to evaluate the biotransformation of flubendazole (FLU) in adult nematodes of *H. contortus*. For this purpose, *in vitro* (subcellular fractions of *H. contortus* homogenate) as well as *ex vivo* (live nematodes cultivated in flasks with medium) experiments were used.

2. Material and methods

2.1. Chemicals

Albendazole was purchased from Sigma-Aldrich (Czech Republic). Albendazole sulfoxide and albendazole sulfone were obtained from Toronto Research Chemicals Inc. (Canada). Flubendazole and its two main metabolites (reduced flubendazole and hydrolysed flubendazole) were provided by Janssen Pharmaceutica (Czech Republic). All other chemicals (HPLC or analytical grade) were obtained from Sigma-Aldrich (Czech Republic).

2.2. Collection of parasite material

H. contortus ISE strain used in this study is anthelminthic-susceptible inbred strain of SE strain (Roos et al., 2004), which was isolated from the field before benzimidazole anthelmintics were on the market. Third stage larvae (L3) of ISE strain were kind gift of Dr. Frank Jackson, Moredun Research Institute, Edinburgh, UK. Six parasite-free lambs (3–4-month old) were orally infected with 5000 L3 larvae of *H. contortus*. Seven weeks after infection the animals were stunned and exsanguined immediately in agreement with Czech slaughtering rules for farm animals. Adult nematodes were removed from sheep abomasum using agar method described by Van Wyk et al. (1980).

2.3. Preparation of subcellular fractions

Freshly isolated *H. contortus* adults were washed repeatedly in 0.1 M phosphate-buffered saline pH 7.4 and rapidly homogenised at the w/v ratio of 1:6 in 0.1 M sodium phosphate buffer, pH 7.4, using a Potter–Elvehjem homogeniser and sonication with Sonopuls (Bandeline, Germany). The subcellular fractions were isolated by fractional ultracentrifugation of the homogenate with the same buffer. Fraction A (20,000 $\times$ *g* sediment) corresponds to mitochondrial fraction in animal tissues fractionation. Fraction B and C consist of 105,000 $\times$ *g* sediment and supernatant (microsomes like and cytosol like). A re-washing step (followed by a second ultracentrifugation) was included at the end of the Fraction B preparation procedure. Fraction A, finally resuspended in 0.1 M sodium phosphate buffer, pH 7.4, Fraction B, finally resuspended in this buffer containing 20% glycerol (v/v), and Fraction C were stored at -80 °C. Protein concentrations were assayed using the bicinchoninic acid method according to the Sigma protocol.

2.4. Cultivation of H. contortus in medium

Live nematodes were cultivated as described Kotze and McClure (2001) with mild modification. Isolated nematodes were washed three times by phosphate buffer saline and held for 1 h in RPMI medium (pH 6.8, 38 °C) containing 2.5 $\mu g\ ml^{-1}$ amphotericin B, 100 $U\ ml^{-1}$ penicillin, and 100 $\mu g\ ml^{-1}$ streptomycin. Groups of approximately 50 nematodes were then placed into 5 ml of incubation medium (RPMI medium, pH 6.8, containing 0.8% glucose, 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[4-butanesulfonic acid] (HEPES), 0.25 $\mu g\ ml^{-1}$ amphotericin B, 10 $U\ ml^{-1}$ penicillin, and 10 $\mu g\ ml^{-1}$ streptomycin) in glass flasks and incubated at 38 °C in a humid atmosphere with 10% CO_2.

2.5. Biotransformation of FLU in vitro

The Fractions A–C were incubated with or without FLU (10 μM). The reaction mixture (total volume of 0.3 ml) contained 50 μl of fractions containing 0.4–0.6 mg of proteins, substrate pre-dissolved in dimethyl sulfoxide (concentration of DMSO in reaction mixture was 1%), NADPH or NADH (1 mM) and 0.1 M Na-phosphate buffer, pH 7.4. The blank samples contained 50 μl of 0.1 M sodium phosphate buffer, pH 7.4, instead of fractions or 50 μl of 10-min-boiled fractions. In kinetic study, concentrations range 0.25–25.0 μM of FLU, 1 mM NADPH and 50 μl of Fraction C were used. All incubations were carried out at 37 °C for 30 min under aerobic conditions. The product formation was linear up to 60 min. At the end of incubation, 30 μl of ammonium solution (concentrated) and 700 μl of cooled ethyl acetate were added, shaken (3 min, vortex) and centrifuged (10 min, 10000 × *g*). Supernatants were evaporated and stored under −20 °C until HPLC analyses.

2.6. Biotransformation of FLU ex vivo

At the beginning of incubation, the 2.5 ml of medium was removed from each flask with nematodes and the same volume of fresh medium with FLU was added. FLU was pre-dissolved in DMSO. The concentration of DMSO in medium was 0.1%. Nematodes were incubated in medium with FLU (2.0, 5.0, or 10.0 μM) for 24 h. In blank samples, medium with FLU but without nematodes was incubated. After incubation, medium was taken up, placed into plastic tubes, frozen and stored under −80 °C. The nematodes were repeatedly washed up, transfer into plastic tubes, frozen and stored under −80 °C. Before analysis, nematodes were quickly homogenised in 0.1 M phosphate buffer (pH 7.4) using Sonopuls. Medium or nematodes homogenate were alkalised and liquid–liquid extraction followed by the same procedure as was described above.

2.7. HPLC analysis of FLU and its metabolites

Chromatographic analyses were performed with Shimadzu liquid chromatograph consisted of degasser GT-154, solvent delivery module LC-10ADvp, auto-injector SIL-10ADvp, column oven CTO-10Avp, UV/VIS photodiode array detector SPD-M10Avp, spectro-fluorimetric detector RF-10AXL and system controller SCL-10Avp. The chromatograph was controlled by Shimadzu software CLASS-VP. A LiChroCART 250 mm × 4 mm chromatographic column packed with LiChrospher 60 RP-select B, 5 μm was used. The column was protected with a pre-column 4 mm × 4 mm packed with the same stationary phase. Following chromatographic conditions were used: an isocratic mobile phase was a mixture of acetonitrile and 0.025 M KH_2PO_4 buffer pH 3 (3:7) delivered at a flow rate of 0.7 ml/min. Using the photodiode array detector, chromatograms were recorded at 246 and 300 nm (scan 195–380 nm). With spectrofluorimetric detector excitation wavelength was 290 nm and emission wavelength was 320 nm. FLU-H, FLU-R, albendazole (I.S.) and FLU were detected with the photodiode array detector. The spectrofluorimetric detector served for improving the sensitivity of FLU-R detection (FLU-H and FLU are not fluorescent). Fifty microlitre of each sample was injected. Duration of the complete analysis was 22 min.

2.8. HPLC separation of FLU-R enantiomers

The chromatographic method used for the determination of flubendazole and its two metabolites has been described previously (Nobilis et al., 2007). The chromatographic system was composed of an SCM1000 solvent degasser, P4000 quaternary gradient pump, AS3000 autosampler with a 100-μl sample loop, UV6000 LP photodiode array detector (UV-PDA) with Light Pipe Technology, SN4000 system controller and a data station (Intel-Pentium 4 CPU 1.6 GHz, RAM 256 MB, HDD 40 GB) with the ChromQuest 4 analytical software (Thermo Electron, Inc., San Jose, CA., USA). A Daicel 250–4.6 mm chromatographic column packed with Chiralcel OD-R (Daicel Chemical Industries, Ltd., Japan) and mobile phase consisting of acetonitrile—1 M aqueous $NaClO_4$ (4:6, v/v) were employed for chiral chromatographic separations. Flow rate was 0.5 ml min^{-1}. UV detection was performed in dual wavelength mode (246 and 300 nm).

2.9. Enzyme assays

Enzyme assays were tested in the fractions of *H. contortus* homogenate. Each enzyme assay was performed in triplicates. The amount of organic solvents in the final reaction mixtures did not exceed 0.1% (v/v).

The activities of reductases of carbonyl group were tested using the following substrates: metyrapone (dissolved in redistilled water), D,L-glyceraldehyde (dissolved in dimethylsulfoxide), and daunorubicin (dissolved in redistilled water). The concentrations of substrates, NADPH and potassium phosphate buffer, pH 6.0 (or 8.5 for daunorubicin) were 1 mM, 0.3 mM and

0.1 M, respectively. The fractions (10–50 μl, containing 60–400 μg of protein) was added into reaction mixture (total volume 1 ml). Spectrophotometric determination (detection wavelength 340 nm, 25 °C) of NADPH consumption in the reaction mixture served for the assessment of reductase activities (Feldsted and Bachur, 1980; Maser and Oppermann, 1997; Palackal et al., 2002; Ohara et al., 1995).

Reductases of oracin were assayed as described (Wsol et al., 2003). Briefly, Fractions A, B or C (100 μl) were incubated with 0.3 mM oracin and 1 mM NADPH in a total buffer volume of 0.3 ml. Incubations (37 °C, 30 min) were terminated by alkalisation and incubates were extracted into ethyl acetate. The extracts were evaporated to dryness and dry samples were dissolved in the mobile phase prior to their HPLC injection. The HPLC separation of dihydrooracin enantiomers was performed using a 250 mm × 4.6 mm ODR Chiralcel column. The mobile phase was prepared by mixing buffer (0.3 M sodium perchlorate, pH 3.00 set by $HClO_4$) with acetonitrile (69:31, v/v). HPLC separation was performed at 25 °C with a flow rate of 0.5 ml/min. Dihydrooracin was detected with fluorescence detector using an excitation wavelength of 340 nm, and an emission wavelength of 418 nm. Oracin was detected with UV-vis detector at a detection wavelength of 280 nm.

Enzyme activities for the model substrate 1-acenaphthenol (substrate of dihydrodiol dehydrogenases, ACNO) were determined using methods described by Palackal et al. (2002) with modifications. The velocity of substrate dehydrogenation was determined spectrophotometrically by measuring the change in absorbance of a cofactor ($NADP^+$) at 340 nm. A final 1.0 ml system contained 1 mM acenaphthenol dissolved in DMSO (1% of organic solvent in final mixture), 2.0 mM $NADP^+$, 50 μl Fractions A, B or C and 0.1 M Tris-HCl buffer pH 8.9.

3. Results

3.1. Biotransformation of flubendazole (FLU) in H. contortus in vitro

All fractions of *H. contortus* homogenate were incubated with FLU and coenzyme NADPH or NADH. Only one FLU metabolite – FLU with reduced carbonyl group (FLU-R) – was detected. Structure of FLU and FLU-R is shown in Fig. 1. FLU-R arises solely in Fraction C and its formation is strictly NADPH-dependent. No metabolite was formed in samples where NADH was used as coenzyme or in samples without coenzymes. In blank samples (without *H. contortus* fraction or with these fractions inactivated by 10-min boiling) no traces of FLU-R were found.

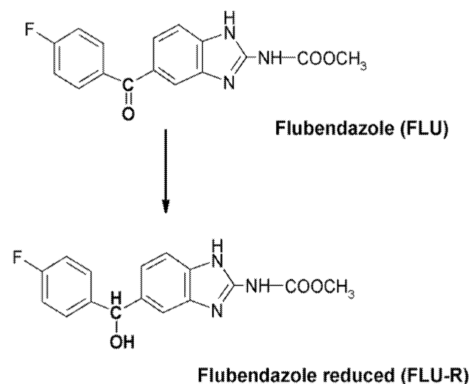


Fig. 1. Structure of FLU and its main metabolite FLU-R.

3.2. Kinetics of FLU reduction in H. contortus in vitro

FLU in various concentrations (0.25–25.0 μM) was incubated with Fraction C of *H. contortus* homogenate and coenzyme NADPH. The amount of FLU-R formed in incubation mixture was expressed as reaction velocity. The direct plot of reaction velocity versus substrate concentration is presented in Fig. 2. The curve fits well the Michaelis–Menten equation. Using GraphPad Prism 5.0 software, the values of basic kinetic parameters, apparent maximal velocity $V'_{max} = 39.8 \pm 2.1\,nM\,min^{-1}$, and apparent Michaelis constant $K'_m = 1.5 \pm 0.3\,\mu M$ were calculated. These kinetic parameters allow characterization of enzyme reaction in multi-enzymatic systems. Apparent maximal velocity V'_{max} indicates the rate of product

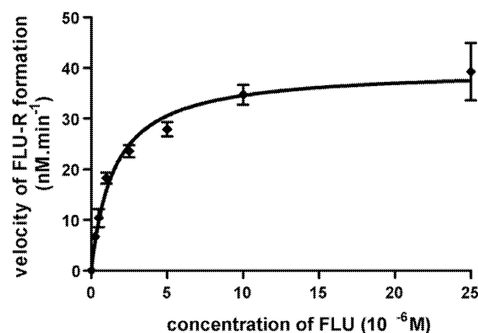


Fig. 2. Kinetics of FLU reduction in Fraction C from *H. contortus* homogenate.

formation at enzyme saturation with substrate, and apparent Michaelis constant K'_m expresses the affinity of enzyme toward substrate. Ratio of these kinetic parameters represents so-called intrinsic clearance.

3.3. Reduction of FLU in H. contortus ex vivo

The live nematodes were cultivated for 24 h in a glass flask with medium. At the commencement of the experiments, fresh medium with 2.0, 5.0, or 10.0 μM initial concentrations of FLU were added, nematodes were gently shaken and first medium samples were taken (time 0) and analyzed. In time 0 samples, approximately 50% of FLU initial concentration was detected, probably because of FLU adsorption to flask and nematodes' surface and binding to medium contents. During experiments, other medium samples were collected (time 2, 8, and 24 h) and analyzed. The results are demonstrated in Figs. 3 and 4. Time-dependent decrease of parent drug FLU in medium with nematodes was observed. The initial concentration of FLU affected the velocity of decrease: the fastest concentration decrease was found in samples with the highest initial concentration of FLU. FLU-R was the only Phase I metabolite of FLU found in medium with *H. contortus*. Its formation increased with increasing FLU concentration and increasing duration of incubation. No FLU reduction was detected in medium without nematodes.

Chiral analyses showed considerable stereospecificity of FLU reduction. *Ex vivo H. contortus* preferentially formed (−)-FLU-R enantiomer. The average ratio of FLU-R enantiomers 90:10 was found. This ratio did not significantly change based on the incubation time or initial concentration of substrate in the medium.

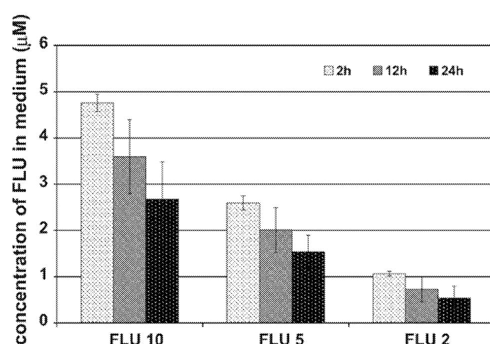


Fig. 3. Decrease of FLU concentration during 24-h incubation of live *H. contortus* nematodes in medium with FLU. The initial concentrations of FLU were 2.0, 5.0 or 10.0 μM.

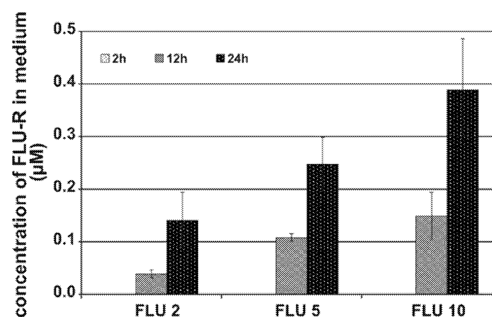


Fig. 4. Formation of FLU-R during 24-h incubation of live *H. contortus* nematodes in medium with FLU. The initial concentrations of FLU were 2.0, 5.0 or 10.0 μM.

3.4. Activity of carbonyl-reducing enzymes toward model xenobiotics in H. contortus in vitro

Activities of carbonyl-reducing enzymes were tested in subcellular fractions of *H. contortus* homogenate. Metyrapone, acenaphenol, D,L-glyceraldehyde, daunorubicin, and oracin, which are relatively specific for main human carbonyl-reducing enzymes, were used as substrates. The results (see Table 1) proved the extensive ability of *H. contortus* enzymes to reduce the carbonyl group of compounds. Different reduction potential of separate fractions was observed: while Fraction C transformed all substrates used, in Fraction A only daunorubicin at pH 8.5 was reduced. The activities of *H. contortus* enzymes toward individual substrates also differed: D,L-glyceraldehyde and daunorubicin at pH 8.5 were the best substrates in Fraction C and Fractions A and B, respectively. The stereospecificity of oracin reduction was tested in Fraction C. The ratio of dihydrooracin enantiomers was 63:37 with preferential formation of (−)-dihydrooracin enantiomer.

