Elucidation of the activity, pharmacokinetics, and toxicity profiles of fasciocidal drug candidates 1,2,4-trioxolane OZ78 and 1,2,4,5-tetraoxane MT04

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Carla Martina Kirchhofer

Kriegstetten (SO)

Basel, 2012

Originaldokument gespeichert auf dem Dokumentenserver der Universität Basel
edoc.unibas.ch

Dieses Werk ist unter dem Vertrag „Creative Commons Namensnennung-Keine kommerzielle Nutzung-Keine Bearbeitung 2.5 Schweiz“ lizenziert. Die vollständige Lizenz kann unter
creativecommons.org/licences/by-nc-nd/2.5/ch
eingesehen werden.

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Dr. Jennifer Keiser
Prof. Dr. Marcel Tanner
Prof. Dr. Elaine Holmes

Basel, den 27. März 2012

Prof. Dr. Martin Spiess
Dekan

creative commons

Namensnennung-Keine kommerzielle Nutzung-Keine Bearbeitung 2.5 Schweiz

---

Sie dürfen:

das Werk vervielfältigen, verbreiten und öffentlich zugänglich machen

Zu den folgenden Bedingungen:

Namensnennung. Sie müssen den Namen des Autors/Rechteinhabers in der von ihm festgelegten Weise nennen (wodurch aber nicht der Eindruck entstehen darf, Sie oder die Nutzung des Werkes durch Sie würden entlohnt).

Keine kommerzielle Nutzung. Dieses Werk darf nicht für kommerzielle Zwecke verwendet werden.

Keine Bearbeitung. Dieses Werk darf nicht bearbeitet oder in anderer Weise verändert werden.

- Im Falle einer Verbreitung müssen Sie anderen die Lizenzbedingungen, unter welche dieses Werk fällt, mitteilen. Am Einfachsten ist es, einen Link auf diese Seite einzubinden.
- Jede der vorgenannten Bedingungen kann aufgehoben werden, sofern Sie die Einwilligung des Rechteinhabers dazu erhalten.
- Diese Lizenz lässt die Urheberpersönlichkeitsrechte unberührt.

Die gesetzlichen Schranken des Urheberrechts bleiben hiervon unberührt.

Die Commons Deed ist eine Zusammenfassung des Lizenzvertrags in allgemeinverständlicher Sprache: http://creativecommons.org/licenses/by-nc-nd/2.5/ch/legalcode.de

Haftungsausschluss:
Die Commons Deed ist kein Lizenzvertrag. Sie ist lediglich ein Referenztext, der den zugrundeliegenden Lizenzvertrag übersichtlich und in allgemeinverständlicher Sprache wiedergibt. Die Deed selbst entfaltet keine juristische Wirkung und erscheint im eigentlichen Lizenzvertrag nicht. Creative Commons ist keine Rechtsanwaltsgesellschaft und leistet keine Rechtsberatung. Die Weitergabe und Verlinkung des Commons Deeds führt zu keinem Mandatsverhältnis.

Quelle: http://creativecommons.org/licenses/by-nc-nd/2.5/ch/ Datum: 3.4.2009

Table of Contents

Acknowledgements

This PhD thesis was carried out from November 2008 to March 2012 at the Swiss Tropical and Public Health Institute in collaboration with several scientific research institutes.
Therefore, I am indebted to many people for their long-lasting support and encouragement, which were invaluable for the successful completion of this research. In the following paragraphs some of them are gratefully acknowledged. However, I am aware of the fact that there are many more and my words cannot express the gratitude and respect I feel for all of them.

First and foremost I want to thank my advisor Prof Dr. Jennifer Keiser. It has been an honor to me to be one of her PhD students. I learned how scientific work has to be done. She was always there for her students and was encouraging when the motivation was low. The enthusiasm for her research was motivational for me, even during tough times. I am also thankful for the excellent and critical atmosphere during many discussions we had.

This is also a great opportunity for me to express my respect to Prof. Dr. Jörg Huwyler. I appreciate his scientific inputs and the support he gave me during my LC/MS studies. His encouraging words helped in difficult times. It was a big honor to work with such an enthusiastic professor.

I also acknowledge Prof. Dr. Stephan Krähenbühl. He supported me in my cell studies. He improved the work with good ideas and gave me the unique opportunity to use the excellent research facilities in his lab.

My thanks are also addressed to Prof. Dr. Marcel Tanner, the Faculty Responsible, which gave me the opportunity to do my PhD at the Swiss Tropical and Public Health Institute.

I do not want to miss the opportunity to thank my group at the Swiss Tropical and Public Health Institute, Basel, and especially Mireille Vargas, as she helped me greatly with the

animal studies and encouraged me through thick and thin. My thanks also go to Urs Duthaler, Theresia Manneck, Lucienne Tritten, Katrin Ingram, Benjamin Speich, Isabel Meister, Roberto Adelfio, Lorenzo Hofer, Monika Marxer, Gordana Panic, and Angelika Silbereisen. It was a great pleasure to work in the lab with all of them and I am very happy to have had the opportunity to get to know them.

My sincere thanks go to many friends and colleagues for scientific discussions, advice and continuous support, which I have always greatly appreciated, among them Dr. Karin Brecht who introduced me to the secrets of cell studies and Massimiliano Donzelli who helped me a lot with the analytical measurements and problems.
My thanks are also addressed to Prof. Dr. Jonnathan Vennerstrom. He supplied us with synthetic peroxides and gave useful information about the physico-chemical properties of the latter compounds.

Furthermore, my sincerest thanks also go to all the Rosental head of units and lab mates: Georgios Imanidis, Berndt Joost, Martin Kuentz, Michael Lanz, Martin Cavegn, Yvonne Arnold, Venkateshwar Rao Nalluri, Constantinos Markopoulos, Cordula Stillhart, Ursula Thormann, Fabienne Thoenen and Martin Studer. I really enjoyed my time there.

I do not want to miss the opportunity to thank all the members of the Pharmaceutical Technology division of the University of Basel supervised by Jörg Huwyler. Special thanks go to Le-Ha Dieu and Pascal Detampel. They greatly aided both with my work and mentally. Thanks are also addressed to Rainer Alles, Christina Erb, André Ziegler, Maxim Puchkov, Susanne Schenk, Claudia Suenderhauf, Felix Hammann, Swen Seeland, Stefan Jenzer, Helene Kettiger and Stefan Winzap.

Another team I would like to address my thanks to is the clinical pharmacology/toxicology division of the University Hospital at Basel; namely Linda Simmler, Swarna Maseneni, Réjane Morand, Andrea Felser, Annalisa Bonifacio, Beatrice Vetter, and Jamal Bouitbir.

I also want to express my thanks to Olivier Braissant from the Laboratory of Biomechanics & Biocalorimetry (LOB2) at the University of Basel for the help with

calorimetric studies and Francesca Trapani for her advice and help in histopathologic observations.

I wish to thank the Swiss National Science Foundation for the financial support of my project (project number: PPOOA-114941).

Last but by no means least, I would like to thank my parents for all their love and encouragement and the opportunity to start and pursue a career in science, very special thanks go to my big brother and my beloved boyfriend: my loving, supportive, encouraging and patient Stefan, who always helped me, had a lot of sympathy and never doubted me.

Thank you all

* * *

It's not that I am so smart.
It's just that I stay with problems longer.
(Albert Einstein)

Summary

Fascioliasis is a disease belonging to the most neglected tropical diseases. Causative agents are the food borne trematodes *Fasciola hepatica* and *Fasciola gigantica*, whereas *F. hepatica* is especially of human concern. *F. hepatica* and *F. gigantica* are setteled in the liver, bile ducts, and gallbladder and are therefore termed as liver flukes.
About 2.4 to 17 million people are infected worldwide with *F. hepatica* leading to a global burden of about 35'206 disability-adjusted life years (DALYs). Fascioliasis is a disease not only of human concern, but affects also farm animals, especially sheep and cattle.

Triclabendazole is the only known drug active against juvenile and adult *F. hepatica* infection. There are no alternative drugs available characterized by a similar activity and safety profile as triclabendazole. Resistance against triclabendazole has already been described in sheep and is spreading over the world. However, to date no resistance in humans has been detected. Nonetheless, screening for new drugs is urgent.

OZ78 was originally synthesized for malaria drug discovery and is a peroxidic compound. This synthetic peroxide has shown promising *in vitro* and *in vivo* activity against juvenile and adult *F. hepatica*. Moreover, OZ78 has shown to be active against a triclabendazole resistant *F. hepatica* strain (Oberon isolate).

In the framework of this PhD thesis preclinical investigations were carried out to further strengthen our knowledge on the potential of the synthetic compounds OZ78 and the related derivative MT04 for the treatment of *F. hepatica* infection. The aim was to find a lead candidate for further studies and to get a better understanding of the drug properties (pharmacokinetic / pharmacodynamic) of these substances.

Different promising trioxanes and tetraoxanes were tested *in vitro* and *in vivo* in more detail with regard to *F. hepatica* activity and results compared to OZ78 (1,2,4-trioxolane). MT04 (1,2,4,5-tetraoxane) was found to be even more active *in vitro* and *in vivo* (in rats) against adult and juvenile *F. hepatica* infection than OZ78. In contrast to

OZ78, MT04 has two peroxide bridges. A dose of 50 mg/kg was enough to reach a complete worm burden reduction of 100% against adult *F. hepatica* in rats, and for juvenile treatment, a dose of 100 mg/kg was required.

In parallel, OZ78 has been tested in sheep according to activity, tolerability, and pharmacokinetic profiles. OZ78 failed to cure fascioliasis in sheep after an application of 50 mg/kg orally or subcutaneously - no reduction in faecal egg count or worm burden could be measured.

To elucidate pharmacokinetic parameters of OZ78 in sheep plasma, a liquid chromatography mass spectrometry (LC/MS) method was developed and validated in terms of accuracy, precision, stability, and selectivity. OZ78 and OZ352 (Internal standard, I.S.) had to be detected in negative mode, according to the mass of deprotonated parent compounds. Protein precipitation was used for sample clean up, recoveries of three concentrations of OZ78 (5, 1.25, and 0.3125 µg/ml) and I.S. (2 µg/ml) were 84.4%, 69.6%, 78.6% and 85.8%, respectively. Calibration line ranged from 5 µg/ml to 0.15625 µg/ml. The developed method demonstrated to be accurate, precise, and selective.

The pharmacokinetic profile of OZ78 following oral application was characterised by a maximal plasma concentration (C_{max}) of 45.8±13 µg/ml after 1 h (T_{max}). An estimated elimination half-life ($t_{1/2}$) of 1.0±0.1 h and a mean area under the plasma time curve (AUC) of 116.2±47 µg h/ml were calculated for the oral administration. On the other hand, following subcutaneous treatment with OZ78, C_{max} and T_{max} were 13.7±6.1 µg/ml and 0.9±0.4 h, respectively. The α and β half-lives were 4.5±4.3 h and 56.5±36 h, respectively and the mean AUC was 219.1±74 µg h/ml.

Further studies were needed to determine the difference in therapeutic outcomes between rats and sheep. The LC/MS method for OZ78 in sheep plasma was adapted and validated for rat plasma measurements. In addition the OZ78 LC/MS method could be approximated, validated, and used for MT04 measurement in sheep and in rat plasma.

OZ78 and MT04 were applied at a dose of 50 mg/kg to rats, and after different time-points, samples were collected. Studies with MT04 according to efficacy and pharmacokinetics in sheep are outstanding, but are planned.

Summary

Following oral administration of 50 mg/kg in rats, the C_{max} of MT04 and OZ78 were 49.8±9.1 and 70.1±19.1 µg/ml after 2.7±1.2 h and 1.6±1.6 h (T_{max}), respectively. The AUCs were estimated at 31'258.8±6'232.7 and 29'794.1±3'990.6 µg min/ml for MT04 and OZ78, respectively. Mean elimination half-lives ($t_{1/2}$) of MT04 and OZ78 were 6.4±5.7 h and 2.5±1.5 h, respectively.

Toxicity of OZ78 and MT04 was determined on a liver cell line (HepG2). Different viability and toxicity assays were done with OZ78 or MT04 combined with different iron containing solutions (haemin, Fe(II), and Fe(III)) to get a better understanding of the toxicity profiles of these substances. Reactive oxygen species (ROS) production of MT04 and OZ78 was measured in combination with these iron-solutions. OZ78 was only moderately toxic combined with iron, whereas the combination of MT04 with iron resulted in a significant toxicity. Additionally, a high ROS signal could be measured especially in combination with haemin for both substances.

In summary, OZ78 and MT04 are promising lead candidates for the development of anti-*Fasciola* drugs. OZ78 and MT04 were active against juvenile and adult infection in rats. The therapy failure in sheep after oral treatment can be explained by a too short exposure of the flukes to the drug. Another application has to be tested in sheep. To date, it is not clear if the applied doses are toxic and, more studies especially in animals are needed. However, ROS formation seems to be responsible for activity of these substances.

Zusammenfassung

Fasziolose ist eine Erkrankung, die zu den vernachlässigten Tropenkrankheiten zählt. Verursacher sind die durch Ernährung übertragbaren Trematoden *Fasciola hepatica* und *Fasciola gigantica*, wobei *F. hepatica* besonders für Menschen Besorgnis erregend ist.
F. hepatica und *F. gigantica* leben in der Leber, Gallengängen und Gallenblase, und werden deshalb auch als Leberegel bezeichnet.
Etwa 2.4 bis 17 Millionen Menschen sind weltweit mit *F. hepatica* infiziert, was zu einer weltweiten Bürde von etwa 35'206 behinderungsbedingten Lebensjahren führt. Fasziolose ist nicht nur eine Erkrankung von Menschen, sondern auch landwirtschaftliche Nutztiere wie Schafe und Rinder sind betroffen.

Triclabendazol ist die einzige Substanz, die gegen juvenile und adulte *F. hepatica* Infektion wirkt. Es gibt keine alternativen Medikamente mit gleicher Aktivität und gleichem Sicherheitsprofil wie Triclabendazol. Resistenzen gegen Triclabendazol sind bereits in Schafen vorhanden und verbreiten sich über die Welt. Bis jetzt sind aber noch keine Resistenzen im Menschen beschriebe worden. Trotzdem ist es wichtig nach neuen Medikamenten zu suchen.

OZ78 wurde ursprünglich für die Malariabehandlung synthetisiert und gehört zu den Peroxiden. Dieses synthetische Peroxid hat versprechende Aktivitäten *in vitro* und *in vivo* gegen juvenile und adulte *F. hepatica* gezeigt. Überdies war OZ78 auch gegen einen Triclabendazol resistenten Stamm (Oberon Isolat) aktiv.

Im Rahmen meiner Dissertation, wurden präklinische Untersuchungen durchgeführt um das Wissen über die versprechenden Substanzen OZ78 und MT04 in der Behandlung von *F. hepatica* Infektion zu erweitern. Das Ziel war eine neue Leitsubstanz für weitere Studien zu finden und die Wirkweise der Substanzen besser zu verstehen (Pharmakokinetik / Pharmakodynamik).

In einer *in vitro* und *in vivo* Studie wurden verschiedene Trioxane und Tetraoxane auf ihre *F. hepatica* Aktivität im Detail getestet und mit OZ78 (1,2,4-Trioxolan) verglichen.

Aus dieser Studie kam hervor, dass MT04 (1,2,4,5-Tetraoxan) noch aktiver *in vitro* und *in vivo* (in Ratten) gegen adulte und juvenile *F. hepatica* Infektionen war als OZ78. Im Unterschied zu OZ78, hat MT04 zwei Peroxid-Brücken. Eine Dosis von 50 mg/kg genügte um eine komplette Reduktion der Wurmbelastung von 100% gegen adulte *F. hepatica* in Ratten zu erreichen, für eine juvenile Behandlung war eine Dosis von 100 mg/kg nötig.

Parallel wurde OZ78 im Schaf getestet und Aktivität, Toleranz und Pharmakokinetik untersucht. Aber OZ78 schaffte es nicht Fasziolose im Schaf nach einer Anwendung von 50 mg/kg OZ78 oral oder subkutan zu heilen, keine Reduktion von Stuhleiern oder Würmern konnte gemessen werden.

Um die pharmakokinetischen Parameter von OZ78 im Plasma zu bestimmen, musste eine Flüssigchromatographische Massenspektrometrische (LC/MS) Methode entwickelt und auf Genauigkeit, Präzision, Stabilität, und Selektivität validiert werden. OZ78 und OZ352 (interner Standard, I.S.) musste im Negativ-Modus nach der Masse der deprotonierten Muttersubstanz detektiert werden. Eine Proteinfällung wurde zur Probenvorbereitung angewendet. Die Wiederfindungen von OZ78 (5, 1.25, und 0.3125 µg/ml) und von I.S. (2 µg/ml) waren 84.4%, 69.6%, 78.6%, und 85.8%. Kalibrationskurve reichte von 5 µg/ml zu 0.15625 µg/ml. Die entwickelte Methode war genau, präzis, und selektiv.

Das Pharmakokinetik-Profil von OZ78 nach oraler Injektion zeigte eine maximale Plasma Konzentration von (C_{max}) 45.8±13 µg/ml nach 1 h (T_{max}) auf. Die Halbwertszeit ($t_{1/2}$) betrug 1.0±0.1 h und eine mittlere Fläche unter der Plasma Zeit Kurve (AUC) von 116.2±47 µg h/ml wurde für die orale Anwendung berechnet. Auf der anderen Seite, nach subkutaner Injektion mit OZ78, wurden ein C_{max} und ein T_{max} von 13.7±6.1 µg/ml und 0.9±0.4 h berechnet. Die α und β Halbwertszeit waren 4.5±4.3 h und 56.5±36 h, und die mittlere AUC war 219.1±74 µg h/ml.

Weitere Studien waren nötig um den Unterschied im Behandlungsergebnis zwischen Ratte und Schaf zu erklären. Die LC/MS Methode von OZ78 für Schafplasma wurde für Rattenplasma angepasst und validiert. Zusätzlich konnte die OZ78 LC/MS Methode modifiziert, validiert und für MT04 Messung in Schaf und Ratten Plasma verwendet werden.

OZ78 und MT04 wurden in einer Dosis von 50 mg/kg Ratten verabreicht und nach verschiedenen Zeitpunkten wurden Samples gezogen. Studien mit MT04 betreffend Aktivität und Pharmakokinetik im Schaf sind noch ausstehend, sind aber in Planung.

Nach oraler Applikation von 50 mg/kg in Ratten, wurden C_{max} Werte von MT04 und OZ78 49.8±9.1 und 70.1±19.1 µg/ml nach 2.7 h±1.2 und 1.6±1.6 h (T_{max}) gemessen. Die berechnete AUC ergab 31'258.8±6'232.7 und 29'794.1±3'990.6 µg min/ml für MT04 und OZ78. Mittlere Halbwertszeiten ($t_{1/2}$) für MT04 und OZ78 waren 6.4 ±5.7 h und 2.5±1.5 h.

Zuletzt wurde die Toxizität von OZ78 und MT04 mit einer Leberzelllinie (HegG2) bestimmt. Verschiedene Toxizitäts- und Viabilitäts Assays wurden mit OZ78 oder MT04 kombiniert mit verschiedenen Einsenlösungen (Hämin, Fe(II) und Fe(III)) angewandt um das Toxizitätsprofil besser zu verstehen. Reaktive Sauerstoffspezies (ROS) Produktion von MT04 und OZ78 wurden in Kombination mit diesen Eisenlösungen gemessen. OZ78 war nur mässig toxisch kombiniert mit Eisen, wobei eine signifikante Toxizität für MT04 in Kombination mit Eisen gefunden werden konnte. Ein hohes ROS Signal wurde für beide Substanzen vor allem in Kombination mit Haemin gemessen.

Zusammengefasst, OZ78 und MT04 sind vielversprechende „Lead"-Kandidaten in der Entwicklung eines Medikaments gegen *Fasziola* Infektionen. OZ78 und MT04 waren aktiv gegen juvenile und adulte Infektion in der Ratte. Das Therapieversagen in Schafen kann durch eine zu geringe Exposition der Saugwürmer im Schaf mit der Substanz erklärt werden. Eine andere Applikation im Schaf ist noch ausstehend. Bis jetzt lässt sich nicht sagen, wie toxisch die verwendeten Dosen sind, mehr Studien im Tier werden dafür benötigt. Die Aktivität der Substanzen scheint von der ROS Produktion abhängig zu sein.

Table of Abbreviations

ADME	**Absorption, distribution, metabolism, and elimination**
AK	**Adenylate Kinase**
AM	**Artemether**
AS	**Artesunate**
AUC	**Area under the plasma concentration time curve**
Cl	**Clearance**
C_{max}	**Maximal plasma concentration**
CUR	**Curtain gas**
CYP	**Cytochrome**
DALYs	**Disability-adjusted life years**
DCF	**Dichlorodihydrofluorescein**
DFO	**Deferoxamine mesylate**
DHA	**Dihydroartemisinin**
DMEM	**Dulbecco's Modified Eagle's Medium**
DMSO	**Dimethyl sulfoxide**
DP	**Declustering potential**
ECT	**Efficacy control test**
ELISA	**Enzyme linked immunosorbent assay**
EP	**Entrance potential**
ESI	**Electrospray ionization**
F	**Bioavailability**
FP	**Focusing potential**
GC	**Gas chromatography**
GLP	**Good laboratory practice**
GMP	**Good manufacturing practice**
HILIC	**Hydrophilic interaction liquid chromatography**
HPLC	**High pressure liquid chromatography**
i.m.	**intramuscular**
IS	**Ion spray voltage**
I.S.	**Internal standard**
KW	**Kruskal-Wallis test**
LC	**Liquid chromatography**

LC/MS	**Liquid chromatography mass spectrometry**
LC-MS/MS	**Liquid chromatography tandem mass spectrometry**
LLOQ	**Lower limit of quantification**
MS	**Mass spectrometry**
MW	**Molecular weight**
NEAA	**Non-essential amino acids**
NEB	**Nebulizer gas**
NTD	**Neglected tropical disease**
OZ	**Secondary ozonide**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PD	**Pharmacodynamic**
PK	**Pharmacokinetic**
QC	**Quality control**
ROS	**Reactive oxygen species**
RRE	**Relative recovery**
RSD	**Relative standard deviation**
SAR	**Structure-activity relationship**
SD	**Standard deviation**
SE	**Standard error**
SEM	**Scanning electron microscopy**
SPE	**Solid phase extraction**
$t_{1/2}$	**Elimination half-life**
TBZ	**Triclabendazole**
TEM	**Transmission electron microscopy**
TIC	**Total iron chromatogramm**
T_{max}	**Time to achieve maximal plasma concentration**
ULOQ	**Upper limit of quantification**
USFDA	**US Food and Drug Administration**
V_d	**Apparent volume of distribution**
WBR	**Worm burden reduction**

Chapter 1

General Introduction

Fascioliasis: Biology, Control, and Treatment

General Introduction

Fascioliasis: Biology, Control, and Treatment

1. *Fasciola hepatica*, the causative agent of fascioliasis

1.1. Parasitism and helminths

Parasites are defined as organisms, which live entirely or partially, permanently, or temporarily at the cost of another organism. Parasites can be viruses, bacteria, protozoa, fungi, or helminths. [1]

Parasitic helminths are worm-like organisms with a complex eukaryotic cell build [2, 3], belonging to the group of endoparasites [1, 4]. Endoparasites live in the body of another species (host) [1].

Trematode is the name for a taxonomic class of multicellular eukaryotic helminths. They are categorized into two main groups: tissue flukes, which are also called food-borne trematodes (liver, lung, and intestinal flukes) or blood flukes [5-7]. An overview about the different groups is given in Fig. 1.

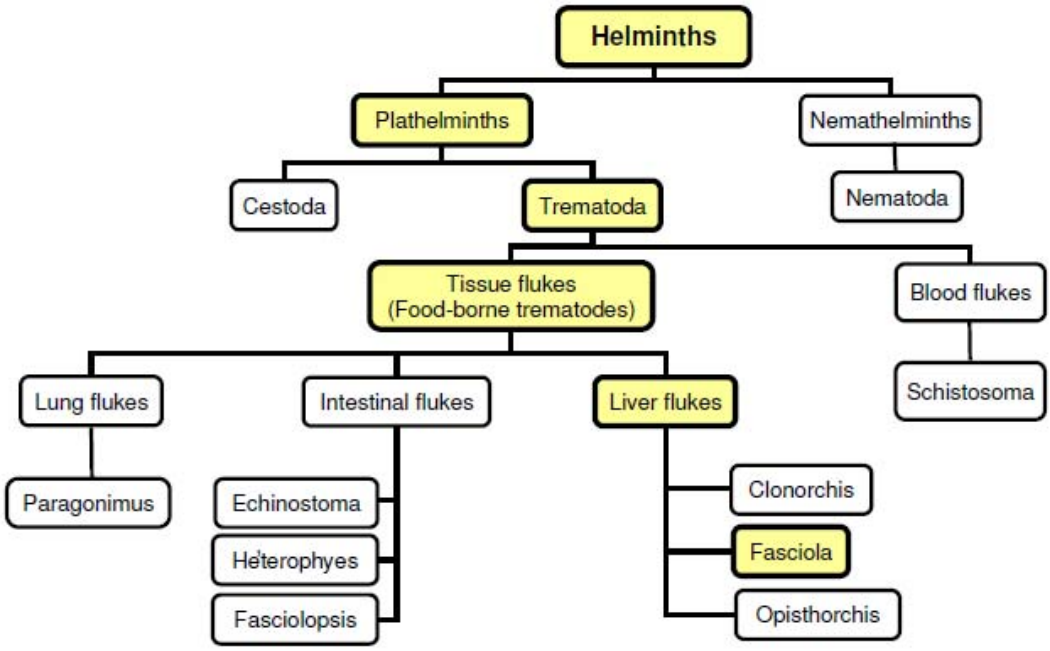


Fig. 1: Taxonomic overview about helminths [1, 6, 8].

A characteristic of food-borne trematodes is an oral and ventral sucker [6]. Food-borne trematodes do not possess a respiratory or circulatory system [5].

The liver fluke *Fasciola hepatica,* belonging to the group of food-borne trematodes, is at the centre of this thesis and will be discussed in more detail in the following paragraphs [7].

F. hepatica is a flat and leaf-shaped worm. Its size is up to 30 mm in length and 13 mm in width for adult flukes [9, 10]. The worm is hermaphroditic [11]. An adult worm can live for many years in the bile ducts of the host and release eggs [10, 11]. Fasciocidal eggs have an ovoid shape and measure 75 to 140 µm [9]. A picture of an adult worm and an egg are presented in Fig. 2.

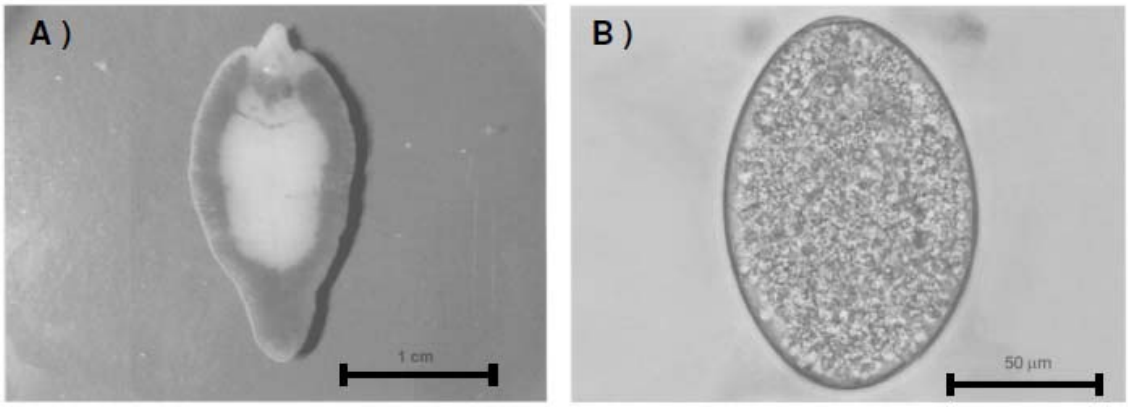


Fig. 2: Picture of an adult F. hepatica (A) and a fasciocidal egg (B) [9].

1.2. Food-borne trematodiases

Food-borne trematodiases are parasitic infections caused by trematode worms (flukes), with an estimated number of about 70 to 80 species being infectious to humans [6, 12]. Food-borne trematodiases belong to the neglected tropical diseases (NTDs) [13-15], which is a group of vector- and non-vector-borne diseases including helminthic, protozoan, bacterial, and fungal infections [16].

Importantly, food-borne trematodes are of considerable public health significance typically in poor and rural settings, with about 56 million people affected and 750 million people at risk worldwide [6, 12, 15, 17, 18]. The global burden of these diseases is estimated to be 665'352 disability-adjusted life years (DALYs) [12]. Infection occurs through eating contaminated aquatic plants, fish or drinking contagioned water [5, 7, 8].

1.3. Fascioliasis

The worms *F. hepatica* and *F. gigantica* live in the liver, bile ducts or gallbladder of the host, which causes the disease fascioliasis (also termed fasciolosis) [9, 10, 19, 20]. This hepatic parasite infests humans and many animal species such as ruminants like sheep and cattle, but also pigs and donkeys [20, 21]. The estimated number of infections in humans ranges from 2.4 to 17 million people [22]. This disease is a considerable public

health problem and belongs to the major zoonotic diseases [23]. In 2004, fascioliasis was added to the list of important helminthiases [19].

Fascioliasis can be caused either by *F. hepatica* or *F. gigantica*, where the majority of documented cases in humans is caused by *F. hepatica* [8, 24].

1.3.1. Life Cycle of *F. hepatica*

Fasciola hepatica needs two hosts in order to complete its life-cycle: the first intermediate host is a fresh-water snail of the family *Lymnaeidea* and the end hosts are herbivorous animals or humans [6, 9, 19]. The life cycle of *Fasciola hepatica* is shown in Fig. 3.

Human infections with *Fasciola* occur through consumption of unwashed aquatic vegetables or freshwater plant species contaminated with metacercariae [20, 25]. Metacercariae are the contagious stage of the parasite [24]. Consumed metacercariae excyst in the intestine, penetrate the intestinal wall and enter the peritoneal cavity [24]. The immature parasite (juvenile fluke) in the liver tissue causes the acute disease. The immature worms feed especially from the liver tissue [10]. After three to four months, the parasite migrates to the bile ducts where sexual maturation to the adult stage takes place [10, 24]. Adult worms excrete eggs via stools of the host and after further development, optimally at 15 to 25°C, the miracidial stage is reached [9]. Miracidia hatch in fresh water and reproduce after penetration of aquatic snails [10, 11]. Within the snail several different reproduction stages (miracidium, sporocyst, redial generations) occur [6, 11]. The final stage, cercariae encyst as metacercarie on plants and the life cycle starts again [11].

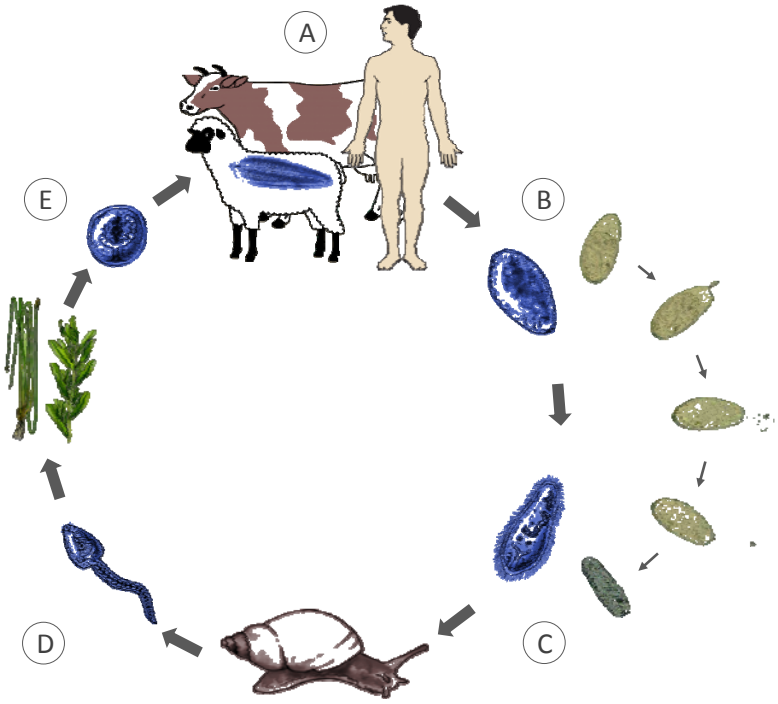


Fig. 3: Life cycle of F. hepatica: end host: animals or humans (A); eggs excreted (B); miracidia hatching (C); cercariae released from the intermediate host (D); metacercariae (infectious stage) (E) [26].