4. Discussion

Metabolism of FLU either in *H. contortus* or in other helminths has not been studied till now. In hosts the biotransformation of FLU is extensive and follows similar metabolic pathways in various animal species. Ketoreduction and carbamate hydrolysis are the major Phase I metabolic pathways in birds, pigs, or sheep (http://www.emea.europa.eu/htms/vet/mrls/a-zmrl.htm#; Moreno et al., 2004). The same biotransformation pathways were described for mebendazole—other benzimidazole anthelmintics with structures like FLU without fluorine atom. In this project, biotransformation of FLU was tested in *H. contortus in vitro* as well as *ex vivo*. Results showed that only one of the two

Table 1
Specific activities of reductases/dehydrogenases toward selected substrates tested in subcellular Fractions A (mitochondria like), B (microsomes like), and C (cytosole like) of *H. contortus* homogenate

	Specific activities (nmol min^{-1} mg^{-1})		
	Fraction A	Fraction B	Fraction C
Metyrapone reductase	ND	ND	1.07 ± 0.39
D,L-glyceraldehyde reductase	ND	1.40 ± 0.21	5.62 ± 0.43
Daunorubicin reductase (pH 6.0)	ND	ND	0.29 ± 0.08
Daunorubicin reductase (pH 8.5)	4.65 ± 1.18	1.82 ± 0.33	0.41 ± 0.09
Oracin reductase	ND	0.07 ± 0.01	0.50 ± 0.02
Acenaphthenol dehydrogenase	ND	ND	2.72 ± 0.43

The data represent the mean ± S.D. from 3–5 samples. ND = not detected.

known FLU metabolites – FLU with reduced carbonyl group (FLU-R) – was formed. Thus, reduction represents the main biotransformation pathway of FLU in *H. contortus.* Reduction of albendazole sulfoxide was observed in *F. hepatica*, *M. expansa*, and *A. suum* subcellular fractions (Solana et al., 2001). In all species tested these reactions were NADPH-independent and almost the same activities in microsomal and cytosolic fractions were found. In the experiments conducted by the authors, reduction of FLU occurred only in Fraction C (like cytosol) and strictly required coenzyme NADPH. Based on the kinetic data, basic kinetic parameters of FLU reduction were calculated. Their values indicate that this reaction is not very fast (velocity approximately nM min^{-1}), but reductases possess relatively high affinity to FLU. In pigs and pheasants, the velocity was much higher (approximately μM min^{-1}) but the affinity to FLU was almost the same (Szotakova et al., submitted for publication). Moreover, the reduction of FLU in *H. contortus* was stereospecific with preferential formation of (−)-FLU-R enantiomer. This finding is very interesting because in pig or pheasant, (+)-FLU-R is the only FLU-R enantiomer formed *in vitro* (Nobilis et al., 2007).

Reduction of carbonyl group is generally considered as a deactivation pathway that protects organisms against the toxic effect of reactive aldehydes and ketones. Because of reduced mebendazole lacked any anthelmintic activity (Prieto et al., 1991; Dayan, 2003), FLU reduction probably also represents deactivation, although, no exact information about anthelmintic activity of FLU-R is available. Also, the information about reductases of carbonyl group of xenobiotics in helminths is negligible. In mammals, carbonyl-reducing enzymes are located both in cytosol and in membranes of endoplasmatic reticulum, and they require NADPH or NADH as coenzymes. The enzymes participating in reduction of the carbonyl are classified into three protein classes: medium chain dehydrogenases (MDR), short chain dehydrogenases (SDR), and aldo-keto reductases (AKR) (Jez et al., 1997; Penning, 2004). The substrates of reductases of the carbonyl group are not only xenobiotics, but also a number of endogenous substances. Hence, the study of the enzymes reducing the carbonyl group is important not only for prediction of metabolism of drugs with carbonyl group in the organisms, but also for understanding the way in which they can be affected (Bauman et al., 2004). Human reductases of the carbonyl group are intensively studied at present. For evaluation of activities of individual families or subfamilies of carbonyl-reducing enzymes several model substrates were commonly used. D,L-glyceraldehyde and daunorubicin (at pH 8.5) are good substrates for human AKR1A enzymes, while daunorubicin at pH 6.0 is preferentially reduced by action of carbonyl reductase and AKR1C enzymes. Acenaphthenol represents the typical substrate for enzymes from AKR1C subfamily. Metyrapone is reduced by AKR1C, AKR1A, and carbonyl reductase in cytosol and by 11β-hydroxysteroid dehydrogenase in microsomes (Kawamura et al., 1999; Maser and Oppermann, 1997; Ohara et al., 1995; Palackal et al., 2002). Almost all carbonyl-reducing enzymes are able to reduce oracin in human (Wsol et al., 2003; Martin et al., 2006).

In *H. contortus*, all model substrates were metabolised and the specific activities of carbonyl-reducing enzymes were similar to the activities found in farm animal species (Szotakova et al., 2004). These results prove that *H. contortus* is able to effectively reduce aldehydes or ketones. In this way, carbonyl-reducing enzymes protect *H. contortus* against the toxic effect of xenobiotics with the carbonyl group. Inhibitors of these enzymes could increase the efficacy of anthelmintics-bearing carbonyl group (FLU or mebendazole) similar to synergism of rotenone by piperonyl butoxide via the inhibition of nematode oxidative detoxication pathways (Kotze et al., 2006). Improving one's knowledge about drugs bio-

transformation and drug-metabolizing enzymes in parasitic helminths could improve the pharmacotherapy of helminthoses.

Acknowledgement

Financial support of this project was provided by Grant Agency of Czech Republic, Grant No. 524/06/1345.

References

Barrett, J., 1997. Helminth detoxification mechanisms. J. Helminthol. 71, 85–89.

Barrett, J., 1998. Cytochrome P450 in parasitic protozoa and helmints. Comp. Biochem. Physiol. 121, 181–183.

Bauman, D.R., Steckelbroeck, S., Penning, T.M., 2004. The roles of aldo-keto reductases in steroid hormone action. Drugs News Perpect. 17, 563–578.

Dayan, A.D., 2003. Albendazole, mebendazole and praziquantel. Review of non-clinical toxicity and pharmacokinetics. Acta Trop. 86, 141–159.

Feldsted, R.L., Bachur, N.R., 1980. Mammalian carbonyl reductases. Drug Metab. Rev. 11, 1–60.

Jez, J.M., Flynn, T.G., Penning, T.M., 1997. A new nomenclature for the aldo-keto reductase superfamily. Biochem. Pharmacol. 54, 639–647.

Kawamura, M., Eisenhofer, G., Kopin, I.J., Kador, P.F., Lee, Y.S., Tsai, J.Y., Fujisawa, S., Lizak, M.J., Sinz, A., Sato, S., 1999. Aldose reductase, a key enzyme in the oxidative deamination of norepinephrine in rats. Biochem. Pharmacol. 58, 517–524.

Kotze, A.C., McClure, S.J., 2001. *Haemonchus contortus* utilises catalase in defence against exogenous hydrogen peroxide in vitro. Int. J. Parasitol. 31, 1563–1571.

Kotze, A.C., Dobson, R.J., Chandler, D., 2006. Synergism of rotenone by piperonyl butoxide in *Haemonchus contortus* and *Trichostrongylus colubriformis in vitro*: potential for drug-synergism through inhibition of nematode oxidative detoxificiation pathways. Vet. Parasitol. 136, 275–282.

Maser, E., Oppermann, U.C.T., 1997. The 11 beta-hydroxysteroid dehydrogenase system, a determinant of glucocorticoid and mineralocorticoid action - Role of type-1 11 beta-hydroxysteroid dehydrogenase in detoxification processes. Eur. J. Biochem. 249, 365–369.

Martin, H.J.O., Breyer-Pfaff, U., Wsol, V., Venz, S., Block, S., Maser, E., 2006. Purification and characterization of AKR1B10 from human liver: role in carbonyl reduction of xenobiotics. Drug Metab. Dispos. 34, 464–470.

Moreno, L., Alvarez, L., Mottier, L., Virkel, G., Bruni, S.S., Lanusse, C., 2004. Integrated pharmacological assessment of flubendazole potential for use in sheep: disposition kinetics, liver metabolism and parasite diffusion ability. J. Vet. Pharm. Ther. 27, 299–308.

Mottier, L., Virkel, G., Solana, H., Alvarez, L., Salles, J., Lanusse, C., 2004. Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidized metabolites into *Fasciola hepatica*. Xenobiotica 34, 1043–1057.

Nobilis, M., Jira, T., Lisa, M., Holcapek, M., Szotakova, B., Lamka, J., Skalova, L., 2007. Achiral and chiral high-performance liquid chromatographic determination of flubendazole and its metabolites in biomatrices using UV photodiode-array and mass spectrometric detection. J. Chromatogr. A 1149, 112–120.

Ohara, H., Miyabe, Y., Deyashiki, Y., Matsuura, K., Hara, A., 1995. Reduction of drug ketones by dihydrodiol dehydrogenases, carbonyl reductase and aldehyde reductase of human liver. Biochem. Pharmacol. 50, 221–227.

Palackal, N.T., Lee, S.H., Harvey, R.G., Blair, I.A., Penning, T.M., 2002. Activation of polycyclic aromatic hydrocarbon trans-dihydrodiol proximate carcinogens by human aldo-keto reductase (AKR1C) enzymes and their functional overexpression in human lung carcinoma (A549) cells. J. Biol. Chem. 277, 24799–32480.

Penning, T.M., 2004. Introduction and overview of the Aldo-keto reductase superfamily. ACS Symp. Ser. 865, 3–20.

Prieto, J.G., Justel, A., Del Estal, J.L., Barrio, J.P., Alvarez, A.I., 1991. Comparative study on gastric absorption of albendazole and mebendazole in rats. Comp. Biochem. Physiol. 100C, 397–400.

Robinson, M.W., Lawson, J., Trudgett, A., Hoey, E.M., Fairweather, I., 2004. The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. Parasitol. Res. 92, 205–210.

Roos, M.H., Otsen, M., Hoekstra, R., Veenstra, J.G., Lenstra, J.A., 2004. Genetic analysis of inbreeding of two strains of the parasitic nematode *Haemonchus contortus*. Int. J. Parasitol. 34, 109–115.

Solana, H.D., Rodriguez, J.A., Lanusse, C.E., 2001. Comparative metabolism of albendazole and albendazole sulphoxide by different helminth parasites. Parasitol. Res. 87, 275–280.

Szotakova, B., Baliharova, V., Lamka, J., Nozinova, E., Wsol, V., Velik, J., Machala, M., Neca, J., Soucek, P., Susova, S., Skalova, L., 2004. Comparison of *in vitro* activities of biotransformation enzymes in pig, cattle, goat and sheep. Res. Vet. Sci. 76, 43–51.

Van Wyk, J.A., Gerber, H.M., Groeneveld, H.T., 1980. A technique for recovery of nematodes from ruminants by migration from gastrointestinal ingesta gelled in agar: large-scale application. J. Vet. Res. 47, 147–158.

Wsol, V., Szotakova, B., Skalova, L., Maser, E., 2003. Stereochemical aspects of carbonyl reduction of the original anticancer drug oracin by mouse liver microsomes and purified 11- beta-hydroxysteroid dehydrogenase type 1. Chem-Biol. Interact. 143, 459–468.

III.

LC-MS-MS identification of albendazole and flubendazole metabolites formed *ex vivo* by *Haemonchus contortus*

Cvilink, V., Skálová, L., Szotáková, B., Lamka, J., Kostiainen, R., Ketola, R. A.

Anal Bioanal Chem (2008) 391:337-343

Anal Bioanal Chem
DOI 10.1007/s00216-008-1863-9

ORIGINAL PAPER

LC–MS–MS identification of albendazole and flubendazole metabolites formed *ex vivo* by *Haemonchus contortus*

Viktor Cvilink · Lenka Skálová · Barbora Szotáková · Jiří Lamka · Risto Kostiainen · Raimo A. Ketola

Received: 19 October 2007 / Revised: 14 December 2007 / Accepted: 9 January 2008
© Springer-Verlag 2008

Abstract Resistance of helminth parasites to common anthelminthics is a problem of increasing importance. The full mechanism of resistance development is still not thoroughly elucidated. There is also limited information about helminth enzymes involved in metabolism of anthelminthics. Identification of the metabolites formed by parasitic helminths can serve to specify which enzymes take part in biotransformation of anthelminthics and may participate in resistance development. The aim of our work was to identify the metabolic pathways of the anthelminthic drugs albendazole (ABZ) and flubendazole (FLU) in *Haemonchus contortus*, a world-wide distributed helminth parasite of ruminants. ABZ and FLU are benzimidazole anthelminthics commonly used in parasitoses treatment. In our *ex vivo* study one hundred living adults of *H. contortus*, obtained from the abomasum of an experimentally infected lamb, were incubated in 5 mL RPMI-1640 medium with 10 μmol L^{-1} benzimidazole drug (10% CO_2, 38 °C) for 24 h. The parasite bodies were then removed from the medium. After homogenization of the parasites, both parasite homogenates and medium from the incubation were separately extracted using solid-phase extraction. The extracts were analyzed by liquid chromatography–mass spectrometry (LC–MS) with electrospray ionization (ESI) in positive-ion mode. The acquired data showed that *H. contortus* can metabolize ABZ via sulfoxidation and FLU via reduction of a carbonyl group. Albendazole sulfoxide (ABZSO) and reduced flubendazole (FLUR) were the only phase I metabolites detected. Concerning phase II of biotransformation, the formation of glucose conjugates of ABZ, FLU, and FLUR was observed. All metabolites mentioned were found in both parasite homogenates and medium from the incubation.

Keywords Mass spectrometry · Drug metabolism · Xenobiotics · Glucose conjugation · Biotransformation · Parasitic helminth

Abbreviations

ABZ	Albendazole
ABZSO	Albendazole sulfoxide
$ABZSO_2$	Albendazole sulfone
DMSO	Dimethyl sulfoxide
ESI	Electrospray ionization
FLU	Flubendazole
FLUH	Hydrolyzed flubendazole
FLUR	Reduced flubendazole
HEPES	*N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)

V. Cvilink · L. Skálová · B. Szotáková
Department of Biochemical Sciences,
Faculty of Pharmacy, Charles University,
Heyrovského 1203,
500 05 Hradec Králové, Czech Republic
e-mail: viktor.cvilink@faf.cuni.cz

J. Lamka
Department of Pharmacology and Toxicology,
Faculty of Pharmacy, Charles University,
Heyrovského 1203,
500 05 Hradec Králové, Czech Republic

R. Kostiainen · R. A. Ketola
Division of Pharmaceutical Chemistry,
Faculty of Pharmacy, University of Helsinki,
P.O. Box 56, 00014 Helsinki, Finland

R. A. Ketola (✉)
Drug Discovery and Development Technology Center,
Faculty of Pharmacy, University of Helsinki,
P.O. Box 56, 00014 Helsinki, Finland
e-mail: raimo.ketola@helsinki.fi

HPLC	High-performance liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
MS–MS	Tandem mass spectrometry
QqQ	Triple-quadrupole mass spectrometer
RPMI	Roswell Park Memorial Institute
SPE	Solid-phase extraction
UDP-glucose	Uridine-5′-diphosphoglucose

Introduction

Benzimidazole anthelminthics, used to control worm infections in humans and animals, have been on the market for a long time, as they were introduced in 1962 [1]. Metabolism of albendazole ([5-(propylthio)-1*H*-benzimidazol-2-yl] methylcarbamate) and flubendazole ([5-(4-fluorobenzoyl)-1*H*-benzimidazol-2-yl] methylcarbamate; chemical structures in Fig. 1) has been investigated in humans [2, 3], rats [4–7], poultry [8], sheep [9, 10] and cattle [11–13]. Although a great deal of information on the metabolism of anthelminthics has been obtained in vertebrates, little is known about their metabolism in parasitic helminths.

In parasitic helminths, oxidation of ABZ to ABZSO, and triclabendazole sulfoxide to triclabendazole sulfone in *Fasciola hepatica* [14–16], and epoxidase activities towards aldrin in *Haemonchus contortus* [17], were reported. In addition, ABZ sulfoxidation was reported in *Moniezia expansa* and *Ascaris suum* [14]. Reduction of flubendazole to the reduced flubendazole was detected in *ex vivo* incubation with *Moniezia benedeni* [10]. Reduction of albendazole sulfoxide was observed in *Fasciola hepatica, Moniezia expansa*, and *Ascaris suum* subcellular fractions [14].

Haemonchus contortus is a highly pathogenic worldwide distributed helminth parasite of small ruminants. This blood sucking parasite is found in the host's abomasum and causes significant production losses due to anaemia, hypoalbuminaemia, and often depressed total protein content [18].

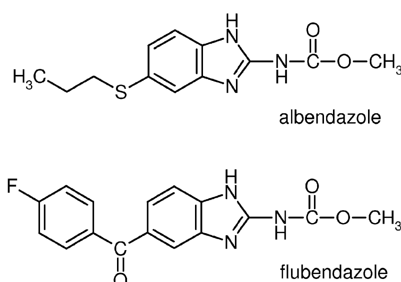


Fig. 1 Chemical structures of albendazole and flubendazole

The anthelminthic action of benzimidazole compounds in the parasite is based on binding to specific β-tubulin receptors. This results in inhibition of tubuline assembly and depolymerization of microtubules, ending in immobilization and death of the parasite [19, 20]. Resistance to the benzimidazole group of anthelminthics is mainly caused by the structural changes of the β-tubulin receptor [21]. However, the influence and additive effects of other mechanisms (altered drug influx–efflux or enzyme induction) may play a significant role in resistance development.

Liquid chromatography coupled with mass spectrometry is a rapid, powerful, and flexible analytical technique which plays an important role in identification, structural characterization, and quantitative analysis of drugs and their metabolites [22].

The objective of our work was to find and identify, by use of liquid chromatographic–mass spectrometric techniques, phase I and phase II metabolites of the anthelminthic drugs albendazole and flubendazole formed in *ex vivo* incubations by the parasitic helminth *Haemonchus contortus*. Consequently, metabolic pathways of albendazole and flubendazole in these incubations are proposed.

Experimental

Chemicals and reagents

ABZ was purchased from Sigma–Aldrich (Prague, Czech Republic). ABZSO and $ABZSO_2$ were obtained from Toronto Research Chemicals (Toronto, Canada). FLU and its two metabolites (FLUR, FLUH) were provided by Janssen Pharmaceutica (Prague, Czech Republic). All other chemicals (HPLC or analytical grade) were obtained from Sigma–Aldrich. Water was purified with a Milli-Q System (Millipore, Bedford, USA).

Stock standard solutions were prepared in DMSO–acetonitrile–water 5:40:55 (*v*/*v*) and stored at 4°C. Working standard solutions were prepared by diluting the corresponding volume of stock standard solution with 5% (*v*/*v*) acetonitrile in water. The concentration of working standard solution was 1 μg mL^{-1} for all standards.

Collection of parasite

H. contortus ISE strain used in this study was an anthelminthic-susceptible inbred strain of SE strain [23], which was isolated from the field before benzimidazole anthelminthics were on the market and had no history of exposure to anthelminthics. Third stage larvae (L3) of ISE strain were a kind gift from Dr Marián Várady, Parasitological Institute, Slovak Academy of Sciences, Košice, Slovakia. Six parasite-free lambs (3–4 months old) were orally infected with

5,000 L3 larvae of *H. contortus*. Seven weeks after infection the animals were stunned and exsanguined immediately in agreement with Czech slaughtering rules for farm animals. Adult nematodes were removed from sheep abomasa using the agar method described by van Wyk [24].

Cultivation of *Haemonchus contortus* in medium

Isolated *H. contortus* nematodes were washed three times with phosphate-buffered saline and held for 1 h in RPMI-1640 medium (pH 6.8, 38 °C) containing 2.5 μg mL^{-1} amphotericin B, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Groups of approximately 100 nematodes were then placed in 5 mL incubation medium (RPMI-1640 medium, pH 6.8, containing 0.8% glucose, 10 mmol L^{-1} HEPES, 0.25 μg mL^{-1} amphotericin B, 10 U mL^{-1} penicillin, and 10 μg mL^{-1} streptomycin) in glass flasks and cultivated at 38 °C in a humid atmosphere with 10% CO_2.

Biotransformation of ABZ and FLU *ex vivo*

At the beginning of incubation of *H. contortus*, the 2.5 mL medium from each flask with nematodes was replaced with the same volume of fresh medium containing 10.0 μmol L^{-1} ABZ or FLU (pre-dissolved in DMSO). The final concentration of DMSO in medium was 0.1% (*v*/*v*). After incubation for 24 h the medium was placed in plastic tubes, frozen, and stored at −80 °C. The nematodes were repeatedly washed, transferred into plastic tubes, frozen, and stored at −80 °C. Before analysis, nematodes were homogenised for 6×10 s in cooled 0.1 mol L^{-1} phosphate buffer (pH 7.4) using Sonopuls (Bandelin, Germany). Medium samples or nematode homogenates were extracted using SPE. In biological blank samples, nematodes were incubated in medium without anthelminthics; in chemical blank samples, medium with ABZ or FLU but without nematodes was incubated.

Sample preparation and solid-phase extraction

The sample (1 mL) was acidified with 1 μL 98% formic acid and centrifuged at approximately 3000 *g* for 3 min. The supernatant was loaded on to a Waters Oasis MCX (1 cc, 30 mg, 30 μm particles; Waters, Dublin, Eire) extraction cartridge previously conditioned by washing with 1 mL acetonitrile and 1 mL purified water. In the next step, the cartridge was washed with 1 mL 2% (*v*/*v*) aqueous formic acid and with 1 mL 1% aqueous ammonia in 30% (*v*/*v*) acetonitrile. Compounds of interest were eluted with 1 mL 4% aqueous ammonia in 80% (*v*/*v*) acetonitrile. The eluate was evaporated to dryness under a stream of nitrogen, redissolved in 100 μL 5% (*v*/*v*) acetonitrile in water, and 20 μL of the final solution was injected into the analytical column.

Liquid chromatography

Experiments were performed with an HP1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, an autosampler, and a column compartment. The column employed was SymmetryShield RP18 (3×100 mm, 3.5 μm; Waters, Milford, USA). The mobile phase consisted of solvent A (0.1% (*v*/*v*) formic acid in water) and solvent B (0.1% (*v*/*v*) formic acid in acetonitrile). A 500 μL min^{-1} flow rate linear gradient of 5–35% B was developed over 15 min. Eluent percentages at various timepoints are presented in Table 1. The mobile phase was postcolumn-split (ratio 1:10) prior to the MS measurement.

Mass spectrometry

An API3000 (Applied Biosystems/MDS Sciex, Toronto, Ontario, Canada) triple-quadrupole mass spectrometer with a turbo ion-spray source, operated in positive-ion mode, was used for analyte detection. Nitrogen produced by a Whatman (Haverhill, USA) 75-72 generator was used as the curtain and collision gas and purified air as the nebulizing gas (Atlas Copco CD 2, Wilrijk, Belgium). Optimized ion-source parameters were set for benzimidazole drug analysis during sample data acquisition: declustering potential 45 V, focusing potential 260 V, entrance potential 8.5 V, and collision cell exit potential 15 V. The spraying capillary voltage was 5000 V, the nebulizer gas flow rate was 1.5 L min^{-1}, and the curtain gas flow rate was 0.8 L min^{-1}. Turbo gas was heated to 300 °C. Data were acquired and processed using Analyst software, version 1.4, the full-scan data were processed using Metabolite ID software, version 1.3 (both Applied Biosystems/MDS Sciex).

Flow injection experiments

Flow injection MS and MS–MS experiments were performed using a microsyringe pump (Harvard Apparatus, Naticle, USA) with a flow rate of 15 μL min^{-1} of benzimidazole working standard solution. The spectra were

Table 1 Eluent composition during LC–MS analysis

Timepoints	Eluent percentages
0 min	5% B
0–15.0 min	5–35% B
15.0–16.0 min	35–70% B
16.0–17.9 min	70% B
17.9–18.0 min	70–5% B
18.0–26.0 min	5% B

Table 2 Parent drugs and their metabolites, retention times, product ions, and precursor ion intensities detected in full-scan and MS–MS measurements of *ex vivo* experiments

Compound	Precursor ion $[M + H]^+$ (*m/z*)	t_R (min)	Product ions (*m/z*, relative abundance)	Precursor ion intensity (cps)
ABZ	266	14.8	266(2), 234(89), 191(100), 159(32)	6.8×10^6
ABZSO	282	9.1	240(0.8), 208(100), 191(20), 159(26)	8.5×10^6
ABZ glucoside 1	428	14.1	266(100), 234(75), 191(10)	8.5×10^5
ABZ glucoside 2	428	14.9	266(94), 234(100), 191(4)	7.2×10^5
FLU	314	17.8	314(5), 282(100), 123(19), 95(4)	7.1×10^6
FLUR	316	10.5	316(2), 284(100), 160(22), 125(16), 97(18)	5.5×10^6
FLU glucoside	476	14.1	314(100), 282(79), 123(58)	5.1×10^5
FLUR glucoside	478	9.1	298(100), 284(0.5), 266(42), 238(0.8)	4.4×10^6

recorded over the mass range of *m/z* 100–650 in MS experiments; for MS–MS experiments the range was *m/z* 50–350. The nebulizing gas and the curtain gas values, the ion source voltages, and the ion optics parameters were optimized to yield the maximum intensity of the protonated molecules, and abundance of molecule-specific product ions as high as possible. Full-scan spectra and MS–MS fragmentation patterns of all six standard compounds were measured and structures of the product ions were tentatively elucidated (results not presented here).

Results and discussion

LC–MS detection of metabolites in samples

The search was carried out by comparing the chromatograms obtained from real samples with those of their respective biological and chemical blank samples. Full-scan data were acquired over the mass range *m/z* 50–650, processed with Metabolite ID software and sought for possible metabolites according to the biotransformation table of the software. The detected peaks were then selected for MS–MS analysis, and the product ion spectra obtained were examined and compared with the product ion spectra of standards. The structures of metabolites were tentatively elucidated by a combination of analysis of their full-scan spectra, MS–MS spectra, and chromatographic behaviour (retention times). Phase I metabolites ABZSO and FLUR were detected in both the incubation medium samples and parasite homogenate samples. In contrast, neither $ABZSO_2$ nor FLUH were detected in the samples. Phase II metabolites—glucose conjugates of ABZ, FLU, and FLUR—were detected and their structures confirmed in our experiments (see below). A summary of all metabolites detected is presented in Table 2. Representative extracted ion

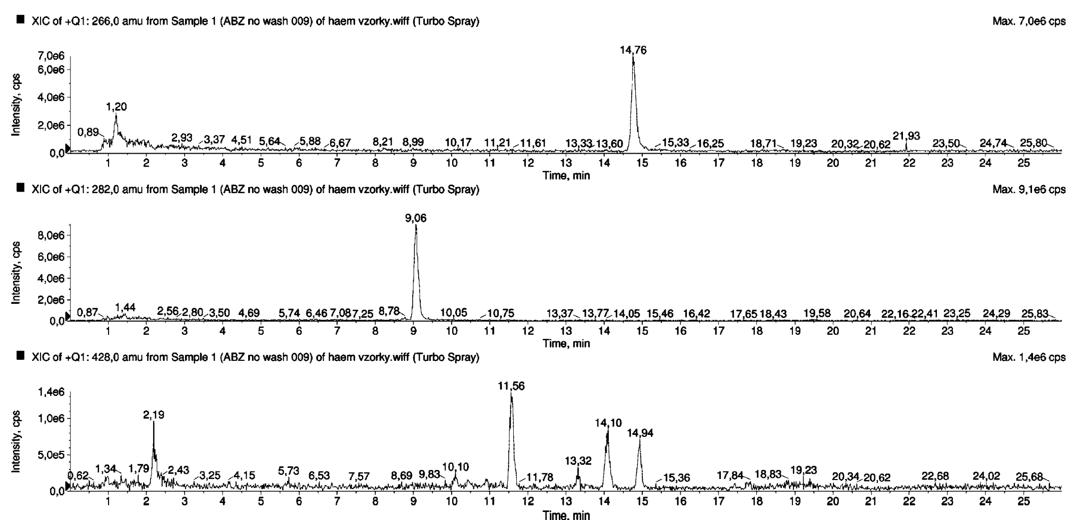


Fig. 2 Representative extracted-ion chromatograms of ABZ and its metabolites detected: ABZ (*m/z* 266), ABZSO (*m/z* 282), and ABZ-glucosides (*m/z* 428)

chromatograms of parent compounds and their metabolites detected in our experiments are shown in Figs 2. and 3.

Conjugation with glucose

Although conjugation with glucose cannot be considered a common metabolic pathway and, compared with other phase II conjugation reactions, is not generally labelled as a major route of metabolism of xenobiotics, it has been described in species across the animal and plant kingdoms. Glucose conjugates as biotransformation products of various xenobiotics have been detected in mice [25–30] and humans [31, 32]. In plants, glucosidation plays a substantial role in metabolism of both endogenous molecules (e.g. plant hormones, secondary metabolism products) regulating their bioactivity, stability, solubility, transport properties [33], and of xenobiotics, including agricultural chemicals [34–36]. Focussing on helminths only O'Hanlon reported glucose conjugation as a detoxification mechanism in metabolism of ecdysteroids in nematodes *Ascaris suum* and *Parascaris equorum* [37, 38]. Therefore in parasitic helminths the significance and importance of metabolism via glucose conjugation has not yet been evaluated. Here we provide the evidence supported by LC–MS–MS analysis that another nematode group member, *H. contortus*, is capable of forming glucose conjugates as products of metabolism of xenobiotics.

LC–MS–MS analysis of glucose conjugates

The LC–MS–MS analysis of *m*/*z* 428 (two peaks eluting at 14.07 min and 14.92 min) afforded product ions at *m*/*z* 266, 234, and 191. The ion at *m*/*z* 266 ($[M + H - 162]^+$, protonated ABZ) was formed by the loss of anhydroglucose, the ions at *m*/*z* 234 and 191 were typical of the ABZ product ion spectrum. We suggest that the two *m*/*z* 428 ions are glucose conjugates of ABZ formed by the attachment of a glucose molecule to two different sites of the parent compound, thus forming two positional isomers, eluting at different retention times. The product ion spectrum of *m*/*z* 476 (eluting at 14.11 min) yielded ions at *m*/*z* 314, 282, and 123. The ion at *m*/*z* 314 was formed by the loss of anhydroglucose ($[M + H - 162]^+$, protonated FLU), and the ions at *m*/*z* 282 and 123 were characteristic of the product ion spectrum of FLU. This metabolite could be assigned as a glucoside of flubendazole. However, as in the case of ABZ glucosides, it is not possible to determine the exact position of the glucose moiety from the MS–MS data obtained.

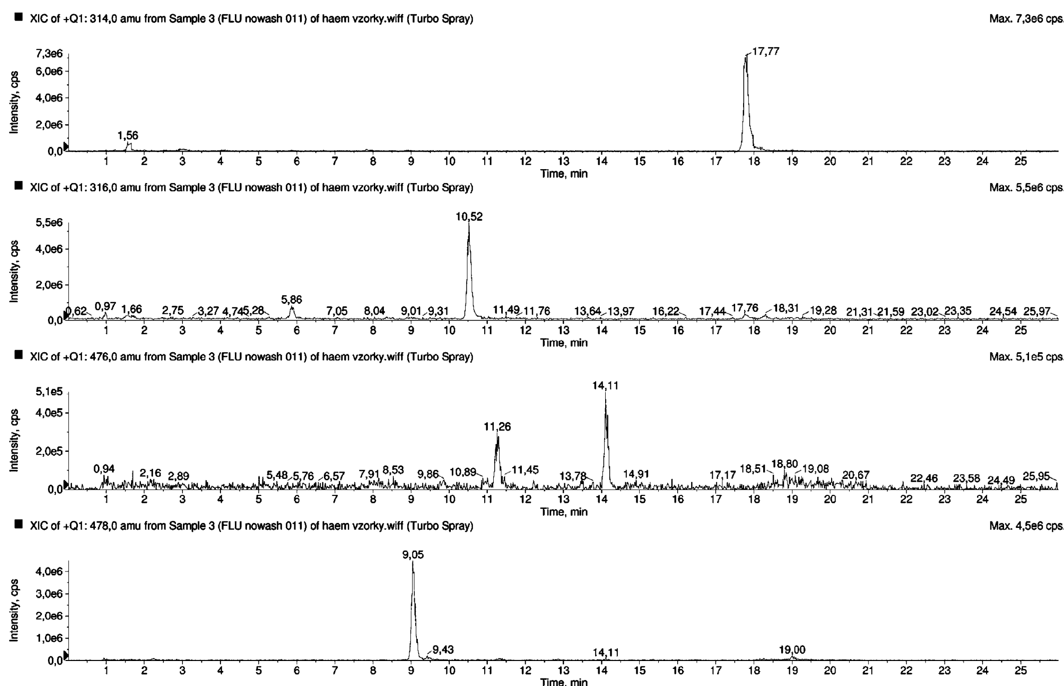


Fig. 3 Representative extracted ion chromatograms of FLU and its metabolites detected: FLU (*m*/*z* 314), FLUR (*m*/*z* 316), FLU-glucoside (*m*/*z* 476), and FLUR-glucoside (*m*/*z* 478)

In addition, an abundant peak showing a protonated molecule at *m*/*z* 478 (eluting at 9.06 min) was observed. In the MS–MS analysis, product ions at *m*/*z* 298, 284, 266, and 238 were observed. The loss of 180 u (*m*/*z* 478 → *m*/*z* 298) could be associated with acyl or benzylglucosides [39] undergoing the loss of one glucose moiety. The product ion at *m*/*z* 266 arose by loss of methanol from the ion at *m*/*z* 298. The product ion at *m*/*z* 238 (abundance 0.8%) was formed by a loss of 60 u ($HCOOCH_3$) from the ion at *m*/*z* 298. The ion at *m*/*z* 284 (abundance 0.5%) was formed by concurrent loss of anhydroglucose and methanol from the precursor ion at *m*/*z* 478. Based on these observations this metabolite was assigned as reduced flubendazole *O*-glucoside and its fragmentation in MS–MS experiments is shown in Fig. 4. All glucose conjugates mentioned were observed in both the incubation medium samples and parasite homogenate samples.