1.3.2. Prevalence of *F. hepatica*

The disease caused by *F. hepatica* was reported to be of European origin [10]. The fast adaptation to new hosts, as well as to new environmental conditions is a special feature of these worms and has resulted in a wide distribution [10]. Hence, fascioliasis has the widest latitudinal, longitudinal, and altitudinal distribution known of a vector-born infectious disease [23].

Human fascioliasis has been increasing over the last years and infections have been found in 51 countries and on all continents except Antarctica [11, 24, 27]. In Bolivia, Peru, Iran, Portugal, Egypt, and France prevalence rates are high; a common factor of all these countries is the temperate climate [11, 24]. Human endemic areas are spread from below sea level to very high altitudes [10]. The distribution pattern of fascioliasis can be described as patchy, and there is a relationship between intermediate host distribution and climatic conditions [20].

However, no clear correlation could be found between the occurrence of fascioliasis in humans and in livestock [27].

1.3.3. Pathology of fascioliasis

Two phases of fascioliasis were distinguished according to the stage of the worms in the host organism: acute or invasive phase (worm migrates through the liver into the bile ducts) and chronic or obstructive phase (worm lodges in the bile ducts or the gallbladder) [9-11]. The acute phase is caused by the juvenile stage of the worms and the chronic phase by the adult worms [28].

During the acute phase, patients suffer particularly from dyspepsia, fever, abdominal pain, weight loss, malaise and other gastrointestinal disturbances [6, 24]. The acute phase reflects the penetration of the parasite through the liver parenchyma, lasting two to four months [9, 10, 24].

The chronic phase is dominated by sub clinical symptoms or symptoms, which are difficult to distinguish from other liver diseases (such as cholangitis, cholecystitis) [24]. This phase is represented by inflammation and destruction of the biliary duct [11]. Clinical manifestation can be biliary colic, epigastric pain, nausea and jaundice among others [10].

Since the worm does not multiply in the end host, there is a direct correlation between the ingested larvae and the infection intensity [7].

1.3.4. Diagnosis and treatment

Diagnosis can be made by direct detection of eggs in the stool (e.g. Kato Katz thick smears, sedimentation), which is the gold standard diagnosis [8, 13, 29]. Detection of eggs by microscope examination is the most widely used technique despite variation in sensitivity, but this technique is easy to perform and are low in cost [6, 29]. Another direct diagnostic technique is called FLOTAC technique, which allows the analysis of larger amounts of stool samples using a flotation cleaning step [13]. Hence, the diagnostic sensitivity of this method is frequently increased compared to other coprodiagnostic methods as for instance Kato-Katz or sedimentation [29].

On the basis of molecular and immunological methods, a broad range of other techniques (enzyme-linked immonosorbent assay (ELISA), polymerase chain reaction (PCR)) were developed. However, these techniques are in most cases too expensive and obviously not suitable for low-income countries [6].

Chemotherapy remains the main tool in combating fascioliasis, since no effective vaccine is available to date [30, 31].

The anthelmintic drug triclabendazole is the first choice treatment for infection with *F. hepatica*. It is a benzimidazole drug (chemical name is [6-chloro-5-(2,3-dichlorophenoxy)-2methylthiobenzimiadzole]), which is the best tolerated and most efficacious chemotherapeutic agent against fascioliasis [27, 30, 32-34]. The precise mechanism of action is not yet elucidated, but it is known that triclabendazole has two major sulfonated metabolites (triclabendazole sulfoxide and sulfone) [30]. Triclabendazole sulfoxide damages the tegument of *Fasciola* leading to its disintegration. In addition, activity against the parasite's microtubule system was described, and a decline in DNA synthesis was documented [30]. The activity against both juvenile and adult flukes is a big advantage of this drug [33]. Egaten® is the brand product for human use [25], and Fasinex® is the product for animals [35]. The WHO recommends a single dose of 10 mg/kg [6, 36]. Note that the drug is registered in only four countries (Ecuador, Egypt, France, and Venezuela) for human application [37].

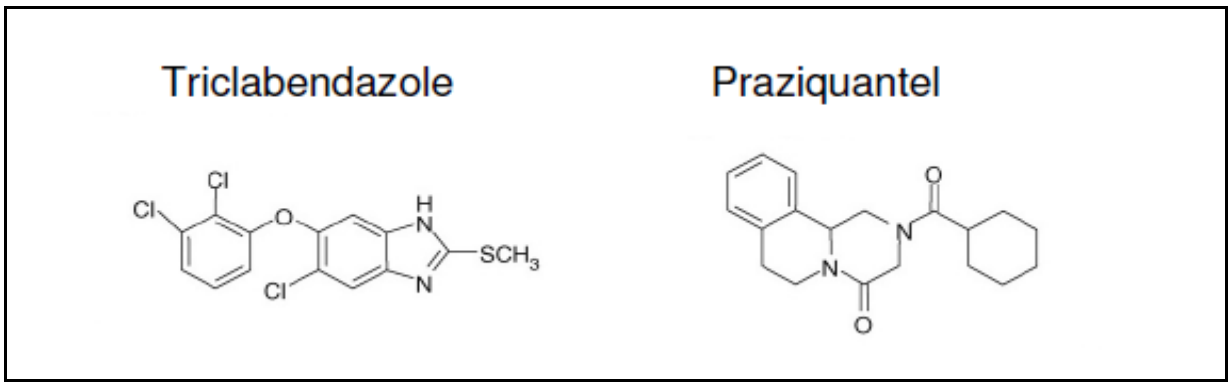


Fig. 4: Molecular structure of Triclabendazole and Praziquantel [37].

The spreading triclabendazole resistance in animals, first documented in sheep in Australia, is a major problem [38-40]. However, to date no resistance has been described in humans [37]. There are no alternative drugs available that are characterized by a similar activity and safety profile [5, 6].

Bithionol (chemical name (2,2-thiobis-[4,6-dichlorophenol])) is a bacteriostatic agent and was used for many years to treat fascioliasis. However, the drug shows frequent adverse effects, and hence is no longer applied in humans [6, 30, 37].

Praziquantel, the drug of choice against food-borne trematodiases, has no activity against *Fasciola* [8, 11, 30, 37]. High cure rates are obtained with praziquantel against

clonorchiasis, opisthorchiasis, paragonimiasis and intestinal fluke infections, but the response against fascioliasis in terms of cure and egg reduction rates is only moderate to low [13].

The molecular structures of the two important drugs treating infections with food-borne trematodes are shown in Fig. 4.

1.3.5. Investigation on potency of new promising drugs against fascioliasis

Novel drugs should exhibit a cure rate and egg reduction rate of more than 80% and 90% against acute and chronic infections, respectively. Moreover, an anthelmintic should be well tolerated, cheap, stable under tropic conditions, and effective in a single dose regimen [37].

To date, the efficacy controlled test (ECT) is the most widely used test to screen for new anthelmintic compounds. This test compares the worm burden of treated and control animals after a defined time post treatment [41].

For *in vitro* drug screening, worms were incubated in a medium supplemented with antibiotics and drug solution. At different time-points the viability of the worms was screened dependent on the worm species. For *Fasciola* screening the worms were inspected under a microscope (motility), or pictures with a scanning electron microscope (SEM) were taken and analyzed (morphological changes)[42]. Treatment efficacy was determined against control worms [42-45].

1.4. Secondary ozonides – a new drug class

Many helminths, including *F. hepatica* are blood feeders similarly to *P. falciparum* (malaria parasite) and therefore it is very worthwhile to test antimalarial drugs against helminth diseases [3, 46-48].

Artemisinin and its derivatives, artesunate and artemether, are the first choice treatment for uncomplicated malaria [49, 50]. However, the use of artemisinin is limited, because of its poor solubility in oil and water [46]. Dihydroartemisinin (reduced lactol derivative) and the new semi-synthetic generation artemisinins (e.g. artesunate, artemether, arteether) are more potent than artemisinin, but have poor

pharmacokinetic properties [3, 37, 49]. Furthermore, only low yields of artemisinin can be gained by extraction of *Artemisia annua*, which leads to erratic artemisinin prices [3]. Artemisinin and the derived semi-synthetic substances belong to the group of first generation endoperoxides [46]. Their main pharmacophore is the sesquiterpen trioxane ring [3]. Second generation endoperoxides are synthetic agents derived from the artemisinin core structure [46]. The ozonides (1,2,4-trioxanes), whose sesquiterpene trioxane rings are substituted with an adamantine ring, have shown to be potent against *P. falciparum* in nanomolar concentration. Importantly, they are more stable than the first generation endoperoxides [49, 51].

It is proposed that activation of synthetic and semi-synthetic endoperoxides takes place in the presence of haeme-iron, as intermediate stage carbonic centered radicals are built, which then react with different parasite proteins and haeme [52-54]. Despite this knowledge the mechanism of action these substances stays controversial and more studies are needed [54].

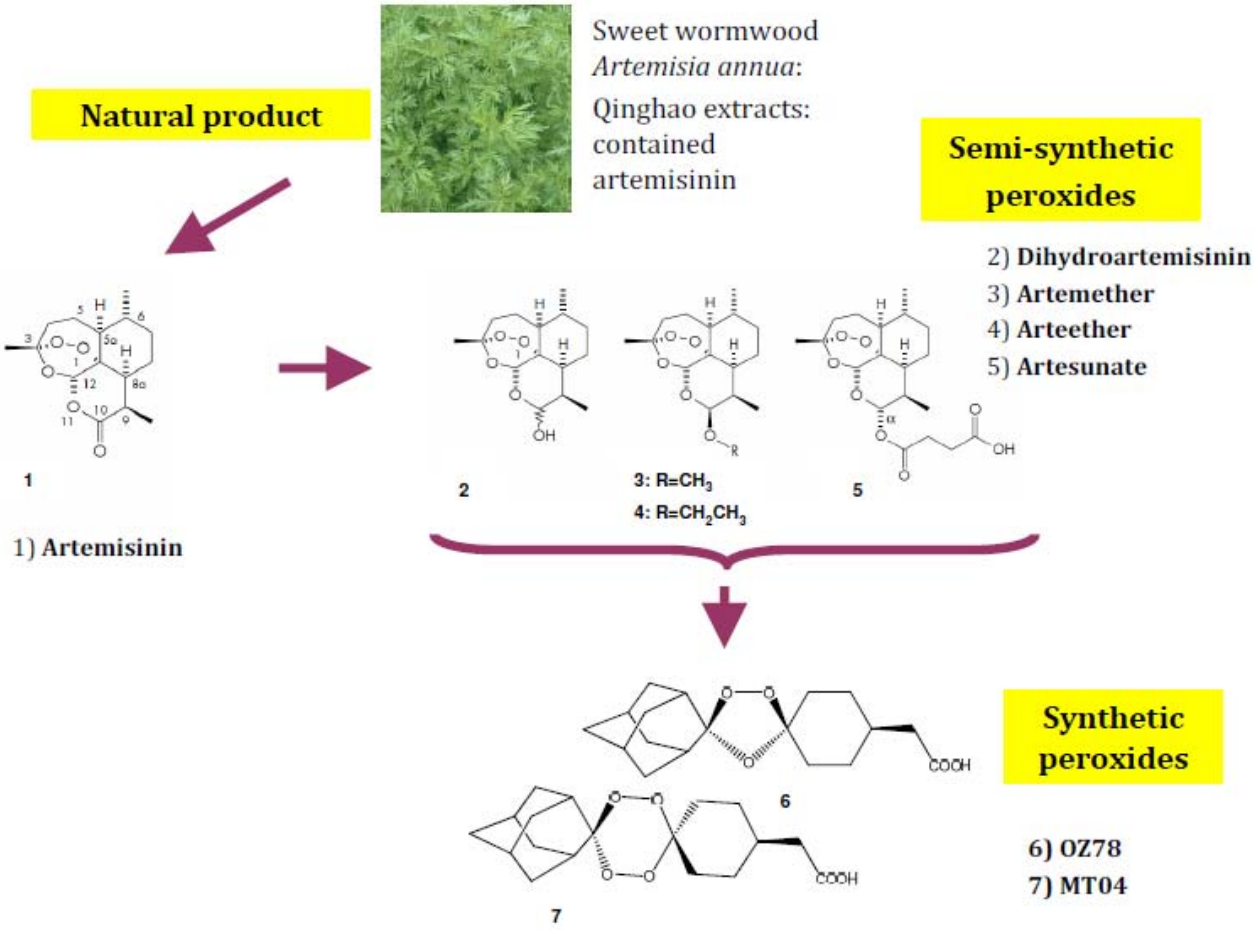


Fig. 5: The pathway from the natural product to the synthetic substances OZ78 and MT04.

The semi-synthetic derivatives artesunate and artemether and the synthetic candidate OZ78 (1,2,4-trioxolane) showed promising cure rates against *F. hepatica in vitro* and *in vivo* (rat), where OZ78 was found to be the best candidate amongst them [37].
Newer investigations of a recently published structure-activity-relationship (SAR) study have shown that a tetraoxane substructure is even more active in combating fascioliasis than a trioxolane ring (OZ78) [47]. Especially the tetraoxane MT04 was found to be a new promising candidate [47]. The pathway from the natural product to these synthetic substances is presented in Fig. 5.
Artesunate has already been tested in a randomized clinical trial against acute symptomatic fascioliasis in 100 Vietnamese patients [55]. The use of artesunate in combating fascioliasis seems to be an opportunity because patients treated with artesunate were more likely to be free of abdominal pain at hospital discharge compared to triclabendazole-treated patients. However, the endpoint response rate of triclabendazole was higher than for artesunate and hence further studies are needed [55]. Artemether has also been tested against chronic *F. hepatica* infected humans in Egypt, but no effect could be found at a dose regime such as the one used against malaria [56].

2. Preclinical studies in Drug Development

2.1. Preclinical phase

Preclinical studies are an important segment during drug development; they include toxicity, pharmacokinetic, and pharmacodynamic studies in animals. Preclinical studies are the last stage before entering the human phase of studies [1]. An overview about the drug development segments is given in Fig. 6.

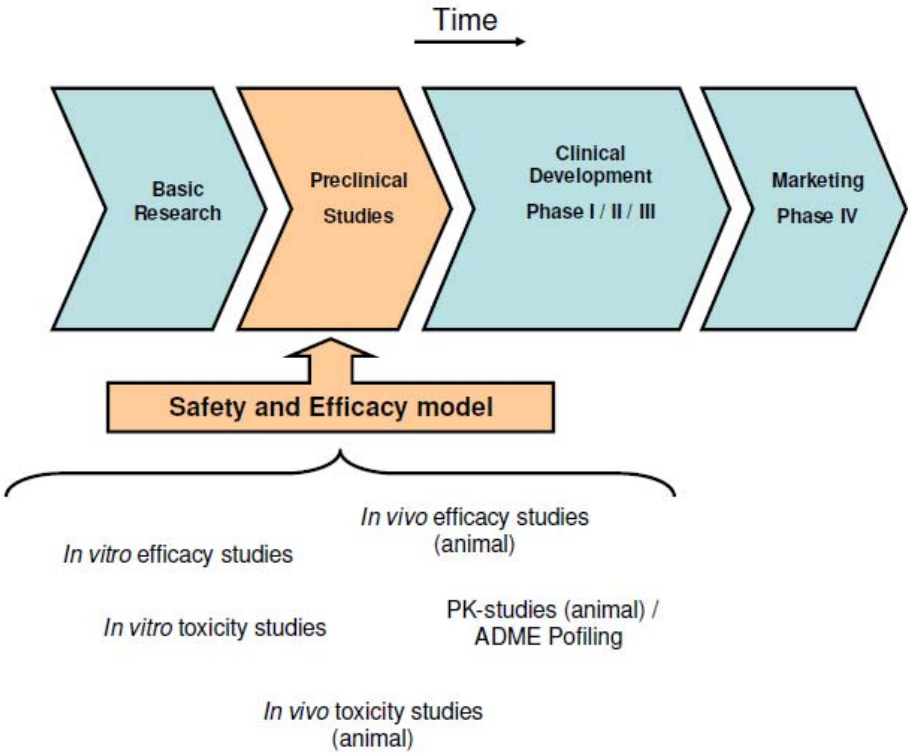


Fig. 6: Overview of the drug development pipeline emphasizing the preclinical part [57, 58].

The drug development process can be summarized shortly as follows.

In the basic research phase, many substances are screened for a defined disease (high-throughput screening). Target identification, lead identification, and lead optimization are the analytical activities during basic research. The aim of these tests is to avoid false positive substances and to lower costs [58, 59]. In helminths drug discovery, substances have to be directly tested *in vitro* and *in vivo* against the parasite and disease, respectively, as no other methods or models are available for drug screening.

In preclinical studies the focus lies on process research, formulation, metabolism and toxicity [58]. To date, it is not possible to predict the complete toxicity profile derived from the structure of a molecule, therefore animal studies are needed for complete studies [59]. In preclinical studies, regulatory issues are addressed such as Food and Drug Administration (FDA) guidelines, good manufacturing practice (GMP), and good laboratory practice (GLP) regulations [58].

After preclinical studies have been conducted, a good candidate with low toxicity and good activity can be tested in humans in clinical trials (Phase I-III). From the gained knowledge in preclinical studies, a safe dose for human use can be calculated [60].

Once a safe and effective drug is found, and all necessary dossiers are available the drug can be marketed (Phase IV) [59].

2.2. Pharmacokinetic (PK) studies

Pharmacokinetic studies (PK) are used to characterize a new potential drug according to absorption, distribution, metabolism, and elimination (ADME) in the body. PK studies describe the reaction of the body to the applied substance [1, 57].

Important PK parameters are: time to achieve maximal plasma concentration (T_{max}), maximal plasma concentration (C_{max}), elimination half-life ($t_{1/2}$), area under the plasma concentration time curve (AUC), clearance (CL), apparent volume of distribution (V_d), and oral bioavailability (F) [61].

During the drug development process PK studies are first done in animals and then later in humans.

An important analytical technique to measure generated PK-samples is liquid chromatography/mass spectrometry (LC/MS), since this method is reliable for low sample volumes [62].

3. Liquid chromatography/mass spectrometry (LC/MS)

3.1. Liquid chromatography/mass spectrometry system

Liquid chromatography mass spectrometry (LC/MS) is an analytical measurement technique. The basis of this analytical technique is the chemical separation of substances by liquid chromatography (LC) and the detection of molecules in a highly sensitive mass spectrometer (MS) based on ionized mass or fragment mass. LC/MS can be applied for quantitative and qualitative measurements of different substances [58, 62].

In Fig. 7 a basic LC/MS system is shown. In detail, the LC/MS system contains a high pressure liquid chromatography (HPLC) pumping system, an injector, and a column for separation coupled to a mass spectrometer [62]. The MS-system is equipped with the

following elements: sample inlet, ionization source, mass analyzers and a detector [63]. The connection of the two systems occurs through evaporation ionization of the liquid phase. The whole system is controlled by a remote system. The measured signal contains information about signal intensity in volts versus ion fragment mass/charge versus time. The information can be shown as total ion chromatogram (TIC, which is defined as a signal of all ions present versus time = cps, counts per second) or as a spectrum, which is a plot showing the signal strength versus mass/charge at a defined point in time.

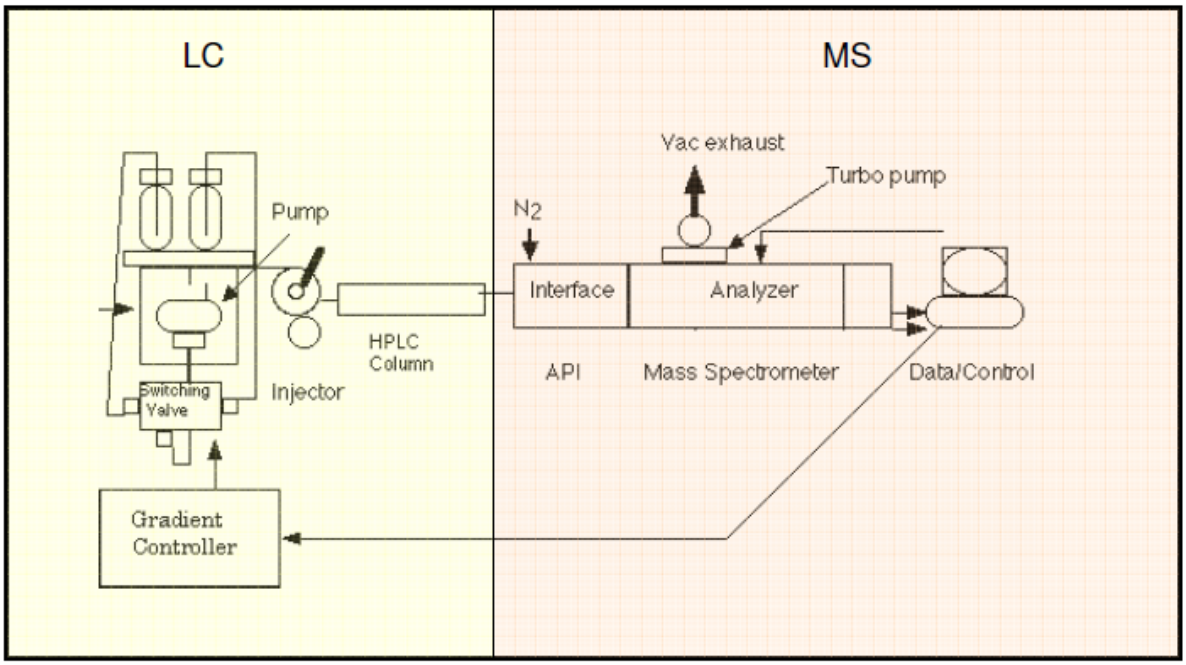


Fig. 7: Basic system of a LC/MS [62].

Over the past 40 years HPLC has been used for substance separation, analysis, and purification. Before HPLC came into use, gas chromatography (GC) was applied for a long time as a separation technique. However, in the last years the LC/MS has shown to be superior to GC/MS, since not all compounds were volatile enough for GC or were degraded by temperature in the GC column oven. [62]

After separation of the molecules, different MS-techniques can be used for analyte detection. The mass to charge ratio (m/z) is a common unit used to detect the molecules in spectrometry. Different spectrometers can be applied: for example quadrupole, ion trap or Fourier transform [63]. In this thesis a triple quadrupole (= tandem) mass spectrometer (MS/MS) was applied for the analysis of peroxidic fasciocidal drugs. This

instrument enables not only the detection of ionized molecules or fragments of molecules, but also the analysis of molecule specific fragmentation processes within the spectrometer. Importantly, selectivity and sensitivity of the method is mostly improved with a MS/MS compared to single MS analytics. Finally, for compound identification, the superior system LC/MS/MS is needed [62].

3.2. LC/MS application

LC/MS or LC/MS/MS are analytical techniques with a broad range of applications. Overall, there are three main different application fields:

First, drugs or other small molecules can be quantified or identified in different biological matrices. For instance, quantification of substances in plasma is required to determine their pharmacokinetic (PK) profile. Short analysis time can be reached and a high through-put is possible. A second field of mass spectrometry is the elucidation of the peptide sequence of proteins. Finally a third important field of application covers the metabolite and trace contaminates studies. [58, 62]

Aim and Objectives

The following five objectives were accomplished to further strengthen our knowledge about treatment of fascioliasis with synthetic peroxides:

1. To analyze the activity of selected OZ78 derivatives against juvenile and adult *F. hepatica* and *E. caproni* (*chapter 2*).

2. To develop and validate a sensitive and selective LC/MS method for the analysis of OZ78 for the prospective determination of pharmacokinetic parameters in sheep (*chapter 3*).

3. To determine the anthelmintic activity of OZ78 in sheep and to analyze pharmacokinetic parameters of OZ78 in sheep infected with *F. hepatica* (*chapter 4*).

4. To develop and validate a sensitive and selective LC/MS method to measure MT04 in rat and sheep plasma, and to adapt the OZ78 LC/MS sheep method for rat plasma (*chapter 5*).

5. To study the toxic effect of OZ78 and MT04 in comparison to the established substances artesunate and dihydroartemisinin (*chapter 6*).

References

[1] de Gruyter W, *Hunnius Pharmazeutisches Wörterbuch*. 9. Auflage ed. 2004, Berlin: H.P.T.Ammon.

[2] Maizels RM, Yazdanbakhsh M, Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol 2003;3:733-44.

[3] Keiser J, Utzinger J, Artemisinins and synthetic trioxolanes in the treatment of helminth infections. Curr Opin Infect Dis 2007;20:605-12.

[4] Sachs JL, Essenberg CJ, Turcotte MM, New paradigms for the evolution of beneficial infections. Trends Ecol Evol 2011;26:202-9.

[5] Keiser J, Utzinger J, Food-borne trematodiases. Clin Microbiol Rev 2009;22:466-83.

[6] Keiser J, Utzinger J, Chemotherapy for major food-borne trematodes: a review. Expert Opin Pharmacother 2004;5:1711-26.

[7] Doughty BL, *Schistosomes and other trematodes (Chapter 88)*, in *Medical Micriobiology*. 1996.

[8] Toledo R, Esteban JG, Fried B, Current status of food-borne trematode infections. Eur J Clin Microbiol Infect Dis 2012;

[9] Saba R, Korkmaz M, Inan D, Mamikoglu L, Turhan O, Gunseren F, et al., Human fascioliasis. Clin Microbiol Infect 2004;10:385-7.

[10] Mas-Coma S, Human Fascioliasis: Epidemiological patterns in human endemic areas of south america, africa and asia. Southeast Asian J Trop Med Public Health 2004;35 (Suppl 1):1-11.

[11] Garcia HH, Moro PL, Schantz PM, Zoonotic helminth infections of humans: echinococcosis, cysticercosis and fascioliasis. Curr Opin Infect Dis 2007;20:489-94.

[12] Furst T, Keiser J, Utzinger J, Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. Lancet Infect Dis 2011;

[13] Keiser J, Duthaler U, Utzinger J, Update on the diagnosis and treatment of food-borne trematode infections. Curr Opin Infect Dis 2010;23:513-20.

[14] Hotez PJ, Molyneux DH, Fenwick A, Ottesen E, Ehrlich Sachs S, Sachs JD, Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria. PLoS Med 2006;3:e102.

[15] Sripa B, Kaewkes S, Intapan PM, Maleewong W, Brindley PJ, Food-borne trematodiases in Southeast Asia epidemiology, pathology, clinical manifestation and control. Adv Parasitol 2010;72:305-50.

[16] Fenwick A, *Host-Parasite Relations and Implications for Control*, in *Advances in Parasitology*. 2009. p. 247-261.

[17] Utzinger J, Raso G, Brooker S, De Savigny D, Tanner M, Ornbjerg N, et al., Schistosomiasis and neglected tropical diseases: towards integrated and sustainable control and a word of caution. Parasitology 2009;136:1859-74.

[18] Toledo R, Bernal MD, Marcilla A, Proteomics of foodborne trematodes. Journal of Proteomics 2011;74:1485-1503.

[19] Mas-Coma S, Bargues MD, Valero MA, Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol 2005;35:1255-78.

[20] Mas-Coma MS, Esteban JG, Bargues MD, Epidemiology of human fascioliasis: a review and proposed new classification. Bull World Health Organ 1999;77:340-6.

[21] O'Neill JF, Johnston RC, Halferty L, Brennan GP, Keiser J, Fairweather I, Adult triclabendazole-resistant Fasciola hepatica: morphological changes in the

tegument and gut following in vivo treatment with artemether in the rat model. J Helminthol 2009;83:151-63.
[22] Keiser J, Utzinger J, Emerging foodborne trematodiasis. Emerg Infect Dis 2005;11:1507-14.
[23] Mas-Coma S, Epidemiology of fascioliasis in human endemic areas. J Helminthol 2005;79:207-16.
[24] Robinson MW, Dalton JP, Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos Trans R Soc Lond B Biol Sci 2009;364:2763-76.
[25] Savioli L, Chitsulo L, Montresor A, New opportunities for the control of fascioliasis. Bull World Health Organ 1999;77:300.
[26] Duthaler U, *Artemisinins for the Treatment of Fascioliasis: Progress in Preclinical and Diagnostic Research*, in *Phil.nat.* 2011, University of Basel: Basel.
[27] Esteban JG, Bargues MD, Mas-Coma S, Geographical distribution, diagnosis and treatment of human fascioliasis: a review. Research an d reviews in parasitology 1998;58:13-48.
[28] Moxon JV, Flynn RJ, Golden O, Hamilton JV, Mulcahy G, Brophy PM, Immune responses directed at egg proteins during experimental infection with the liver fluke Fasciola hepatica. Parasite Immunol 2010;32:111-24.
[29] Duthaler U, Rinaldi L, Maurelli MP, Vargas M, Utzinger J, Cringoli G, et al., Fasciola hepatica: comparison of the sedimentation and FLOTAC techniques for the detection and quantification of faecal egg counts in rats. Exp Parasitol 2010;126:161-6.
[30] Keiser J, Engels D, Buscher G, Utzinger J, Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin Investig Drugs 2005;14:1513-26.
[31] Fairweather I, Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet Parasitol 2011;180:133-43.
[32] Fairweather I, Triclabendazole progress report, 2005-2009: an advancement of learning? J Helminthol 2009;83:139-50.
[33] Boray JC, Crowfoot PD, Strong MB, Allison JR, Schellenbaum M, Von Orelli M, et al., Treatment of immature and mature Fasciola hepatica infections in sheep with triclabendazole. Vet Rec 1983;113:315-7.
[34] McKellar QA, Kinabo LD, The pharmacology of flukicidal drugs. Br Vet J 1991;147:306-21.
[35] Wolff K, Eckert J, Schneiter G, Lutz H, Efficacy of triclabendazole against Fasciola hepatica in sheep and goats. Vet Parasitol 1983;13:145-50.
[36] WHO, Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control. Geneva Switzerland 2006;1-31.
[37] Keiser J, Utzinger J, Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. Trends Parasitol 2007;23:555-62.
[38] Moll L, Gaasenbeek CP, Vellema P, Borgsteede FH, Resistance of Fasciola hepatica against triclabendazole in cattle and sheep in The netherlands. Vet Parasitol 2000;91:153-8.
[39] Overend DJ, Bowen FL, Resistance of Fasciola hepatica to triclabendazole. Aust Vet J 1995;72:275-6.
[40] Alvarez-Sanchez MA, Mainar-Jaime RC, Perez-Garcia J, Rojo-Vazquez FA, Resistance of Fasciola hepatica to triclabendazole and albendazole in sheep in Spain. Vet Rec 2006;159:424-5.

[41] Scarcella S, Fiel C, Guzman M, Alzola R, Felipe A, Hanna RE, et al., Reproductive disruption in Fasciola hepatica associated with incomplete efficacy of a new experimental formulation of triclabendazole. Vet Parasitol 2011;176:157-64.
[42] Keiser J, In vitro and in vivo trematode models for chemotherapeutic studies. Parasitology 2010;137:589-603.
[43] Duthaler U, Smith TA, Keiser J, In vivo and in vitro sensitivity of Fasciola hepatica to triclabendazole combined with artesunate, artemether, or OZ78. Antimicrob Agents Chemother 2010;54:4596-604.
[44] Keiser J, Shu-Hua X, Tanner M, Utzinger J, Artesunate and artemether are effective fasciolicides in the rat model and in vitro. J Antimicrob Chemother 2006;57:1139-45.
[45] Keiser J, Morson G, Fasciola hepatica: Surface tegumental responses to in vitro and in vivo treatment with the experimental fasciolicide OZ78. Exp Parasitol 2008;119:87-93.
[46] Meshnick SR, Taylor TE, Kamchonwongpaisan S, Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiol Rev 1996;60:301-15.
[47] Wang X, Zhao Q, Vargas M, Dong Y, Sriraghavan K, Keiser J, et al., The activity of dispiro peroxides against Fasciola hepatica. Bioorg Med Chem Lett 2011;21:5320-3.
[48] Lowther J, Robinson MW, Donnelly SM, Xu W, Stack CM, Matthews JM, et al., The importance of pH in regulating the function of the Fasciola hepatica cathepsin L1 cysteine protease. PLoS Negl Trop Dis 2009;3:e369.
[49] O'Neill PM, Posner GH, A medicinal chemistry perspective on artemisinin and related endoperoxides. J Med Chem 2004;47:2945-64.
[50] White NJ, Qinghaosu (artemisinin): the price of success. Science 2008;320:330-4.
[51] Vennerstrom JL, Arbe-Barnes S, Brun R, Charman SA, Chiu FC, Chollet J, et al., Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 2004;430:900-4.
[52] Creek DJ, Charman WN, Chiu FC, Prankerd RJ, Dong Y, Vennerstrom JL, et al., Relationship between antimalarial activity and heme alkylation for spiro- and dispiro-1,2,4-trioxolane antimalarials. Antimicrob Agents Chemother 2008;52:1291-6.
[53] Creek DJ, Charman WN, Chiu FC, Prankerd RJ, McCullough KJ, Dong Y, et al., Iron-mediated degradation kinetics of substituted dispiro-1,2,4-trioxolane antimalarials. J Pharm Sci 2007;96:2945-56.
[54] Bousejra-El Garah F, Wong MH, Amewu RK, Muangnoicharoen S, Maggs JL, Stigliani JL, et al., Comparison of the reactivity of antimalarial 1,2,4,5-tetraoxanes with 1,2,4-trioxolanes in the presence of ferrous iron salts, heme, and ferrous iron salts/phosphatidylcholine. J Med Chem 2011;54:6443-55.
[55] Hien TT, Truong NT, Minh NH, Dat HD, Dung NT, Hue NT, et al., A randomized controlled pilot study of artesunate versus triclabendazole for human fascioliasis in central Vietnam. Am J Trop Med Hyg 2008;78:388-92.
[56] Keiser J, Sayed H, El-Ghanam M, Sabry H, Anani S, El-Wakeel A, et al., Efficacy and safety of artemether in the treatment of chronic fascioliasis in egypt: exploratory phase-2 trials. PLoS Negl Trop Dis 2011;5:e1285.
[57] Gallo JM, Pharmacokinetic/ pharmacodynamic-driven drug development. Mt Sinai J Med 2010;77:381-8.
[58] Lee MS, Kerns EH, LC/MS applications in drug development. Mass Spectrom Rev 1999;18:187-279.