Tang [32] reported that supplementation of human liver cells with UDP-glucose resulted in involvement of UDP-glucuronosyltransferase UGT2B7 in the glucosidation of a new endothelin ET_A receptor antagonist. In our experiments only intact, not activated, glucose molecules were added into the incubation medium. This implies that *H. contortus* possesses enzymatic systems capable of UDP-glucose synthesis and of transporting the glucose moiety to the aglycon, thus giving rise to glucoside metabolites.

Constant neutral loss scans

The finding that *H. contortus* is capable of glucose conjugation was further supported by LC–MS–MS measurements, using a constant neutral loss scans, where both ions at *m*/*z* 428 (ABZ-glucoside) and *m*/*z* 476 (FLU-glucoside) readily lost 162 u (anhydroglucose), and the ion at *m*/*z* 478 (FLUR-*O*-glucoside), being a benzylglucoside, easily lost 180 u (a glucose moiety). In contrast, in LC–MS–MS measurements using a constant neutral loss scan of 176 u (a glucuronide moiety), glucuronide conjugates were not observed, indicating that *H. contortus* lacks enzymatic systems capable of glucuronidation.

Conclusions

The metabolic pathways of benzimidazole anthelminthics albendazole and flubendazole in the parasitic nematode

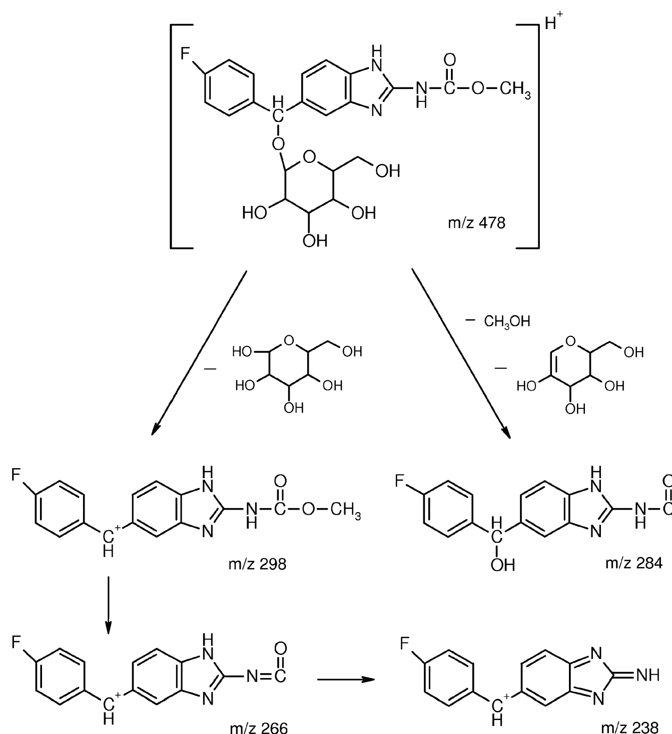


Fig. 4 Proposed fragmentation pathways of FLUR-*O*-glucoside

Haemonchus contortus were tentatively assigned and presented. The results demonstrated the ability of *H. contortus* to metabolize albendazole and flubendazole using both phase I and phase II biotransformation enzymes. *H. contortus* metabolized albendazole via sulfoxidation, and flubendazole via reduction of the carbonyl group. The formation of glucose conjugates represents phase II biotransformation of albendazole, flubendazole, and reduced flubendazole in *H. contortus*. Glucose conjugation of albendazole sulfoxide was not detected. All detected metabolites were identified by means of an LC–MS–MS technique, by comparison of mass spectra of metabolites produced by *H. contortus* and corresponding mass spectra of standard compounds (if available), including their retention times in preceding LC separation. The presence of glucose conjugates was confirmed by using constant neutral loss scans. This is the first piece of evidence that glucose conjugation represents the main conjugation pathway of benzimidazole anthelminthics in a helminth parasite. Although no quantitation had been performed, the extent of glucosidation was at least approximately comparable with that of oxidation or reduction. However, the significance of metabolism via glucose conjugation requires further examination, and the detection of the exact location of conjugation.

Acknowledgements This project was kindly supported by the Czech Science Foundation (GA ČR, grant No. 524/06/1345) and the Fund of Mobility of Charles University.

References

1. Kerboeuf D, Blackhall W, Kaminsky R, von Samson-Himmelstjerna G (2003) Int J Antimicrob Agents 22:332–346
2. Penicaut B, Maugein P, Maisonneuve H, Rossignol JF (1983) Bull Soc Pathol Exot Filiales 76:698–708
3. Marriner SE, Morris DL, Dickson B, Bogan JA (1986) Eur J Clin Pharmacol 30:705–708
4. Delatour P, Garnier F, Benoit E, Longin C (1984) J Vet Pharmacol Ther 7:139–145
5. Fargetton X, Galtier P, Delatour P (1986) Vet Res Commun 10:317–324
6. Moroni P, Buronfosse T, Longinsauvageon C, Delatour P, Benoit E (1995) Drug Metab Dispos 23:160–165
7. Solana HD, Sallovitz JM, Najle R, Rodriguez JA, Lanusse CE (2000) Methods Find Exp Clin Pharmacol 22:83–88
8. De Ruyck H, Daeseleire E, Grijspeerdt K, De Ridder H, Van Renterghem R, Huyghebaert G (2004) Br Poult Sci 45:540–549
9. Marriner SE, Bogan JA (1980) Am J Vet Res 41:1126–1129
10. Moreno L, Alvarez L, Mottier L, Virkel G, Bruni SS, Lanusse C (2004) J Vet Pharmacol Ther 27:299–308
11. Prichard RK, Hennessy DR, Steel JW, Lacey E (1985) Res Vet Sci 39:173–178
12. Galtier P, Alvinerie M, Delatour P (1986) Am J Vet Res 47:447–450
13. Lanusse CE, Nare B, Prichard RK (1993) Xenobiotica 23:285–295
14. Solana HD, Rodriguez JA, Lanusse CE (2001) Parasitol Res 87:275–280
15. Robinson MW, Lawson J, Trudgett A, Hoey EM, Fairweather I (2004) Parasitol Res 92:205–210
16. Mottier L, Virkel G, Solana H, Alvarez L, Salles J, Lanusse C (2004) Xenobiotica 34:1043–1057
17. Kotze AC (2000) J Parasitol 86:873–876
18. Wahab AR, Suhaila Abd H (2007) Trop Biomed 24:23–27
19. Lacey E (1990) Parasitol Today 6:112–115
20. Mottier L, Alvarez L, Ceballos L, Lanusse C (2006) Exp Parasitol 113:49–57
21. Sangster N (1996) Parasitology 113(Suppl):S201–216
22. Kostiainen R, Kotiaho T, Kuuranne T, Auriola S (2003) J Mass Spectrom 38:357–372
23. Roos MH, Otsen M, Hoekstra R, Veenstra JG, Lenstra JA (2004) Int J Parasitol 34:109–115
24. van Wyk JA, Gerber HM, Groeneveld HT (1980) Onderstepoort J Vet Res 47:147–158
25. Arima N, Kato Y (1990) J Pharmacobiodyn 13:719–723
26. Arima N, Kato Y (1990) J Pharmacobiodyn 13:739–744
27. Neighbors SM, Soine WH (1995) Drug Metab Dispos 23:548–552
28. Egestad B, Green G, Sjoberg P, Klasson-Wehler E, Gustafsson J (1996) J Chromatogr B Biomed Appl 677:99–109
29. Chmela Z, Vesely J, Lemr K, Rypka M, Hanus J, Havlicek L, Krystof V, Michnova L, Fuksova K, Lukes J (2001) Drug Metab Dispos 29:326–334
30. Cervenkova K, Belejova M, Chmela Z, Rypka M, Riegrova D, Michnova K, Michalikova K, Surova I, Brejcha A, Hanus J, Cerny B, Fuksova K, Havlicek L, Vesely J (2003) Physiol Res 52:467–474
31. Shipkova M, Strassburg CP, Braun F, Streit F, Grone HJ, Armstrong VW, Tukey RH, Oellerich M, Wieland E (2001) Br J Pharmacol 132:1027–1034
32. Tang C, Hochman JH, Ma B, Subramanian R, Vyas KP (2003) Drug Metab Dispos 31:37–45
33. Li Y, Baldauf S, Lim EK, Bowles DJ (2001) J Biol Chem 276:4338–4343
34. Wetzel A, Sandermann H, Jr (1994) Arch Biochem Biophys 314:323–328
35. Messner B, Thulke O, Schaffner AR (2003) Planta 217:138–146
36. Cole DJ, Edwards R (2000) Secondary metabolism of agrochemicals in plants. In Roberts T (ed) Metabolism of agrochemicals in plants, Chapter 4. Wiley, New York
37. O'Hanlon GM, Howarth OW, Rees HH (1987) Biochem J 248:305–307
38. O'Hanlon GM, Cleator M, Mercer JG, Howells RE, Rees HH (1991) Mol Biochem Parasitol 47:179–187
39. Levsen K, Schiebel HM, Behnke B, Dotzer R, Dreher W, Elend M, Thiele H (2005) J Chromatogr A 1067:55–72

IV.

Phase I biotransformation of albendazole in lancet fluke (*Dicrocoelium dendriticum*)

Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L.

in press

ARTICLE IN PRESS

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Research in Veterinary Science xxx (2008) xxx–xxx

www.elsevier.com/locate/rvsc

Phase I biotransformation of albendazole in lancet fluke (*Dicrocoelium dendriticum*)

V. Cvilink *, B. Szotáková, V. Křížová, J. Lamka, L. Skálová

Department of Biochemical Sciences, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, Hradec Králové CZ-500 05, Czech Republic

Accepted 9 May 2008

Abstract

Dicroceliosis, a lancet fluke infection, is a frequent parasitosis of small ruminants and the anthelmintic drug albendazole (ABZ) is effective in control of this parasitosis. The aim of our project was to study the metabolism of ABZ and ABZ sulphoxide (ABZ.SO) in lancet fluke. Both *in vitro* (subcellular fractions of fluke homogenates) and *ex vivo* experiments (adult flukes cultivated in medium) were performed for this purpose. ABZ was metabolised *in vitro* by lancet fluke NADPH-dependent enzymes by two oxidative steps (sulphoxidation and sulphonation). The apparent kinetic parameters of these reactions have been determined. In the *ex vivo* experiments, only ABZ sulphoxidation was observed. The stereospecificity in ABZ sulphoxidation *in vitro* was slight, with preferential formation of (+)-ABZ.SO enantiomer. In contrast (−)-ABZ.SO formation predominated in *ex vivo* experiments. Sulphoreduction of ABZ.SO occurred neither *in vivo* nor *ex vivo*. The detection of ABZ oxidative metabolites indicates the presence of drug metabolising oxidases in lancet fluke.
© 2008 Elsevier Ltd. All rights reserved.

Keywords: Drug metabolism; Anthelmintics; Dicrocoeliosis; Stereospecificity

1. Introduction

Biotransformation enzymes serve as an efficient defence against potential negative effects of xenobiotics. The xenobiotics (drugs, food additives, and environmental contaminants) usually undergo biotransformation and thus they can be more easily excreted from the organism. The parent compounds generally differ from their metabolites in physico-chemical properties, biological activity, and pharmacokinetic behaviour. Therefore, the activity of the biotransformation enzymes substantially affects both desired and undesired effects of xenobiotics.

The biotransformation enzymes of parasitic helminths may to a certain extent protect these organisms from toxic effects of anthelmintics and the ability to metabolise anthelmintics can represent an advantageous defence strategy of the parasites (Robinson et al., 2004; Kotze et al., 2006). The biotransformation of benzimidazole anthelmintics by parasitic worms has not yet been thoroughly investigated. Oxidation of albendazole (ABZ, Fig. 1) and triclabendazole in *Fasciola hepatica*, *Haemonchus contortus*, *Moniezia expansa*, and *Ascaris suum* was reported (Kotze, 2000; Solana et al., 2001; Mottier et al., 2004; Robinson et al., 2004). Sulphoreduction of albendazole sulphoxide (ABZ.SO) to ABZ was described in *F. hepatica*, *M. expansa*, and *A. suum* (Solana et al., 2001). Clearly, metabolic activities differed among the helminth species studied. There is a lack of information on ABZ metabolism in *Dicrocoelium dendriticum*, although this anthelmintic drug is an important tool for the control of lancet fluke infections in small ruminants.

In mammals, a pronounced stereospecificity in ABZ oxidation has been described: (−)-ABZ.SO was preferentially formed in rat and deer species, while (+)-ABZ.SO predominated in sheep, cattle, pig, and mouflon (Delatour et al., 1990, 1991a, b; Velík et al., 2005). In helminths, chiral aspects of ABZ oxidation have not yet been studied.

* Corresponding author. Tel.: +420 495067449; fax: +420 495067168. *E-mail address:* viktor.cvilink@faf.cuni.cz (V. Cvilink).

0034-5288/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2008.05.006

Please cite this article in press as: Cvilink, V. et al., Phase I biotransformation of albendazole in lancet fluke ..., Res. Vet. Sci. (2008), doi:10.1016/j.rvsc.2008.05.006

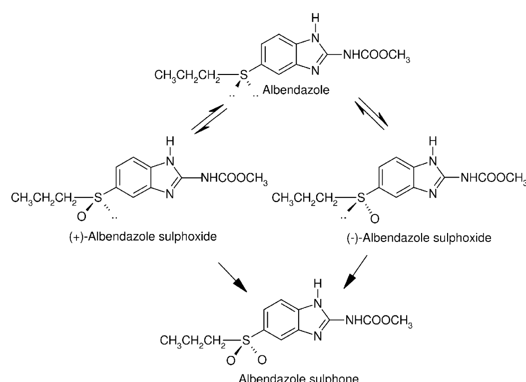


Fig. 1. Phase I biotransformation of ABZ.

The aim of the present study was to evaluate the *in vitro* and *ex vivo* metabolic fate of ABZ in adult lancet flukes (*D. dendriticum*).

2. Materials and methods

2.1. Chemicals

Albendazole was purchased from Sigma–Aldrich (Prague, Czech Republic). Albendazole sulphoxide and albendazole sulphone were obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Liquid sterile-filtered RPMI medium (Roswell Park Memorial Institute medium) and all other chemicals (HPLC or analytical grade) were obtained from Sigma–Aldrich.

2.2. Collection of parasite material

Dicrocoelium dendriticum adults were isolated from naturally infected mouflon ewes (*Ovis musimon*, $n = 5$, aged 5–7 years) breeding in game enclosure Vlkov (Czech Republic), which is situated in Central Bohemia near to Podebrady town. The game enclosure covers an area of 145 ha. The principal wild ruminants are mouflon (120 heads), fallow deer (*Dama dama*, 30 heads), and roe deer (*Capreolus capreolus*, 30 heads). The infected mouflon ewes were culled and immediately exsanguined according to Czech law. After the removal of liver from the abdominal cavity, the organs were coated with polyethylene sac, immersed in the warm saline solution (0.9% NaCl in water, 38 °C), and transported to the laboratory (up to 60 min). The liver tissue was cut and repeatedly flushed with a saline solution (38 °C) to collect the fluke adults. All collected lancet flukes were combined and used in the following experiments.

2.3. Preparation of subcellular fractions

Freshly isolated *D. dendriticum* adults were washed repeatedly with 0.1 M phosphate buffered saline (pH 7.4) and quickly homogenised in 0.1 M sodium phosphate buffer (pH 7.4, ratio 1:6, w/v) using a Potter-Elvehjem homogeniser and sonication with Sonopuls (Bandelin, Germany). The subcellular fractions were isolated by the fractional ultracentrifugation of the homogenate in the same buffer. During the first centrifugation, un-homogenised pieces and the nuclei were removed. Fraction A (20,000*g* sediment of the second centrifugation) corresponds to the mitochondrial fraction in vertebrate tissues fractionation. Fractions B and C correspond to 105,000*g* sediment and supernatant (microsomes like and cytosol like). A re-washing step with the same buffer (followed by the next centrifugation) was included at the end of the Fraction B preparation procedure. Fractions A and B were eventually resuspended in 0.1 M sodium phosphate buffer (pH 7.4) and in 0.1 M sodium phosphate (pH 7.4) buffer containing 20% (v/v) glycerol, respectively. All fractions were stored at −80 °C. Protein concentrations were assayed using the bicinchoninic acid method according to the Sigma protocol.

2.4. Cultivation of D. dendriticum in medium

Freshly isolated living flukes were washed three times with a phosphate buffered saline (pH 7.4) and cultivated at 38 °C in plastic flasks in 5 mL of RPMI medium (pH 7.4, containing 10 U mL^{-1} penicillin and 10 µg mL^{-1} streptomycin) under humid atmosphere with 5% CO_2.

2.5. Biotransformation of ABZ and ABZ.SO in vitro

Fractions A–C were incubated with either ABZ (10 µM) or ABZ.SO (10 µM). The total volume of the reaction mixture (0.3 mL) contained 50 µL of the Fraction (containing 0.4–0.6 mg of proteins), 3 µL of substrate pre-dissolved in dimethylsulphoxide (DMSO), NADPH or NADH (1.0 mM), and 0.1 M sodium phosphate buffer (pH 7.4). The blank samples contained 50 µL of 10-min boiled Fraction or 50 µL of 0.1 M sodium phosphate buffer (pH 7.4) instead of the Fraction. In the kinetic study, concentrations of 0.25–25.0 µM for ABZ, 1.0 mM for NADPH, and 50.0 µL of Fraction A or B were used. The concentration of DMSO in all reaction mixtures and blank samples was 1% (v/v) to reach the precise concentration of ABZ and ABZ.SO which are only slightly soluble in water. All incubations were carried out under aerobic conditions at 37 °C for 30 min. The product formation was linear up to 60 min. At the end of the incubation, 30 µL of ammonium solution (concentrated) and 700 µL of cooled ethyl acetate were added, the mixture was shaken (3 min, vortex) and centrifuged (10 min, 10,000*g*). Supernatants were evaporated using an Eppendorf 5310 concentrator (Hamburg, Germany) and the residue was stored under −20 °C until HPLC analysis.

2.6. Biotransformation of ABZ and ABZ.SO ex vivo

At the beginning of the incubation, 2.5 mL of medium was removed from each flask with flukes and the same vol-

Please cite this article in press as: Cvilink, V. et al., Phase I biotransformation of albendazole in lancet fluke ..., Res. Vet. Sci. (2008), doi:10.1016/j.rvsc.2008.05.006

ume of fresh medium containing either ABZ or ABZ.SO was added. ABZ was pre-dissolved in DMSO. The final concentration of DMSO in medium was only 0.1% (v/v) to prevent its harmful impact on living flukes. The flukes were incubated in medium with ABZ (5.0, 10.0 or 20.0 μM) or ABZSO (10 μM) for 48 h. After the incubation, medium was taken up, placed into plastic tubes, frozen, and stored under −80 °C. More than 60% of the flukes were alive after the 48 h experiment. The vivid flukes were suckered on to the flask walls. The flukes were repeatedly washed up, transferred into plastic tubes, frozen, and stored under −80 °C. In chemical blank samples, medium containing either ABZ or ABZ.SO but not the flukes was incubated. In biological blank samples, the flukes were incubated in a drug-free medium. Prior to the analysis, fluke bodies were quickly homogenised in 0.1 M phosphate buffer (pH 7.4) using Sonopuls. Medium from the incubation and fluke homogenate were alkalised and extracted under the same conditions as described in Section 2.5.

2.7. HPLC analysis of ABZ and its metabolites

Achiral HPLC analysis was carried out using a Shimadzu LC-10ADvp solvent delivery module, a Shimadzu SIL-10ADvp autoinjector, a Shimadzu RF-10Axl fluorescence detector ($\lambda_{EX} = 290$ nm, $\lambda_{EM} = 320$ nm), and a Shimadzu CTO-10ACvp column oven fitted with a LiChroCART 250-3 (LiChrospher 60 RP-select B, 250 mm × 3 mm, 5 μm) reverse-phase HPLC column (Hewlett Packard, USA) equipped with a LiChroCART 4-4 (LiChrospher 60 RP-select B, 4 mm × 4 mm, 5 μm) guard column (Merck, Germany). The mobile phase A consisted of acetonitrile −25 mM potassium phosphate buffer (pH 3.0, 35:65, v/v). The flow rate was 0.5 mL min^{-1} in isocratic mode. Data were processed using the Shimadzu Class VP integrator software, version 6.12 SP2. The compounds were identified according to the retention times of reference standards. All experiments were carried out at 25 °C.

2.8. Linearity, accuracy, precision of the analytical method

The linearity of the method was evaluated using calibration samples of five different analyte concentrations. Calibration curves in ranges 0.18–3.55 nmol mL^{-1} (ABZ.SO), 0.80–420.45 pmol mL^{-1} ($ABZ.SO_2$), and 7.54–37.69 nmol mL^{-1} (ABZ) have been constructed. The calibration curves were obtained by least-squares linear regression.

The control samples were prepared in order to obtain accuracy (percentage of recovery) and precision (R.S.D.) of the analytical method. Three kinds of control samples at three different analyte concentrations ($n = 5$) were prepared for this purpose: the spiked Fraction A samples, the spiked biological blank samples, and the spiked RPMI incubation medium. All control samples were prepared as described in Section 2.5 (the incubation step was skipped).

2.9. HPLC separation of ABZ.SO enantiomers

During the achiral HPLC analysis, the ABZ.SO chromatographic peak fractions were collected into vials. The collected fractions were evaporated to dryness using an Eppendorf 5310 concentrator and redissolved in 120 μL 0.2% (v/v) 2-propanol in water.

One hundred microlitres of each sample were injected into a Shimadzu HPLC system fitted with a Chiral-AGP column (150 mm × 4 mm, 5 μm), equipped with a Chiral-AGP guard column (10 mm × 4 mm, 5 μm; both Chrom-Tech, Hägersten, Sweden). The mobile phase B consisted of 2-propanol – 0.01 M phosphate buffer (pH 6.9, 0.2:99.8, v/v). The flow rate was 0.9 mL min^{-1} in isocratic mode. All experiments were carried out at 25 °C. This chiral chromatographic method was adapted from Delatour et al. (1990).

3. Results

3.1. Biotransformation of ABZ and ABZ.SO in D. dendriticum in vitro

All fractions of *D. dendriticum* homogenate were separately incubated with either ABZ or ABZ.SO and either NADPH or NADH coenzyme. Using NADH, no metabolites were formed in any incubation. Two main ABZ metabolites of phase I biotransformation, ABZ.SO and $ABZ.SO_2$, were formed in Fraction A (mitochondria like) incubations with NADPH. ABZ.SO was the only ABZ metabolite detected in Fraction B (microsomes like) incubations with NADPH. Neither of ABZ metabolites were formed in Fraction C (cytosol like). When ABZ.SO was used as a substrate, $ABZ.SO_2$ formation was observed, but in Fraction A only. Sulphoreduction of ABZ.SO did not occur in any Fraction. Trace amounts of ABZ.SO (approximately 8–10-fold lower than in incubates) were detected in blank samples. The amount of ABZ.SO formed by this non-enzymatic conversion was subtracted from the ABZ.SO amount detected in enzymatic incubations. No $ABZ.SO_2$ was detected in blank samples.

3.2. Kinetics of ABZ sulphoxidation in D. dendriticum in vitro

Fractions A and B were incubated with NADPH coenzyme and various concentrations of ABZ (0.25–25.0 μM). The amounts of ABZ.SO and $ABZ.SO_2$ formed in the incubation mixtures were expressed as reaction velocities. The direct plots of reaction velocity vs. substrate concentration are presented in Figs. 2–4. The curves fit well the Michaelis–Menten equation. The values of basic kinetic parameters, apparent maximal velocity V'_{max} and apparent Michaelis constant K'_m, were calculated using GraphPad Prism 5.0 software (see Table 1). Corresponding specific activities for the ABZ sulphoxidation in Fractions A and B (under substrate saturation) were 52.0 and 23.7 pmol min^{-1} mg^{-1}

Please cite this article in press as: Cvilink, V. et al., Phase I biotransformation of albendazole in lancet fluke ..., Res. Vet. Sci. (2008), doi:10.1016/j.rvsc.2008.05.006

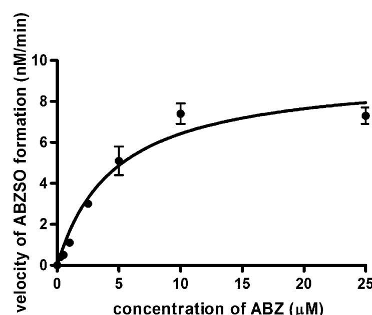


Fig. 2. Kinetics of ABZ.SO formation in Fraction B (microsomes like) from *D. dendriticum* homogenate. Data represent the mean ± SD ($n = 4$).

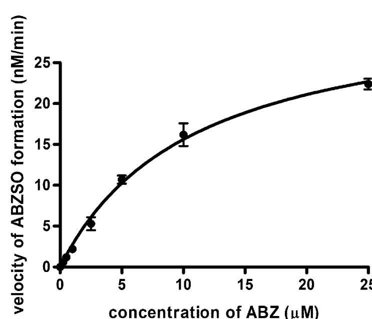


Fig. 3. Kinetics of ABZ.SO formation in Fraction A (mitochondria like) from *D. dendriticum* homogenate. Data represent the mean ± SD ($n = 4$).

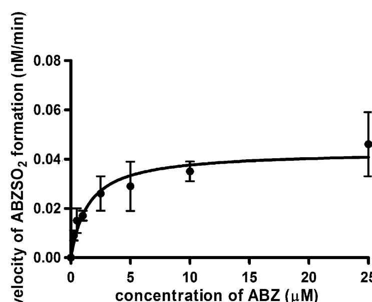


Fig. 4. Kinetics of ABZ.SO_2 formation in Fraction A (mitochondria like) from *D. dendriticum* homogenate. Data represent the mean ± SD ($n = 4$).

of protein, respectively, and 0.68 pmol min^{-1} mg^{-1} of protein for the ABZ.SO_2 formation (in Fraction A).

3.3. Biotransformation of ABZ and ABZ.SO in D. dendriticum ex vivo

The living flukes were cultivated in glass flasks with medium containing 5.0, 10.0, 20.0 μM ABZ, or 10.0 μM ABZ.SO for 48 h. Medium samples were taken up 4, 8, 24, and 48 h from the beginning of the incubation and analysed. A time-dependent consumption of ABZ was observed. ABZ.SO was the only phase I ABZ metabolite detected in medium from the incubation as well as in fluke homogenates. In medium, its formation increased with increasing ABZ concentration and increasing duration of the incubation. The results are demonstrated in Fig. 5. Time- and concentration-dependent ABZ sulphoxidation was also observed in blank samples. However, the intensity of this process was approximately five times lower than that in medium from the incubation. Neither ABZ.SO further sulphoxidation nor sulphoreduction was detected in lancet fluke *ex vivo*. The amount of ABZ.SO formed by the non-enzymatic conversion was subtracted from the ABZ.SO amount detected in medium.

Table 1
Apparent kinetic parameters of ABZ sulphoxidation in *Dicrocoelium dendriticum in vitro*

	Fraction A (mitochondria like)		Fraction B (microsomes like)	
	K'_m (μM)	V'_{max} (nmol L^{-1} min^{-1})	K'_m (μM)	V'_{max} (nmol L^{-1} min^{-1})
Formation of ABZSO	10.9 ± 1.1	32.6 ± 1.6	4.7 ± 0.7	9.4 ± 0.5
Formation of ABZSO_2	1.5 ± 0.4	0.043 ± 0.003	ND	ND

3.4. HPLC analysis of ABZ and its metabolites

Under given chromatographic conditions the retention times were 5.1 min (ABZ.SO), 7.1 min (ABZ.SO_2), and 17.2 min (ABZ). Linearity of the method was confirmed using calibration samples over the ranges of 0.18–3.55 nmol mL^{-1} (ABZ.SO), 0.80–420.45 pmol mL^{-1} (ABZ.SO_2), and 7.54–37.69 nmol mL^{-1} (ABZ). The r^2-values were 0.998 for ABZ.SO and ABZ.SO_2. For ABZ it was 0.999. The lowest concentration level of each analyte on the calibration curve has been accepted as its limit of quantification (LOQ), meeting all LOQ designation requirements (www.fda.gov/cder/guidance/4252fnl.htm). For ABZ.SO, ABZ.SO_2, and ABZ the LOQ were 0.18 nmol mL^{-1}, 0.84 pmol mL^{-1}, and 7.54 nmol mL^{-1}, respectively.

3.5. Accuracy, precision, recovery of the analytical method

Accuracy and precision for ABZ.SO and ABZ.SO_2 were determined by performing replicate analysis of spiked control samples ($n = 5$); three different concentration levels were used: for ABZ.SO they were 0.5 nmol mL^{-1} (140.6 ng mL^{-1}), 1.0 nmol mL^{-1} (281.3 ng mL^{-1}), 2.0 nmol mL^{-1} (562.6 ng mL^{-1}), for ABZ.SO_2 the levels were 30 pmol mL^{-1} (8.91 ng mL^{-1}), 100 pmol mL^{-1} (29.7 ng mL^{-1}), and 300 pmol mL^{-1} (89.2 ng mL^{-1}). Accuracy was calculated as a recovery of control samples anal-

Please cite this article in press as: Cvilink, V. et al., Phase I biotransformation of albendazole in lancet fluke ..., Res. Vet. Sci. (2008), doi:10.1016/j.rvsc.2008.05.006

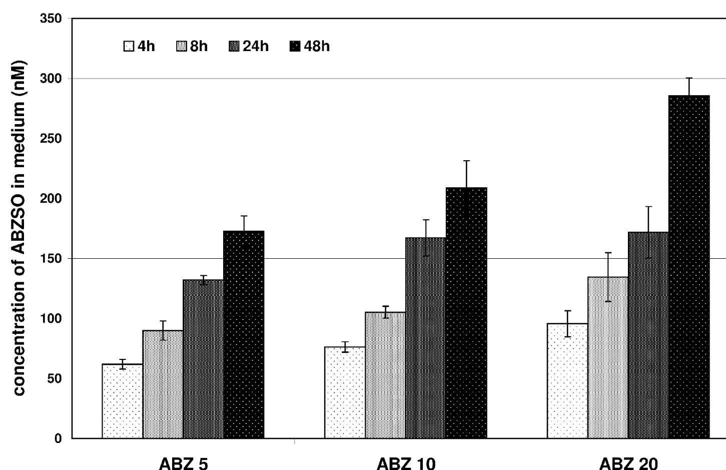


Fig. 5. Formation of ABZ.SO during the 48-h *ex vivo* incubation of *D. dendriticum* adults with ABZ (initial concentrations 5.0, 10.0, and 20.0 μM). Data represent the mean ± SD ($n = 3$).

Table 2
Intra-day precision and accuracy (spiked Fraction A control samples)

Concentration added (ng/mL) ABZSO/ABZSO$_2$	Concentration found (ng/mL) ± SD	Precision (R.S.D.%)	Accuracy (%)	*n*
140.6/8.91	124.89 ± 3.34/7.66 ± 0.52	2.67/6.77	88.83/85.99	5
281.3/29.7	260.70 ± 8.55/27.18 ± 1.46	3.28/5.37	92.68/91.52	5
562.6/89.2	504.70 ± 10.92/80.60 ± 2.87	2.16/3.56	89.71/90.36	5

Table 3
Intra-day precision and accuracy (spiked biological blank samples)

Concentration added (ng/mL) ABZSO/ABZSO$_2$	Concentration found (ng/mL) ± SD	Precision (R.S.D.%)	Accuracy (%)	*n*
140.6/8.91	125.73 ± 3.24/7.72 ± 0.47	2.58/6.07	89.14/86.64	5
281.3/29.7	249.97 ± 4.86/26.79 ± 0.93	1.94/3.46	88.86/90.19	5
562.6/89.2	515.22 ± 8.27/81.77 ± 1.42	1.61/1.73	91.58/91.67	5

Table 4
Intra-day precision and accuracy (spiked RPMI incubation medium)

Concentration added (ng/mL) ABZSO/ABZSO$_2$	Concentration found (ng/mL) ± SD	Precision (R.S.D.%)	Accuracy (%)	*n*
140.6/8.91	120.54 ± 4.32/7.88 ± 0.55	3.58/6.98	85.73/88.44	5
281.3/29.7	255.94 ± 5.55/26.18 ± 0.78	2.17/2.98	90.98/88.15	5
562.6/89.2	508.39 ± 13.48/81.19 ± 1.58	2.65/1.95	90.36/91.02	5

yses. Precision was determined as the R.S.D. of values obtained. For ABZ the parameters were not assessed. Intra-day accuracy and precision data are shown in Tables 2–4. Inter-day variation tests were not performed.

3.6. Chiral analysis, stereospecificity in ABZ sulphoxidation

Under conditions described, the retention times were 6.7 min for (−)-ABZ.SO and 15.4 min for (+)-ABZ.SO. The identity of enantiomers was determined according to Lienne et al. (1989).

Chiral analysis showed moderate but significant stereospecificity in ABZ sulphoxidation. In *in vitro* incubations, (+)-ABZ.SO was the preferentially formed enantiomer in both microsomes like and mitochondria like subcellular fractions. On the other hand, (−)-ABZ.SO was the dominating enantiomer formed in *ex vivo* experiments. The ratio of ABZ.SO enantiomers detected is shown in Table 5.