[59] Mutschler E, Geisslinger G, Kroemer HK, Schäfer-Korting M, *Mutschler Arzneimittelwirkungen Lehrbuch der Pharmakologie und Toxikologie.* 8 ed. 2001, Stuttgart: Wissenschaftliche Verlaggesellschaft mbH Stuttgart.
[60] Zhou Q, Gallo JM, The pharmacokinetic/pharmacodynamic pipeline: translating anticancer drug pharmacology to the clinic. Aaps J 2011;13:111-20.
[61] Raaflaub VJ, *Pharmakokinetik. Ein Leitfaden für Autodidakten*. 1985, Basel: Editiones Roche.
[62] McMaster MC, *LC/MS a practical user's guide*. 2005, United States of America: Wiley.
[63] de Hoffman E, Stroobant V, *Mass Spectrometry Principles and Applications*. Third ed. 2007: Wiley.

Chapter 2

Activity of OZ78 analogues against *Fasciola hepatica* and *Echinostoma caproni*

Carla Kirchhofer[a,b], Mireille Vargas[a,b], Olivier Braissant[c], Yuxiang Dong[d], Xiaofang Wang[d], Jonathan L. Vennerstrom[d], Jennifer Keiser[a,b,*]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O.Box, CH-4002 Basel, Switzerland

[b] University of Basel, P.O.Box, CH-4003 Basel, Switzerland

[c] Laboratory of Biomechanics and Biocalorimetry, Biozentrum/Pharmazentrum, University of Basel, Basel, Switzerland

[d] University of Nebraska Medical Center, College of Pharmacy, 986025 Nebraska Medical Center, Omaha, NE68198-6025, USA

Published in Acta Tropica, 118 (2011) 56–62

Acta Tropica 118 (2011) 56–62

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Activity of OZ78 analogues against *Fasciola hepatica* and *Echinostoma caproni*

Carla Kirchhofer[a,b], Mireille Vargas[a,b], Olivier Braissant[c], Yuxiang Dong[d], Xiaofang Wang[d], Jonathan L. Vennerstrom[d], Jennifer Keiser[a,b,*]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland
[b] University of Basel, P.O. Box, CH-4003 Basel, Switzerland
[c] Laboratory of Biomechanics and Biocalorimetry, Biozentrum/Pharmazentrum, University of Basel, Basel, Switzerland
[d] University of Nebraska Medical Center, College of Pharmacy, 986025 Nebraska Medical Center, Omaha, NE 68198-6025, USA

ARTICLE INFO

Article history:
Received 14 December 2010
Received in revised form 3 February 2011
Accepted 4 February 2011
Available online 21 February 2011

Keywords:
Fasciola hepatica
Echinostoma caproni
Synthetic peroxides
In vivo studies
In vitro studies
Microcalorimetry

ABSTRACT

The rapid spread of triclabendazole resistance in veterinary medicine is an important motivation for the discovery and development of novel fasciocidal drugs. The aim of this study was to characterize the fasciocidal properties of 1,2,4,5-tetraoxane (MT04 and MT14) and 1,2,4-trioxane (ST16 and ST28) analogues of the fasciocidal drug candidate OZ78, a 1,2,4-trioxolane. Dose response relationships were determined against juvenile and adult *Fasciola hepatica* in rats and *Echinostoma caproni* in mice. The temporal effects of MT04, MT14, ST16, and ST28 compared to OZ78 on the viability of *F. hepatica* were tested *in vitro*. The heat flow of OZ78 and MT04 treated flukes was studied with isothermal microcalorimetry. Finally, surface changes to adult flukes were monitored by scanning electron microscopy (SEM) 18, 24, and 48 h post-treatment of rats with 50 mg/kg MT04. Administration of 50–100 mg/kg of the synthetic peroxides resulted in complete elimination of adult *F. hepatica* from rats. SEM pictures revealed sloughing and blebbing already 18 h post-treatment with MT04. MT04 (100 mg/kg) cured infections with juvenile *F. hepatica*, whereas MT14, ST16, and ST28 showed only low to moderate worm burden reductions. At 300 mg/kg, MT14 was the only compound to completely eliminate worms from *E. caproni* infected mice. MT14 showed the highest activity against juvenile *F. hepatica in vitro*. MT04 was very active against adult *F. hepatica in vitro*, which was confirmed by heat flow measurements. In conclusion, we have identified MT04 as another lead compound with potential against *F. hepatica*, hence further preclinical studies are necessary to determine if MT04 can be considered a drug development candidate.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Fasciola hepatica and *F. gigantica* are hepatic plant-borne trematodes causing fascioliasis (Keiser and Utzinger, 2009; Robinson and Dalton, 2009). Fascioliasis is an important public health problem in many countries on different continents (Bolivia, Chile, Cuba, Ecuador, Egypt, France, Peru, Portugal, and Spain) (Mas-Coma et al., 2007). It has been estimated that more than 91 million people are at risk of infection, with 2.4–17 million infections (Keiser and Utzinger, 2009). In the veterinary field, the economic loss due to fascioliasis of cattle and sheep is enormous (Schweizer et al., 2005).

Today's first line therapy of infections with *Fasciola* spp. is triclabendazole (Fasinex®, Egaten®), a benzimidazole anthelminthic, which is highly effective against immature and mature flukes. The drug is widely available in veterinary medicine but registered in only four countries for the treatment of human fascioliasis (Fairweather, 2009; Fairweather and Boray, 1999). Triclabendazole-resistant *F. hepatica* populations, which have emerged on different continents in sheep and cattle, are of major concern in Australia and North Europe (Moll et al., 2000). Interestingly, resistance to this drug is poorly studied and documented in some parts of the world, such as the Andean Region, where triclabendazole is widely used in cattle (Espinoza et al., 2010).

The rapid spread of triclabendazole resistance is an important motivation for fasciocidal drug discovery. Recent studies have shown that the artemisinins and the synthetic 1,2,4-trioxolane (ozonide) OZ78 have potent flukicidal activity (Halferty et al., 2009; Keiser et al., 2006; Vennerstrom et al., 2004). In an effort to identify more effective trematocidal synthetic peroxides, a structurally diverse library of OZ78 analogues was recently studied. It was found that a peroxide group, a spiroadamantane substructure and an acidic functional group (or an ester prodrug) were required for fasciocidal activity (Zhao et al., 2010). We also observed that 1,2,4-trioxane and 1,2,4,5-tetraoxane isosteres are usually more effective than the corresponding 1,2,4-trioxolanes (unpublished observation).

* Corresponding author at: Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, Socinstr. 57, CH-4002 Basel, Switzerland. Tel.: +41 61 284 8218; fax: +41 61 284 8105.
E-mail address: Jennifer.Keiser@unibas.ch (J. Keiser).

0001-706X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2011.02.003

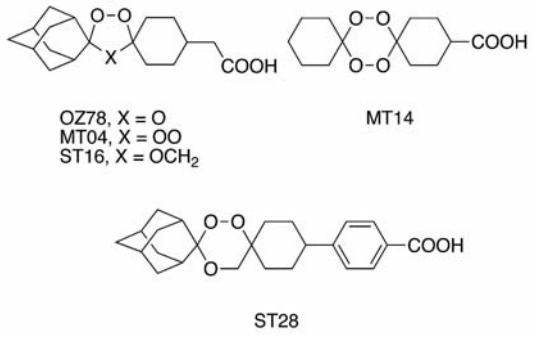


Fig. 1. Chemical structures of MT04, MT14, ST16, ST28, and OZ78.

The aim of the present work was to study and compare the fasciocidal activity of synthetic peroxides MT04, MT14, ST16, and ST28 (Fig. 1). We determined dose–response relationships against juvenile and adult *F. hepatica in vitro* and *in vivo*. We studied the *in vivo* effect of the compounds against the intestinal fluke *Echinostoma caproni*, a non-haematophagous feeder to determine the contribution of haemoglobin digestion to the activity of these peroxides. Finally, scanning electron microscopy (SEM) and isothermal microcalorimetry were used to characterize the fasciocidal properties of MT04 in greater detail.

2. Materials and methods

2.1. Ethical clearance, parasites and host–parasite model

All animal studies were carried out at the Swiss Tropical and Public Health Institute (Basel, Switzerland) and were approved by Swiss and cantonal authorities (permission: 2070). Female Wistar rats ($n = 124$, age: 4–5 weeks, weight: ~150 g) and female NMRI mice ($n = 36$, age: 3–4 weeks, weight: ~20 g) were purchased from Harlan Laboratories (Horst, The Netherlands). Animals were kept in groups of 5 (rats) and 10 (mice) in macrolon cages in environmentally controlled conditions (temperature: ~25 °C; humidity: ~70%; 12 h light/dark cycle) and acclimatized for one week. They had free access to water and rodent diet.

Metacercariae (Pacific Northwest Wild Strain) of *F. hepatica* were purchased from Baldwin Aquatics (Monmouth, OR, USA). Metacercarial cysts of *E. caproni* were obtained from infected *Biomphalaria glabrata* snails kept in our laboratories.

2.2. Test compounds

OZ78, MT04, MT14, ST16, and ST28 were synthesized following literature methods (Tang et al., 2005). The chemical structures of MT04, MT14, ST16, ST28, and OZ78 are depicted in Fig. 1.

For the *in vivo* studies MT04, MT14, ST16, and ST28 were suspended in 7% (v/v) Tween-80 and 3% (v/v) ethanol. Stock solutions of MT04, MT14, ST16, ST28, and OZ78 were prepared in 60% DMSO (v/v) for *in vitro* studies.

2.3. In vivo studies

2.3.1. F. hepatica infection

Approximately 20 metacercarial cysts of *F. hepatica* were orally administered to each rat using the gavage technique. Three (juvenile infection) and eight (adult infection) weeks post-infection, groups of 3 to 6 rats were treated orally with MT04, MT14, ST16, and ST28 at single doses of 25–100 mg/kg. Untreated rats served as controls. One week after treatment, rats were killed using CO_2. The livers of rats harbouring juvenile flukes were flattened and examined for the presence of worms. Adult *F. hepatica* flukes were harvested from the livers and excised bile ducts and placed in Petri dishes. The worm count and the viabilities of all flukes recovered were recorded. The egg counts were not determined during the course of infection.

2.3.2. E. caproni infection

Approximately 35 metacercarial cysts of *E. caproni* were applied to each mouse using the gavage technique. Two weeks post-infection, 5 groups of 3–5 mice were treated orally with MT04, MT14, ST16, and ST28 at single 150–300 mg/kg oral doses. Untreated mice served as control. Seven days after treatment, mice were euthanized by CO_2. At necropsy, all *E. caproni* were removed from the pylorus to the ilecaecal valve of the mice and counted.

2.4. SEM observations

Three rats were infected orally with 20 *F. hepatica* metacercariae each. Eight weeks post-infection, the rats were treated with MT04 (50 mg/kg). At 18, 24, and 48 h post-treatment, respectively one rat was killed by CO_2. Flukes were collected from the livers and bile ducts and fixed for 24 h in 2.5% glutaraldehyde in PBS buffer at room temperature. The specimens were then thoroughly washed with buffer, dehydrated with ethanol and critically point dried (Bomar SPC-900; Tacoma, USA). Flukes were placed on aluminum stubs, sputter-coated with gold of 20 nm (Baltec Med 020, Tucson, USA) and observed in a high-resolution SEM (Philips XL30 ESEM; Eindhofen, The Netherlands) at an accelerating voltage of 5 kV.

2.5. In vitro studies

Adult and juvenile *F. hepatica* flukes were recovered from livers and bile ducts of infected rats. In addition, adult *F. hepatica* collected from infected bovine livers obtained from the local slaughterhouse (Basel, Switzerland) were used. The worms were quickly washed with 0.9% (w/v) NaCl and placed in 6 or 12-well plates (Costar).

Culture medium in each well contained RPMI 1640 (Gibco) at 37 °C, which was supplemented with antibiotics (50 μg/ml streptomycin and 50 IU/ml penicillin; Gibco) and 80 μg/ml of a haemin solution. The haemin solution was prepared as follows: 5 mg haemin was dissolved in 1 ml of 0.1 M aqueous solution of NaOH, and 3.95 ml of PBS (pH = 7.4) and 0.05 ml of 1 M HCl were added to adjust the pH to 7.1–7.4 (Keiser and Morson, 2008). Cultures were kept at 37 °C in an atmosphere of 5% CO_2.

To monitor the temporal drug effect of MT04, MT14, ST16, ST28, and OZ78 *in vitro*, 3–6 flukes were incubated for 72 h in the presence of 50 μg/ml of the test drugs. At 24, 48, and 72 h, worms were examined using a dissecting microscope. For the adult worms, a viability scale ranging from 4 (normal movements) to 1 (death; no movement observed for two min using a microscope) was used. The experiment was repeated 2–4 times. For the juvenile worms, we applied a viability scale from 3 (normal movements observed using a microscope) to 1 (death; no movement observed for two min using a microscope).

2.6. Microcalorimetry

A multi-channel isothermal multi-calorimeter (Model "TAM III", TA instruments, New Castle, DE) was used to monitor the heat-production of *F. hepatica* over time as a result of their metabolic activity. The calorimeter was set at 37 °C two days before the start of the experiment. All materials used were sterilized and drug solutions were sterile filtered (0.2 μm).

Worms recovered from the bile ducts of infected rats were washed and placed in 20 ml glass ampoules containing 3 ml cul-

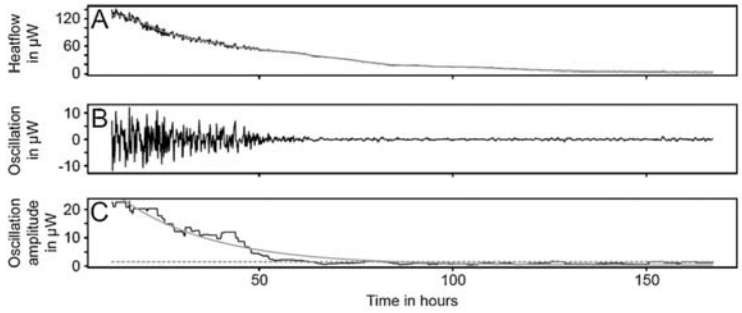


Fig. 2. Noise analysis of adult *F. hepatica*: (A) heat-flow curve of a sample containing 1 adult worm, showing the occurrence of noise (i.e., random oscillations) over time (black curve). Oscillation amplitude values follow exponential decay (grey curve); (B) magnification of oscillations derived from (A); and (C) maximum values of the amplitude over a window of 20 min during the entire course of the experiment. The intersection of the background noise of the calorimetric system (grey dotted line) with the smoothed sample curve (grey curve) is the endpoint and corresponds to the calculated death of the worm.

ture medium supplemented with antibiotics, haemin-solution, and drug solution as described above. The heat-flow of 4 flukes (incubated in 50 μg/ml OZ78), 5 flukes (incubated in 50 μg/ml MT04) and 5 controls was recorded every 10 min over 96 h. Drug effects were analysed by comparing the heat-flow curves of medium containing dead worms or medium only, worms alive with no treatment, and worms incubated in drug solution. Inhibition of activity of adult *F. hepatica* was calculated by comparing (random) oscillation amplitudes, which were derived from the worm motor activities (Fig. 2) of untreated and treated worms (Manneck et al., 2011).

2.7. Statistical analysis

Statistical analyses were performed with version 2.4.5 of StatsDirect statistical software (StatsDirect Ltd; Cheshire, UK). Average worm burdens were expressed as arithmetic means. The Kruskal–Wallis (KW) test was applied to compare the medians of the responses between the treatment and control groups. A difference in median was considered to be significant at a level of 5%. Analyses of noise amplitudes for calorimetric measurements were performed using R software and Microsoft Excel® (R Development Core Team, 2008).

3. Results

3.1. Effect of MT04, MT14, ST16, and ST28 against adult and juvenile F. hepatica harboured in rats

OZ78 analogues MT04, MT14, ST16, and ST28 were first administered as single 100 mg/kg oral doses to rats infected with adult *F. hepatica*. This dose was chosen based on previous findings documented for OZ78 (ED_{50} of 23 mg/kg and ED_{99} of 99 mg/kg) (Duthaler et al., 2010). In a next step, doses were titrated down to 50 and 25 mg/kg. Compound efficacies from these experiments are summarized in Table 1. At 100 mg/kg, all compounds were completely curative. At 50 mg/kg, MT04, ST16, and ST28 resulted in worm burden reductions of 100%, respectively, whereas MT14 produced only a 61% worm burden reduction. At the lowest dose administered (25 mg/kg), MT04, ST16, and ST28 effected worm burden reductions of 71, 88, and 0%, respectively.

Since a fasciocidal drug development candidate should have a broad spectrum of activity, activities against juvenile *F. hepatica* were studied. Compound efficacies of MT04, MT14, ST16, and ST28 administered at 50 and 100 mg/kg oral doses to rats infected with juvenile *F. hepatica* are presented in Table 2. Administration of 100

Table 1
Worm burden reductions achieved against adult *F. hepatica* harboured in rats following the administration of MT04, MT14, ST16, and ST28 at different doses.

Treatment	Dose (mg/kg)	No. of rats investigated	No. of rats cured	Mean worm burden	Total flukes recovered		Total worm burden reduction (%)	KW	P
					Live	Dead			
Control	–[1]	7	0	7.7	54	0	–		
	–[2]	7	0	7	49	0	–		
	–[3]	5	0	2	10	0	–		
	–[4]	5	0	7.2	36	0	–		
	–[5]	5	0	4	20	0	–		
	–[6]	7	0	7.6	53	0	–		
MT04	25[1]	4	0	2.25	9	0	70.8	17.06	<0.001
	50[4]	4	4	0	0	3	100		
	100[4]	4	4	0	0	7	100		
MT14	50[2]	4	1	2.75	11	3	60.7	11.96	<0.001
	100[6]	3	3	0	0	8	100		
ST16	25[3]	4	3	0.25	1	0	87.5	17.48	<0.001
	50[2]	4	4	0	0	1	100		
	100[2]	3	3	0	0	1	100		
ST28	25[5]	4	1	5	20	0	0	2.63	0.105
	50[5]	3	3	0	0	7	100		
	100[5]	3	3	0	0	0	100		

KW: Kruskal–Wallis. Superscript number matches control group with the corresponding treatment group.

Table 2
Worm burden reductions achieved against juvenile *F. hepatica* harboured in rats following the administration of MT04, MT14, ST16, and ST28 at two different doses.

Treatment	Dose (mg/kg)	No. of rats investigated	No. of rats cured	Mean worm burden	Total flukes recovered		Total worm burden reduction (%)	KW	P
					Live	Dead			
Control	–[1]	7	0	7	49	0	–		
	–[2]	4	0	6.75	27	0	–		
	–[3]	3	0	8.33	25	0	–		
MT04	50[1]	4	0	2.75	11	0	60.7	13.26	<0.001
	100[2]	6	6	0	0	2	100		
MT14	50[3]	4	0	7.75	31	0	7.0	0.52	0.469
	100[3]	4	0	6.75	27	0	19.0		
ST16	50[2]	4	0	5.75	23	0	14.8	1.44	0.230
	100[2]	4	0	3.75	15	0	44.4		
ST28	50[3]	4	0	10.5	42	0	0	0.17	0.679
	100[3]	4	0	4.5	18	0	46.0		

KW: Kruskal–Wallis. Superscript number matches control group with the corresponding treatment group.

and 50 mg/kg MT04 resulted in worm burden reductions of 100 and 61%, respectively, an outcome almost identical to that previously observed for OZ78, which at the same doses, decreased worm burden by 100 and 67% (Keiser et al., 2006). A significant difference was observed between MT04 treated and untreated rats in the juvenile infection model (KW = 13.26; $P < 0.001$). On the other hand, low to moderate worm burden reductions (0–46%) were observed for ST16, ST28, and MT14.

3.2. Effect of OZ78 analogues against E. caproni

We assessed the efficacies of the 4 OZ78 derivatives against the non-blood feeder *E. caproni* to obtain further insight into the mechanism of action of these compounds. In more detail, our goal was to determine whether trematocidal activity entirely depends on haeme iron-mediated reactivity or whether also other targets are involved. In addition, as juvenile *F. hepatica* show a preference for hepatic cells rather than blood (Dawes, 1961) we were wondering whether there would be a relationship between an echinostomicidal activity and activity against juvenile *F. hepatica.* At 300 mg/kg, MT04, ST16, and ST28 showed no activity against *E. caproni* in mice. In comparison, 1000 mg/kg OZ78 was required for good echinostomicidal activity (Keiser et al., 2006). On the other hand, a worm burden reduction of 100% was observed with MT14 at 300 mg/kg (Table 3).

3.3. In vitro activity against juvenile and adult F. hepatica

The temporal effects of MT04, MT14, ST16, ST28, and OZ78 (50 μg/ml) on adult *F. hepatica in vitro* collected from rats and bovine are presented in Fig. 3A and B. Control *Fasciola* showed normal movements at all examination time points. Flukes obtained from rats incubated in the presence of MT04 showed reduced activities at the 24 h time point (mean viability: 2.3). Twenty-four hours later, only minimal viability was observed (mean viability: 1.6). 72 h post-incubation with MT04, all flukes were dead. Bovine flukes incubated with 50 μg/ml MT04 showed strongly reduced viabilities 72 h post-incubation (mean viability: 1.6). Flukes incubated with ST28 and OZ78 showed reduced movements 72 h post exposure (mean viabilities rat flukes: 2.1 and 2.0, respectively and mean viabilities bovine flukes: 1.9 and 1.8, respectively). Slightly contradictory results were observed with ST16: while flukes obtained from rats were highly affected by the drug 72 h post-incubation (mean viability: 1.3) a less pronounced effect on *Fasciola* obtained from bovines was observed at this examination time point (mean viability: 2.1). Finally, the majority of worms incubated with MT14 had died 72 h post-exposure (mean viability: 1.2 rat flukes and mean viability: 1.1 bovine flukes).

The fasciocidal activities of the test drugs against juvenile *F. hepatica in vitro* are presented in Fig. 3C. Control flukes were alive for 72 h. Incubation with MT14 (50 μg/ml) resulted in death of all *F. hepatica* 48 h post-incubation. MT04, ST16, and OZ78 showed no effect against juvenile flukes *in vitro* (mean viability after 72 h: 2.7, 2.8 and 2.4, respectively). *F. hepatica* incubated in ST28 showed reduced movements after 72 h (mean viability: 1.5).

3.4. Microcalorimetry of adult F. hepatica

Thermogenic noise value curves of control adult *F. hepatica* and worms incubated with 50 μg/ml MT04 and OZ78 are depicted in

Table 3
Worm burden reductions achieved against adult *E. caproni* harboured in mice following the administration of MT04, MT14, ST16, and ST28 at different doses.

Treatment	Dose (mg/kg)	No. of mice investigated	No. of mice cured	Mean worm burden	Total flukes recovered		Total worm burden reduction (%)	KW	P
					Live	Dead			
Control	–[1]	7	0	19.9	139	0	–		
	–[2]	5	0	24.2	121	0	–		
	–[3]	5	0	29.6	148	0	–		
MT04	300[3]	4	1	13.5	54	0	54.4	5.46	0.020
MT14	150[2]	5	1	15	75	0	38.0	5.59	0.018
	300[1]	3	3	0	0	0	100		
ST16	300[2]	3	0	25.7	77	0	0	0.02	0.882
ST28	300[2]	4	0	20	80	0	17.4	1.54	0.215

KW: Kruskal–Wallis. Superscript number matches control group with the corresponding treatment group.

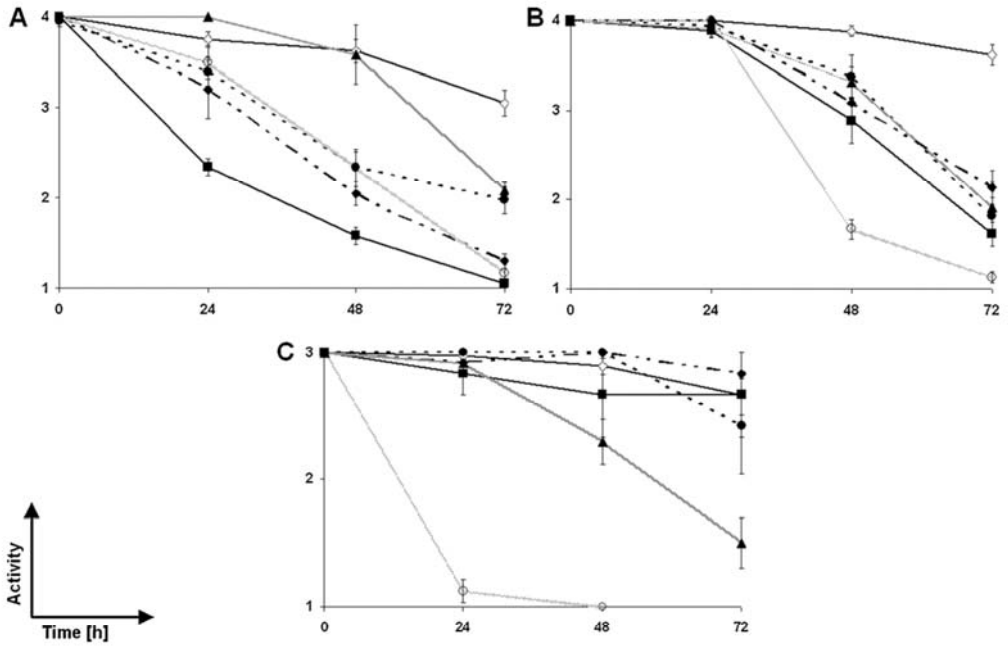


Fig. 3. (A) *In vitro* activity of MT04, MT14, ST16, and ST28 at a concentration of 50 μg/ml against adult *F. hepatica* (obtained from rats) compared to control worms and worms incubated with OZ78 (50 μg/ml). (B) *In vitro* activity of MT04, MT14, ST16, and ST28 at a concentration of 50 μg/ml against adult *F. hepatica* (obtained from bovine livers) compared to control worms. (C) *In vitro* activity of 50 μg/ml MT04, MT14, ST16, and ST28 against juvenile *F. hepatica* compared to control worms and worms incubated with OZ78 50 μg/ml. Black line with white diamond: control; black dotted line with black circle: OZ78; black line with black square: MT04; grey line with white circle: MT14; dotted and dashed black line with black diamond: ST16; grey line with black triangle: ST28. The limits of the whiskers correspond to the standard error of the mean values per time point.

Fig. 4. Consistently low signals of 1.46 μW were measured for dead worms or medium only (data not shown). The intersection of the sample amplitude curve (following exponential decay) with the background signal noise of dead worms (1.46 μW) was set as an endpoint of worm motility. Worms incubated with MT04 and OZ78 were dead after 29.6 h and 43.4 h, respectively. Control worms were viable for 69.3 h.

3.5. *In vivo* SEM observations

SEM studies were only performed with MT04 since it was the most efficacious analogue of OZ78. At 18 h post-treatment with 50 mg/kg of MT04, 6 flukes were collected from a rat and processed for SEM. Disruption of the tegument was visible, in particular on the anterior region of *F. hepatica* where blebbing and sloughing were

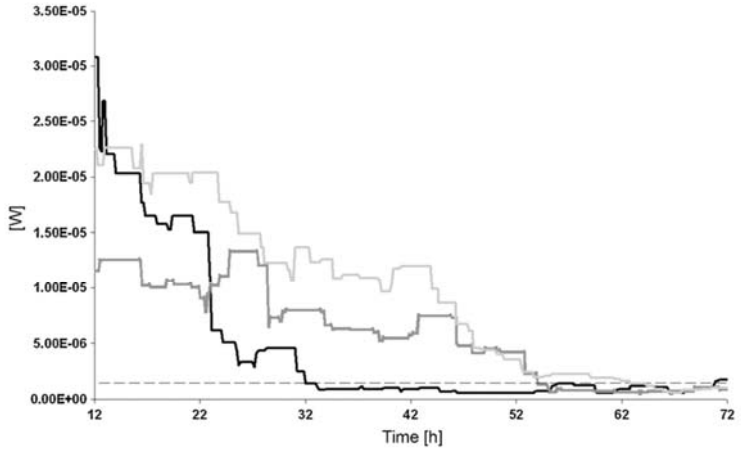


Fig. 4. Absolute noise values of untreated and treated worms (OZ78 50 μg/ml and MT04 50 μg/ml). Dotted black line: instrument background; black line: MT04; dark-grey shaded line: OZ78; light-grey line: control.

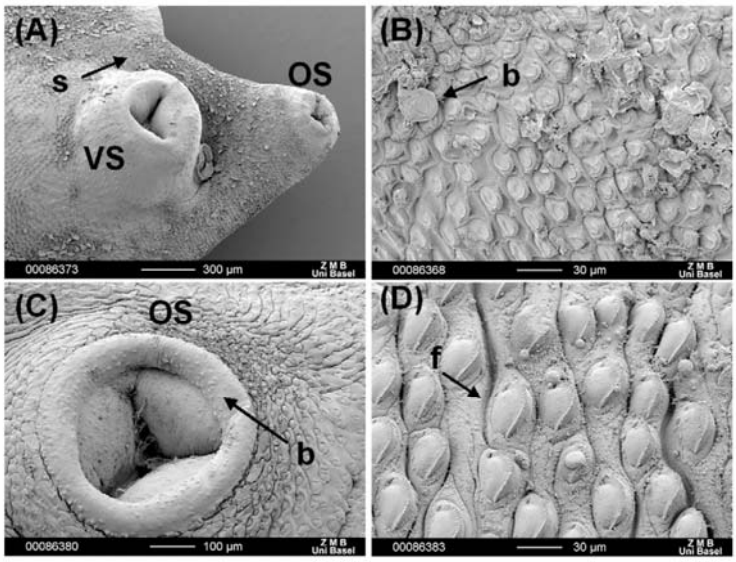


Fig. 5. (A and B) SEM observation of adult *F. hepatica* 18 h post treatment with 50 mg/kg MT04. (A) Disruption and sloughing (s) of the tegument near the oral sucker (OS). (B) Blebbing (b) observed on the tegument. (C and D) SEM observation of adult *F. hepatica* 24 h post treatment with 50 mg/kg MT04. (C) Blebs in the OS region and (D) furrows (f) visible in the mid body region.

observed (Fig. 5A and B). Twenty-four hours post-treatment, we collected 2 dead specimens and 1 *F. hepatica* that showed minor activity. Similar abnormalities such as blebbing and furrowing, which had not progressed further in severity, were observed on these worms (Fig. 5C and D). Forty-eight hours post-treatment only dead *F. hepatica* were recovered and since flukes were broken they were not processed for SEM analyses.

4. Discussion

Triclabendazole is an ideal fasciocidal drug as it is orally active against both juvenile and adult *F. hepatica* (Fairweather and Boray, 1999). However, since drug resistance is spreading it is imperative that novel fasciocidal drugs are discovered and developed. The synthetic ozonides seem to offer an excellent starting point as recent studies showed that OZ78 is active against adult and juvenile *F. hepatica in vitro* and *in vivo*, including resistant isolates (Keiser and Utzinger, 2007). In the present work, the fasciocidal activities of 4 OZ78 analogues were studied in greater detail.

MT04 had the highest activities against both juvenile and adult *F. hepatica in vivo*. MT04 was superior to OZ78, in particular against adult *F. hepatica*. A single 50 mg/kg oral dose of MT04 achieved complete worm burden reductions against adult *F. hepatica* in rats, while 100 mg/kg doses of OZ78 were required to cure *F. hepatica* infected rats (Keiser et al., 2006). Forty-eight hours after treatment with 50 mg/kg MT04, only dead flukes were recovered from a rat. Flukes collected at earlier time points showed disrupted teguments including sloughing and blebbing and some flukes had already died. Comparable tegumental alterations (blebs, sloughing, and furrows) were also seen 24–72 h after treatment with 100 mg/kg OZ78 (Keiser and Morson, 2008). The main difference observed between the two drugs was the onset of action. 18–24 h after treatment with MT04, *F. hepatica* showed reduced viabilities or had already died, whereas dead worms were collected from OZ78-treated rats 72 h post-treatment (Keiser and Morson, 2008). Whether differences in *in vivo* efficacy and the onset of action between the two compounds derive from pharmacodynamic or pharmacokinetic parameters is not clear, but it is evidently a function of their two different peroxide heterocycles. In this respect, O'Neill et al. (2010) have recently shown that the red blood cell stability of tetraoxanes is higher than that of the corresponding trioxolanes (ozonides). The mechanism of action of the secondary ozonides against *Fasciola* spp. has not yet been elucidated. However, a formation of carbon-centered radicals, similar to the antimalarial mechanism of action might play a role (Dong et al., 2010).

Our *in vitro* studies on adult *F. hepatica* confirmed the excellent flukicidal activity of MT04. After 72 h, the majority of adult worms incubated in presence of 50 µg/ml MT04 were dead. It is interesting to note that OZ78 and MT04 did not show any effect against juvenile *F. hepatica in vitro* in line with results obtained with OZ78 in a recent study (Duthaler et al., 2010). Why juveniles are affected *in vivo*, but not *in vitro* is not known, but drug metabolism may account for these differences. A good relationship with regard to compound sensitivity was observed between the *F. hepatica* Pacific Northwest wild strain harboured in rats and bovine slaughterhouse isolates, although flukes obtained from infected bovine livers were slightly less susceptible to the test drugs.

We speculated that a drug effect against *E. caproni* might point to an activity against juvenile *F. hepatica*, since both parasites do not feed on large quantities of blood (Dawes, 1961; Keiser and Utzinger, 2007). However, no relationship was observed between drug sensitivities on echinostomes and juvenile *F. hepatica*. Though ST16 and ST28 lacked activity against both parasite stages, MT14 had activity against *E. caproni*, while lacking activity against juvenile *F. hepatica in vivo*. On the other hand, MT04 revealed no activity against echinostomes but cured infections with juvenile *F. hepatica*.

We have shown for the first time that heat flow measurements are an excellent tool to study the effects of fasciocidal drugs. The usefulness of this method to study drug effects on helminths has recently been demonstrated for another trematode, namely *Schistosoma mansoni* (Manneck et al., 2011). In the present work, heat flow measurements confirmed data obtained by morphological *in vitro* testing. Microcalorimetry showed that worms incubated

in MT04 died earlier than worms incubated with OZ78. Compared to our standard *in vitro* assays, untreated worms died earlier (69 h), which might be due to a lack of oxygen in the calorimetry vials. Further studies are currently ongoing in our laboratories, including experiments with juvenile flukes and the reference drug triclabendazole in order to validate and standardize the use of microcalorimetry to study drug effects on *Fasciola* spp.

In conclusion, this assessment of 4 promising synthetic peroxide derivatives of OZ78 has identified MT04 as another lead compound with potential against *F. hepatica* and perhaps other haemoglobin-degrading flukes. We anticipate that ongoing pharmacokinetic and mechanism of action studies with MT04 should provide the necessary data to determine if MT04 can be considered a drug development candidate.

Acknowledgements

This investigation received financial support from the Swiss National Science Foundation (project no. PPOOA–114941) and the NIH (Grant R21AI076783).

We are thankful to Mrs. Evi Bieler at the Center for Microscopy of University Basel for expert help with the SEM pictures. Thanks are also addressed to Urs Duthaler for his laboratory support and scientific help.

References

Dawes, B., 1961. Juvenile stages of *Fasciola hepatica* in the liver of the mouse. Nature 190, 646–647.

Dong, Y., Wittlin, S., Sriraghavan, K., Chollet, J., Charman, S.A., Charman, W.N., Scheurer, C., Urwyler, H., Santo Tomas, J., Snyder, C., Creek, D.J., Morizzi, J., Koltun, M., Matile, H., Wang, X., Padmanilayam, M., Tang, Y., Dorn, A., Brun, R., Vennerstrom, J.L., 2010. The structure–activity relationship of the antimalarial ozonide arterolane (OZ277). J. Med. Chem. 53, 481–491.

Duthaler, U., Smith, T.A., Keiser, J., 2010. *In vivo* and *in vitro* sensitivity of *Fasciola hepatica* to triclabendazole combined with artesunate, artemether, or OZ78. Antimicrob. Agents Chemother. 54, 4596–4604.

Espinoza, J.R., Terashima, A., Herrera-Velit, P., Marcos, L.A., 2010. Human and animal fascioliasis in Peru: impact in the economy of endemic zones. Rev. Peru Med. Exp. Salud Publica 27, 604–612.

Fairweather, I., Boray, J.C., 1999. Fasciolicides: efficacy, actions, resistance and its management. Vet. J. 158, 81–112.

Fairweather, I., 2009. Triclabendazole progress report, 2005–2009: an advancement of learning? J. Helminthol. 83, 139–150.

Halferty, L., O'Neill, J.F., Brennan, G.P., Keiser, J., Fairweather, I., 2009. Electron microscopical study to assess the *in vitro* effects of the synthetic trioxolane OZ78 against the liver fluke, *Fasciola hepatica*. Parasitology 136, 1325–1337.

Keiser, J., Utzinger, J., Tanner, M., Dong, Y., Vennerstrom, J.L., 2006. The synthetic peroxide OZ78 is effective against *Echinostoma caproni* and *Fasciola hepatica*. J. Antimicrob. Chemother. 58, 1193–1197.

Keiser, J., Utzinger, J., 2007. Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. Trends Parasitol. 23, 555–562.

Keiser, J., Morson, G., 2008. *Fasciola hepatica*: surface tegumental responses to *in vitro* and *in vivo* treatment with the experimental fasciolicide OZ78. Exp. Parasitol. 119, 87–93.

Keiser, J., Utzinger, J., 2009. Food-borne trematodiases. Clin. Microbiol. Rev. 22, 466–483.

Manneck, T., Braissant, O., Ellis, W., Keiser, J., 2011. *Schistosoma mansoni*: antischistosomal activity of the four optical isomers and the two racemates of mefloquine on *schistosomula* and adult worms *in vitro* and *in vivo*. Exp. Parasitol. 127, 260–269.

Mas-Coma, S., Bargues, M.D., Valero, M.A., 2007. Plant-borne trematode zoonoses: fascioliasis and fascioliopsiasis. In: Food-Borne Parasitic Zoonoses. Fish and Plant-Borne Parasites , In: Murrell, K.D., Fried, B. (Eds.), World Class Parasites, vol. 11. Springer, New York, NY, pp. 293–334.

Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The Netherlands. Vet. Parasitol. 91, 153–158.

O'Neill, P.M., Amewu, R.K., Nixon, G.L., Bousejra ElGarah, F., Mungthin, M., Chadwick, J., Shone, A.E., Vivas, L., Lander, H., Barton, V., Muangnoicharoen, S., Bray, P.G., Davies, J., Park, B.K., Wittlin, S., Brun, R., Preschel, M., Zhang, K., Ward, S.A., 2010. Identification of a 1,2,4,5-tetraoxane antimalarial drug-development candidate (RKA 182) with superior properties to the semisynthetic artemisinins. Angew. Chem. Int. Ed. Engl. 49, 5693–5697.

R Development Core Team, 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Robinson, M.W., Dalton, J.P., 2009. Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos. Trans. R. Soc. Lond. B: Biol. Sci. 364, 2763–2776.

Schweizer, G., Braun, U., Deplazes, P., Torgerson, P.R., 2005. Estimating the financial losses due to bovine fasciolosis in Switzerland. Vet. Rec. 157, 188–193.

Tang, Y., Dong, Y., Wang, X., Sriraghavan, K., Wood, J.K., Vennerstrom, J.L., 2005. Dispiro-1,2,4-trioxane analogues of a prototype dispiro-1,2,4-trioxolane: mechanistic comparators for artemisinin in the context of reaction pathways with iron(II). J. Org. Chem. 70, 5103–5110.

Vennerstrom, J.L., Arbe-Barnes, S., Brun, R., Charman, S.A., Chiu, F.C., Chollet, J., Dong, Y., Dorn, A., Hunziker, D., Matile, H., McIntosh, K., Padmanilayam, M., Santo Tomas, J., Scheurer, C., Scorneaux, B., Tang, Y., Urwyler, H., Wittlin, S., Charman, W.N., 2004. Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 430, 900–904.

Zhao, Q., Vargas, M., Dong, Y., Zhou, L., Wang, X., Sriraghavan, K., Keiser, J., Vennerstrom, J.L., 2010. Structure–activity relationship of an ozonide carboxylic acid (OZ78) against *Fasciola hepatica*. J. Med. Chem. 53, 4223–4233.

Chapter 3

Development and validation of a liquid chromatography/mass spectrometry method for pharmacokinetic studies of OZ78, a fasciocidal drug candidate

Carla Kirchhofer[a,b], Jennifer Keiser[a,b,*], Jörg Huwyler[c,d]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O.Box, CH-4002Basel, Switzerland

[b] University of Basel, P.O.Box, CH-4003Basel, Switzerland

[c] Institute of Pharma Technology, University of Applied Sciences, Northwestern Switzerland, Gründenstrasse40, CH-4132Muttenz, Switzerland

[d] Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland

Published in Journal of Chromatography B, 878 (2010) 2770–2774

Journal of Chromatography B, 878 (2010) 2770–2774

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of a liquid chromatography/mass spectrometry method for pharmacokinetic studies of OZ78, a fasciocidal drug candidate

Carla Kirchhofer[a,b], Jennifer Keiser[a,b,*], Jörg Huwyler[c,d]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland
[b] University of Basel, P.O. Box, CH-4003 Basel, Switzerland
[c] Institute of Pharma Technology, University of Applied Sciences, Northwestern Switzerland, Gründenstrasse 40, CH-4132 Muttenz, Switzerland
[d] Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland

ARTICLE INFO

Article history:
Received 11 May 2010
Accepted 14 August 2010
Available online 21 August 2010

Keywords:
OZ78
Fascioliasis
Secondary ozonides
Trioxolanes
Liquid chromatography
Mass spectrometry

ABSTRACT

Fascioliasis is a zoonotic disease of considerable public health and great veterinary significance and new drugs are needed. OZ78 is a promising fasciocidal drug candidate. In order to support the development of OZ78, including pharmacokinetic (PK) studies an accurate, precise, and selective liquid chromatography/mass spectrometry (LC/MS) method for OZ78 was developed for sheep plasma and validated in accordance with the US Food and Drug Administration Guidance on Bioanalytical Method Validation. Protein precipitation was used for sample clean up. Separation was performed through a Phenomenex C8(2) analytical column (50.0 mm × 2.0 mm, 5 μm) with a mobile phase of acetonitrile (buffer B) and 5 mM ammonium formate (buffer A) at a flow-rate of 0.3 mL/min and a gradient from 20% to 95% acetonitrile. The mass spectrometer was operated under selected ion monitoring, and orifice voltage set to −4.1 kV and ion spray temperature to 400 °C. Nitrogen was used as a nebulizer, curtain, and collision gas. OZ78 was monitored at 321.4 *m/z* (deprotonated parent compound, M-). The validated linear dynamic range was between 156.25 ng/mL and 5 μg/mL and the achieved correlation coefficient (r^2) was greater than 0.99. The validation results demonstrated that the developed LC/MS method is precise, accurate, and selective for the determination of OZ78 in sheep plasma. The method was successfully applied to the evaluation of the PK profile of OZ78 in sheep.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The secondary ozonide, 1,2,4-trioxolane OZ78 belongs to a new class of synthetic peroxide antimalarials [1]. In contrast to the semisynthetic artemisinins, currently the most important antimalarials available [2], 1,2,4-trioxolanes are characterized by excellent biopharmaceutical properties and low toxicities [1]. OZ277 combined with piperaquine is undergoing phase III clinical testing [3]. Another trioxolane antimalarial candidate, OZ439, is currently in phase IIa trials [4].

The 1,2,4-trioxolanes do not only possess antiplasmodial but also trematocidal activities, since hemoglobin metabolism is common in plasmodia and several trematodes including *Fasciola* spp. [3]. A recent structure-activity study evaluating 26 OZ derivatives in the *Fasciola hepatica* rat model, showed OZ78 to have an optimized ozonide structure for efficacy against *F. hepatica*. At a single oral dose of 100 mg/kg, OZ78 cured acute (immature flukes) and chronic (adult flukes) *F. hepatica* infections in rats, including infections with resistant isolates [5]. Addition of a new drug in the current drug armamentarium for the treatment of trematode infections, which are neglected tropical diseases [6,7], would be most welcome. Currently the treatment of fascioliasis in humans relies on one single drug, triclabendazole. Though triclabendazole resistance has not been shown in humans, resistance to this drug is widely spreading in sheep and cattle [8,9]. In veterinary medicine additional drugs are commonly used in the treatment of infections with *Fasciola* spp. including albendazole, closantel, hexachlorophene, mebendazole, nitroxynil, rafoxanide, and triclabendazole [9]. Different analytical methods have been established in order to quantify these drugs and their corresponding metabolites in biological fluids or tissues. For example, a HPLC with fluorescence detection has recently been developed to screen closantel and rafoxanide in animal muscles [10].

An efficacy and tolerability study with OZ78 in target animals, *F. hepatica* infected sheep, was recently conducted in Australia.

Abbreviations: ESI, electrospray ionization; IS, internal standard; LC/MS, liquid chromatography/mass spectrometry; LLOQ, lowest limit of quantification; OZ, ozonide; PK, pharmacokinetic; QC, quality control; RSD, relative standard deviation; USFDA, US Food and Drug Administration.

* Corresponding author at: Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland. Tel.: +41 61 284 8218; fax: +41 61 284 8105.
E-mail address: jennifer.keiser@unibas.ch (J. Keiser).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.08.020

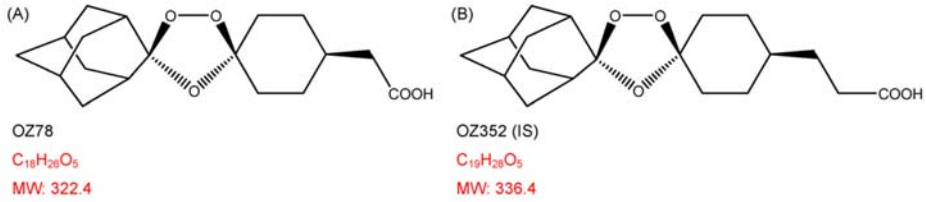


Fig. 1. Structures of (A) OZ78 and (B) OZ352 [internal standard (IS)].

Sheep were administered single oral or subcutaneous doses of 50 mg/kg OZ78. Tolerability, egg reduction rates, and worm burden reductions were recorded. In addition, serum samples were collected at selected time points [12]. It was the aim of the present study to develop and validate a LC/MS method to quantify OZ78 in sheep plasma to support analysis of the pharmacokinetic (PK) parameters of OZ78 of this study and future preclinical trials.

2. Experimental

2.1. Chemicals and reagents

OZ78 and OZ352 (internal standard, IS) were synthesized at the College of Pharmacy, University of Nebraska Medical Center (Omaha, USA). The chemical structures of OZ78 and OZ352 are depicted in Fig. 1.

Acetonitrile and methanol were of HPLC grade and were obtained from Bisolve (Valkenswaard, Netherlands) and J.T. Baker (Deventer, Netherlands), respectively. Ammonium formate was purchased from Fluka Analytical (Buchs, Switzerland). Ultrapure water was obtained from a Millipore Milli-Q water purification system and applied for the preparation of mobile phase. Blank sheep serum and plasma were obtained from Novartis Animal Health (Kemps Creek, Australia) and the local slaughterhouse (Basel, Switzerland).

2.2. LC/MS/MS system and conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-20AD pumps, a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), and an online DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan). Chromatographic separation was performed through a Phenomenex C8(2) (50.0 mm × 2.0 mm, 5 μm, Brechbühler AG, Schlieren, Switzerland) column coupled with a Phenomenex security guard used at room temperature. The following gradient was used: 0–4 min, B 20–35%; 4–7 min, B 35–65%; 7–8 min, B 65–95%; 8–10 min, 95%; 10–12 min, B 95–20%; 12–14 min B 20%, where A was 5 mM ammonium formate in ultra pure water and B was 100% acetronitrile. The flow-rate of the mobile phase was set at 0.3 mL/min.

Table 1
LC/MS working parameters.

Parameter	Value
Source temperature	400 °C
Nebulizer gas (NEB)	12 L/min
Curtain gas (CUR)	9 L/min
Declustering potential (DP)	−51 V
Focusing potential (FP)	−335 V
Entrance potential (EP)	−11 V
Ion spray voltage (IS)	−4.1 kV
Polarity of analysis	Negative
Detected mass for OZ78 (*m/z*)	322.4/321.4
Detected mass for IS (*m/z*)	336.4/335.4

An API 365 triple–quadrupole mass spectrometer (PE Biosystems, Foster City, CA) equipped with a turbo ion spray source was operated in negative ionization mode. The major working parameters are summarized in Table 1. Instrument control and data analyses were performed using the Analyst 1.4.2. software package (PE Biosystems, Foster City, CA).

2.3. Standard, QC and IS preparation

Stock solutions (OZ78 150 μg/mL, IS 150 μg/mL) were prepared in methanol. Appropriate volumes of stock solutions were serially

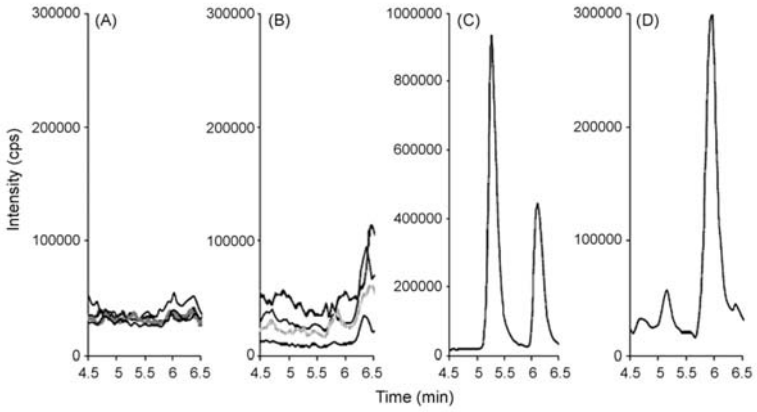


Fig. 2. Chromatograms of OZ78 and the internal standard OZ352 covering a retention time of 4.5–6.5 min: (A) blank sheep serum ($n = 4$), (B) blank sheep plasma ($n = 4$), (C) mobile phase spiked with OZ78 5 μg/mL and OZ352 (IS) 2 μg/mL, and (D) spiked plasma at concentrations of 0.3125 μg/mL (OZ78) and 2 μg/mL (OZ352).

Table 2
Intra- and inter-assay accuracy and precision.

Nominal concentration (μg/mL)	Intra-assay ($n = 5$)			Inter-assay ($n = 20$)		
	Mean concentration (μg/mL)	RSD (%)	Accuracy (%)	Mean concentration (μg/mL)	RSD (%)	Accuracy (%)
0.3125	0.30	11.07	95.52	0.29	6.27	92.68
	0.29	3.87	91.54			
	0.28	4.66	91.04			
	0.29	5.29	93.19			
1.25	1.28	3.71	102.11	1.23	5.99	98.48
	1.21	6.00	96.79			
	1.17	6.77	93.91			
	1.26	4.63	101.13			
5	5.24	3.54	104.73	4.79	8.09	95.90
	4.65	4.78	93.06			
	4.51	5.06	90.15			
	4.87	9.59	97.43			

diluted with methanol to obtain OZ78 working solutions from 4.7 to 150 μg/mL or a working IS solution at 60 μg/mL.

Calibration samples were freshly prepared and included in each analytical run by the dilution of working solutions with blank sheep plasma, resulting in concentrations of 5000, 2500, 1250, 625, 312.5, and 156.25 ng/mL.

Blank sheep plasma was used for method development and the preparation of calibrators and quality control (QC) samples.

2.4. Plasma sample extraction procedure

A 300 μL plasma aliquot was vortex-mixed with 10 μL of IS working solution. For protein precipitation 900 μL of ice-cooled methanol was added to each sample and vortex-mixed for 1 min. The samples were cooled on ice for 10 min and subsequently centrifuged for 15 min at 16,100 × g (Eppendorf centrifuge 5415 R, Hamburg, Germany) at 4 °C. The supernatant was transferred to a 1.5 mL microtube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 μL methanol and 150 μL acetonitrile in water (30:70, v/v), vortex-mixed, and then transferred to an auto-sampler vial. The auto-sampler rack was cooled to 6 °C. Finally, a 20 μL aliquot of each sample was injected into the LC/MS/MS system for analysis.

2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, recovery, and stability according to the US Food and Drug Administration (USFDA) guidelines [11].

2.5.1. Selectivity

Frozen blank sheep serum and plasma were examined for potential interferences with endogenous substances using the proposed extraction procedure but without adding any internal standard working solution.

2.5.2. Calibration curves

Four calibration curves were established using the Internal Standard method, plotting the peak-area ratios of OZ78 to the IS vs. the concentration of OZ78. Six point standard calibration curves, covering the range of concentrations expected in sheep (156.25, 312.5, 625, 1250, 2500, and 5000 ng/mL) were calculated and fitted by a linear regression.

2.5.3. Lowest limit of quantification (LLOQ)

The lowest limit of quantification (LLOQ) was chosen as the minimum concentration in plasma samples which could be determined with a standard deviation (SD) below 15%.

2.5.4. Accuracy and precision

Intra- and inter-day assay precisions were calculated using the relative standard deviation (RSD%). Intra- and inter-day assay accuracies were calculated as the ratio of the measured concentration to the nominal concentration multiplied by 100%.

Data from replicate analysis of QC samples at three different concentrations (312.5, 1250, and 5000 ng/mL, $n = 5$) on four different days were used to calculate the intra-day precision and accuracy as well as the inter-assay precision and accuracy. The USFDA recommended acceptance criterion (percentage of deviation between theoretical and back-calculated concentrations less than ±15%) was used in our study [11].

2.5.5. Recovery

Absolute recovery of OZ78 was determined by comparing the absolute peak areas obtained from QC samples with those of OZ78 spiked plasma after extraction at three concentrations (312.5, 1250, and 5000 ng/mL, $n = 4$). The recovery value of the IS was determined at a single concentration of 2000 ng/mL.

2.5.6. Stability

Stability studies included autosampler stability, bench-top stability, and freeze-thaw stability. For the autosampler stability QC samples (1.25 and 5 μg/mL, $n = 3$) were measured over a period of 30 h.

Freeze–thaw stability was tested by the analysis of 1.25 and 5 μg/mL of QC samples ($n = 3$) in two freeze and thaw cycles (1 h RT/1 h −80 °C/1 h RT/1 h −80 °C).

Bench-top stability was determined by analyzing QC samples (1.25 and 5 μg/mL, $n = 3$) stored over a time period of 4 h at RT. Quality control samples of 1.25 and 5 μg/mL (injected immediately after preparation) served as control.

The solutions were accepted as stable with a deviation of not more than ±15%.

Table 3
Absolute recovery (ARE) of OZ78 and IS.

Analyte	Nominal concentration (μg/mL)	ARE ($n = 4$)	
		Mean (%)	RSD (%)
OZ78	0.3125	84.36	15.33
	1.25	69.58	7.93
	5	78.57	7.71
IS	2	85.84	6.90

Table 4
Stability analysis of quality control samples for OZ78 in sheep plasma ($n = 3$).

Nominal concentration (μg/mL)	Calculated concentration (μg/mL)	RSD (%)	Accuracy (%)
Autosampler stability (6 °C for 30 h after processing)			
1.25	1.21	6.22	97.01
5	5.43	9.96	108.67
Bench-top stability (room temperature for 4 h)			
1.25	1.29	11.44	103.25
5	5.46	6.93	109.22
Freeze–thaw stability (two cycles)			
1.25	1.27	8.51	101.85
5	5.39	3.18	107.87

2.6. Pharmacokinetic study

Details of the pharmacokinetic study have been presented elsewhere [12]. Briefly, serum samples were collected at Yarrandoo R & D Centre, Novartis Animal Health, Kemps Creek, NSW 2178, Australia. Twelve sheep were selected from the Yarrandoo Merino lamb mob (3 months of age at commencement, weights 20.8–30.2 kg). On day −2, all study animals were bled for pre-treatment pharmacokinetic data. On day 0, blood samples were withdrawn from the jugular vein into vacutainers from all sheep of the group treated with 50 mg/kg OZ78 subcutaneously and from all sheep of the group treated with 50 mg/kg OZ78 orally at 0.5, 1, 2, 4, 8, 24, 36, and 168 h post-treatment.

3. Results and discussion

3.1. Method development

OZ78 is a novel fasciocidal drug candidate, for which to date no analytical method has been developed and validated. In the framework of developing the antimalarial drug development candidate OZ277, levels of OZ78 were quantified in rat plasma using negative electrospray ionization (ESI), however only few details of the analytical method had been presented [1]. A recently developed analytical method for the related molecule OZ277, characterized by an amide functional group and its polar metabolites was based on hydrophilic interaction chromatography in combination with mass spectrometry using positive ESI [13]. However, this method is not suitable for the determination of OZ78 in biofluids, due to its acidic functional group.

In a first step, an IS had to be chosen. A number of OZs sharing structural similarities with OZ78 were evaluated as possible IS. Best results were obtained with OZ352. As depicted in Fig. 1, the only difference between OZ352 and OZ78 is an extension of the connecting alkyl link of the carboxylic acid functional group in OZ352.

After the IS had been selected, the MS conditions were optimized. MS optimization was performed by directly infusing solutions of OZ78 and OZ352 (IS) (10 μg/mL in methanol) into the electrospray injection unit of a mass spectrometer at a constant flow-rate of 10 μL/min. Quadrupole full scans (Q1 scans) were carried out in negative ion detection mode to optimize ESI conditions. As expected, no peak was detected with acceptable sensitivity in the positive mode. The mass spectra of OZ78 and OZ352 revealed base peaks at m/z 321.4 and 335.4, respectively, corresponding to the deprotonated parent molecules.

The MS parameters were set to maximize the amounts of deprotonated parent ions produced (Table 1).

In a second step, the LC conditions were refined. Different tests were done with varying concentrations of ammonium formate, and hence different pH values. Best results were obtained with 5 mM ammonium formate at a pH of 6.0 ± 0.3, resulting in symmetric peaks with a good ratio of height to width. An increase in mobile phase polarity with time was selected to achieve baseline separation of the OZs.

Protein precipitation is one of the easiest and fastest methods for processing biological samples such as plasma [14]. For the separation of analytes from endogenous components different solvents were evaluated. Finally, methanol at a ratio of 3:1 (precipitant to plasma) was chosen. To get a better response (higher sensitivity) the samples were evaporated to dryness under a stream of nitrogen and resuspended in a smaller volume of methanol and acetonitrile in water (30:70, v/v). In order to minimize contamination of the source of the MS, eluents were diverted to the instrument with a limited time window (4.5–6.5 min).

3.2. Method validation

3.2.1. Selectivity

In the majority of chromatograms of blank plasma and serum samples, no visible interferences were seen (Fig. 2(A) and (B)). Thus, no endogenous peaks were detected at the retention time of OZ78 (5.2 ± 0.25 min) or OZ352 (6.1 ± 0.3 min) as presented in Fig. 2(C). In some plasma from European sheep, there were contaminating signals near the IS peak (Fig. 2(B)). However, these signals did not interfere with IS peak integration and data evaluation (Fig. 2(D)).

3.2.2. Linearity and LLOQ

The calibration curve was linear over a concentration range from 156.25 to 5000 ng/mL in sheep plasma with a coefficient of correlations (r^2) above 0.994. A signal-to-noise ratio of 3 at 156.25 ng/mL was reached. This concentration was defined as LLOQ since the standard deviation at this concentration was lower than 15% and the calibration line was still linear. It should be noted that the sensitivity of the used API 365 mass spectrometer is suboptimal when the instrument is operated in the negative mode. However, to evaluate plasma samples in the present project the obtained LLOQ was considered to be acceptable.

3.2.3. Accuracy and precision

Four batches of QC samples at three concentrations were analyzed in terms of accuracy and precision. The intra- and inter-assay deviations are shown in Table 2. Intra- and inter-batch precisions were below 11.1% and 8.1%, respectively, with accuracy ranging from 90.2% to 104.7%. These values were within the suggested range of 15% for accurate and precise methods.

3.2.4. Extraction recovery

Extraction recoveries of three concentrations of OZ78 and IS were 84.4, 69.6, 78.6 and 85.8%, respectively. The recovery data are shown in detail in Table 3.

3.2.5. Stability

The results obtained from autosampler, freeze–thaw, and bench-top stability studies indicate that samples were stable under

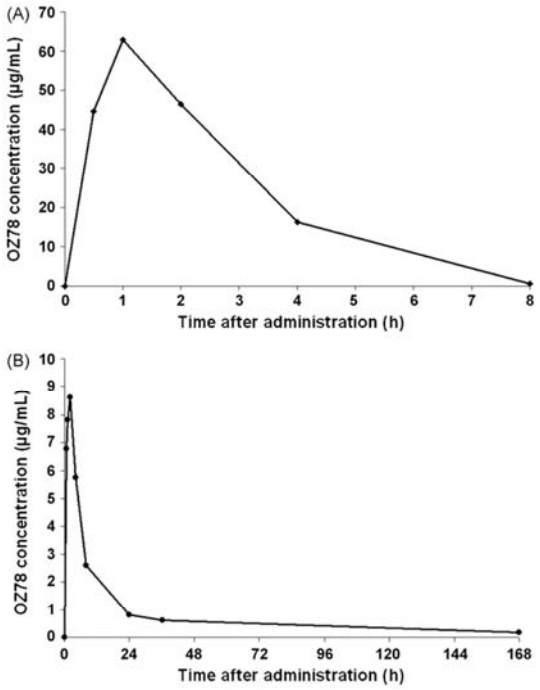


Fig. 3. Serum concentration–time profile of OZ78 in sheep, (A) following oral administration of 50 mg/kg OZ78 and (B) following subcutaneous administration of 50 mg/kg OZ78.

the different conditions (Table 4). Accuracy of all stability samples was within 109.2% with precision below 11.4%.

3.3. Application of the method

The validated method was used to analyze serum samples from ongoing PK studies with sheep receiving oral or subcutanous OZ78 (50 mg/kg). The usefulness of the method could be demonstrated. It was possible to measure the concentration vs. time profile of all animals. PK parameters were calculated, which have been presented elsewhere [12]. In Fig. 3(A) and (B) we present a representative serum concentration vs. time profile of an oral and a subcutaneous treated sheep.

4. Conclusions

This analytical LC/MS method has proven to be accurate, precise, simple, and sensitive. It was possible to monitor concentrations of OZ78 as low as 156.25 ng/mL in sheep plasma or serum. The validated method was successfully used to support a PK study in *F. hepatica* infected sheep [12]. Further work with this method is currently ongoing. With minor variations, the method will be validated, for example for the determination of OZ78 in bile fluids and rat plasma and adapted for potential novel fasciocidal OZ derivatives.

Acknowledgments

CK and JK are grateful to the Swiss National Science Foundation for financial support (project no. PPOOA-114941). We are thankful to Prof. S. Krähenbühl for fruitful discussions and scientific support.