Please cite this article in press as: Cvilink, V. et al., Phase I biotransformation of albendazole in lancet fluke ..., Res. Vet. Sci. (2008), doi:10.1016/j.rvsc.2008.05.006

ARTICLE IN PRESS

Table 5
Ratio of ABZSO enantiomers formed during *in vitro* and *ex vivo* experiments

		(−)-ABZSO (%)	(+)-ABZSO (%)	SD
In vitro	Fraction A (mitochondria like)	44.4	55.6	0.4
	Fraction B (microsomes like)	43.2	56.8	0.9
Ex vivo	Fluke homogenate	66.9	33.1	2.7
	Medium	52.9	47.1	1.2

4. Discussion

The oxidative metabolism of drugs and other xenobiotics in helminths was unknown for a long time and helminth parasites were considered to be the only organisms lacking cytochrome P450 (Barrett, 1997, 1998). Later, several studies have brought unambiguous evidence in favour of enzymatic oxidation of certain xenobiotics. Kotze (1997) detected 7-ethoxycoumarin *O*-deethylase and aldrin epoxidase activities in *H. contortus* subcellular fractions and significant inhibition of these activities by common cytochrome P450 inhibitors (carbon monoxide and piperonyl butoxide). Alvinerie et al. (2001) demonstrated oxidative metabolism of moxidectin in *H. contortus* homogenate and reported its inhibition by carbon monoxide. Saeed et al. (2002) reported *Schistosoma mansoni* and *Schistosoma haematobium* had been able to metabolise typical cytochrome P450 substrates. Kotze et al. (2006) demonstrated rotenone toxicity synergism by piperonyl butoxide in *Trichostrongylus colubriformis* and *H. contortus* which indirectly suggested the role of cytochrome P450 in rotenone deactivation by nematodes. The oxidation of ABZ and triclabendazole was detected in *F. hepatica*, *M. expansa*, and *A. suum* (Solana et al., 2001; Mottier et al., 2004; Robinson et al., 2004; Alvarez et al., 2005). Although the examined helminths were able to oxidise ABZ or triclabendazole, their metabolic activities significantly differed among the helminth species.

In our project, biotransformation of ABZ in *D. dendriticum* was tested both *in vitro* and *ex vivo*. Sulphoxidation of ABZ occurred in Fractions A (mitochondria like) and B (microsomes like). The specific activity of ABZ oxidases and apparent maximal velocity of ABZ sulphoxidation were significantly higher in mitochondria than in microsomes. On the contrary, microsomal oxidases exerted a higher affinity (lower apparent Michaelis constant K'_m) to ABZ than the mitochondrial ones. No ABZ.SO formation was observed in *D. dendriticum* Fraction C (cytosol like) while in a related species, *F. hepatica*, sulphoxidation of ABZ in both microsomal and cytosolic fractions occurred (Solana et al., 2001). Triclabendazole sulphoxidation by *F. hepatica* microsomal fraction has also been reported (Mottier et al., 2004; Alvarez et al., 2005). The specific activity of triclabendazole sulphoxidation was comparable with the specific activities of *D. dendriticum* ABZ oxidases determined in our *in vitro* experiments.

Ex vivo incubations of living *D. dendriticum* adults with ABZ yielded time- and concentration-dependent formation of ABZ.SO. An efflux of ABZ.SO into medium was observed. The extent of ABZ.SO formation was low as only 2.5% of the initial ABZ concentration were metabolised during the incubation. Since ABZ.SO itself is an anthelmintically active compound, any extent of ABZ.SO formation would not decrease the ABZ efficacy.

As ABZ is a prochiral substrate, a chiral discrimination in its biotransformation in *D. dendriticum* can be assumed. In *in vitro* experiments, a slight but significant predomination of (+)-ABZ.SO was observed. In *F. hepatica*, *M. expansa*, and *A. suum*, the *in vitro* sulphoxidation of ABZ was not stereospecific (Solana et al., 2001). On the other hand, a pronounced stereospecificity in ABZ oxidation has been described in mammalian microsomes. (−)-ABZ.SO was preferentially formed in rat and deer species while (+)-ABZ.SO predominated in sheep, cattle, pig, and mouflon (Delatour et al., 1990, 1991a, b; Velík et al., 2005). In mammals, the formation of (−)-ABZ.SO is catalyzed by cytochrome P450 (particularly P4501A), whereas (+)-ABZ.SO formation depends on activity of flavin-containing monooxygenases (Delatour et al., 1991a, b; Moroni et al., 1995). It is likely that oxidases of a different structure, less stereospecific, with a more open entry to the binding place, participate in the ABZ sulphoxidation in helminths.

In *ex vivo* incubations, (−)-ABZ.SO was found to be the prevailing enantiomer both in medium and particularly in fluke homogenates. This shift in ABZ.SO enantiomer ratio could stem from (+)-ABZ.SO stronger binding to the flukes' macromolecules and/or from enantioselective efflux via specific transporters. Solana et al. (2002) described the ABZ.SO enantioselective binding to cytosolic proteins of *F. hepatica*, *M. expansa*, and *A. suum*. An active efflux of anthelmintics from these helminth species has also been reported (Mottier et al., 2006). On the other hand, the ABZ.SO transfer into *F. hepatica* was not enantioselective as the ratio of ABZ.SO enantiomers found in helminth bodies corresponded to the ABZ.SO ratio detected in plasma and bile of the hosts (Alvarez et al., 2000).

The second step of ABZ oxidation, sulphonation, was observed in *D. dendriticum* Fraction A (mitochondria like) incubations. This is the first piece of evidence that helminth enzymes are able to deactivate ABZ via the formation of the biologically inactive ABZ.SO_2. However, the rate was so low that there cannot be assumed any significant protective role of this reaction. When ABZ.SO was used as a substrate, ABZ.SO_2 was formed in Fraction A *in vitro* incubations only; no sulphonation was detected in *ex vivo* experiments. In the case of triclabendazole, much more intensive sulphonation was reported in *F. hepatica* (Robinson et al., 2004). *F. hepatica* was able to effectively convert triclabendazole sulphoxide to inactive triclabendazole sulphone and the extent of the reaction was significantly

Please cite this article in press as: Cvilink, V. et al., Phase I biotransformation of albendazole in lancet fluke ..., Res. Vet. Sci. (2008), doi:10.1016/j.rvsc.2008.05.006

higher in the triclabendazole-resistant than in the susceptible strain. In contrast to *M. expansa* (Solana et al., 2001), *F. hepatica* specimens were unable to reduce ABZ.SO to ABZ. Neither in *in vitro* nor in *ex vivo* experiments with *D. dendriticum* the sulphoreduction of ABZ.SO was observed. It appears that reverse metabolism of ABZ.SO does not occur in lancet flukes.

All the results demonstrate that drug metabolising enzymes and their activities significantly differ among the helminth species studied. This fact should be taken into account when trying to understand the variable efficacies of anthelmintics against different helminth parasites. Complete knowledge of drug uptake and drug metabolism in helminths is necessary so that the control of parasitoses, including dicrocoeliosis, becomes more effective.

Acknowledgements

Financial support of this project was provided by the Grant Agency of Czech Republic, Grant No. 524/07/0611.

References

Alvarez, L.I., Imperiale, F.A., Sanchez, S.F., Murno, G.A., Lanusse, C.E., 2000. Uptake of albendazole and albendazole sulphoxide by *Haemonchus contortus* and *Fasciola hepatica* in sheep. Veterinary Parasitology 94, 75–89.

Alvarez, L.I., Solana, H.D., Mottier, M.L., Virkel, G.L., Fairweather, I., Lanusse, C.E., 2005. Altered drug influx/efflux and enhanced metabolic activity in triclabendazole-resistant liver flukes. Parasitology 131, 501–510.

Alvinerie, M., Dupuy, J., Eeckhoutte, C., Sutra, J.F., Kerboeuf, D., 2001. In vitro metabolism of moxidectin in *Haemonchus contortus* adult stages. Parasitology Research 87, 702–704.

Barrett, J., 1997. Helminth detoxification mechanisms. Journal of Helminthology 71, 85–89.

Barrett, J., 1998. Cytochrome P450 in parasitic protozoa and helminths. Comparative Biochemistry and Physiology 121, 181–183.

Delatour, P., Benoit, E., Caude, M., Tambute, A., 1990. Species differences in the generation of the chiral sulfoxide metabolite of albendazole in sheep and rats. Chirality 3, 156–160.

Delatour, P., Benoit, E., Besse, S., Boukraa, A., 1991a. Comparative enantioselectivity in the sulphoxidation of albendazole in man, dogs and rats. Xenobiotica 2, 217–221.

Delatour, P., Garnier, F., Benoit, E., Caude, I., 1991b. Chiral behaviour of the metabolite albendazole sulphoxide in sheep, goats and cattle. Research in Veterinary Science 2, 134–138.

Kotze, A.C., 1997. Cytochrome P450 monooxygenase activity in *Haemonchus contortus* (Nematoda). International Journal for Parasitology 27, 33–40.

Kotze, A.C., 2000. Oxidase activities in macrocyclic-resistant and -susceptible *Haemonchus contortus*. Journal of Parasitology 86, 873–876.

Kotze, A.C., Dobson, R.J., Chandler, D., 2006. Synergism of rotenone by piperonyl butoxide in *Haemonchus contortus* and *Trichostrongylus colubriformis in vitro*: potential for drug-synergism through inhibition of nematode oxidative detoxificiation pathways. Veterinary Parasitology 136, 275–282.

Lienne, M., Caude, M., Rosset, R., Tambuté, A., 1989. Direct resolution of anthelmintic drug enantiomers on Chiral-AGP protein-bonded chiral stationary phase. Journal of Chromatography A 472, 265–270.

Moroni, P., Buronfosse, T., Longin-Sauvageon, C., Delatour, P., Benoit, E., 1995. Chiral sulfoxidation of albendazole by the flavin adenine dinucleotide-containing and cytochrome P450-dependent monooxygenases from rat liver microsomes. Drug Metabolism and Disposition 2, 160–165.

Mottier, L., Virkel, G., Solana, H., Alvarez, L., Salles, J., Lanusse, C., 2004. Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidised metabolites into *Fasciola hepatica*. Xenobiotica 34, 1043–1057.

Mottier, L., Alvarez, L., Ceballos, L., Lanusse, C., 2006. Drug transport mechanisms in helminth parasites: passive diffusion of benzimidazole anthelmintics. Experimental Parasitology 113, 49–57.

Robinson, M.W., Lawson, J., Trudgett, A., Hoey, E.M., Fairweather, I., 2004. The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. Parasitology Research 92, 205–210.

Saeed, H.M., Mostafa, H.M., ÓConnor, P.J., Rafferty, J.A., Doenhoff, M.J., 2002. Evidence for the presence of active cytochrome P450 systems in *Schistosoma mansoni* and *Schistosoma haematobium* adult worms. FEBS Letters 519, 205–209.

Solana, H.D., Rodriguez, J.A., Lanusse, C.E., 2001. Comparative metabolism of albendazole and albendazole sulphoxide by different helminth parasites. Parasitology Research 87, 275–280.

Solana, H.D., Sallowitz, J.M., Lanusse, C.E., Rodriguez, J.A., 2002. Enantioselective binding of albendazole sulphoxide to cytosolic proteins from helminth parasites. Methods and Findings in Experimental and Clinical Pharmacology 24, 7–13.

Velík, J., Baliharová, V., Skálová, L., Szotáková, B., Wsól, V., Lamka, J., 2005. Liver microsomal biotransformation of albendazole in deer, cattle, sheep and pig and some related wild breeds. Journal of Veterinary Pharmacology and Therapeutics 28, 377–384.

Please cite this article in press as: Cvilink, V. et al., Phase I biotransformation of albendazole in lancet fluke ..., Res. Vet. Sci. (2008), doi:10.1016/j.rvsc.2008.05.006

V.

LC-MS identification of benzimidazole anthelminthics metabolites formed *ex vivo* by *Dicrocoelium dendriticum*

Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L.

submitted

LC-MS identification of benzimidazole anthelminthics metabolites formed *ex vivo* by *Dicrocoelium dendriticum*

Viktor Cvilink*, Barbora Szotáková, Veronika Křížová, Jiří Lamka, Lenka Skálová

Viktor Cvilink, Barbora Szotáková, Veronika Křížová, Lenka Skálová

Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

Jiří Lamka

Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

* Corresponding author. Tel.: +420495067449, fax: +420495067168
E-mail address: viktor.cvilink@faf.cuni.cz (Viktor Cvilink)

Abstract

With further use of chemical agents in control of parasitic infections, an increased number of drug resistance occurences to antiparasitic drugs has been reported. Induction of enzymes responsible for detoxification of given drugs can contribute to drug resistance development in a parasitic organism. The identification of formed metabolites allows the characterization of the enzymes participating in biotransformation and possibly in drug resistance development.

The objective of our work was to find and identify phase I and phase II metabolites of the anthelminthic drugs albendazole (ABZ), flubendazole (FLU) and mebendazole (MEB) formed in *ex vivo* incubations by parasitic helminth *Dicrocoelium dendriticum*, a parasite of wild ruminants and other grazing animals, using liquid chromatography-mass spectrometric techniques.

In the *ex vivo* study, approximately 50 living *D. dendriticum* adults were incubated in 5 mL RPMI-1640 medium in presence of 10.0 μmol L^{-1} benzimidazole drug (5% CO_2, 38°C) for 24 hours. The parasite bodies were then removed from the medium. After homogenization of parasites, both parasite homogenates and medium from the incubation were separately extracted using solid phase extraction. The extracts were analyzed using liquid chromatography-mass spectrometry with electrospray ionization.

The results showed that *D.dendriticum* enzymatic systems are capable of phase I oxidation and reduction as well as phase II conjugation reactions. Detected phase I metabolites comprised albendazole sulphoxide, reduced flubendazole and reduced mebendazole. As for phase II metabolites, methylderivatives of flubendazole, reduced flubendazole and reduced mebendazole were observed. Employing phase I and II enzymatic systems *D. dendriticum* can alter the structure of a given drug and potentially decrease its therapeutic effect.

Keywords

Mass spectrometry, drug metabolism, xenobiotics, biotransformation, parasitic helminth, albendazole, flubendazole, mebendazole

Abbreviations

ABZ: albendazole; ABZSO: albendazole sulfoxide; BZD: benzimidazole; DFLU: deoxydimethylflubendazole; DMEBR: reduced dimethylmebendazole; DMSO: dimethysulfoxide; ESI: electrospray ionization; FLU: flubendazole; FLUR: reduced flubendazole; LC-MS: liquid chromatography-mass spectrometry; MEB: mebendazole; MEBR: reduced mebendazole; MFLUR: reduced monomethylflubendazole; MS/MS: tandem mass spectrometry; MS^n: multiple stage mass spectrometry; RPMI: Roswell Park Memorial Institute; SPE: solid phase extraction

Introduction

Benzimidazole anthelminthics represent a large group of agents used to combat the parasitic infections of man and animals. Being marketed since 1960s, many instances of resistance of parasitic helminths to benzimidazoles have been reported; resistance negatively influences the efficacy of anthelminthic treatment. Various mechanisms of resistance development may take place, some of which have been clarified and some of which still remain equivocal or unknown. One of the possible ways of resistance development is the induction (increased expression) of the enzymes responsible for biotransformation of a drug which results in an accelerated detoxification and prevents from reaching the desired therapeutic levels in given organism. Moreover, it is well known that a repeated contact of an organism with subtherapeutic drug levels significantly increases the activity of its biotransformation enzymes either by the increased expression or protein stabilization.

In order to find out which enzymes are involved in BZD biotransformation, to identify the responsible enzymatic systems, and thus uncover the site of possible therapeutic counterattack, we investigated the BZD metabolism in a trematode *Dicrocoelium dendriticum*, an endoparasite of wild ruminants. We employed the versatile LC-MS technique, which plays a dominant role in metabolic profiling, to elucidate the metabolic fate of selected benzimidazole anthelminthics in *D. dendriticum*. In the presented project, metabolism of albendazole ([5-(propylthio)-1H-benzimidazol-2-yl] methylcarbamate), flubendazole ([5-(4-fluorobenzoyl)-1H-benzimidazol-2-yl] methylcarbamate) and mebendazole (5-benzoyl-1H-benzimidazol-2-yl) methylcarbamate; chemical structures in Fig. 1) was investigated.

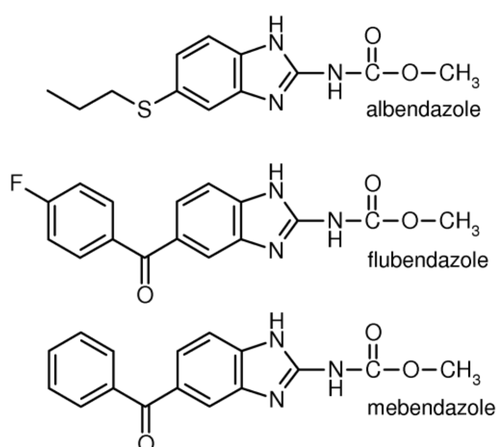


Fig. 1 Chemical structures of albendazole, flubendazole and mebendazole

Experimental

Chemicals and reagents

ABZ was purchased from Sigma-Aldrich (Prague, Czech Republic). ABZSO was provided by Toronto Research Chemicals Inc. (Toronto, Canada). FLU, FLUR, MEB and MEBR were obtained from Janssen Pharmaceutica (Prague, Czech Republic). All other chemicals (HPLC or analytical grade) were obtained from Sigma-Aldrich (Prague, Czech Republic). Water was purified with a Milli-Q System (Millipore, Bedford, USA).

Collection of parasite material

D. dendriticum adults were isolated from naturally infected mouflon ewes (*Ovis musimon*, n = 5, aged 5-7 years) breeding in game enclosure Vlkov (Czech Republic). The infected mouflon ewes were culled and immediately exsanguined according to the Czech law. After the removal of liver from the abdominal cavity, the organs were coated with polyethylene sac, immersed in the saline solution (0.9% NaCl in water, 38°C) and transported to the laboratory (up to 60 minutes). The liver tissue was cut and repeatedly flushed with a saline solution (38°C) to collect the fluke adults. All collected lancet flukes were combined and used in following experiments.