References

[1] J.L. Vennerstrom, S. Arbe-Barnes, R. Brun, S.A. Charman, F.C. Chiu, J. Chollet, Y. Dong, A. Dorn, D. Hunziker, H. Matile, K. McIntosh, M. Padmanilayam, J. Santo Tomas, C. Scheurer, B. Scorneaux, Y. Tang, H. Urwyler, S. Wittlin, W.N. Charman, Nature 430 (2004) 900.
[2] N.J. White, Science 320 (2008) 330.
[3] Y. Dong, S. Wittlin, K. Sriraghavan, J. Chollet, S.A. Charman, W.N. Charman, C. Scheurer, H. Urwyler, J. Santo Tomas, C. Snyder, D.J. Creek, J. Morizzi, M. Koltun, H. Matile, X. Wang, M. Padmanilayam, Y. Tang, A. Dorn, R. Brun, J.L. Vennerstrom, J. Med. Chem. 53 (2010) 481.
[4] J. Möhrle, MMV, 2010, http://www.mmv.org/research-development/project-portfolio/oz-439, accessed 30/08/2010.
[5] Q. Zhao, M. Vargas, Y. Dong, L. Zhou, X. Wang, K. Sriraghavan, J. Keiser, J.L. Vennerstrom, J. Med. Chem. 53 (2010) 4223.
[6] J. Keiser, J. Utzinger, Clin. Microbiol. Rev. 22 (2009) 466.
[7] P.J. Hotez, D.H. Molyneux, A. Fenwick, E. Ottesen, S. Ehrlich Sachs, J.D. Sachs, PLoS Med. 3 (2006) e102.
[8] I. Fairweather, J. Helminthol. 83 (2009) 139.
[9] I. Fairweather, J.C. Boray, Vet. J. 158 (1999) 81.
[10] H.S. Yeung, W.O. Lee, Y.T. Wong, J. Sep. Sci. 33 (2010) 206.
[11] FDA guidance for industry bioanalytical method validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001.
[12] J. Keiser, C. Kirchhofer, M. Haschke, J. Huwyler, Y. Dong, J.L. Vennerstrom, K. Vanhoff, R. Kaminsky, N. Malikides, Vet. Parasitol. (2010), doi:10.1016/j.vetpar.2010.06.031.
[13] R.K. Bhamidipati, J. Morizzi, F.C. Chiu, D.M. Shackleford, S.A. Charman, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 2989.
[14] L. Novakova, H. Vlckova, Anal. Chim. Acta 656 (2009) 8.

Liquid chromatography/mass spectrometry method for phamacokinetic studies of OZ78

Swiss TPH
Swiss Tropical and Public Health Institute
Schweizerisches Tropen- und Public Health-Institut
Institut Tropical et de Santé Publique Suisse

Associated Institute of the University of Basel

C. Kirchhofer[1], J. Keiser[1], J. Huwyler[2]

[1] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH–4002 Basel, Switzerland

[2] Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, CH-4056 Basel, Switzerland

Introduction and Aim

Fascioliasis is an important public health problem in many countries on different continents. Today, triclabendazole is the first choice for therapy, but the rapid spread of triclabendazole resistance in veterinary medicine should be an important motivation for fasciocidal drug discovery and development. OZ78 is a promising fasciocidal drug candidate. In order to support the development of OZ78, we developed an accurate, precise, and selective liquid chromatography/mass spectrometry (LC/MS) method for OZ78 in sheep plasma. The validation was done in accordance with the US Food and Drug Administration Guidance on Bioanalytical Method Validation.

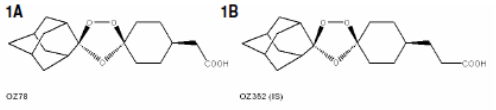


Fig. 1: Structures of (A) OZ78 and (B) OZ352 (internal standard).

Materials and Methods

OZ78 and OZ352 were synthesized at the College of Pharmacy, University of Nebraska Medical Center (Omaha, USA).

The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-20AD pumps, a CTC HTS PAL auto sampler (CTC Analytics, Zwingen, Switzerland), and an online DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan). Chromatographic separation was performed through a Phenomenex C8(2) (50.0 mm x 2.0 mm, 5 µm, Brechbühler AG, Schlieren, Switzerland) column coupled with a Phenomenex security guard used at room temperature. The following gradient was used: 0-4 min, B 20-35%; 4-7 min, B 35-65%; 7-8 min, B 65-95%; 8-10 min, 95%; 10-12 min, B 95-20%; 12-14 min 20%, where A was 5 mM ammonium formate in ultra pure water and B was 100% acetronitrile. The flow rate of the mobile phase was set at 0.3 mL/min.

An API 365 triple-quadrupole mass spectrometer (PE Biosystems, Foster City, CA) equipped with a turbo ion spray source was operated in negative ionization mode. Instrument control and data analyses were performed using the Analyst 1.4.2. software package (PE Biosystems, Foster City, CA).

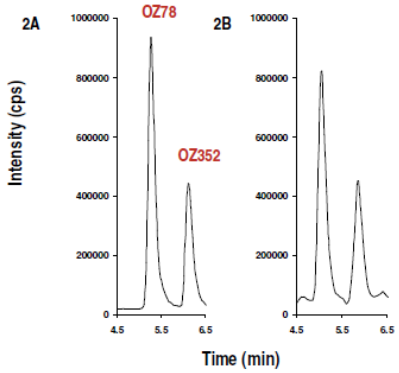


Fig. 2: Chromatograms of OZ78 and the internal standard OZ352 covering a retention time of 4.5 minutes to 6.5 minutes: (A) mobile phase spiked with OZ78 5 µg/mL and OZ352 (IS) 2 µg/mL and (B) plasma at high concentration (final concentration OZ78 5 µg/mL and OZ352 (IS) 2 µg/mL).

Results

The method is selective as confirmed by analysis of different sheep plasma and sensitive. Over the considered concentration range (150 ng/mL to 5 µg/mL) the regression coefficient (r2) of the calibration curves were always greater than 0.99.The validation results demonstrated that the developed LC/MS method is precise, accurate, and selective for the determination of OZ78 in sheep plasma. The validated method was used to analyze serum samples from ongoing PK studies with sheep who were orally or subcutaneously administered 50 mg/kg of OZ78. In Figure 3(A) and (B) we present a representative serum concentration vs. time profile of an oral and a subcutaneous treated sheep.

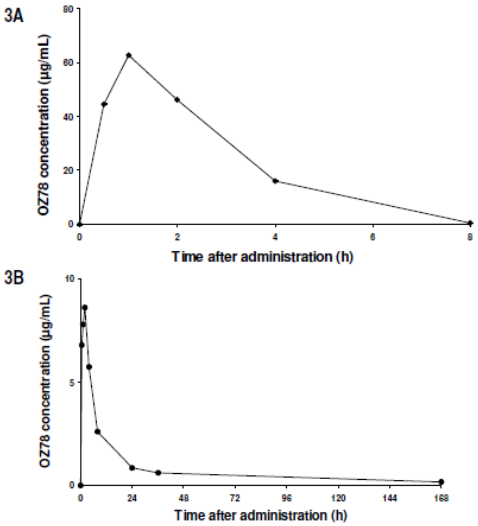


Fig. 3: Serum concentration-time profile of OZ78 in sheep, (A) following oral administration of 50 mg/kg OZ78 and (B) following subcutaneous administration of 50 mg/kg OZ78.

Outlook

The validated method was successfully used to support a PK study in *F. hepatica* infected sheep. Further work with this method is currently ongoing. With minor variations, the method will be validated, for example for the determination of OZ78 in bile fluids and rat plasma and adapted for potential novel fasciocidal OZ derivatives. A further aim is to determine PK data in other animals than sheep.

References

Kirchhofer, C., Keiser, J., Huwyler, J., 2010. Development and validation of a liquid chromatography/mass spectrometry method for pharmacokinetic studies of OZ78, a fasciocidal drug candidate. J Chromatogr B Analyt Technol Biomed Life Sci. 878, 2770-4.

Chapter 4

Efficacy, safety, and pharmacokinetics of 1,2,4-trioxolane OZ78 against an experimental infection with *Fasciola hepatica* in sheep

Jennifer Keiser[a,b,*], Carla Kirchhofer[a,b], Manuel Haschke[c], Jörg Huwyler[d], Yuxiang Dong[e], Jonathan L. Vennerstrom[e], Kathleen Vanhoff[f], Ronald Kaminsky[g], Nick Malikides[f]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, 4051 Basel, Switzerland

[b] University of Basel, 4003 Basel, Switzerland

[c] Division of Clinical Pharmacology & Toxicology, UniversityHospital Basel,4031 Basel, Switzerland

[d] Institute of Pharma Technology, University of Applied Sciences, Northwestern Switzerland, 4132 Muttenz, Switzerland

[e] College of Pharmacy, University of Nebraska Medical Center, Nebraska, NE68198-6025,USA

[f] Novartis Animal Health, Yarrandoo Research Station, 245 Western Road, Kemps Creek, NSW 2178, Australia

[g] Novartis Centre de Recherche Samté Animale SA, 1566St-Aubin(FR), Switzerland

Published in Veterinary Parasitology, 173 (2010) 228–235

Veterinary Parasitology 173 (2010) 228–235

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Efficacy, safety and pharmacokinetics of 1,2,4-trioxolane OZ78 against an experimental infection with *Fasciola hepatica* in sheep

Jennifer Keiser[a,b,*], Carla Kirchhofer[a,b], Manuel Haschke[c], Jörg Huwyler[d], Yuxiang Dong[e], Jonathan L. Vennerstrom[e], Kathleen Vanhoff[f], Ronald Kaminsky[g], Nick Malikides[f]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, 4051 Basel, Switzerland
[b] University of Basel, 4003 Basel, Switzerland
[c] Division of Clinical Pharmacology & Toxicology, University Hospital Basel, 4031 Basel, Switzerland
[d] Institute of Pharma Technology, University of Applied Sciences, Northwestern Switzerland, 4132 Muttenz, Switzerland
[e] College of Pharmacy, University of Nebraska Medical Center, Nebraska, NE 68198-6025, USA
[f] Novartis Animal Health, Yarrandoo Research Station, 245 Western Road, Kemps Creek, NSW 2178, Australia
[g] Novartis Centre de Recherche Santé Animale SA, 1566 St-Aubin (FR), Switzerland

ARTICLE INFO

Article history:
Received 19 March 2010
Received in revised form 13 June 2010
Accepted 21 June 2010

Keywords:
Fasciola hepatica
Fascioliasis
OZ78
Ozonide
1,2,4-Trioxolane
Sheep
Lamb
Experimental infection
Worm burden
Egg count
Safety
LC–MS
Pharmacokinetics

ABSTRACT

The synthetic peroxide OZ78 is an effective flukicide in the rodent model, but the potential of OZ78 in target animals has not been studied to date. In the present study, OZ78 was administered at 50 mg/kg orally and subcutaneously to sheep harbouring an experimental *Fasciola hepatica* infection and the efficacy, tolerability and pharmacokinetic profiles were monitored. OZ78 given orally or subcutaneously revealed no effect neither on faecal egg counts nor on worm burdens. Apart from significant subcutaneous swelling at the injection sites of most of the treated animals, no other treatment related adverse events occurred. OZ78 had no significant effect on any haematological, coagulation or clinical chemistry variables tested. Following oral administration, a mean C_{max} of 45.8 ± 13 μg/ml was reached after 1 h. An estimated elimination half-life of 1.0 h and a mean AUC of 116.2 ± 47 μg min/ml was calculated for the oral administration. Following subcutaneous treatment with OZ78 C_{max} and t_{max} were 13.7 ± 6.1 μg/ml and 0.9 ± 0.4 h, respectively. The α and β half-lives were 4.5 ± 4.3 h and 56.5 ± 36 h, respectively and the mean AUC was 219.1 ± 74 μg min/ml. Further studies are needed to determine whether the excellent activity observed with OZ78 in the rat model can be translated into efficacy in larger mammals.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Fasciola hepatica is a digenetic trematode flatworm of considerable agricultural and economic importance worldwide, with estimated losses as high as US$ 3 billion annually (Keiser and Utzinger, 2009; Mas-Coma et al., 2009). In addition, fascioliasis is a serious public health problem, with an estimated 17 million people infected and up to 180 million at risk (Keiser and Utzinger, 2005; Keiser and Utzinger, 2009). In the absence of an effective vaccine, chemotherapy remains the mainstay of control. The benzimidazole derivative triclabendazole currently is the treatment of choice for fascioliasis due to its high efficacy against juvenile and adult *F. hepatica*. However, development of resistance to triclabendazole is increasing such that alternative treatment options are warranted (Fairweather, 2009; Keiser et al., 2005).

* Corresponding author at: Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box. CH-4002 Basel, Switzerland. Tel.: +41 61 284 8218; fax: +41 61 284 8105.
E-mail address: jennifer.keiser@unibas.ch (J. Keiser).

0304-4017/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2010.06.031

In the past few years, we have demonstrated that the semisynthetic artemisinin derivatives artemether and artesunate, today the most commonly used antimalarials, are effective fasciolicides in the treatment of rodent and sheep infections (Keiser et al., 2008, 2006a, 2010). Since the artemisinins have short half-lives and must be administered over a period of several days in the treatment of malaria, many synthetic antimalarial peroxides have been prepared with the ultimate goal to identify a more stable, rapid-acting, fully synthetic, artemisinin-like molecule (Tang et al., 2004). One promising class of potent antimalarial synthetic peroxides is the ozonides (1,2,4 trioxolanes (OZs)) (Tang et al., 2004; Vennerstrom et al., 2004). Several studies have characterized the trematocidal properties of these ozonides (Halferty et al., 2009; Keiser and Utzinger, 2007; Xiao et al., 2007; Zhao et al., 2010). For example, ozonide OZ78 is an effective flukicidal drug in the rodent model. Complete *F. hepatica* burden reductions were achieved with a single oral dose of 100 mg/kg in rats harbouring juvenile and adult *F. hepatica* infections (Keiser et al., 2006b). Importantly, OZ78 also resolved a triclabendazole resistant *F. hepatica* infection in rats (Keiser et al., 2007). Additionally, OZ78 showed an acceptable toxicological profile, based on an exploratory tolerance study in rats and *in vitro* (Vennerstrom et al., 2004).

The aim of this study was to determine the efficacy of OZ78 when administered orally and subcutaneously to sheep at 50 mg/kg for the control of mature *F. hepatica*. Additionally, the pharmacokinetic profiles and tolerability of OZ78 were investigated following both routes of drug administration.

2. Materials and methods

2.1. Study site and regulatory

The study was conducted in an indoor animal housing facility at Yarrandoo Research and Development Centre, Novartis Animal Health, Kemps Creek, NSW, 2178, Australia. The centre has a certificate of accreditation as an animal research establishment. The study was conducted in accordance with local and international guidelines (Anon., 2001a,b,c,d; Wood et al., 1995).

2.2. Study design

In week −13, twelve lambs were selected (3 months of age at commencement, body weights 20.8–30.2 kg). All lambs underwent a general health inspection by a veterinarian and only clinically healthy lambs were included in the study. Faecal examinations were performed to ensure all lambs were not infected with *F. hepatica*, and at most were harbouring only low levels of infection with nematodes. Each lamb was orally infected with 150–200 freshly harvested *F. hepatica* metacercariae mixed with water and dispensed over the back of the tongue (Veterinary Health Research [VHR] Sunny Corner susceptible strain) in week −10. Sufficient time was subsequently allowed to ensure that chronic *F. hepatica* had established. Faecal samples, collected directly from the rectum were sampled on days −6 and −2, processed using an in-house sedimentation

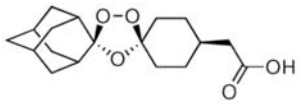


Fig. 1. Chemical structure of OZ78.

(modified McMaster) method. Two grams of faeces was mixed and vigorously stirred with tap water. The sample was strained through a 75 μm mesh sieve into a 100 ml medicine bottle. The sample was allowed to stand for 4 min with the supernatant subsequently discarded. The sedimentation process was repeated and the supernatant discarded down to a level of 1.5 ml. Zinc sulphate (1.5 ml) was added to the sediment, the sample shaken and both chambers of a Whitlock paracytometer filled. All eggs seen in one field of each chamber were counted and multiplied by five to obtain the number of eggs per gram (epg).

On the basis of faecal egg burden (day −2) and body weight (day −2), the animals were randomly allocated on day 0 to either the untreated control group (Group 1; $n=4$) or the two treated groups (Groups 2 and 3; $n=4$ lambs each) using a randomization software developed in-house. Treatment of Groups 2 and 3 occurred once on day 0.

Twenty days post-treatment another faecal sample was taken and the egg count determined. Lambs were euthanized on day 21 post-treatment. The liver, with gall bladder and bile duct intact with a portion of the small intestine, were removed from each animal. The bile ducts and small intestine were opened carefully and the flukes removed. Each liver was then sliced, squeezed and soaked in warm tap water. Individual slices were squeezed between the fingers to express flukes. Flukes were transferred to Petri dishes. Entire flukes as well as head and tail portions were counted. Total counts comprised the number of entire flukes and what ever was the greater, head or tail portions.

2.3. Treatment

OZ78 (Fig. 1) was obtained from Ranbaxy Laboratories Limited, India. The oral drug solution (20%, w/v) was prepared with 25% (w/v) solutol HS 15 (Macrogol 15 Hydroxystearate Ph.Eur./CAS-No. 70142-34-6), 20% (w/v) NMP (*N*-methylpyrrolidone Ph.Eur./CAS 70142-34-6) and Arlasolve DMI (Dimethylisosorbide/CAS 5306-85-4) ad 100 ml. For the subcutaneous administration, a 20% (w/v) solution was prepared with 20 ml Lipoid S 100, 10 ml ethanol, 30 ml NMP and Arlasolve DMI ad 100 ml.

Body weights of sheep were determined on calibrated scales two days prior to treatment. On the treatment day and before feeding, individual animals were treated once with 50 mg OZ78/kg orally (Group 2) and 50 mg OZ78/kg subcutaneously (Group 3) by the onsite veterinarian. All treatments were administered from disposable plastic syringes, measured by volume and rounded to the nearest 0.2–0.5 ml. Subcutaneous injections were administered on the neck behind the ear using new 18 G, 1 in. needles attached to disposable syringes. Six ml was the maximum volume administered at each injection site. Once the sheep had been treated they were returned to their pens and fed within 1 h.

The use of any other treatment with efficacy against *F. hepatica* was prohibited during the study.

2.4. Safety assessment

All lambs were individually examined pre- and post-treatment, were observed daily, and on the day of product administration, were observed more frequently and any adverse events, whether related to the treatment or not were identified and recorded. The sheep treated subcutaneously with OZ78 were examined daily from days 1 to 7 to check for transient injection site swellings. Finally, food intake was observed on treatment day and continued until day 7. All attempts were made to have the lambs complete the study.

2.5. Clinical pathology

Blood specimens for analysis of clinical chemistry and haematological variables (summarized in Table 1) were obtained on days −2, 1 and 7. The blood specimens were collected into sodium citrate tubes (1× 3.5 ml for coagulation variables), gel serum tubes (1× 5 ml for clinical chemistry variables) and EDTA tubes (2× 4 ml for haematology variables). All EDTA blood specimens were placed on mixing rollers and 2 blood smears per lamb were prepared on clean glass slides and stained, one with Wright Giemsa stain and the other with New Methylene Blue stain. Blood specimens collected into citrate tubes were immediately centrifuged at 2440 × *g* for 15 min (used at approximately 24 °C; Centra QP8R, Thermo IEC) and the plasma separated and retained in labelled vials. Finally, blood specimens collected into gel tubes were centrifuged at 1800–2000 × *g* for 10–15 min ensuring that the gel had moved between serum and red blood cells. All blood specimens were analyzed on the day of collection.

2.6. Pharmacokinetic analysis

On day −2 blood was obtained from all study animals to determine pre-treatment pharmacokinetic data. On day 0, blood specimens were collected from the jugular vein into vacutainers from all sheep in Groups 2 and 3 at 0.5, 1, 2, 4 and 8 h following oral drug administration and at 0.5, 1, 2, 4, 8, 24, 36 and 168 h following subcutaneous treatment. Blood samples were allowed to clot for at least 2 h at room temperature and then centrifuged at 1800–2000 × *g* for 10–15 min. The serum was separated into two labeled aliquots that were frozen immediately at −80 °C. One aliquot of each serum sample was shipped frozen to the Swiss Tropical and Public Health Institute. All samples were kept frozen at ≤80 °C until assayed.

Details of the analytical LC/MS method will be presented elsewhere (Kirchhofer et al., submitted for publication). Briefly, a 300 μl aliquot of each thawed serum sample was transferred to a microtube and spiked with 10 μl of an internal standard (OZ352; 60 μg/ml; synthesized by J. Vennerstrom, University of Nebraska Medical Center, Nebraska, USA). Proteins were precipitated by addition of 900 μl of ice-cold methanol. Samples were vortexed, incubated on ice for 10 min followed by centrifugation at 4 °C (15 min at 16,000 × *g*). Supernatants were collected and taken to dryness under a stream of nitrogen. The residue was dissolved in methanol:acetonitrile:water = 50:45:105 (v/v) and subjected to liquid chromatography (HPLC)/mass spectroscopy (MS) analysis: The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-20AD pumps, a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an online Degasys DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan). Chromatographic separation was performed on a Phenomenex C8(2) analytical column (50 mm × 2.0 mm, 5 μm; Phenomenex, Brechbühler AG, Schlieren) with a mobile phase of acetonitrile and 5 mM ammonium formate. The flow rate was 0.3 ml/min using a gradient over 14 min from 20% to 95% acetonitrile. Analytes were detected and quantified using a Sciex API 365 triple quadrupole mass spectrometer (PE Biosystems, Foster City, CA) equipped with a turbo ion spray source. The instrument was operated in negative mode under selected ion monitoring. The orifice voltage was −4100 V and the ionspray temperature was 400 °C. Nitrogen was used as a nebuliser and curtain gas. OZ78 was monitored at 321.4 *m/z* (parent compound, M-). The method was validated following the FDA bioanalytical guidelines on bioanalytical method validation (http://www.fda.gov).

2.7. Statistical analysis

Arithmetic means, geometric means and ranges were summarized for *F. hepatica* burdens and egg counts. Percentage efficacy was determined using the formula:

$$\text{Efficacy}\,[\%] = \frac{100 \times (C - T)}{C}$$

C and *T* are the *F. hepatica* egg count means for the control and treated groups, respectively.

Statsdirect statistical software (version 2.4.5, Statsdirect Ltd., Cheshire, UK) was used for the statistical analyses. The Kruskal–Wallis (KW) test was used to compare the medians of the *F. hepatica* worms and egg counts as well as differences in the clinical pathology variables pre- and post-treatment among treated and control groups. Differences in medians were considered to be significant at a significance level of 0.05.

PK parameters were estimated using WinNonlin (Version 5.2, Pharsight Corporation, USA). For the oral data a non-compartmental analysis was used. Area under the plasma concentration–time curve (AUC) was calculated using the linear trapezoidal rule with extrapolation from the last measured concentration to time infinity. The elimination rate constant λz was estimated by linear regression of the natural logarithm of the concentration values in the elimination phase. Another parameter obtained by the model was the elimination half-life ($t_{1/2}$) ($t_{1/2} = 0.693/\lambda$). C_{max} and t_{max} were taken directly from the primary data.

A two-compartment model was used for the observed plasma concentration vs. time data for lambs treated subcutaneously. Pharmacokinetic parameters obtained by fitting the model to the observed data included $t_{1/2\lambda1}$ and $t_{1/2\lambda2}$ (half-lives of the distribution and terminal elimination phase), C_{max} and AUC. The above indices were determined for each individual animal and their arithmetic mean (±SD) was calculated.

Table 1
Clinical pathology variables measured in treated and control sheep.

Parameter	Reference range[a]	Control (mean ± SD)		Oral treatment (mean ± SD)		Subcutaneous treatment (mean ± SD)	
		Before	After	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Haemoglobin (g/l)	90–150 g/l	98 ± 9	106 ± 2	82 ± 8	80 ± 0.2	92 ± 10	90 ± 0.2
Haematocrit (l/l)	0.27–0.45 l/l	0.27 ± 0.03	0.30 ± 0.01	0.23 ± 0.02	0.22 ± 0.00	0.25 ± 0.02	0.25 ± 0.00
Mean corpuscular haemoglobin concentration (g/l)	310–340 g/l	361 ± 8	358 ± 2	365 ± 16	362 ± 0	369 ± 13	363 ± 1
Platelets ($\times 10^9$ l^{-1})	250–750 $\times 10^9$ l^{-1}	802 ± 147	712 ± 98	837 ± 92	782 ± 10	1057 ± 287	1101 ± 87
Eosinophils (%)	0.0–10.0%	11.4 ± 8.6	9.3 ± 1.4	10.3 ± 7.2	11.3 ± 1.2	15.1 ± 12.3	13.6 ± 0.2
Natrium (mmol/l)	145–152 mmol/l	147.5 ± 1.7	141.9 ± 0.2	149.0 ± 2.9	143.1 ± 0.6	146.8 ± 1.3	142.0 ± 0.9
Potassium (mmol/l)	3.9–5.4 mmol/l	5.5 ± 0.6	5.0 ± 0.3	5.9 ± 0.7	4.9 ± 0.0	5.5 ± 0.4	4.8 ± 0.0
Chloride (mmol/l)	95–103 mmol/l	110 ± 2	106 ± 1	114 ± 4	108 ± 1	111 ± 4	108 ± 1
Magnesium (mmol/l)	0.90–1.26 mmol/l	0.82 ± 0.07	0.83 ± 0.00	0.77 ± 0.04	0.74 ± 0.09	0.75 ± 0.03	0.78 ± 0.04
Calcium (mmol/l)	2.88–3.20 mmol/l	2.69 ± 0.12	2.65 ± 0.01	2.64 ± 0.08	2.50 ± 0.21	2.60 ± 0.09	2.61 ± 0.00
Cholesterol (mmol/l)	1.05–1.55 mmol/l	1.53 ± 0.37	1.62 ± 0.08	1.55 ± 0.20	1.86 ± 0.58	1.88 ± 0.28	1.74 ± 0.02
Creatinine (mg/dl)	1.2–1.9 mg/dl	0.78 ± 0.05	0.76 ± 0.02	0.69 ± 0.45	0.69 ± 0.00	0.70 ± 0.07	0.72 ± 0.00
Total bilirubin (μmol/l)	1.71–8.55 μmol/l	3.5 ± 0.6	3.6 ± 0.3	2.9 ± 0.5	3.6 ± 0.5	3.7 ± 1.1	3.9 ± 0.3
γ-Glutamate transpeptidase (U/l)	20–52 U/l	252.9 ± 85.1	201.7 ± 4.1	223.4 ± 59.7	207.4 ± 53.2	381.3 ± 189.3	284.2 ± 27.2
Lactate dehydrogenase (U/l)	240–440 U/l	855.8 ± 366.4	780.3 ± 3.9	795.8 ± 266.5	721.5 ± 87.5	835.2 ± 230.9	796.6 ± 42.8
Total bile acids (μmol/l)	<25 μmol/l	156.2 ± 212.2	134.2 ± 21.7	113.1 ± 45.8	115.3 ± 53.7	138.3 ± 65.9	118.7 ± 31.9
Alkaline phosphatase (U/l)	70–390 U/l	206.3 ± 18.0	166.9 ± 10.9	293.8 ± 133.5	244.5 ± 27.1	300.4 ± 129.1	232.7 ± 4.9
Creatine kinase (U/l)	Unpublished	155.4 ± 27.6	593.2 ± 944.6	333.5 ± 316.6	163.1 ± 53.4	198.3 ± 86.2	156.6 ± 19.9
Alanine aminotransferase (U/l)	22–38 U/l	6.0 ± 0.8	7.9 ± 1.0	7.5 ± 1.7	8.1 ± 0.5	7.8 ± 5.1	9.9 ± 0.5

[a] Radostits et al. (1999).

3. Results

3.1. Safety assessment

3.1.1. General health observations, body weight and adverse events

All sheep treated with OZ78 subcutaneously (Group 3) developed various degrees of non-painful epidermal and/or sub-dermal swelling occurring 4–24 h post-treatment. On day 2, the non-painful swelling was larger in diameter and height (2 cm depth and up to 7 cm in diameter) and was firmer. From day 3 to 7 the dermal swelling slowly decreased in size and became harder. One lamb treated subcutaneously showed signs of discomfort, inappetence and depression. This sheep was given 1.5 ml Tolfedine (tolfenamic acid) intramuscularly and recovered. No other treatment-related adverse events occurred. There were no significant clinical or veterinary observations found in treated or control animals throughout the study. Feed intake of sheep was normal. Significant weight loss was noted in the controls and treated animals.

3.2. Clinical pathology

3.2.1. Haematology variables

There were no noteworthy effects of OZ78 treatment on red blood cell count, white blood cell count, reticulocyte count, mean corpuscular volume, mean corpuscular haemoglobin, neutrophil and basophil counts and percent lymphocyte and monocytes (data not shown).

Results for haemoglobin, haematocrit, mean corpuscular haemoglobin concentration and platelets are summarized in Table 1. Treatment with OZ78 had no significant effect on any of these variables. The mean haematocrit was lower in animals treated orally (0.22 l/l) or subcutaneously (0.25 l/l) with OZ78 than in control animals (0.30 l/l) ($P > 0.05$), although the haematocrit values in the 2 treatment groups already were below the reference range before treatment.

Values for the mean corpuscular hemoglobin concentrations were above the reference range (310–340 g/l) for all treated and control animals before (361–369 g/l) and after treatment (358–363 g/l), but there was no significant difference between mean values for treated animals and controls.

Similarly, values for platelet concentration were above the reference range ($250–750 \times 10^9\ l^{-1}$) for all lambs before ($802–1057 \times 10^9\ l^{-1}$) and for both treatment groups ($782–1101 \times 10^9\ l^{-1}$) after treatment.

Finally, the mean eosinophil percentage (Table 1) and mean absolute eosinophil count were above their respective reference ranges for all control and treated animals before and for the OZ78 treated lambs after treatment.

3.2.2. Coagulation variables

The overall post-treatment mean for APTT time, PT time and fibrinogen concentration were similar to pre-treatment values and were not outside the reference range at any examination time point for all animals (data not shown).

Table 2
Effect of oral and subcutaneous OZ78 on *F. hepatica* faecal egg counts in sheep, expressed as arithmetic and geometric mean eggs per gram of faeces (epg).

Treatment group	Dose (mg/kg)	Route of administration	Pre-treatment (EPG)[a] Arithmetic mean (SD)	Pre-treatment (EPG)[a] Geometric mean	Post-treatment (EPG)[b] Arithmetic Mean (SD)	Post-treatment (EPG)[b] Geometric mean	% Reduction
1 (Control)	–	–	179.6 (196)	49	2002.3 (1197)	1530.2	–
2	50	Oral	453.6 (314)	209.9	3310.8 (1545)	3044.4	0
3	50	Subcutaneous	417.1 (707)	70.44	1804 (1426)	681.3	0

[a] Data from day −7 to −2.
[b] Data from day 20.

Table 3
Worm burden reductions of *F. hepatica* in sheep after OZ78 treatment .

Treatment group	Dose (mg/kg)	Route of administration	Animals cured[a]	Mean worm burden (SD)	Total worm burden reduction (%)	KW	*P*
1 (Control)	–	–	0	208.5 (64.3)	–		
2	50	Oral	0	251.5 (62.7)	0	0.083	0.773
3	50	Subcutaneous	0	209.5 (65.4)	0	0	>0.999

[a] Number of animals without *F. hepatica*.

3.2.3. Clinical chemistry variables

There were no significant differences in phosphate, glucose, amylase, albumin, total protein, and blood urea nitrogen and aspartate aminotransferase between treated and control animals and all values were within the reference ranges (data not shown). Chloride, alanine aminotransferase, creatinine, total bile acids, γ-glutamate transpeptidase (GGT), lactate dehydrogenase (LDH), creatine kinase, cholesterol, magnesium and calcium, summarized in Table 1, were outside the reference range (either above or below) before and after treatment for each group. There was a slight decrease in mean sodium concentration ($P > 0.05$) in all groups after treatment. Finally, the control group showed a significantly higher value for creatine kinase post-treatment (593 U/l versus 163 U/l and 156 U/l, respectively).

3.3. Pathology

In all animals, the liver appeared grossly enlarged, friable and discoloured with engorged bile ducts. The mesenteric lymph nodes were enlarged, especially those positioned close to the liver. In all subcutaneously treated lambs, variably sized chronic, fibrous, caseous or abscessed masses were noted at the injection sites of the neck.

3.4. Effect of OZ78 on F. hepatica faecal egg burden

The effect of OZ78 on the *F. hepatica* egg count is summarized in Table 2. On sampling points day −7 and −2, lambs passed between 0 and 2091 epg. A slightly higher pre-treatment epg (i.e. arithmetic and geometric mean egg counts) was calculated for the orally treated group when compared to the control and subcutaneous treatment groups. However, these values were not statistically significant. Twenty days post-treatment, significantly higher epgs when compared to baseline were recorded for all sheep analyzed ($P = 0.0008$). The untreated control group produced an average of 2002 epg on day 20 post-infection, compared to 3310 and 1804 epg recorded from orally and subcutaneously treated lambs respectively. Hence, both treatments revealed no effect on faecal egg counts.

3.5. Effect of OZ78 on F. hepatica worm burden

The *F. hepatica* recovery data at post-mortem is presented in Table 3. The untreated control group harboured a mean of 208 *F. hepatica* in their liver bile ducts. The number of *F. hepatica* recovered from treated sheep was no different from untreated lambs (oral: 251 flukes; $P = 0.773$ and subcutaneous: 209 flukes; $P > 0.999$). *F. hepatica* collected from treated lambs revealed neither morphological alterations nor reduced movements.

3.6. Pharmacokinetic parameters

The serum concentration–time profiles of OZ78 given orally are presented in Fig. 2a. The PK parameters are summarized in Table 4. Mean peak concentration of 45.8 ± 13 μg/ml (C_{max}) was reached after 1 h (t_{max}). The estimated elimination half-life was 1.0 h and the mean AUC was 116.2 ± 47 μg min/ml.

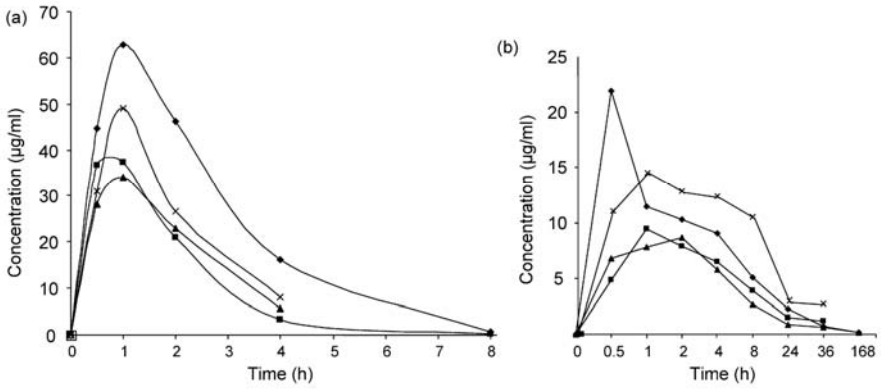


Fig. 2. Pharmacokinetic profiles following oral (a) and subcutaneous administration (b).

Table 4
Pharmacokinetic parameters following oral and subcutaneous administration of OZ78.

Group	C_{max} (μg/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC (μg min/ml)	$t_{1/2\lambda1}$	$t_{1/2\lambda2}$
Oral OZ78	45.8 ± 13	1.0	1.0 ± 0.1	116.2 ± 47		
Subcutaneous OZ78	13.7 ± 6.1	0.9 ± 0.4		219.1 ± 73.5	4.5 ± 4.3	56.5 ± 35.8

Serum concentration profiles after subcutaneous administration are given in Fig. 2b. Mean peak concentration of 13.7 ± 6.1 μg/ml was reached after 0.9 h. The α and β (terminal) half-lives were 4.5 ± 4.3 h and 56.5 ± 36 h, respectively. The mean AUC was 219.1 ± 74 μg min/ml.

4. Discussion

OZ78, highly effective against the Cullompton, Oberon and wild Pacific Northwest *F. hepatica* isolates in the rat model administered orally at 100 mg/kg (Keiser et al., 2006b, 2007), failed to show an effect in lambs experimentally infected with *F. hepatica*. The lack of efficacy also occurred at relatively high doses of OZ78 (50 mg/kg) administered to lambs using two different routes of drug administration. Although it is difficult to translate drug doses from one animal species to another, the FDA recommended method of normalization of the body surface area (http://www.fda.gov) was applied to estimate a starting dose in sheep. Given that a 100 mg/kg dose in rats corresponded to 15 mg/kg in humans, 25 mg/kg in pigs or 33 mg/kg in dogs, it was considered that a dose of 50 mg/kg would be sufficient if not higher than necessary to yield a pharmacological effect in the lambs used in this study.

There are a number of reasons that might explain the lack of efficacy observed. First, there might be differences among *F. hepatica* isolates in the level of sensitivity to OZ78. OZ78 had not been tested before against the "susceptible Sunny Corner isolate" maintained by VHR used for infection of sheep in this study. However, to our knowledge, significant variations in the drug sensitivity patterns of *Fasciola* spp. have not been reported to date with the exception of isolates resistant and susceptible to triclabendazole (Fairweather, 2005; Keiser et al., 2005). Nonetheless, the efficacy of OZ78 should be studied against this isolate *in vitro* and in the rat model. A second reason for the lack of efficacy might be that concentrations of OZ78 above the MIC_{90} were not attained at the site of infection, the bile ducts and gallbladder. It has been demonstrated that large concentrations of triclabendazole and its main metabolites are present in the bile duct of sheep and influence its flukicidal potency (Hennessy et al., 1987). It is possible that in our study none of the sheep were exposed to sufficiently high OZ78 concentrations in the bile and/or effective levels were not maintained for a sufficiently long period to achieve a parasitological response. Previous *in vitro* studies showed that a minimum concentration of 10 μg/ml of OZ78 was required to induce significant tegumental damage and death. However, the exact duration of drug incubation associated with parasitological response has not been studied (Keiser et al., 2006b). Hence, more in depth *in vitro* studies are warranted. In addition, the disposition of OZ78 and its metabolites in bile should be studied both in rats (where OZ78 achieved high worm burden reductions) and sheep. There might be substantial differences in the biliary excretion of OZ78 in rats and sheep, similar to what has been demonstrated for several drugs (Abou-El-Makarem et al., 1967).

Though bile concentrations, protein-binding or metabolism of OZ78 have not been studied to date, serum levels of OZ78 were monitored in the current study. Mean maximum serum concentrations were 45 μg/ml 1 h following oral drug administration to the lambs. Following subcutaneous treatment mean serum levels of 13 μg/ml were obtained. OZ78 had a short half-life of 1 h following oral administration in these lambs. In sheep treated subcutaneously a mean terminal half-life of 57 h was estimated due to slow release of OZ78 from the application site. It might be useful to study the serum concentration–response relation of OZ78 and main PK parameters in *F. hepatica*-infected rats and compare these results with the data obtained from sheep in the current study.

Apart from significant subcutaneous swelling at the injection sites of most of the treated animals, no other treatment-related adverse events occurred. There were no significant clinical or veterinary observations found in treated animals throughout the study. Low haematocrit (or anemia) (0.22 l/l and 0.25 l/l post-treatment, respectively), which was also reflected with low mean haemoglobin concentrations for animals treated orally and subcutaneously with OZ78 (80 g/l and 90 g/l post-treatment, respectively), may have been the result of low grade inflammatory disease, red blood cell loss or red blood cell destruction in animals before treatment and was not reflective of a treatment effect. High values of the mean corpuscular haemoglobin concentration observed in all animals pre- and post-treatment can accompany red cell destruction, which may help to explain the anemia in all treated animals (but not controls) before and after treatment. It is possible that many animals entered the study with some form of sub-clinical haemolytic anemia. High platelet concentrations observed in all sheep might have been the result of epinephrine or stress induced splenic contraction. The high GGT and LDH values observed before and after treatment in all groups may suggest biliary and secondary hepatic cell damage present prior to commencement of the study. Finally, the high value for creatine kinase in the controls post-treatment (593 U/l versus 163 U/l and 156 U/l, respectively), possibly reflects some muscle inflammation or damage (trauma) in one or more animals.

In contrast to the findings of this study, two other peroxidic drugs, artemether and artesunate showed a high efficacy in sheep harbouring a natural *F. hepatica* infection, when administered intramuscularly. Intramuscular artesunate at 40 mg/kg reduced faecal egg count and worm burden by 97.8% and 87.1%, respectively; whereas artemether achieved a worm burden reduction of 91%

when a single intramuscular dose of 160 mg/kg was given (Keiser et al., 2008, 2010). However, artemether also failed to reduce the worm burden when administered orally (Keiser et al., 2008). Nonetheless, the discrepancy in the lack of activity seen with subcutaneous OZ78 and high efficacy observed with artemether and artesunate intramuscularly are difficult to explain, in particular as OZ78 was superior to the artemisinin derivatives in *F. hepatica* infected rats. One possible explanation might be that sheep treated with the artemisinins were characterized by much lower infection intensities. In addition, different drug vehicles were used (e.g. artemether was dissolved in peanut oil). Differences in biliary excretion of peroxidic drugs might also play a role. A recent study showed that the majority of dihydroartemisinin was excreted via bile (Maggs et al., 1997; Xie et al., 2009).

In conclusion, single 50 mg/kg oral and subcutaneous doses of OZ78 lacked activity against *F. hepatica* infections in experimentally infected lambs. Further studies are needed, such as in depth PK studies, evaluations of different dosing regimens or higher doses to determine whether the high activity observed with OZ78 in the rat model can be translated into efficacy in larger mammals. In addition, the ongoing lead optimization with ozonides and related derivatives (Zhao et al., 2010) may lead to a superior fasciocidal drug candidate qualified to be tested in sheep in the near future.

Conflict of interest statement

The authors have no conflict of interest to declare.

Acknowledgements

J. Keiser and C. Kirchhofer are grateful to the Swiss National Science Foundation (SNF) (project PPOOA-114941) for financial support. The SNF had no influence in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

References

Abou-El-Makarem, M.M., Millburn, P., Smith, R.L., Williams, R.T., 1967. Biliary excretion in foreign compounds. Species difference in biliary excretion. Biochem. J. 105, 1289–1293.

Anon., 2001a. Efficacy Standards for Ruminant Anthelmintics in Australia. The Australian Pesticides and Veterinary Medicines Authority.

Anon., 2001b. VICH Guideline 7. Efficacy of Anthelmintics: General Requirements. International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products, Brussels, Belgium.

Anon., 2001c. VICH Guideline 9. Good Clinical Practice. International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products, Brussels, Belgium.

Anon., 2001d. VICH Guideline 13. Efficacy of Anthelmintics: Specific Recommendations for Ovines. International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products, Brussels, Belgium.

Fairweather, I., 2005. Triclabendazole: new skills to unravel an old(ish) enigma. J. Helminthol. 79, 227–234.

Fairweather, I., 2009. Triclabendazole progress report, 2005–2009: an advancement of learning? J. Helminthol. 83, 139–150.

Halferty, L., O'Neill, J.F., Brennan, G.P., Keiser, J., Fairweather, I., 2009. Electron microscopical study to assess the in vitro effects of the synthetic trioxolane OZ78 against the liver fluke, *Fasciola hepatica*. Parasitology 136, 1325–1337.

Hennessy, D.R., Lacey, E., Steel, J.W., Prichard, R.K., 1987. The kinetics of triclabendazole disposition in sheep. J. Vet. Pharmacol. Ther. 10, 64–72.

Keiser, J., Engels, D., Büscher, G., Utzinger, J., 2005. Triclabendazole for the treatment of fascioliasis and paragonimiasis. Exp. Opin. Invest. Drugs 14, 1513–1526.

Keiser, J., Rinaldi, L., Veneziano, V., Mezzino, L., Tanner, M., Utzinger, J., Cringoli, G., 2008. Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol. Res. 103, 517–522.

Keiser, J., Shu-Hua, X., Tanner, M., Utzinger, J., 2006a. Artesunate and artemether are effective fasciolicides in the rat model and in vitro. J. Antimicrob. Chemother. 57, 1139–1145.

Keiser, J., Utzinger, J., 2005. Emerging foodborne trematodiasis. Emerg. Infect. Dis. 11, 1507–1514.

Keiser, J., Utzinger, J., 2007. Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. Trends Parasitol. 23, 555–562.

Keiser, J., Utzinger, J., 2009. Food-borne trematodiases. Clin. Microb. Rev. 22, 466–483.

Keiser, J., Utzinger, J., Tanner, M., Dong, Y., Vennerstrom, J.L., 2006b. The synthetic peroxide OZ78 is effective against *Echinostoma caproni* and *Fasciola hepatica*. J. Antimicrob. Chem. 58, 1193–1197.

Keiser, J., Utzinger, J., Vennerstrom, J.L., Dong, Y., Brennan, G., Fairweather, I., 2007. Activity of artemether and OZ78 against triclabendazole-resistant *Fasciola hepatica*. Trans. R. Soc. Trop. Med. Hyg. 101, 1219–1222.

Keiser, J., Veneziano, V., Rinaldi, L., Mezzino, L., Duthaler, U., Cringoli, G., 2010. Anthelmintic activity of artesunate against *Fasciola hepatica* in naturally infected sheep. Res. Vet. Sci. 88, 107–110.

Kirchhofer, C., Keiser, J., Huwyler, J., submitted for publication. Development and validation of a liquid chromatography/mass spectrometry method for pharmacokinetic studies of OZ78, a fasciocidal drug candidate. Journal of Chromatography B.

Maggs, J.L., Madden, S., Bishop, L.P., O'Neill, P.M., Park, B.K., 1997. The rat biliary metabolites of dihydroartemisinin, an antimalarial endoperoxide. Drug Metab. Dispos. 25, 1200–1204.

Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. Chapter 2. Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv. Parasitol. 69, 41–146.

Radostits, O.M., Gay, C.C., Blood, D.C., Hinchcliff, K.W., 1999. Reference laboratory values. Veterinary Medicine. A Textbook of the Diseases of Cattle, Sheep, Pigs. In: Goats and Horses. WB Saunders, London, UK.

Tang, Y., Dong, Y., Vennerstrom, J.L., 2004. Synthetic peroxides as antimalarials. Med. Res. Rev. 24, 425–448.

Vennerstrom, J.L., Arbe-Barnes, S., Brun, R., Charman, S.A., Chiu, F.C., Chollet, J., Dong, Y., Dorn, A., Hunziker, D., Matile, H., McIntosh, K., Padmanilayam, M., Santo Tomas, J., Scheurer, C., Scorneaux, B., Tang, Y., Urwyler, H., Wittlin, S., Charman, W.N., 2004. Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 430, 900–904.

Wood, I.B., Amaral, N.K., Bairden, K., Duncan, J.L., Kassai, T., Malone Jr., J.B., Pankavich, J.A., Reinecke, R.K., Slocombe, O., Taylor, S.M., et al., 1995. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). Vet. Parasitol. 58, 181–213.

Xiao, S.H., Keiser, J., Chollet, J., Utzinger, J., Dong, Y., Vennerstrom, J.L., Tanner, M., 2007. The in vitro and in vivo activities of synthetic trioxolanes on major human schistosome species. Antimicrob. Agents Chemother. 51, 1440–1445.

Xie, L.H., Li, Q., Zhang, J., Weina, P.J., 2009. Pharmacokinetics, tissue distribution and mass balance of radiolabeled dihydroartemisinin in male rats. Malar. J. 8, 112.

Zhao, Q., Vargas, M., Dong, Y., Zhou, L., Wang, X., Sriraghavan, K., Keiser, J., Vennerstrom, J.L., 2010. The structure-activity relationship of an ozonide carboxylic acid (OZ78) against *Fasciola hepatica*. J. Med. Chem. 53, 4223–4233.

Chapter 5

Pharmacokinetics of the fasciocidal drug candidates MT04 and OZ78 in uninfected rats and *in vitro* pharmacodynamic studies

Carla Kirchhofer[a,b], Mireille Vargas[a,b], Jörg Huwyler[c], Jennifer Keiser[a,b,*]

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel, Switzerland

[2]University of Basel, P.O. Box, CH–4003 Basel, Switzerland

[3]Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland.

*Corresponding author:

J. Keiser, Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland. Phone: +41 61 284-8218; Fax: +41 61 284-8105.

E-mail address: jennifer.keiser@unibas.ch (J. Keiser).

Accepted in International Journal for Parasitology: Drugs and Drug Resistance

Abstract

OZ78 and MT04 are promising drug candidates against fascioliasis (fasciolosis). We determined basic pharmacokinetic (PK) parameters of OZ78 and MT04 in uninfected rats. Rats were treated with single oral doses of 50 mg/kg OZ78 or MT04. Blood samples were withdrawn at selected time points post treatment and the plasma concentrations were quantified by a validated liquid chromatography / mass spectrometry (LC/MS) method. The LC/MS method for MT04 and OZ78, initially developed for sheep plasma analysis, was adapted for rat plasma. *In vitro* pharmacodynamic studies with *Fasciola hepatica* incubated in solutions of either test agent complemented our work. The adapted and validated method was precise and accurate to measure OZ78 and MT04 in rat plasma. Accuracies for MT04 ranged from 87.9% to 104.7% with precisions not exceeding 14.3%. Precisions for OZ78 were lower than 9.8% and accuracies were between 88.4% and 105.3%. Following oral administration, maximum plasma concentrations (C_{max}) of MT04 and OZ78 were 49.8 and 70.1 µg/ml after 2.7 h and 1.6 h, respectively ($p>0.05$). The estimated areas under the plasma time curves (AUCs) were comparable for MT04 and OZ78. Mean elimination half-lives ($t_{1/2}$) of MT04 and OZ78 covered a range from 1 to 7 h. *In vitro* studies demonstrated that the fasciocidal activity of MT04 and OZ78 was dependent on the incubation-time, with exposure of flukes for 24 h to the drugs not being sufficient to kill the worms. In conclusion, differences in PK parameters of MT04 and OZ78 were observed in rats. However, further studies (e.g. in infected rats) are necessary to characterize these drugs in greater detail.

Keywords: Fascioliasis, *Fasciola hepatica*, Pharmacokinetics, OZ78, MT04

1. Introduction

Fascioliasis (fasciolosis) caused by the food-borne trematodes *Fasciola hepatica* and *F. gigantica* is a major public health problem and of significant veterinary importance (Mas-Coma et al., 2005; Robinson and Dalton, 2009). Recently the global burden of fascioliasis was calculated for the first time. It has been estimated that 2.6 million people are infected with *Fasciola* spp. and 299'510 people suffer from heavy fascioliasis resulting in 35'206 years lived with disability (Fürst et al., 2012). The benzimidazole triclabendazole is the first choice treatment against fascioliasis in human and veterinary medicine because of its excellent activity against both, adult and juvenile worms (Keiser et al., 2005; Fairweather, 2011). However, the development of triclabendazole resistance can not be ignored and therefore new treatments should be discovered and developed (Fairweather and Boray, 1999; Keiser and Utzinger, 2007).

The ozonides OZ78 and MT04 are promising fasciocidal drug candidates (Vennerstrom et al., 2004; Keiser et al., 2006; Kirchhofer et al., 2011). Both drugs have shown high *in vitro* activities against adult *F. hepatica*. Flukes incubated in MT04 or OZ78 at concentrations of 50 and 100 µg/ml, respectively were dead after 72 h (Keiser and Morson, 2008a; Kirchhofer et al., 2011). More importantly, in rat studies, both compounds were active against juvenile (acute) and adult (chronic) infections at oral doses of 50-100 mg/kg (Keiser et al., 2006; Kirchhofer et al., 2011), whereas MT04 showed slightly superior activity compared to OZ78. The ozonide structures of OZ78 and MT04 are both characterized by an acidic functional group coupled to a peroxidic bridge and a spiroadamantane substructure, features shown to be essential for flukicidal activity in rats (Zhao et al., 2010).

Recently, OZ78 was tested in sheep experimentally infected with *F. hepatica* using doses of 50 mg/kg applied orally and subcutaneously. However, no effect on the worm counts was observed despite high concentrations measured in plasma after both treatments (Keiser et al., 2010).

In depth pharmacokinetic (PK) studies with OZ78 and MT04 in rats might clarify the differences observed in activity between rats and sheep. The aim of the present study was therefore to determine the basic pharmacokinetic (PK) parameters of OZ78 and MT04 in uninfected rats using a recently developed high performance liquid chromatography mass spectrometry (LC/MS) method for OZ78 and a newly adapted

LC/MS method for MT04. Both drugs were applied orally at 50 mg/kg to uninfected rats. In addition, *in vitro* studies with both test agents simulating the observed *in vivo* conditions complemented our work.

2. Materials and methods

2.1. Animal studies

Animal experiments were carried out in accordance with Swiss legislation on animal welfare.

Female Wistar rats (weight: ~150 g) were purchased from Charles River (Germany). The animals were housed in standard cages and maintained under a 12 h light/dark cycle with access to laboratory chow and water *ad libitum*. For kinetic studies, rats had an indwelling cannula implanted in the right jugular vein (Bittner et al., 2003).

2.2. Drugs and drug formulation

OZ78 and MT04 (Fig. 1) were kindly supplied by the College of Pharmacy, University of Nebraska Medical Center (Omaha, USA). Drugs suspensions were prepared in 7% (v/v) Tween 80 and 3% (v/v) ethanol 96% shortly before administration to rats.

OZ352 (Fig. 1, also provided by the College of Pharmacy, University of Nebraska Medical Center) was used as internal standard (IS) for analysis.

Figure 1 near here

2.3. Pharmacokinetic study

Pharmacokinetic profiles were studied in rats treated orally with 50 mg/kg OZ78 (n=4) and MT04 (n=4). Venous blood samples (0.2 ml) were collected using lithium-heparin coated tubes (Sarstedt, Nümbrecht, Germany) at 0, 30, 60, 120, 180, 240, 360, and 1440 minutes post treatment. The blood volume was replaced with an equal volume of 0.9% saline solution. Samples were allowed to stand for 20 min at RT and subsequently centrifuged at 200 rpm for 15 min. Obtained plasma samples were stored at -20 °C prior to analysis.

2.4. Liquid chromatography mass spectrometry analysis

OZ78 and MT04 were analyzed as described previously (Kirchhofer et al., 2010). In brief, analytes were separated by a high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) connected to an API 365 triple-quadrupole mass spectrometer (PE Biosystems, Foster City, CA, USA) with a turbo ion spray interface. A phenomenex C8 (2) column (50 mm x 2.0 mm, 5 µm; Phenomenex, Brechbühler AG, Schlieren,

Switzerland) was used to separate the respective analyte and the internal standard (OZ352).

Acetonitrile combined with 5 mM ammonium formate in water served as mobile phase. A gradient over 14 min was applied at a flow rate of 0.3 ml/min. OZ78 was detected in negative mode at m/z ratio of 321.4 and the internal standard was detected at m/z of 335.4. MT04 was monitored at m/z 337.4 (negative mode, deprotonated parent compound, M-).

Calibration curves of the analyte were prepared by diluting analyte working solutions with blank sheep plasma. Each calibration curve consisted of seven calibrators (10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 µg/ml), one zero sample (plasma sample spiked with 2 µg/ml IS) and one blank plasma sample (plasma sample processed without IS). The calibration curves were expanded in this study to cover a broader concentration range (10'000 – 156.25 ng/ml).

Sample preparation was done by protein precipitation. 900 µl methanol was added to 300 µl plasma followed by vortex-mixing for 1 min and centrifugation for 30 min at 16'100 g and 4°C. The supernatant was dried under nitrogen gas and then reconstituted in a mixture of 50 µl methanol and 150 µl acetonitrile/water (3/7, (v/v)). The reconstituted sample was transferred into an autosampler vial and stored at 6°C in the CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) prior to analysis. A 20 µL aliquot of each sample was injected into the LC-MS/MS system.

2.5. Validation of the analytical method

The analytical methods were developed initially to analyze sheep plasma samples and were validated accordingly. For measurements of rat plasma samples, the LC/MS methods were modified and validated as follows: Rat plasma spiked with OZ78 or MT04 was mixed with sheep plasma (9/1 or 4/1, (v/v)) followed by addition of the internal standard solution. Accuracies were calculated as the ratio of the measured concentration to the nominal concentration. Precisions were calculated using the relative standard deviations (RSD%). Calibration samples were freshly prepared and included in each analytical run. For accuracy and precision, a deviation of <15% was considered acceptable as recommended by US guidelines of the Food and Drug Administration (FDA, 2001). It should be noted, that this method can not be used to analyze rat bile samples due to severe interference with bile salts.

2.6. *In vitro* assay with adult *F. hepatica*

In vitro studies with *F. hepatica* were carried out as described by Kirchhofer et al. (Kirchhofer et al., 2011). Adult flukes were recovered from bile ducts of infected cattle (slaughterhouse, Basel). Flukes were washed with 0.9% (w/v) NaCl and placed in petri dishes. The flukes were rinsed with pre-warmed RPMI 1640 culture medium (Gibco, N.Y., USA) and pre-incubated for several hours in this medium at 37°C in an atmosphere of 5% CO_2. A single adult *Fasciola* was placed per well (6-well plate, Costar, MA, USA) containing 5.4 ml RPMI 1640, supplemented with 1% (v/v) antibiotics (50 µg/ml streptomycin and 50 U/ml penicillin; Sigma, Buchs, Switzerland), 1% (v/v) drug-solution, and 8% (v/v) of a haemin solution (Keiser and Morson, 2008a). Adult *F. hepatica* were incubated in the presence of 50 µg/ml MT04 or OZ78 for 96 h. In a second series of experiments worms were exposed to the drugs for 24 h and then placed in fresh, drug-free medium for another 72 h. Cultures were kept at 37°C in an atmosphere of 5% CO_2.

The viabilities of treated and untreated *Fasciola* were monitored at 0, 24, 48, 72, and 96 h and compared to control worms. Worms were examined using a dissecting microscope. Viability was defined on a scale from 4 (normal activity; movements clearly visible) to 1 (worm dead, no movement observed for two minutes). Results were expressed as means ± standard error (SE).

2.7. Data analysis and statistics

Data analysis was performed with Analyst 1.4.2. software (PE Biosystems). Microsoft Excel 2003 was used for the statistical analysis. Student's t-test was used to compare means of the PK parameters, p-values < 0.05 were considered to be significant. For kinetic parameter estimation WinNonLin (Version 5.2, Pharsight Corporation, USA) was used. Maximal plasma concentration (C_{max}) and time to achieve maximal plasma concentration (T_{max}) were observed values. Area under the plasma concentration time curve (AUC, from 0 to infinity) was calculated by the program using the linear trapezoidal rule. The elimination half life ($t_{1/2}$) was determined by the equation: $t_{1/2}=0.693/\lambda$, where λ was estimated by performing a regression of the natural logarithm of the concentration values in the elimination phase.

3. Results and discussion

Fascioliasis is a disease with a worldwide distribution but nevertheless belongs to the neglected tropical diseases (Hotez et al., 2006; Fürst et al., 2012). Funding for the identification and development of novel control tools, including drug discovery and development, has been limited despite disturbing effects of these diseases on health (Spiegel et al., 2010). This is reflected by the fact that for the treatment of fascioliasis only a single drug, triclabendazole, is available (Keiser et al., 2005). The documented resistance to triclabendazole and the empty drug pipeline emphasize the great need to discover and develop novel drugs for the treatment of fascioliasis (Keiser et al., 2005).

MT04 and OZ78 are promising fasciocidal drug candidates as documented by studies *in vitro* and in rats (Kirchhofer et al., 2011). However, disappointingly OZ78 failed to cure *F. hepatica* infected sheep (Keiser et al., 2010). We were interested to study PK parameters of OZ78 in rats to get a deeper insight on the differences observed between activity of OZ78 in rats and sheep. In addition, it was our goal to compare the PK parameters of OZ78 and MT04 since activities of these drugs slightly varied in rats. At 50 mg/kg per os MT04 achieved a complete elimination of worms in rats, whereas the worm burden reduction of OZ78 was only 52.7% at this dose (Keiser et al., 2006; Kirchhofer et al., 2011). OZ78 at 100 mg/kg per os cured *F. hepatica* infected rats (Keiser et al., 2006).

First, the MT04 method was validated according to the previously published LC/MS method for OZ78 (Kirchhofer et al., 2010). The same LC/MS method was used as described for OZ78, with exception that MT04 was detected at m/z 337.4. The calibration curves of MT04 were linear over a concentration range of 156.25 ng/ml to 10'000 ng/ml in sheep plasma with coefficient of correlations (r2) above 0.99. Accuracy and precision were determined for three batches of QC samples at four concentrations (10, 5, 1.25, and 0.3125 µg/ml). Intra- (n=3) and inter-batch (n=9) precisions were below 13.75% and 9.73%, respectively, with accuracies ranging from 87.2% to 101.7% and 90.5% to 98.9%. These values were within the suggested range of 15% for accurate and precise methods as recommended by USFDA (FDA guidance, 2001). Extraction recoveries of three concentrations (10, 1.25, and 0.3125 µg/ml) of MT04 in sheep plasma were 74.7 (±16.2)%, 81.6 (±13.5)%, and 75.4 (±16.7)% respectively. No interfering signals were observed at the retention time of OZ78 and MT04. Both analytes

were detected at the same retention-time. In some samples interfering peaks near the analyte or IS were visible, however signals did not influence peak integration. Selectivity was therefore ensured for the analytical method.

Dilution of rat plasma samples with sheep plasma is possible as demonstrated by an additional set of validation experiments. Mixed plasma quality control samples (10, 5, 1.25, and 0.3125 µg/ml) represented the expected concentration with a deviation of not more than 15%. Precisions for MT04 or OZ78 were below 14.1% and 13.1%, respectively and accuracies ranged from 89.6% to 111.1% for MT04 and OZ78. Validation results from the diluted rat plasma samples confirmed that the MT04 and OZ78 methods developed for sheep plasma could be used to measure rat samples by diluting rat with sheep plasma. The back calculated concentrations revealed deviations of not more than 15% of the expected concentrations for MT04 and OZ78. Accuracies for MT04 were between 87.9% and 104.7% with precisions not exceeding 14.3%. Precisions for OZ78 were lower than 9.8% and accuracies were between 88.4% and 105.3%. The lower limit of quantitation of the method did not allow to measure samples with concentrations lower than 156.25 ng/ml.

Concentration versus time profiles of MT04 and OZ78 are depicted in Fig. 2 (A) and (B) and the corresponding basic PK parameters are summarized in Table 1. A non compartmental model was used to estimate PK parameters. In our study differences were observed in the drug disposition parameters of MT04 and OZ78. MT04 reached a mean peak concentration of 49.8 ± 9.1 µg/ml (C_{max}) after 2.7 h ± 1.2 (T_{max}), whereas for OZ78 a mean peak concentration of 70.1 ± 19.1 µg/ml (C_{max}) was observed after 1.6 h ± 1.6 (T_{max}). Twenty four hours after treatment only traces of the drugs could be detected in plasma. The estimated half life of MT04 and OZ78 were 6.4 ± 5.7 and 2.5 ± 1.5 hours, respectively. The mean AUC were 31'258.8 ± 6'232.7 and 29'794.1 ± 3'990.6 µg min/ml for MT04 and OZ78. However, our finding should be interpreted with care since few animals were used, the high inter-individual variability of PK parameters observed and the few data-points measured in the elimination phase of the drugs. In addition, no statistical significance was observed between PK parameters calculated for OZ78 and MT04.

Figure 2 near here

Though, the longer half-life of MT04 might contribute to an increased activity, it is difficult to explain the better activity of MT04 entirely with the obtained PK parameters.

It is possible that the double peroxide bridge feature of MT04 plays an important role in activity. It is most likely that, similar to the antimalarial activity, an iron dependent activation of the peroxide core of the artemisinins and synthetic peroxides is the main driver of trematocidal activity (Keiser and Utzinger, 2007).

A clear difference in PK parameters was observed between rats and sheep (Keiser et al., 2010) treated orally with 50 mg/kg OZ78. A limitation of this face to face comparison is that in the present work uninfected rats were used, while PK parameters were derived from sheep, which suffered a chronic *Fasciola* infection. Keiser et al. (2009) have recently shown that PK parameters of artesunate and dihydroartemisinin were dramatically influenced by the *F. hepatica* infection (acute and chronic) in rats (Keiser et al., 2009). However, strikingly the AUC in rats was 4.3 times higher than in sheep (29'794.1 ± 3'990.6 versus 6'972.0 ± 2'820 µg min/ml) ($p = 0.001$). Note that a dose of 50 mg/kg in sheep corresponds to approximately 100 mg/kg in rats if normalized to body surface area (Keiser et al., 2010). A dose of 100 mg/kg OZ78 in rat would presumably result in a higher AUC and thus the difference in AUC between sheep and rat would be even more pronounced. Further differences in PK parameters (e.g. T_{max}, C_{max}, and $t_{1/2}$) between sheep and rat were observed. However, the differences were less pronounced than the AUC level and were not significant (a summary of the sheep data is given in Table 1). Hence, the therapy failure in sheep might be explained by the shorter exposure time of *F. hepatica* to OZ78 when compared to rats. Flukes presumably had insufficient duration of exposure to the drug and therefore were not killed.