Biotransformation of BZD *ex vivo*

Freshly isolated living flukes were washed three times with a phosphate buffered saline (pH 7.4) and cultivated in plastic flasks in 5 mL of RPMI-1640 medium (pH 7.4, 38°C, containing 10 U mL^{-1} penicillin and 10 μg mL^{-1} streptomycin) under humid atmosphere with 5% CO_2.

At the beginning of incubation, 2.5 mL of medium from each flask with flukes was replaced with the same volume of fresh medium containing ABZ, FLU or MEB predissolved in DMSO. The final concentration of DMSO in medium was only 0.1% (v/v) to prevent its harmful impact on living flukes. The flukes were incubated with ABZ, FLU or MEB (10.0 μmol L^{-1}) for 24 h. After the incubation, medium was taken up, placed into plastic tubes, frozen, and stored under -80°C. The flukes were repeatedly washed up, transferred into plastic tubes, frozen, and stored under -80°C. In chemical blank samples, medium containing the BZD drug but not the flukes was incubated. In biological blank samples, the flukes were incubated in a drug-free medium. Prior to the

analysis, fluke bodies were homogenised in 0.1 M phosphate buffer (pH 7.4) using Sonopuls (Bandelin, Germany). Medium from the incubation and the fluke homogenate were extracted using SPE.

Solid phase extraction and sample preparation

Samples were extracted using two SPE methods: the method A was suited for extraction of cationic compounds, the method B was designed to extract the anionic compounds. In the method A, one mL of the sample was acidified with 1 μL 98% formic acid and centrifuged at approximately 3000 g for 3 minutes. The supernatant was loaded on to the Waters Oasis MCX (1cc, 30 mg, 30 μm particles; Waters, Dublin, Ireland) extraction cartridge previously conditioned by washing with 1 mL acetonitrile and 1 mL purified water. In the next step, the cartridge was washed with 1 mL 2% (v/v) aqueous formic acid. Compounds of interest were eluted with 1 mL 4% aqueous ammonia in 80% (v/v) aqueous acetonitrile. Similarly, in the method B, 4 μL 25% (v/v) aqueous ammonia were added to 1 mL of the sample, centrifuged and loaded on to the preconditioned (1 mL acetonitrile and 1 mL water) Waters Oasis MAX (1cc, 30mg, 30 μm particles; Waters, Dublin, Ireland) extraction cartridge. The cartridge was rinsed with 2% (v/v) aqueous ammonia and the retained analytes were eluted with 2% (v/v) formic acid in 80% (v/v) aqueous acetonitrile. The eluates were evaporated to dryness using Eppendorf 5310 concentrator (30°C, Hamburg, Germany), reconstituted in 100 μL 10% (v/v) aqueous acetonitrile and 20 μL of the final solution were injected into the analytical column.

Liquid chromatography

The LC system comprised a Surveyor MS pump and a Surveyor autosampler (both ThermoFinnigan, San Jose, CA, USA). The column employed was SymmetryShield RP18 (2.1×100 mm, 3.5 μm, Waters, Milford, USA). For the MS measurements in positive ion mode, the mobile phase consisted of solvent A (0.1% (v/v) aqueous formic acid) and solvent B (0.1% (v/v) formic acid in acetonitrile). For the MS measurements in negative ion mode, the mobile phase consisted of solvent C (0.1% (v/v) aqueous ammonia) and solvent D (0.1% (v/v) ammonia in acetonitrile). A 120 μL min^{-1} flow rate gradient was developed over 25 minutes; see Table 1). Column compartment temperature was set to 40°C.

0 min	10% B or D
10.0 min	60% B or D
11.0 min	80% B or D
12.9 min	80% B or D
13.0min	10% B or D
25.0 min	10% B or D

Table 1. Eluent composition during the LC analysis

Mass spectrometry

The experiments were performed with an LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an electrospray ion source. Following optimized ion source parameters were set for benzimidazole drug analysis during sample data acquisition in positive ion mode (negative ion mode values in brackets): spray voltage: 5.3 kV; capillary voltage: 25.0 V (-11.0 V); heated capillary temperature: 200°C; tube lens offset voltage: 30.0 V (-19.0 V); sheath gas flow rate: 20.0 arbitrary units; auxiliary gas flow rate: 10.0 arbitrary units. Ion optics settings were as follows: multipole 1 offset voltage: -3.0 V (4.0 V); lens voltage: -16.0 V (10.0 V); multipole 2~~1~~ offset voltage: -8.0 V (2.0 V); multipole RF amplitude: 550 V. Nitrogen was used as both sheath and auxiliary gas, helium was used as the damping gas. Data acquisition and processing were carried out using Xcalibur software (version 1.2).

Fragmentation of parent BZD drugs

All BZD drug working standard solutions were introduced into the mass spectrometer at a flow rate of 5 μL min^{-1} using an inbuilt syringe pump. Stock standard solutions were prepared in a mixture of DMSO:acetonitrile:water (5:40:55; v/v) and stored at 4°C. Working standard solutions were prepared by diluting the corresponding volume of stock standard solution with 10% (v/v) aqueous acetonitrile. The final concentration of each working standard solution was 1 μg benzimidazole drug per mL. The spectra were recorded over the mass range of *m/z* 100–650 in MS experiments, for MS^n experiments the range was variable. The MS and MS^n spectra were measured in order to obtain fragmentation patterns of all parent compounds and thus to facilitate the spectra interpretation of novel unknown metabolites.

Screening for metabolites

The screening was carried out by comparing the chromatograms obtained from incubation samples with those of their respective biological and chemical blank samples. Full-scan data were acquired over the mass range *m/z* 50-650 and processed with Metabolite I.D. software (ThermoFinnigan, CA, USA). The selected compounds, present in incubation samples and absent in blank samples, were then subjected to MS^n analysis. The product ion spectra obtained were examined and compared with the product ion spectra of the standards (if available). The structures of metabolites were tentatively assessed by the combination of their corresponding full-scan spectra, MS^n spectra and chromatographic behaviour.

Results and discussion

All the below discussed measurements were carried out in positive ion mode as the analyte signal was approximately 3-4 fold higher than in the negative ion mode. There were also no anionic metabolites detected when analyzing the samples using the negative ion mode measurements. Retention times, *m/z* ratios and product ions of BZD metabolites detected in MS^n experiments are summarized in Table 2.

metabolite	ret. time (min)	*m/z*	MS stage	product ions (*m/z*, relative abundance)
ABZSO	9.5	282	MS^2	159(20), 191(18), 208(100), 240(2)
FLUR	10.5	316	MS^2	97(12), 125(33), 160(100), 284(48)
MFLUR1	11.1	330	MS^2	174(4), 298(100)
		298	MS^3	97(2), 123(2), 125(3), 174(38), 280(4), 298(100)
MFLUR2	12.1	330	MS^2	270(25), 298(100)
		298	MS^3	123(2), 270(100), 298(3)
		270	MS^4	97(20), 119(49), 123(40), 125(28), 146(100), 252(16), 270(5)
DFLU	9.6	328	MS^2	123(4), 137(28), 160(58), 186(42), 281(89), 296(100)
		296	MS^3	94(10), 109(20), 121(6), 135(4), 137(52), 160(72), 186(83), 201(4), 264(19), 280(11), 281(100), 296(8)
		281	MS^4	160(100), 186(76), 264(53)
		186	MS^4	160(100)
MEBR	9.4	298	MS^2	79(8), 107(28), 160(100), 266(42)
DMEBR	11.3	326	MS^2	105(2), 107(10), 160(37), 276(3), 294(100)
		294	MS^3	91(14), 105(9), 107(34), 160(100), 261(22), 276(32), 294(1)
		276	MS^4	261(100)
		261	MS^4	246(100)

Table 2. MS^n spectra and retention times of detected benzimidazole metabolites

LC-MS^n analysis of ABZ metabolism

Protonated albendazole, the parent drug, was observed at *m/z* 266 (elution time 12.6 min). A possible metabolite was detected at *m/z* 282 eluting at 9.5 min. The MS/MS analysis of *m/z* 282 resulted in product ions at *m/z* 159, 191, 208 and 240 which were typical for the product spectrum of ABZSO. The retention times of the metabolite and ABZSO were identical; the metabolite was assessed as ABZSO. It was the only ABZ phase I and phase II metabolite detected in the incubations. As distinct from our previous experiments with *Haemonchus contortus* [1] there were no glucose conjugates observed in *D. dendriticum* incubations.

LC-MS^n analysis of FLU metabolism

The only phase I metabolite of the parent drug FLU (elution time 14.0 min, *m/z* 314) detected in our experiments was FLUR (*m/z* 316, eluting at 10.5 min). FLUR was determined on the grounds of the FLUR standard and found metabolite MS/MS spectra identity which both featured typical product ions at *m/z* 97, 125, 160 and 284. Also the retention times were the same.

Two ions at *m/z* 330 were detected eluting at 11.1 min (MFLUR1) and 12.1 min (MFLUR2). In both cases, MS/MS analyses revealed a ready loss of 32 u (methanol) which was typical for all benzimidazole anthelminthics and signified a benzimidazole origin. The *m/z* 330 would correspond to a methylderivative of FLUR. The analysis of the ion formed at *m/z* 298 (MS^3 of *m/z* 330@298) yielded, in case of MFLUR1, product ions at *m/z* 97, 123, 125, 174, 280, 281 and 298, almost all of which were characteristic for the FLUR product ion spectrum. The FLUR and MFLUR1 spectra differed in the ion at *m/z* 174, the *m/z* of which was 14 u larger than that of the FLUR most abundant product ion (*m/z* 160, benzimidazol-2-ylcarbamoyl moiety). We tentatively suggest this metabolite is a methylated FLUR metabolite, with the methyl modification located at the benzimidazole core (see Fig. 2). The MS^3 analysis of another possible methylmetabolite MFLUR2 (MS^3 of *m/z* 330@298) resulted in preferential formation of one abundant ion at *m/z* 270. Further analysis of *m/z* 270 afforded product ions at *m/z* 97, 119, 123, 125, 146, 252 and 270. The ions at 97 and 125 were contained in the FLUR product ion spectrum. This fact indicated these fragments were not methylated and the methylation occurred in other parts of the molecule. The *m/z* 160 ion, the most abundant ion for FLUR, was not detected in MFLUR2 measurements. The reason could lie in the site of biotransformation: methylation at the side carbamoyl chain could affect

m/z 160 stability, induce its fragmentation and produce different product ions, giving rise to the ions at *m/z* 270 and *m/z* 146 which were not formed in MFLUR1 spectra. We suggest MFLUR2 is a methylderivative of FLUR with the site of methylation on the side carbamoyl chain. A scheme of MFLUR2 proposed structure and MFLUR2 product ions is depicted in Fig. 3.

An abundant ion at *m/z* 328 (retention time 9.6 min) was observed, in MS/MS experiments readily losing 32 u, giving rise to an ion at *m/z* 296. MS^3 of *m/z* 296 resulted in product ions at *m/z* 94, 109, 121, 135, 137, 160, 186, 201, 264, 280 and 281. MS^4 analysis of the ion at *m/z* 281 produced ions at *m/z* 160, 186 and 264. MS^4 analysis of *m/z* 186 afforded an ion at *m/z* 160. MS^4 of other product ions were unsuccessful either due to low ion signal or low fragmentation of the ion. Taking into account the molecular weight of flubendazole, the potential possible modifications, the variety of product ions and the observed emergence of radical cations, we tentatively proposed the structure of this novel metabolite as deoxydimethylflubendazole (DFLU, Fig. 4).

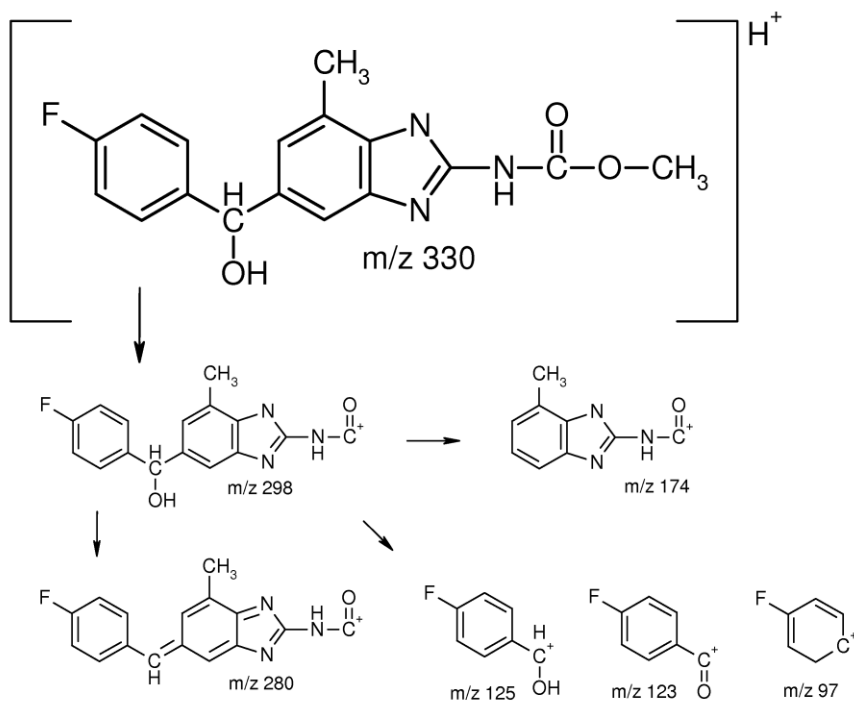


Fig. 2 Proposed structure and MS^n product ions of MFLUR1

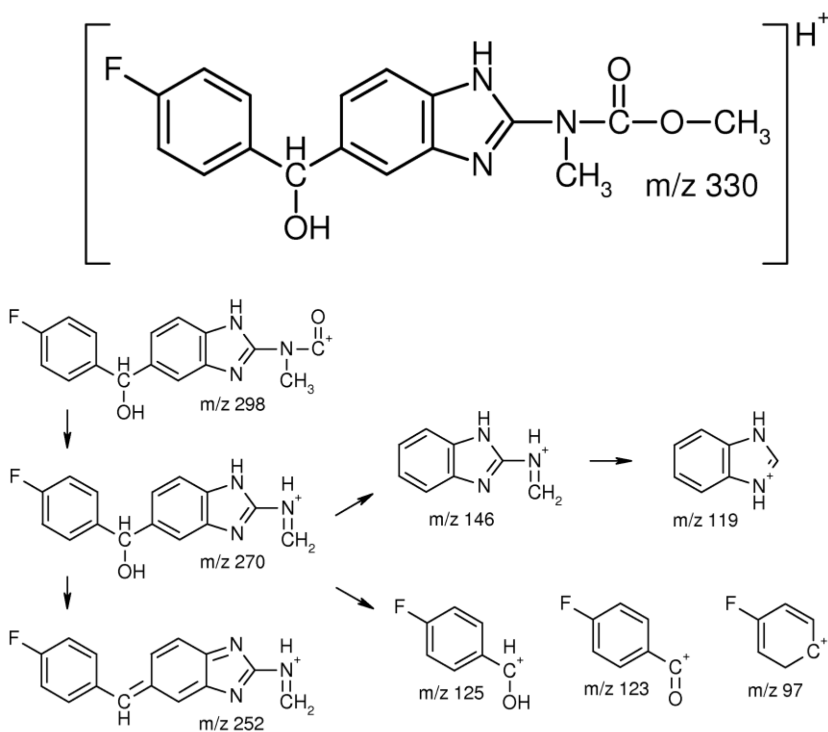


Fig. 3 Proposed structure and MS^n product ions of MFLUR2

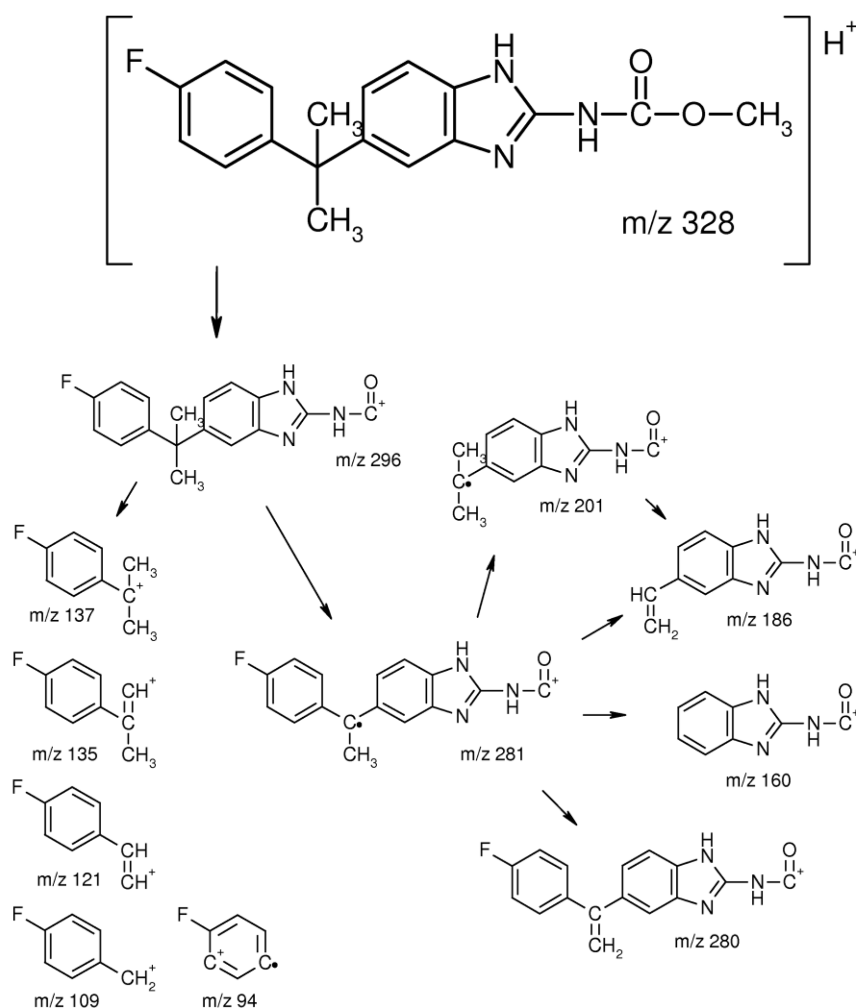


Fig. 4 Proposed structure and MS^n product ions of DFLU

LC-MS^n analysis of MEB metabolism

Mebendazole (MEB) is basically defluorinated flubendazole and thus similarities in fragmentation behaviour could be expected. The parent drug mebendazole (*m/z* 296) eluted at 13.4 min. The major and only phase I metabolite detected was reduced mebendazole (MEBR) at *m/z* 298 eluting at 9.4 min. MS/MS spectra of MEBR contained ions at *m/z* 79, 107, 160 and 266 which, allowing for the absence of fluorine, fully corresponded to FLUR MS/MS product ions. The MEBR identity was confirmed by MS/MS analysis of MEBR standard, yielding identical MS/MS spectra, and by the identity of their retention times. Concerning the phase II metabolism, an ion at *m/z* 326 was observed (retention time 11.3 min). MS/MS experiments showed the *m/z* 326 ion is prone to lose 32 u (methanol) giving rise to an abundant ion at *m/z* 294. Further fragmentation of this ion (MS^3 of 326@294) yielded product ions at *m/z* 91, 105, 107, 160, 261, 276 and 294. The ion at *m/z* 276 was formed