The hypothesis that adequately long plasma levels are required for fasciocidal activity is supported by findings obtained in our pharmacodynamic *in vitro* studies. To approximately simulate the measured *in vivo* conditions, flukes (n=6 for each concentration) were incubated in the presence of MT04 or OZ78 at a concentration of 50 µg/ml for 24 hours (followed by incubation in drug free medium) or for 96 hours and viabilities were recorded (Fig. 3 (A) and (B)). Worms placed in MT04 for 72 h had died, whereas worms incubated in MT04 for 24 h (followed by incubation in drug free medium) were still alive after 96 h displaying a reduced viability. Worms incubated for 96 h in OZ78 50 µg/ml were alive. However, they revealed a strongly reduced viability. Worms incubated in OZ78 for 24 h (followed by incubation in drug free medium) were affected to a nearly similar degree as those incubated for 96 h.

Figure 3 near here

Note that adult *Fasciola* worms are settled in the bile ducts and gallbladder of the host (Robinson and Dalton, 2009). However, in the present study drug levels were determined in plasma and not in bile fluid due to technical limitations of the method. It is most likely that bile concentrations of OZ78 and MT04 in rats are higher or have longer half-lives than the determined plasma levels given the excellent activity of the drugs in rats.

A recently published study in *F. hepatica* infected sheep with structurally related compounds, the semi-synthetic artemisinin (artemether), demonstrated that the treatment outcome was dependent on the route of administration (Keiser et al., 2008b). Artemether applied at a single intramuscular (i.m.) dose of 160 mg/kg showed an efficacy of >90%, whereas oral dosages of 40 or 80 mg/kg had no effect on egg and worm counts (Keiser et al., 2008b). PK parameters were determined from these treatments (Duthaler et al., 2011). It was shown that the AUC after i.m. application was significantly higher than after oral application. It might be therefore worthwhile to test the activity of intramuscular applications of OZ78 and MT04 in sheep.

In conclusion, the established LC/MS method for determination of MT04 is accurate and precise. The MT04 as well as the OZ78 LC/MS methods could be adapted to measure both sheep and rat plasma samples. We analyzed drug disposition parameters for OZ78 and MT04 in uninfected rats, which pointed to differences between the drugs. In addition, comparing PK parameters of OZ78 in rats and sheep revealed a significantly lower AUC in sheep and might therefore provide an explanation for the lack of activity in this species. Further studies in *F. hepatica* infected rats are needed to strengthen these findings. Finally, the analytical methods should be modified in order to be able to determine bile concentrations of OZ78 and MT04.

Conflict of interest

We have no conflict of interest to declare.

Acknowledgments

We thank Massimiliano Donzelli and Prof. Stephan Krähenbühl for their support and helpful suggestions concerning analytical and pharmacokinetic questions. We are grateful to Prof. Jonathan Vennerstrom for the supply of OZ78, MT04, and OZ352.

C.K. and J.K. are supported by the Swiss National Science Foundation (project PPOOA-114941 and PPOOP3_135170).

Legend to Figures

Figure 1: Chemical structures and molecular weights of (A) OZ78, (B) OZ352, and (C) MT04.

Figure 2: Plasma concentration versus time profiles in rats following 50 mg/kg oral doses of OZ78 (A) (n=4) and MT04 (B) (n=4).

Figure 3: *In vitro* viability of adult *F. hepatica*, A) in the presence of 50 µg/ml MT04 for 96 hours (black squares), 50 µg/ml MT04 for 24 hours (followed by incubation in drug free medium for 72 hours) (black squares, dotted line), and B) in the presence of 50 µg/ml OZ78 for 96 hours (black triangles), 50 µg/ml OZ78 for 24 hours (followed by incubation in drug free medium for 72 hours) (black triangles, dotted line) versus control adult *F. hepatica* (white diamonds). Values are means ± standard error (SE).

Table 1: Pharmacokinetic parameters of OZ78 and MT04 in uninfected rats after oral administration of 50 mg/kg. For comparison, disposition parameters following treatment of *F. hepatica* infected sheep with 50 mg/kg are shown.

Treatment	Animal	Dose (mg/kg)	C_{max} (µg/ml)	T_{max} (h)	AUC (µg min/ml)	$T_{1/2}$ (h)
OZ78 oral	rat	50	70.1 (±19.1)	1.6 (±1.6)	29'794.1 (±3'990.6)	2.5 (±1.5)
MT04 oral	rat	50	49.8 (±9.1)	2.7 (±1.2)	31'258.8 (±6'232.7)	6.4 (±5.7)
OZ78 oral[1]	sheep	50	45.8 (±13)	1.0	6'972.0 (±2'820)*	1.0 (±0.1)

Data are presented as mean (±SD)

[1] (Keiser et al., 2010)

* statistical significant, $p < 0.05$.

References

Bittner, B., Gonzalez, R.C., Walter, I., Kapps, M., Huwyler, J., 2003. Impact of Solutol HS 15 on the pharmacokinetic behaviour of colchicine upon intravenous administration to male Wistar rats. Biopharm Drug Dispos. 24, 173-81.

Duthaler, U., Huwyler, J., Rinaldi, L., Cringoli, G., Keiser, J., 2011. Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet Parasitol, doi:10.1016/j.vetpar.2011.11.076.

Fairweather, I., Boray, J.C., 1999. Fasciolicides: efficacy, actions, resistance and its management. Vet J. 158, 81-112.

Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet Parasitol. 180, 133-43.

FDA guidance for industry bioanalytical method validation, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). 2001.

Fürst, T., Keiser, J., Utzinger, J., 2012. Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. Lancet Infect Dis. 12, 210-21.

Hotez, P.J., Molyneux, D.H., Fenwick, A., Ottesen, E., Ehrlich Sachs, S., Sachs, J.D., 2006. Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria. PLoS Med. 3, e102.

Keiser, J., Engels, D., Buscher, G., Utzinger, J., 2005. Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin Investig Drugs. 14, 1513-26.

Keiser, J., Utzinger, J., Tanner, M., Dong, Y., Vennerstrom, J.L., 2006. The synthetic peroxide OZ78 is effective against *Echinostoma caproni* and *Fasciola hepatica*. J Antimicrob Chemother. 58, 1193-7.

Keiser, J., Utzinger, J., 2007. Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. Trends Parasitol. 23, 555-62.

Keiser, J., Morson, G., 2008a. *Fasciola hepatica*: Surface tegumental responses to *in vitro* and *in vivo* treatment with the experimental fasciolicide OZ78. Exp Parasitol. 119, 87-93.

Keiser, J., Rinaldi, L., Veneziano, V., Mezzino, L., Tanner, M., Utzinger, J., et al., 2008b. Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol Res. 103, 517-22.

Keiser, J., Gruyer, M.S., Perrottet, N., Zanolari, B., Mercier, T., Decosterd, L., 2009. Pharmacokinetic parameters of artesunate and dihydroartemisinin in rats infected with *Fasciola hepatica*. J Antimicrob Chemother. 63, 543-9.

Keiser, J., Kirchhofer, C., Haschke, M., Huwyler, J., Dong, Y., Vennerstrom, J.L., et al., 2010. Efficacy, safety and pharmacokinetics of 1,2,4-trioxolane OZ78 against an experimental infection with *Fasciola hepatica* in sheep. Vet Parasitol. 173, 228-35.

Kirchhofer, C., Keiser, J., Huwyler, J., 2010. Development and validation of a liquid chromatography/mass spectrometry method for pharmacokinetic studies of OZ78, a fasciocidal drug candidate. J Chromatogr B Analyt Technol Biomed Life Sci. 878, 2770-4.

Kirchhofer, C., Vargas, M., Braissant, O., Dong, Y., Wang, X., Vennerstrom, J.L., et al., 2011. Activity of OZ78 analogues against *Fasciola hepatica* and *Echinostoma caproni*. Acta Trop. 118, 56-62.

Mas-Coma, S., Bargues, M.D., Valero, M.A., 2005. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol. 35, 1255-78.

Robinson, M.W., Dalton, J.P., 2009. Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos Trans R Soc Lond B Biol Sci. 364, 2763-76.

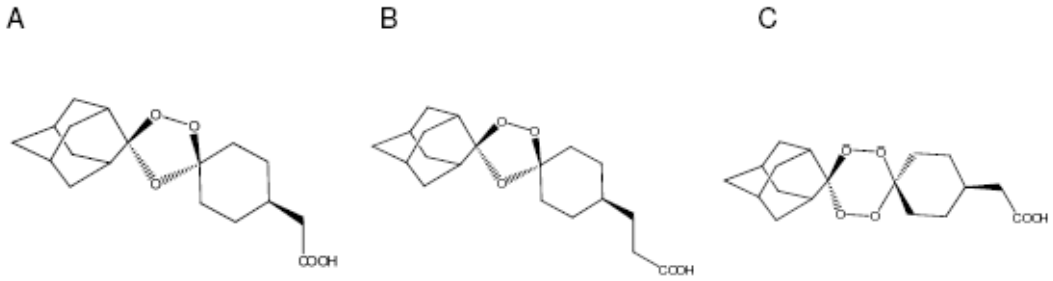
Spiegel, J.M., Dharamsi, S., Wasan, K.M., Yassi, A., Singer, B., Hotez, P.J., et al., 2010. Which new approaches to tackling neglected tropical diseases show promise? PLoS Med. 7, e1000255.

Vennerstrom, J.L., Arbe-Barnes, S., Brun, R., Charman, S.A., Chiu, F.C., Chollet, J., et al., 2004. Identification of an antimalarial synthetic trioxolane drug development candidate. Nature. 430, 900-4.

Zhao, Q., Vargas, M., Dong, Y., Zhou, L., Wang, X., Sriraghavan, K., et al., 2010. Structure-activity relationship of an ozonide carboxylic acid (OZ78) against *Fasciola hepatica*. J Med Chem. 53, 4223-33.

Figures

Figure 1



OZ78
MW: 322.4 g/mol
$C_{18}H_{26}O_5$

OZ352 (IS)
MW: 336.4 g/mol
$C_{19}H_{28}O_5$

MT04
MW: 338.4 g/mol
$C_{18}H_{26}O_6$

Figure 2

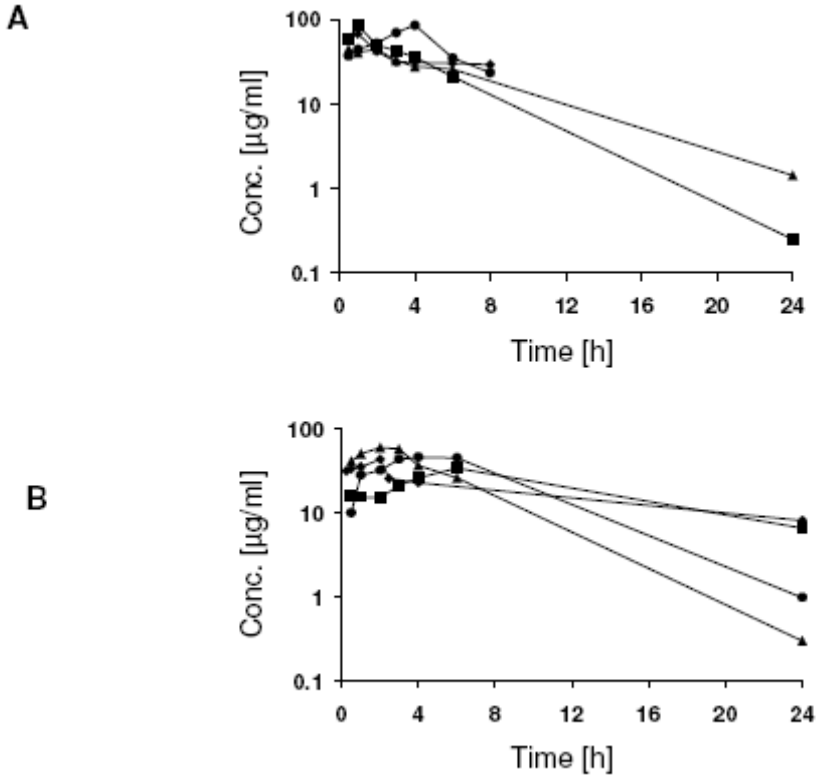
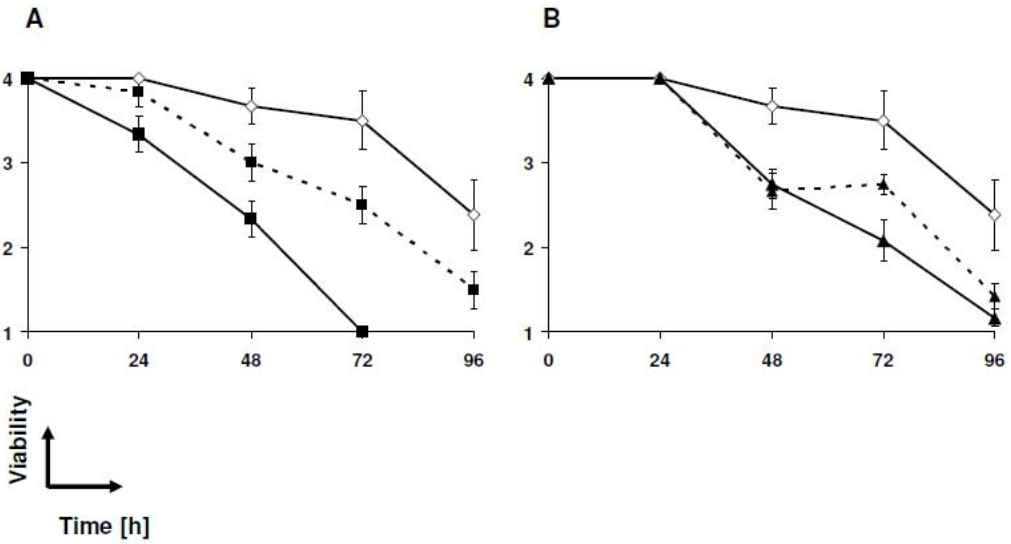


Figure 3



Chapter 6

The role of haemin and iron in the toxicity of two new fasciocidal drug candidates (MT04 and OZ78) compared to artesunate

Carla Kirchhofer[1,2], Jamal Bouitbir[3], Jennifer Keiser[1,2], Stephan Krähenbühl[3], Karin Brecht[3]

[1] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland

[2] University of Basel, P.O. Box, CH-4003 Basel, Switzerland

[3] Division of Clinical Pharmacology & Toxicology, University Hospital, Basel, Switzerland.

Manuscript in preparation

Abstract

OZ78 and MT04 are promising fasciocidal drug candidates. However, in depth toxicity studies have not been carried out to date. In the present work, we studied the liver toxicity of these substances compared to a related peroxidic derivative, the antimalarial drug artesunate (AS).

We tested the toxicity of OZ78 and MT04 at different concentrations (100 and 500 µM) on HepG2 cells in comparison to AS. The role of iron or haemin in inducing toxicity was studied. Two cell assays were applied. Viability and toxicity were determined by adenylate kinase (AK) release and ATP content. Additionally, reactive oxygen species (ROS) were measured after 90 min, 4 h, and 24 h and metabolism was investigated in presence of liver microsomes.

OZ78 showed only moderate toxicity on HepG2 cells in combination with Fe(II) or haemin as expressed by a lower ATP content. MT04 was more toxic both in membrane leakage and in CellTiter-Glo assay. OZ78 and MT04 resulted especially in combination with haemin in a high ROS production. AS was the most toxic substance shown by a high AK release and a low ATP content in presence of iron and alone. MT04 and OZ78 treatment resulted in high ROS values in combination with haemin already after 90 min, while ROS values generated by AS were only after 24 hours comparable with those generated by MT04 or OZ78. Neither OZ78 nor MT04 degraded in presence of liver microsomes, while Dihydroartemisinin (DHA) was rapidly formed after incubation of microsomes with AS.

MT04 and OZ78 might act via ROS generation on *F. hepatica*. Since a toxic effect could be measured for MT04, thorough preclinical toxicity studies are needed for this drug candidate.

Keywords: MT04, OZ78, Toxicity, ROS, Fascioliasis

1. Introduction

Fasciola hepatica is a food-borne-trematode, which infests about 2.4-17 million people worldwide with an estimated global burden of about 35'206 disability adjusted life years (DALYs) [1, 2]. The parasites settle in the liver, bile ducts, and the gallbladder of the host causing fascioliasis [1, 3].

Triclabendazole is first choice therapy against fascioliasis; however, resistance to this anthelmintic drug has already been documented in sheep in Australia and from other continents. It is therefore of great importance to discover new drugs [4, 5].

In our recent work we have shown that OZ78 and MT04 are promising fasciocidal drug candidates [6, 7]. OZ78 is a 1,2,4-trioxolane and belongs to the so called secondary ozonides (OZs) [8]. MT04 is a 1,2,4,5-tetraoxane [9].

Different studies under standardized conditions showed that OZ78 and other trioxolanes react with iron. Especially the reduced form (Fe(II)), which is coordinated in heme, has been reported to build alkylated heme adducts at a high reaction rate via an intermediate step of carbonic-centered radicals [10, 11]. It is proposed that these alkylated heme adducts might be responsible for the biological activity of these synthetic peroxides [10].

Artesunate (AS) is a semi-synthetic, water soluble derivative derived from the endoperoxide artemisinin, a natural extraction product from the plant *Artemisia annua* [12]. Dihydroartemisinin (DHA) is the active metabolite of AS. Both compounds are widely used in the treatment of malaria [12]. In addition, both drugs are also active against the liver fluke *F. hepatica* [13].

The endoperoxide group of the artemisinins acts as pharmacopohore, but it is also supposed to be responsible for neurotoxicity and embryo toxicity in animal studies [14, 15]. Artemisinin and its derivatives are toxic to neuronal cells at micro molar concentration [16, 17].

While the artemisinins have poor pharmacokinetic parameters (low bioavailability and short half-lives) [18], many fully synthetic compounds are characterized by higher bioavailability, higher plasma concentration and hence longer systemic exposure. Indeed, for example OZ78 was reported to have a bioavailability of 74% and half life of 2 hours, approximately twice as long as AS [8, 19]. It is therefore important to define the toxicity of synthetic peroxidic drug candidates. Preliminary studies have shown that

OZ78 was neither mutagenic nor toxic on a lymphoma cell line [8]. To our knowledge, however, no other toxicity studies have been carried out with OZ78 and the tetraoxanes. In this work we aim to elucidate the toxic mode of action of OZ78 and MT04 on the liver cell line, HepG2, compared to AS. We particularly address the role of iron or haemin in inducing toxicity. HepG2 cells are used frequently for the assessment of hepato-cellular toxicity, and since *Fasciola* is a liver fluke this cell-line was applied.

2. Materials and methods

2.1. Materials

OZ78 and MT04 were kindly supplied by the College of Pharmacy, University of Nebraska Medical Centre (Omaha, USA, [20]). Artesunate (AS) was obtained from Mepha AG (Aesch, Switzerland) and α-β-dihydroartemisinin (DHA) was kindly provided from Dafra Pharma (Turnhout, Belgium). Chemical structures are shown in Figure 1.

Triton-X-100 (Sigma-Aldrich, Switzerland) was used as positive control to induce full cell lysis.

The ToxiLight® BioAssay Kit LT007-117 was obtained from Lonza (Basel, Switzerland).

Cell culture plates were purchased from BD Biosiences (Franklin Lakes, NJ).

Human liver microsomes were purchased from BD Gentest (Woburn, MA, USA).

All other chemicals used were ordered from Sigma or Fluka.

Figure 1 near here

2.2. Cells and culture conditions

The human hepatocarcinoma cell line HepG2 was purchased from ATCC. HepG2 cells were maintained in DMEM containing 1 g/L glucose supplemented with 10% fetal bovine serum, 5% non-essential amino acids, and HEPES buffer, pH 7.2 (Invitrogen). Cells were incubated under humidified air containing 5% CO_2 at 37°C.

For cell counting, cells were trypsinized for 5 minutes until they were detached, stained with trypan blue and then counted using a haemocytometer.

2.3. Treatments

Stock solutions of the drugs tested were prepared in DMSO 100% (v/v). Final concentrations of DMSO did not exceed 0.1% (v/v) for all experiments performed. Treatments with OZ78, MT04, or AS (10, 100, or 500 µM) were performed either alone or in combination with 8% (v/v) haemin-, Fe(II)chloride- or Fe(III)chloride-solution for 24 h. The final iron concentration used was 122 µM (stock 1.5 mM) based on previous *in vitro* studies with *Fasciola hepatica* [21].

The haemin solution was prepared as follows: 5 mg haemin was dissolved in 1 ml of 0.1 M aqueous solution of NaOH, 3.95 ml of PBS (pH= 7.4) and 0.05 ml of 1 M HCl were added to adjust the pH to 7.1–7.4 [21]. For the iron-solutions, hydrated salt was used

and solved in ultra pure water. All solutions were sterile filtered (0.2 µm) after preparation.

For positive and negative control, cells were incubated in 0.1% Triton-X or 0.1% DMSO, respectively.

2.4. ToxiLight (adenylate kinase release)

The ToxiLight assay was used to determine the toxicity of AS, MT04, and OZ78 with or without combination of haemin, Fe(II), or Fe(III). Adenylate kinase (AK) release reflects the loss of cell membrane integrity.

ToxiLight assay was performed after 24 hours treatment according to the protocol supplied by the manufacturer.

2.5. ATP content measurements

To assess ATP content the CellTiter GloKit was used (Promega). HepG2 cells were seeded at a density of 20'000 cells/well in white-wall 96-well plates with clear bottom. After 24 h treatment with the respective drugs medium was removed from the cells and once washed with PBS containing Ca^{2+}/Mg^{2+} in order to remove all haemin. Finally 50 µl PBS was added to each well and incubated with 50 µl CellTiter-Glo reagent as described in the protocol provided by the manufacturer. Luminescence was measured using Tecan M200 Infinite Pro.

2.6. Measurement of reactive oxygen species (ROS)

Dichlorodihydrofluorescein (DCF) was purchased at Sigma and was used to assess the formation of reactive oxygen species (ROS). HepG2 cells were seeded in a black-wall 96-well plate with clear bottom as described above. Briefly, 30 min prior to treatment start, DCF was added to the cells at a final concentration of 50 µM. DCF was removed before the drugs were added. ROS was measured after 90 min, 4 h, and 24 h using a Tecan M200 Infinite Pro (excitation 485 nm, emission 535 nm).

2.7. *In vitro* / *ex vivo* worm studies

Adult *F. hepatica* were collected from bovine liver bile ducts obtained from the local slaughterhouse (Basel, Switzerland). The flukes were washed for 12 hours in RPMI 1640 culture medium (Gibco, N.Y., USA) at 37°C in an atmosphere of 5% CO_2. After washing, a single adult worm was placed per well (6-well plate, Costar, MA, USA) containing 5.4 ml

RPMI 1640, supplemented with 1% (v/v) antibiotics (50 µg/ml streptomycin and 50 U/ml penicillin; Sigma, Buchs, Switzerland), 1% (v/v) drug-solution and 8% (v/v) haemin-, Fe(II)- or Fe(III)-solution (prepared as described above). Adult *F. hepatica* were incubated in the presence of 50 µg/ml MT04 or OZ78 combined with the different iron-containing solutions. Drug stocks 5 mg/ml were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Buchs, Switzerland) and stored at -20°C. Control worms were incubated in 1% (v/v) DMSO combined with the different iron-solutions. Cultures were kept at 37°C in an atmosphere of 5% CO_2.

The viabilities of treated and untreated *Fasciola* flukes were monitored at 0, 24, 48, and 72 h. Worms were examined using a dissecting microscope. A viability scale from 1 to 4 was used (with 4 for normal activity; movements were visible without microscope to 1 for worm was dead (no movement observed for two min using a microscope)).

2.8. *In vivo* toxicity studies

All animal studies were carried out at the Swiss Tropical and Public Health Institute (Basel, Switzerland) and were approved by Swiss and cantonal authorities (permission: 2070). Female NMRI mice (age: 3–4 weeks, weight: ~20 g) were purchased from Harlan Laboratories (Horst, The Netherlands). Animals were kept in groups 10 in macrolon-cages in environmentally controlled conditions (temperature: ~25°C; humidity: ~70%; 12 h light/dark cycle) and acclimatized for one week. They had free access to water and rodent diet.

For the *in vivo* studies AS was suspended in 7% (v/v) Tween-80 and 3% (v/v) ethanol. Stocks of AS were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Buchs, Switzerland).

Mice were treated with AS at doses of 200, 400, and 800 mg/kg. Three mice were left untreated for control measurements. After 8 h mice were euthanized by CO_2, blood was collected into microtainer lithium heparin tubes. Blood was tested for following lab parameters: creatine kinase (U/L), total bilirubin (µmol/L), aspartate aminotransferase (U/L), and alkaline phosphatase (U/L). After blood collection, the liver was collected and placed into tubes with formalin solution 10% (v/v) for histopathologic observations.

2.9. Measurement of metabolism in presence of human liver microsomes

Determination of degradation of AS and DHA in comparison to MT04 and OZ78 was tested in the presence of human liver microsomes or heat-inactivated (~70°C, 20 min)

microsomes (referred to as control microsomes) at 37°C. The substances were added to 1.0 ml of reaction mixture consisting of microsomes (0.5 mg/kg), the respective drug 0.1% (v/v), NADPH regenerating system and PBS buffer, pH 7.4.
Adding the substances started the reaction. At the beginning of the experiment (0 min) and after 15, 30, 60, 120 min an aliquot of 100 µl was taken out and transferred into an Eppendorf tube. Here the reaction was stopped adding 300 µl methanol, the sample was mixed and centrifuged at 10'000 g for 30 minutes. Supernatants were taken for LC-MS/MS analysis.

2.10. LC-MS/MS analysis

LC-MS/MS measurements for AS and DHA were done by the method validated by Duthaler et al. [22]. Briefly sample measurements were done with a high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) connected to an API 365 triple-quadrupole mass spectrometer (PE Biosystems, Foster City, CA, USA) with a turbo ion spray interface. AS and DHA were detected in positive ionization mode by selected reaction monitoring (SRM) with a transition of 267.4→163.0. For analyte separation a reversed phase column was used (2.1x10 mm guard cartridge (3 µm) connected to a 2.1x20 mm Atlantis T3 (3 µm) analytical column (Waters, Milford MA, USA)). A gradient was used (mobile phase A: 5 mM ammonium formate plus 0.15% (v/v) formic acid in ultra pure water and mobile phase B: 0.15% (v/v) formic acid in acetonitrile) at a flow rate of 0.3 ml/min.
The analysis for OZ78 and MT04 were done by the methods described by Kirchhofer et al. [23, 24]. The same system as described above for AS and DHA was used. A phenomenex C8(2) (50x2.00 mm, 5µm, Brechbühler AG, Schlieren) was used for chromatographic separation with a gradient and at a flow rate of 0.3 ml/min (mobile A: 5 mM ammonium formate in water and mobile B: acetonitrile). MS was conducted in negative ionization mode. OZ78 was detected at 321.4 m/z and MT04 at 337.4 m/z.

2.11. Statistical analysis

Results were expressed as means +/- standard deviation. Student's t-test was used to compare means, p-values < 0.05 were considered to be significant.

3. Results

3.1. AK release

HepG2 cells treated with OZ78 or MT04 did not result in membrane leakage (Fig. 2) at both concentrations (100 and 500 µM) tested. In contrast, AS at 100 and 500 µM was clearly toxic on the cells, the effect was dose dependent.

In a next step, drug toxicity was tested in the presence of iron (haemin or Fe(II)) in relation to DMSO and DMSO/Fe(II) controls.

The combinations of OZ78 and MT04 with inorganic iron-salt (Fe(II)) showed membrane leakage. The effect was more pronounced for MT04 than for OZ78. MT04 (100 and 500 µM) combined with Fe(II) resulted in a significant (all p-values<0.003) dose dependent, higher AK release compared to DMSO and to DMSO/Fe(II) control. AK release for OZ78 (100 and 500 µM) was significantly (p=0.019 and p=0.003) different from DMSO control. AS (100 and 500 µM) showed high toxicity in AK assay in combination of inorganic iron-salt in HepG2 cells. The combination of AS (500 µM) with Fe(II) was so toxic that AK could not be measured any longer, since it has a short half live and was degraded in the medium. All toxicity signals measured for AS were significantly (all p-values<0.02) different compared to DMSO and DMSO/Fe(II) control.

Figure 2 near here

Results obtained combining compounds with haemin are presented in Fig. 3. Haemin solution had an intensive colour resulting in a quenched signal in all luminescent and colorimetric assays used. This reduction of signal was not due to a decrease in toxicity, but instead a false negative signal was generated. Hence, signals were compared to DMSO/haemin control.

OZ78 (100 and 500 µM) was not toxic in the AK assay in combination with haemin-solution as shown in Fig. 3, whereas MT04 showed a clear significant toxic effect in presence of haemin at 100 µM (1.5 times, p=0.038) and at 500 µM (2 times, p=0.021). AS (100 µM) in combination with haemin-solution was also significantly (p=0.023) toxic on HepG2 cells. AS (500 µM) combined with haemin was so toxic that AK could not be measured any longer, since degradation of latter took place.

No difference in toxicity was observed when a Fe(III) solution instead of Fe(II) solution was combined with drugs (data not shown).

In addition, using a lower concentration of 10 µM of all substances was not toxic either alone or in combination with iron (data not shown).

Figure 3 near here

3.2. ATP content

The toxic effects were more pronounced in the ATP assay (as shown in Fig. 4) than in AK release. Treatment of cells with MT04 and OZ78 at 500 µM resulted in a significant decreased ATP content. At 100 µM no toxicity was observed with the drugs. Exposure to AS resulted in a significantly reduced ATP content at both 100 µM (0.4 times) and 500 µM (nearly zero) compared to control value. Combinations of drugs with Fe(II) salt generated a decrease of ATP content for AS and MT04 at 100 and 500 µM, whereas OZ78 was only significantly toxic at the higher concentration (500 µM) tested in combination with Fe(II). Combinations with haemin resulted for all substances (AS, OZ78, and MT04) tested at both concentrations (100 and 500 µM) in a significant reduced ATP content. The effect was most pronounced for AS, followed by MT04 and OZ78.

Figure 4 near here

3.3. ROS measurement

ROS generation was measured, since for this class of molecules peroxide bond cleavage induced by iron resulting in radical formation is a proposed mechanism of action [10, 11, 17]. ROS were measured after 90 min, 4, and 24 hours and compared to DMSO, DMSO/Fe(II) or DMSO/haemin controls.

As shown in Figure 5A, a high ROS signal was detected after 90 min of treatment with MT04 (500 µM) combined with haemin. Treatments with MT04 (500 µM) without any addition or combined with Fe(II) were also significantly different compared to controls. The same effect was measured for OZ78, all three treatments (500 µM) were significantly different from control measurement. MT04 (500 µM) and OZ78 (500 µM) with haemin or Fe(II) were as well significantly (all p-values<0.025) different to DMSO/haemin or DMSO/Fe(II) control values.

At this early time point (90 min), ROS generation for AS (500 µM) and haemin was less pronounced compared with MT04 or OZ78, but was significantly different in comparison to DSMO control. AS (500 µM) in combination with the different iron solutions (haemin or Fe(II)) was not significantly different to DMSO/haemin or DMSO/Fe(II) controls.

Similar ROS signals were obtained after 4 h treatment (data not shown).

After 24 hours the ROS signal induced by the combination of AS (100 µM and 500 µM) with haemin had increased. In addition, the combinations of MT04 and OZ78 (100 µM) with haemin generated likewise a higher ROS than after 90 min. ROS values after 24 hours are presented in Figure 5B. Overall, the ROS generation was less pronounced for all compounds in combination with Fe(II) than with haemin, only a small increase could be measured after 24 hours compared to control. However, at 500 µM drugs and Fe(II) ROS levels were significantly different to DMSO/Fe(II) control.

Figure 5 A and B near here

3.4. *In vitro* / *ex vivo* worm studies

As already shown by Kirchhofer et al [6], worms incubated in the presence of 50 µg/ml MT04 and haemin were dead after 72 hours, whereas worms incubated with 50 µg/ml OZ78 were still alive revealing a reduced activity (Fig. 6 A). Incubation of worms in Fe(II) or Fe(III) combined with MT04 or OZ78 at a concentration of 50 µg/ml did not result in death of worms, however worms showed minor activity as depicted in Figure 6 B and C.

Figure 6 near here

3.5. *In vivo* studies

In vivo studies were done with AS, the most toxic substance on the basis of AK and ATP assay. AS was applied at different dosages (200, 400, or 800 mg/kg) to mice. In pre-studies, the best time-point (8 h) for clinical diagnostics was determined (data not shown). Artesunate showed no effect on creatine kinase (U/L), total bilirubin (µmol/L), aspartate aminotransferase (U/L), and alkaline phosphatase (U/L) at all dosages. All treatments were well tolerated by mice. Histopathologic observation confirmed these results. The liver tissue was neither damaged nor showed lesions after AS treatment (200-800 mg/kg) as shown in Fig. 7.