by a commonly detected loss of 18 u (water) from the ion at *m/z* 294. MS^4 analysis of the ion at *m/z* 276 gave rise to an ion at *m/z* 261 (a radical cation). The product ion at *m/z* 261 emerged spontaneously even in the MS^3 analysis of *m/z* 294. MS^4 analysis of the ion at *m/z* 261 resulted in formation of an ion at *m/z* 246. Two consecutive 15 u losses were observed. Such a 15 u loss could be explained as a loss of a methylradical. The two 15 u moieties were surprisingly readily cleaved from the molecule. This is distinct from the monomethylderivatives of FLUR, where no such loss was observed, and might indicate the difference in the site of MEB and FLU methylation. However, the supposed methylradicals were cleaved from a relatively large mebendazole fragment making impossible to estimate the site of methylation. Interestingly, no lower mass product ions that contained a 14 u increment were detected. Also no methylradical loss directly from the ion at *m/z* 294 was observed; the loss of 18 u (water) was a prerequisite to allow the methylradical to cleave. This metabolite was assessed as a dimethylderivative of reduced mebendazole (DMEBR). Proposed structures of DMEBR and its product ions are shown in Fig. 5.

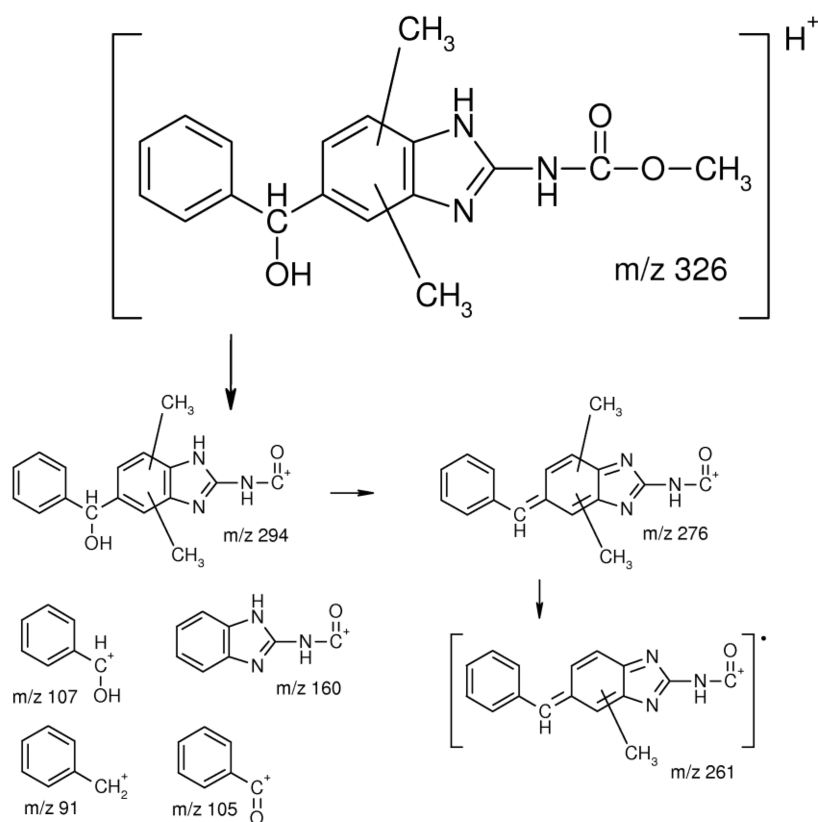


Fig. 5 Proposed structure and MS^n product ions of DMEBR

Helminth xenobiotic metabolism

Generally, there is lack of detailed information on metabolism of xenobiotics in parasitic helminths although much information on metabolism of anthelminthics has been obtained in mammals. In parasitic helminths, phase I biotransformation mainly involves oxidation [2-8]. Reduction and hydrolysis represent further phase I metabolic routes detected in several helminth species [9-12]. Concerning phase II metabolism, most information available is connected with glutathione transferases. Although helminth glutathione transferases are well documented, conjugation of xenobiotics with glutathione has not been reported yet in helminths. The only paper dealing with a certain form of glutathione metabolite is by O'Leary et *al*. [13] who reported the O-demethylation of dichlorvos and subsequent formation of demethylated dichlorvos and S-methylglutathione. Helminth conjugation of eobiotics and xenobiotics with glucose has been detected by O'Hanlon et *al*. [14,15] and Cvilink et *al*. [1], respectively. The conjugation of hydroxythiabendazole (probably with sulfate) was detected when hydroxythiabendazole was used as a substrate in incubations with *Trichostrongylus colubriformis* [16].

The experiments in the presented study showed *D. dendriticum* has its own enzymatic systems by the action of which it is able to actively modify, possibly neutralize and eventually evade the effect of xenobiotics or administered anthelminthics. In addition to phase I oxidation and reduction, which are documented in several helminth species, BZD methylderivatives were detected. Methylation as a phase II metabolic pathway has not been reported in helminths yet. Moreover, the occurring methylmodification indicates *D. dendriticum* also probably has enzymatic systems capable of synthesizing S-adenosylmethionine, the essential cofactor of methylation in mammals. In relation to previous research on *Haemonchus contortus* biotransformation enzymes [1], also *D. dendriticum* was able to oxidize and reduce xenobiotic substrates. *H. contortus* adults considerably metabolized BZD substrates via glucose conjugation; this was not observed in the presented study. In *D. dendriticum*, methylation was the main and only detected phase II metabolic pathway. This finding indicates that there exist substantial interspecies differences in enzymatic systems of parasitic helminths.

In this project, remarkable dissimilarities in metabolism of individual benzimidazole drugs in *D. dendriticum* were demonstrated. Methylation enzymes appeared to be selective, since ABZ remained unaffected by methyltransferases while FLU and MEB were extensively methylated. Moreover, FLU derived metabolites were

not methylated in the same fashion as the MEB derived metabolites. FLU/FLUR methylation yielded two monomethylderivatives with methylgroups in different positions and one deoxydimethylderivative whereas only a dimethylmetabolite of MEBR was found. Surprisingly, MEBR monomethylmetabolites as well as methylderivatives of parent drugs FLU and MEB were not detected.

Conclusions

The presented project contributes to the knowledge of helminth biotransformation enzymes and might help to render the anthelminthic therapy more effective. In the presented study, metabolism of benzimidazole anthelminthics albendazole, flubendazole and mebendazole was investigated by means of liquid chromatography coupled with mass spectrometry. The results showed that *D.dendriticum* biotransformation systems are able to oxidize and reduce xenobiotics as well as further conjugate them with eobiotic compounds. Detected phase I metabolites comprised albendazole sulfoxide, reduced flubendazole and reduced mebendazole. As for phase II metabolites, mono- and dimethylderivatives of reduced flubendazole and dimethylderivatives of reduced mebendazole were observed. Up to our knowledge, this is the first time methylation is reported to be a phase II metabolic pathway in parasitic helminths. Also the selectivities of *D. dendriticum* phase II enzymes to the substrates studied significantly differed. These facts should have implications on the design of new anthelminthic agents to overcome the problem of anthelminthic resistance. *D. dendriticum* has its own enzymatic systems and is ready to employ them in counteracting the anthelminthic exposition. Modifying the structure of the given drug *D. dendriticum* can potentially decrease the desired therapeutic effect.

Acknowledgement

This project was supported by the Czech Science Foundation (GACR, grant No. 524/07/0611) and by the Grant Agency of Charles University (GAUK, grant No. 71808/B/2008).

[1] V. Cvilink, L. Skalova, B. Szotakova, J. Lamka, R. Kostiainen, R.A. Ketola, Anal. Bioanal. Chem. 391 (2008) 337-343.

[2] A.C. Kotze, J. Parasitol. 86 (2000) 873-876.

[3] A.C. Kotze, R.J. Dobson, D. Chandler, Vet. Parasitol. 136 (2006) 275-282.

[4] H.D. Solana, J.A. Rodriguez, C.E. Lanusse, Parasitol. Res. 87 (2001) 275-280.

[5] L. Mottier, G. Virkel, H. Solana, L. Alvarez, J. Salles, C. Lanusse, Xenobiotica 34 (2004) 1043-1057.

[6] M.W. Robinson, J. Lawson, A. Trudgett, E.M. Hoey, I. Fairweather, Parasitol. Res. 92 (2004) 205-210.

[7] V. Cvilink, B. Szotakova, V. Krizova, J. Lamka, L. Skalova, Res. Vet. Sci. (2008) doi:10.1016/j.rvsc.2008.05.006.

[8] L.I. Alvarez, H.D. Solana, M.L. Mottier, G.L. Virkel, I. Fairweather, C.E. Lanusse, Parasitology 131 (2005) 501-510.

[9] W.A. Munir, J. Barrett, Parasitology 91 (Pt 1) (1985) 145-156.

[10] V. Cvilink, V. Kubicek, M. Nobilis, V. Krizova, B. Szotakova, J. Lamka, M. Varady, M. Kubenova, R. Novotna, M. Gavelova, L. Skalova, Vet. Parasitol. 151 (2008) 242-248.

[11] J. Barrett, J Helminthol 71 (1997) 85-89.

[12] W.Y. Precious, J. Barrett, Parasitol. Today 5 (1989) 156-160.

[13] K.A. O'Leary, J.W. Tracy, Exp. Parasitol. 72 (1991) 355-361.

[14] G.M. O'Hanlon, O.W. Howarth, H.H. Rees, Biochem. J. 248 (1987) 305-307.

[15] G.M. O'Hanlon, J.G. Mercer, H.H. Rees, Parasitol. Res. 77 (1991) 271-272.

[16] N.C. Sangster, R.K. Prichard, J. Parasitol. 72 (1986) 798-800.

7.2. Seznam všech publikací

Cvilink, V., Kubíček, V., Nobilis, M., Křížová, V., Szotáková, B., Lamka, J., Várady, M., Kuběnová, M., Novotná, R., Gavelová, M., Skálová, L. (2008). Biotransformation of flubendazole and selected model xenobiotics in *Haemonchus contortus*. *Vet Parasitol.* 151:242-248.

Cvilink, V., Skálová, L., Szotáková, B., Lamka, J., Kostiainen, R., Ketola, R. A. (2008). LC-MS-MS identification of albendazole and flubendazole metabolites formed *ex vivo* by *Haemonchus contortus*. *Anal Bioanal Chem.* 391:337-343.

Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L. (2008). Phase I biotransformation of albendazole in lancet fluke (*Dicrocoelium dendriticum*). *Res Vet Sci*.(in press)

Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L. - LC-MS identification of benzimidazole anthelminthics metabolites formed *ex vivo* by *Dicrocoelium dendriticum* (submitted)

Cvilink, V., Lamka, J., Skálová, L. - Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths (submitted)

Lamka, J., Křížová, V., Cvilink, V., Šavlík, M., Velík, J., Ducháček, L., Szotáková, B., Skálová, L. (2007). A single adulticide dose of albendazole induces cytochromes P4501A in mouflon (*Ovis musimon*) with dicrocoeliosis, Czech Vet. Med., 52 (2007) 343–352

Lamka, J., Křížová, V., Nobilis, M., Prušková, L., Szotáková, B., Cvilink, V., Skálová, L. - Pharmacokinetics of flubendazole and its metabolites in lambs and adult sheep (*Ovis aries*) (submitted)

Skálová, L., Křížová, V., Cvilink, V., Szotáková, B., Štorkánová, L., Velík, J., Lamka, J. (2007). Mouflon (*Ovis musimon*) dicrocoeliosis: effects of parasitosis on the activities of biotransformation enzymes and albendazole metabolism in liver. *Vet Parasitol.* 146:254-262.

Skálová, L., Kubíček, V., Szotáková, B., Cvilink, V., Křížová, V., Várady, M., Škrtelová, M., Prušková, L., Příhodová, V., Nobilis, M., Lamka, J. - Subtherapeutic doses of flubendazole in sheep with experimental haemonchosis: effect on biotransformation enzymes and flubendazole metabolism in *Haemonchus contortus in vitro* (submitted)

7.3. Seznam prezentací na vědeckých setkáních

Skálová, L., Nobilis, M., Cvilink, V., Szotáková, B., Lamka, J. Biotransformation of anthelmintic drug flubendazole in ovine hepatic microsomes and cytosole. Drug Metabolism Reviews 38 suppl. 1, 131 (2006)

Skálová, L., Gavelová, M., Cvilink, V. Biotransformation enzymes of parasites and their role in drug-resistance development. Proceedings, XX. Biochemický sjezd, Piešťany, 77 (2006)

Cvilink V., Kubíček V., Nobilis M., Křížová V., Szotáková B., Lamka J., Várady M., Skálová L. Biotransformation of flubendazole and selected model xenobiotics in *Haemonchus contortus*. XXIV. Xenobiochemické symposium, 22.-24.5.2007 Liptovský Ján, Slovensko. Book of abstracts (2007) P7, 44

Cvilink V., Skálová L., Lamka J., Kostiainen R., Ketola R. Biotransformation of albendazole and flubendazole in *Haemonchus contortus*: glucose conjugate formation. XXIV. Xenobiochemické symposium, 22.-24.5.2007 Liptovský Ján, Slovensko. Book of abstracts (2007) P8, 45

Szotáková B., Křížová V., Cvilink V., Lamka J., Skálová L. *Dicrocoelium dendriticum* – biotransformation of anthelmintics and other drugs. XXIV. Xenobiochemické symposium, 22.-24.5.2007 Liptovský Ján, Slovensko. Book of abstracts (2007) P12, 49

Skálová, L., Cvilink, V., Kubíček, V., Nobilis, M., Szotáková, B., Várady, M., Ketola, R.A., Lamka, J. *Ex vivo* biotransformation of flubendazole in *Haemonchus contortus*. 10th International helminthological symposium, 9.-14.9.2007 Stará Lesná, Slovensko. Book of abstracts 22 (2007)

Szotáková, B., Skálová, L., Cvilink, V., Křížová, V., Lamka, J. Biotransformation of albendazole in *Dicrocoelium dendriticum* both *in vitro* and *ex vivo*. 10th International helminthological symposium, 9.-14.9.2007 Stará Lesná, Slovensko. Book of abstracts 22 (2007)

Cvilink, V., Skálová, L., Szotáková, B.,Lamka, J., Kostiainen, R., Ketola, R.A. - Identification of albendazole and flubendazole metabolites formed by *Haemonchus contortus ex vivo*, 12th International Meeting on Recent Developments in Pharmaceutical Analysis, Biodola, Itálie, 23.-26.9.2007, P150

Cvilink, V., Szotáková, B., Křížová, V., Lamka, J., Skálová, L. - LC-MS identification of benzimidazole anthelminthics metabolites formed *ex vivo* by *Dicrocoelium dendriticum,* 25th Montreux Symposium on LC/MS, Montreux, Švýcarsko, 11.-14.11.2008