Figure 7 near here

3.6. Microsome-studies

Microsomes (normal and heat inactivated) were incubated with OZ78, MT04, AS, and DHA at 10 µM. Degradation was measured over time, results are presented in Table 1. No degradation was observed for OZ78 and MT04. Incubation of microsomes with AS resulted in the formation of DHA, whereas DHA was stable in presence of microsomes.

Table 1 near here

4. Discussion

Fascioliasis is a considerable public health problem and resistance to the only available drug triclabendazole is a big motivation to discover novel drugs [25]. MT04 and OZ78 are promising drug candidates against fascioliasis [6]. In this study, we aimed to get an insight on the toxicity of these drugs (especially liver toxicity since *F. hepatica* is a liver fluke). Furthermore, different iron-solutions (Fe(II) and haemin) were used in order to gain a better understanding of the toxic pathways. For comparison, the structurally related antimalarials AS and DHA, also known for their fasciocidal properties were included in this work [13]. While for malaria treatment only nano-molar concentrations of AS and DHA are used [12, 17] for fasciocidal activity concentrations in the micro-molar range of semi-synthetic (AS and DHA) and fully synthetic substances (OZ78 and MT04) have been measured in sheep [26, 27] and rat studies [8, 28]. Therefore, high concentrations (100 – 500 µM) correlating with fasciocidal activity were selected in the present work.

OZ78 did not result in membrane leakage in HepG2 cells with or without iron, whereas a reduction in viability was measured in the ATP assay for all solutions of OZ78 tested at 500 µM. MT04 showed toxicity in combination with iron (haemin or Fe(II)) in the AK release assay. The toxic effect with MT04 was more pronounced in the CellTiter Glo assay, even MT04 (500 µM) without iron resulted in decreased viability. However, AS was the most toxic substance in this study. AS showed clear signs of toxicity, in particular in combination with iron salt, as presented by a high AK release as well as in a very low ATP content.

The high toxicity of the combination AS with iron was not surprising, since Mercer et al. have recently described an apoptotic process in presence of iron of the artemisinins on fast proliferating cells [29]. Our studies confirmed these results. Cells incubated in the presence of AS were more damaged in the presence of iron than without. The iron dependent toxicity was less pronounced for MT04 or OZ78 as shown in our assays.

As already described, iron might play an important role for this class of molecules in activating and generating radicals [10, 11, 17, 29]. Therefore, we were interested in ROS production of MT04 and OZ78, since for DHA and AS, ROS production has been described in literature [30, 31]. In our studies, MT04 and OZ78 resulted in high ROS values in combination with haemin already after 90 min, while the effect was less

pronounced in combination with Fe(II). In contrast, AS resulted in a lower ROS value compared to MT04 or OZ78 after 90 min. However, after 24 hours all combinations with haemin revealed a similarly high ROS signal.

A possible explanation for the higher ROS signal of MT04 compared to OZ78 might be the presence of the double peroxide bridge in the structure of MT04. The higher ROS generation following MT04 treatment compared to OZ78 might also explain why MT04 has a higher *in vitro* and *in vivo* activity on adult *F. hepatica* than OZ78. The ROS results obtained are also in line with the *in vitro* studies done with *F. hepatica*. OZ78 or MT04 combined with haemin showed the highest activity on the worms *in vitro*. Measurement of the ROS generation in *in vitro* assays with the peroxides might be interesting in order to get a better understanding of the role of ROS in killing *Fasciola*.

Toxicity in mice was only tested with AS, the most toxic drug of this study. AS was applied at different doses (200-800 mg/kg) to mice, but no toxicity was found. Creatine kinase (U/L), total bilirubin (µmol/L), aspartate aminotransferase (U/L), and alkaline phosphatase (U/L) were neither lower nor higher than control values. Despite the high toxic results *in vitro,* the drug seemed to be safe in mice after single dose administration. The safety after multiple doses has not been tested in our study. Published LD50 values of AS in mice were 520 and 475 mg/kg after intravenous and intramuscular administration, respectively [26]. The lack of toxicity in our study is most likely due to the low bioavailability of AS after oral application. Interestingly, in another study three of five *F. hepatica* infected rats died after an AS application of 400 mg/kg [13], which might be explained by an altered drug metabolism in infected rats [28]. Using the body surface area dose conversion scheme, a dose of 400 mg/kg in rats is equivalent to a dose of 800 mg/kg in mice (http://www.fda.gov). Therefore, additional investigations should be carried out in infected animals.

In summary, we believe that the synthetic peroxides MT04 and OZ78 react via ROS formation against *F. hepatica*. More studies are needed to clarify the exact mechanism of action of peroxidic drugs against *F. hepatica* infection and to elucidate the full safety profile. Studies in cells showed toxic effects, in particular for MT04, however whether these effects are relevant is not yet known.

Acknowledgment

J. Keiser and C. Kirchhofer are grateful to the Swiss National Science Foundation (SNF) (project PPOOA- 114941) for financial support. The SNF had no influence in the

collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

We are thankful to Prof. J. Huwyler for scientific support. Thanks are also addressed to Annalisa Bonifacio for her laboratory support.

Legend to Figures

Figure 1:

Molecular structure of (A) OZ78, (B) MT04, (C) Artesunate, and (D) Dihydroartemisinin.

Figure 2:

Adenylate kinase release (AK).

HepG2 cells were treated with OZ78, MT04, or AS at 100 and 500 μM alone or in the presence of iron (II) salt for 24 hours.

Toxicity was assessed measuring adenylate kinase release. Results represent the mean of five independent experiments carried out in triplicates. Triton-X was used as positive control reflecting complete cell lysis.

✶: indicates significance compared to DMSO control, $p < 0.05$.

✧: indicates significance compared to DMSO/Fe(II) control, $p < 0.05$.

Figure 3:

Adenylate kinase release (AK).

HepG2 cells were treated with OZ78, MT04, or AS at 100 and 500 μM in the presence of haemin-solution for 24 hours.

Toxicity was assessed measuring adenylate kinase release. Results represent the mean of five independent experiments carried out in triplicates. Triton-X was used as positive control reflecting complete cell lysis.

✶: indicates significance compared to DMSO control, $p < 0.05$.

Figure 4:

ATP content.

HepG2 cells were treated with OZ78, MT04, or AS at 100 and 500 μM alone or in the presence of iron (II) salt or haemin-solution for 24 hours.

Toxicity was assessed measuring ATP content. Results represent the mean of five independent experiments carried out in triplicates. Triton-X was used as positive control reflecting complete cell lysis.

✶: indicates significance compared to DMSO control, $p < 0.05$.

Figure 5:

ROS generation.

HepG2 cells were treated with OZ78, MT04, or AS at 100 or 500 µM alone or in the presence of iron (II) or haemin. ROS production was measured after 90 min (A) and 24 h (B) incubation with the compounds.

Results are expressed as means of three independent experiments carried out in triplicates.

✶: indicates significance compared to DMSO control, $p < 0.05$.

✧: indicates significance compared to corresponding DMSO control, $p < 0.05$.

Figure 6:

In vitro activity of MT04 (black line), OZ78 (grey line) at a concentration of 50 µg/ml against control (dotted line) adult *F. hepatica* combined with (A) haemin, (B) Fe(II)-solution, and (C) Fe(III)-solution. Values are means ± SEM, n=6.

Figure 7:

Histopathologic observation.

Histopathologic pictures of liver collected from mice treated with AS (200-800 mg/kg) and from control mice (magnification of the pictures x20).

Table 1: Degradation studies of AS, DHA, OZ78, and MT04 with microsomes.

Substance	**Conc.**	**Combination**	**Measurement time-point**	**Percent of start [%]**
AS DHA OZ78 MT04	10 µM	microsomes	120 min	**5.8** *No degradation* *No degradation* *No degradation*
AS DHA OZ78 MT04	10 µM	inactivated microsomes	120 min	*No degradation* *No degradation* *No degradation* *No degradation*

References

[1] Keiser J, Utzinger J, Food-borne trematodiases. Clin Microbiol Rev 2009;22:466-83.

[2] Furst T, Keiser J, Utzinger J, Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. Lancet Infect Dis 2011;

[3] Mas-Coma S, Epidemiology of fascioliasis in human endemic areas. J Helminthol 2005;79:207-16.

[4] Moll L, Gaasenbeek CP, Vellema P, Borgsteede FH, Resistance of Fasciola hepatica against triclabendazole in cattle and sheep in The netherlands. Vet Parasitol 2000;91:153-8.

[5] Fairweather I, Boray JC, Fasciolicides: efficacy, actions, resistance and its management. Vet J 1999;158:81-112.

[6] Kirchhofer C, Vargas M, Braissant O, Dong Y, Wang X, Vennerstrom JL, et al., Activity of OZ78 analogues against Fasciola hepatica and Echinostoma caproni. Acta Trop 2011;118:56-62.

[7] Keiser J, Utzinger J, Tanner M, Dong Y, Vennerstrom JL, The synthetic peroxide OZ78 is effective against Echinostoma caproni and Fasciola hepatica. J Antimicrob Chemother 2006;58:1193-7.

[8] Vennerstrom JL, Arbe-Barnes S, Brun R, Charman SA, Chiu FC, Chollet J, et al., Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 2004;430:900-4.

[9] Wang X, Zhao Q, Vargas M, Dong Y, Sriraghavan K, Keiser J, et al., The activity of dispiro peroxides against Fasciola hepatica. Bioorg Med Chem Lett 2011;21:5320-3.

[10] Creek DJ, Charman WN, Chiu FC, Prankerd RJ, Dong Y, Vennerstrom JL, et al., Relationship between antimalarial activity and heme alkylation for spiro- and dispiro-1,2,4-trioxolane antimalarials. Antimicrob Agents Chemother 2008;52:1291-6.

[11] Creek DJ, Charman WN, Chiu FC, Prankerd RJ, McCullough KJ, Dong Y, et al., Iron-mediated degradation kinetics of substituted dispiro-1,2,4-trioxolane antimalarials. J Pharm Sci 2007;96:2945-56.

[12] White NJ, Qinghaosu (artemisinin): the price of success. Science 2008;320:330-4.

[13] Keiser J, Shu-Hua X, Tanner M, Utzinger J, Artesunate and artemether are effective fasciolicides in the rat model and in vitro. J Antimicrob Chemother 2006;57:1139-45.

[14] Clark RL, White TE, S AC, Gaunt I, Winstanley P, Ward SA, Developmental toxicity of artesunate and an artesunate combination in the rat and rabbit. Birth Defects Res B Dev Reprod Toxicol 2004;71:380-94.

[15] Mercer AE, Copple IM, Maggs JL, O'Neill PM, Park BK, The role of heme and the mitochondrion in the chemical and molecular mechanisms of mammalian cell death induced by the artemisinin antimalarials. J Biol Chem 2011;286:987-96.

[16] Meshnick SR, Taylor TE, Kamchonwongpaisan S, Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiol Rev 1996;60:301-15.

[17] O'Neill PM, Posner GH, A medicinal chemistry perspective on artemisinin and related endoperoxides. J Med Chem 2004;47:2945-64.

[18] Keiser J, Utzinger J, Artemisinins and synthetic trioxolanes in the treatment of helminth infections. Curr Opin Infect Dis 2007;20:605-12.
[19] Mercer AE, The role of bioactivation in the pharmacology and toxicology of the artemisinin-based antimalarials. Curr Opin Drug Discov Devel 2009;12:125-32.
[20] Tang Y, Dong Y, Wang X, Sriraghavan K, Wood JK, Vennerstrom JL, Dispiro-1,2,4-trioxane analogues of a prototype dispiro-1,2,4-trioxolane: mechanistic comparators for artemisinin in the context of reaction pathways with iron(II). J Org Chem 2005;70:5103-10.
[21] Keiser J, Morson G, Fasciola hepatica: tegumental alterations in adult flukes following in vitro and in vivo administration of artesunate and artemether. Exp Parasitol 2008;118:228-37.
[22] Duthaler U, Keiser J, Huwyler J, Development and validation of a liquid chromatography and ion spray tandem mass spectrometry method for the quantification of artesunate, artemether and their major metabolites dihydroartemisinin and dihydroartemisinin-glucuronide in sheep plasma. J Mass Spectrom 2011;46:172-81.
[23] Kirchhofer C, Keiser J, Huwyler J, Development and validation of a liquid chromatography/mass spectrometry method for pharmacokinetic studies of OZ78, a fasciocidal drug candidate. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878:2770-4.
[24] Kirchhofer C, Vargas M, Huwyler J, Keiser J, Pharmacokinetics of the fasciocidal drug candidates MT04 and OZ78 in healthy rats. Manuscript submitted
[25] Overend DJ, Bowen FL, Resistance of Fasciola hepatica to triclabendazole. Aust Vet J 1995;72:275-6.
[26] Keiser J, Veneziano V, Rinaldi L, Mezzino L, Duthaler U, Cringoli G, Anthelmintic activity of artesunate against Fasciola hepatica in naturally infected sheep. Res Vet Sci 2010;88:107-10.
[27] Duthaler U, Huwyler J, Rinaldi L, Cringoli G, Keiser J, Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with Fasciola hepatica. Vet Parasitol 2011;
[28] Keiser J, Gruyer MS, Perrottet N, Zanolari B, Mercier T, Decosterd L, Pharmacokinetic parameters of artesunate and dihydroartemisinin in rats infected with Fasciola hepatica. J Antimicrob Chemother 2009;63:543-9.
[29] Mercer AE, Maggs JL, Sun XM, Cohen GM, Chadwick J, O'Neill PM, et al., Evidence for the involvement of carbon-centered radicals in the induction of apoptotic cell death by artemisinin compounds. J Biol Chem 2007;282:9372-82.
[30] Lu JJ, Meng LH, Cai YJ, Chen Q, Tong LJ, Lin LP, et al., Dihydroartemisinin induces apoptosis in HL-60 leukemia cells dependent of iron and p38 mitogen-activated protein kinase activation but independent of reactive oxygen species. Cancer Biol Ther 2008;7:1017-23.
[31] Du JH, Zhang HD, Ma ZJ, Ji KM, Artesunate induces oncosis-like cell death in vitro and has antitumor activity against pancreatic cancer xenografts in vivo. Cancer Chemother Pharmacol 2010;65:895-902.

Figures

Figure 1

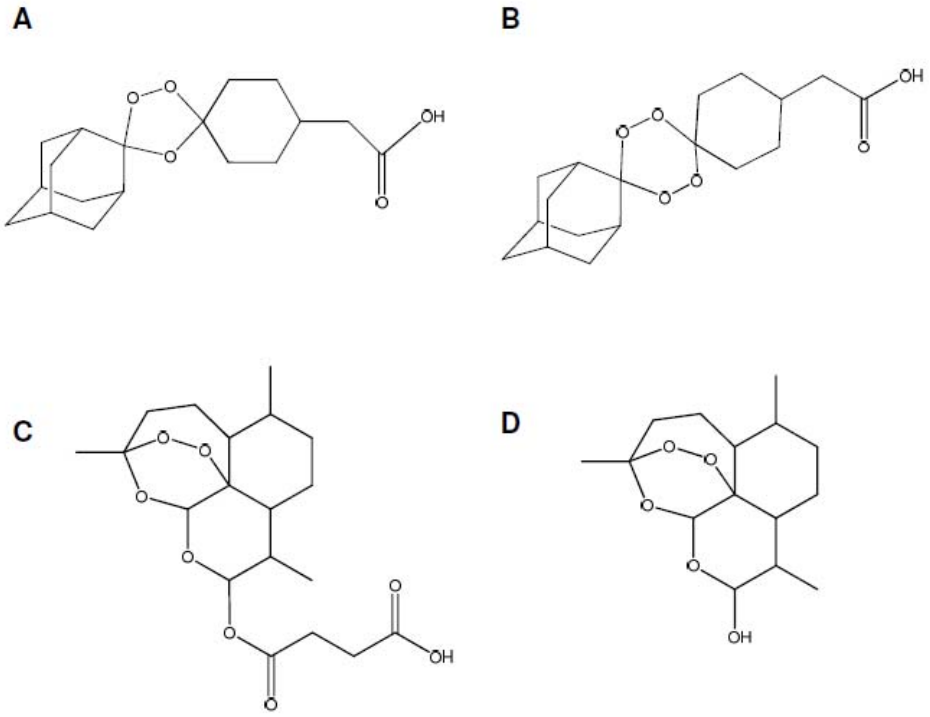


Figure 2

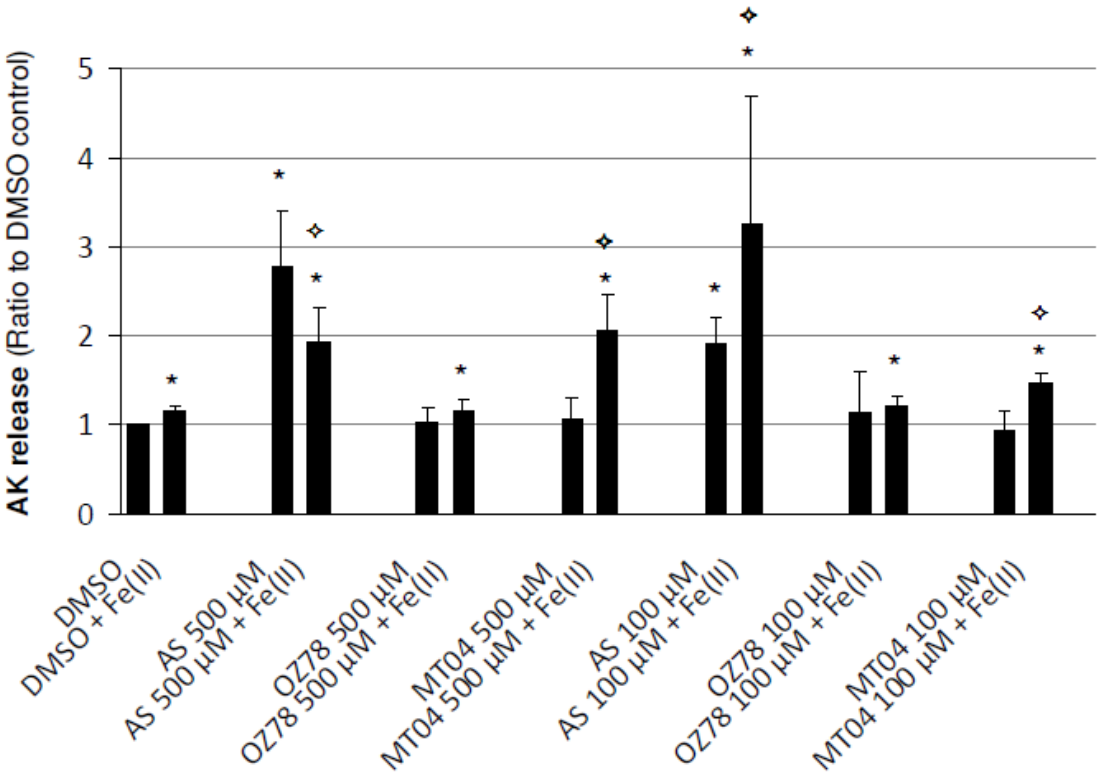


Figure 3

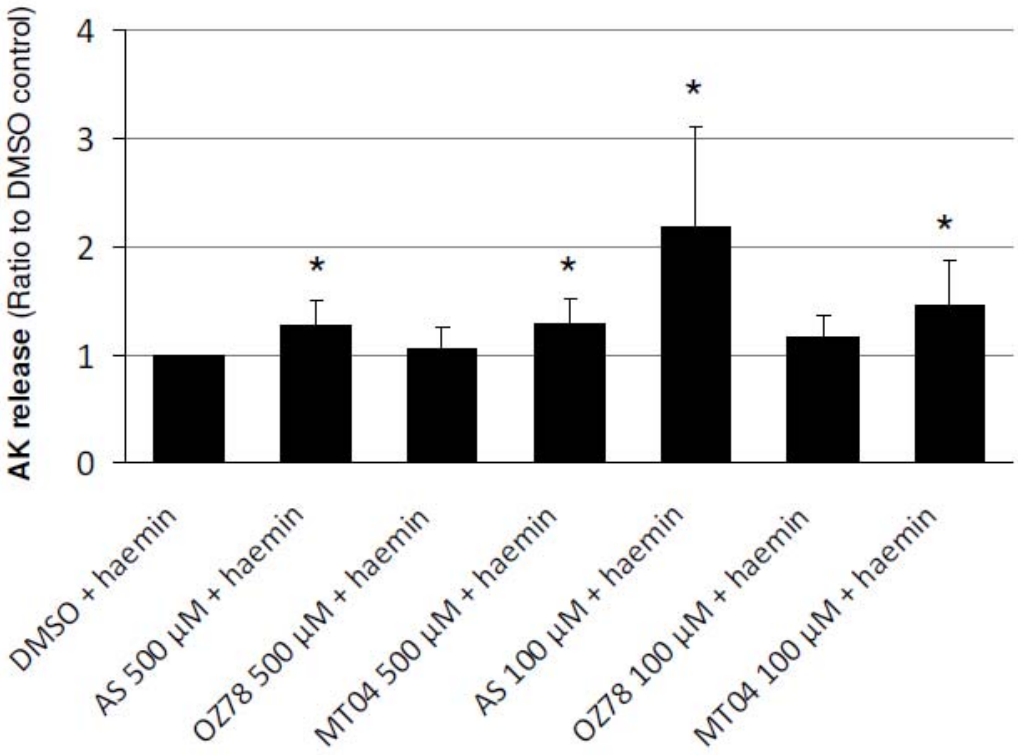


Figure 4

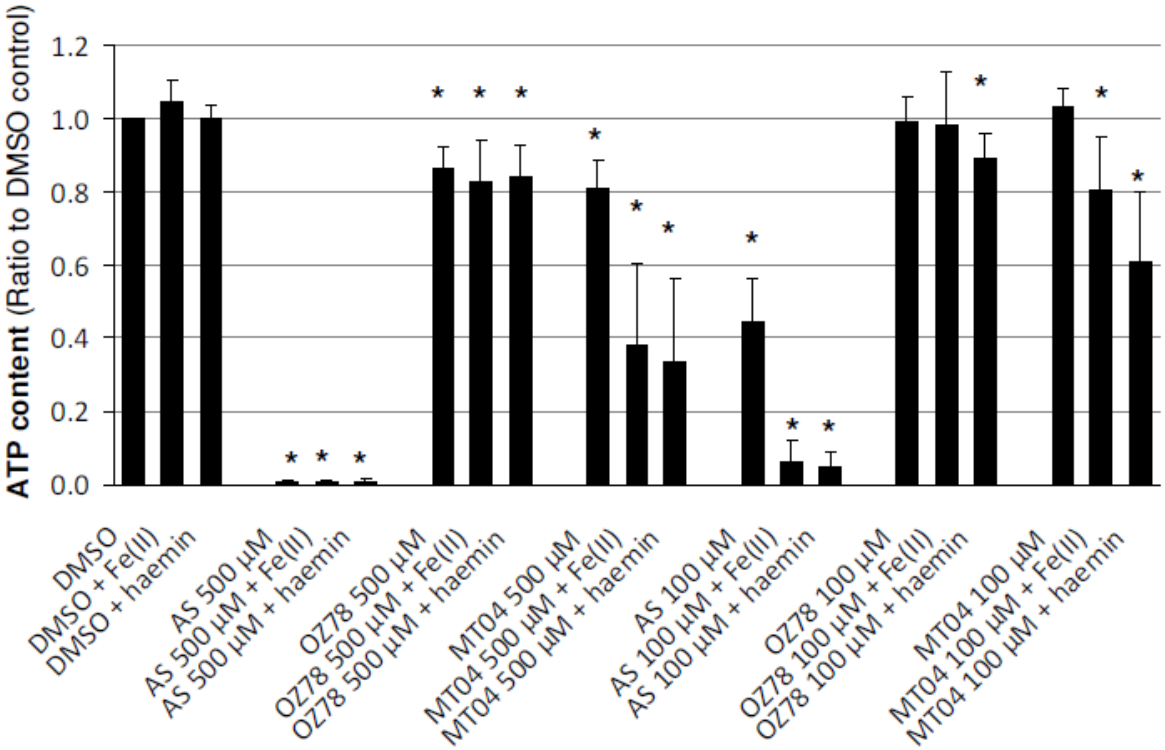

ATP content (Ratio to DMSO control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
DMSO + Fe(II)
DMSO + haemin
AS 500 µM
AS 500 µM + Fe(II)
AS 500 µM + haemin
OZ78 500 µM
OZ78 500 µM + Fe(II)
OZ78 500 µM + haemin
MT04 500 µM
MT04 500 µM + Fe(II)
MT04 500 µM + haemin
AS 100 µM
AS 100 µM + Fe(II)
AS 100 µM + haemin
OZ78 100 µM
OZ78 100 µM + Fe(II)
OZ78 100 µM + haemin
MT04 100 µM
MT04 100 µM + Fe(II)
MT04 100 µM + haemin

Figure 5

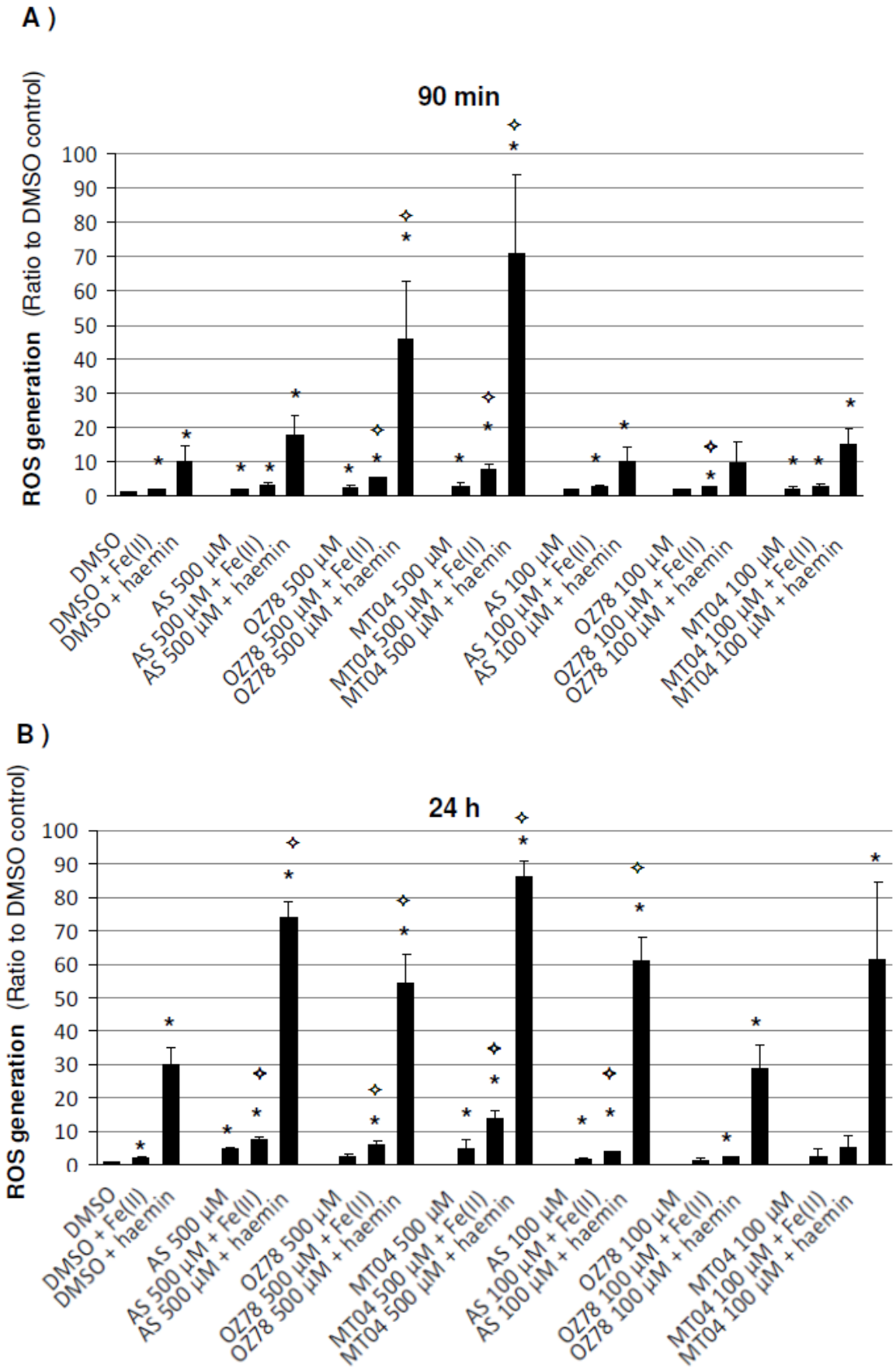

A)
90 min
ROS generation (Ratio to DMSO control)
100
90
80
70
60
50
40
30
20
10
0
DMSO
DMSO + Fe(II)
DMSO + haemin
AS 500 µM
AS 500 µM + Fe(II)
AS 500 µM + haemin
OZ78 500 µM
OZ78 500 µM + Fe(II)
OZ78 500 µM + haemin
MT04 500 µM
MT04 500 µM + Fe(II)
MT04 500 µM + haemin
AS 100 µM
AS 100 µM + Fe(II)
AS 100 µM + haemin
OZ78 100 µM
OZ78 100 µM + Fe(II)
OZ78 100 µM + haemin
MT04 100 µM
MT04 100 µM + Fe(II)
MT04 100 µM + haemin
B)
24 h
ROS generation (Ratio to DMSO control)
100
90
80
70
60
50
40
30
20
10
0
DMSO
DMSO + Fe(II)
DMSO + haemin
AS 500 µM
AS 500 µM + Fe(II)
AS 500 µM + haemin
OZ78 500 µM
OZ78 500 µM + Fe(II)
OZ78 500 µM + haemin
MT04 500 µM
MT04 500 µM + Fe(II)
MT04 500 µM + haemin
AS 100 µM
AS 100 µM + Fe(II)
AS 100 µM + haemin
OZ78 100 µM
OZ78 100 µM + Fe(II)
OZ78 100 µM + haemin
MT04 100 µM
MT04 100 µM + Fe(II)
MT04 100 µM + haemin

Figure 6

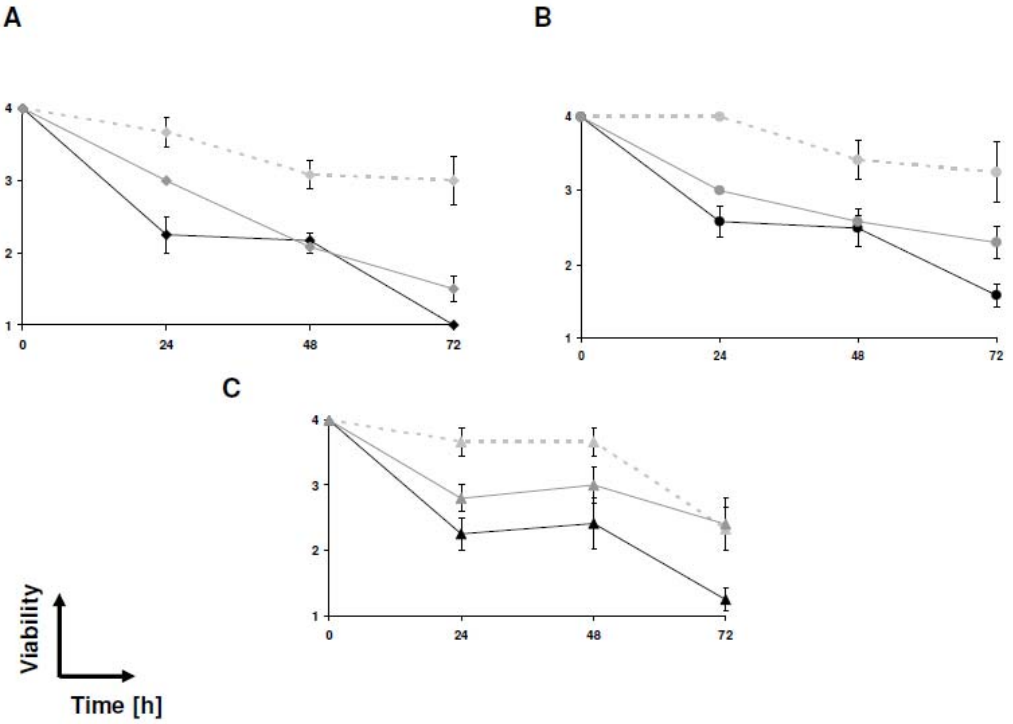
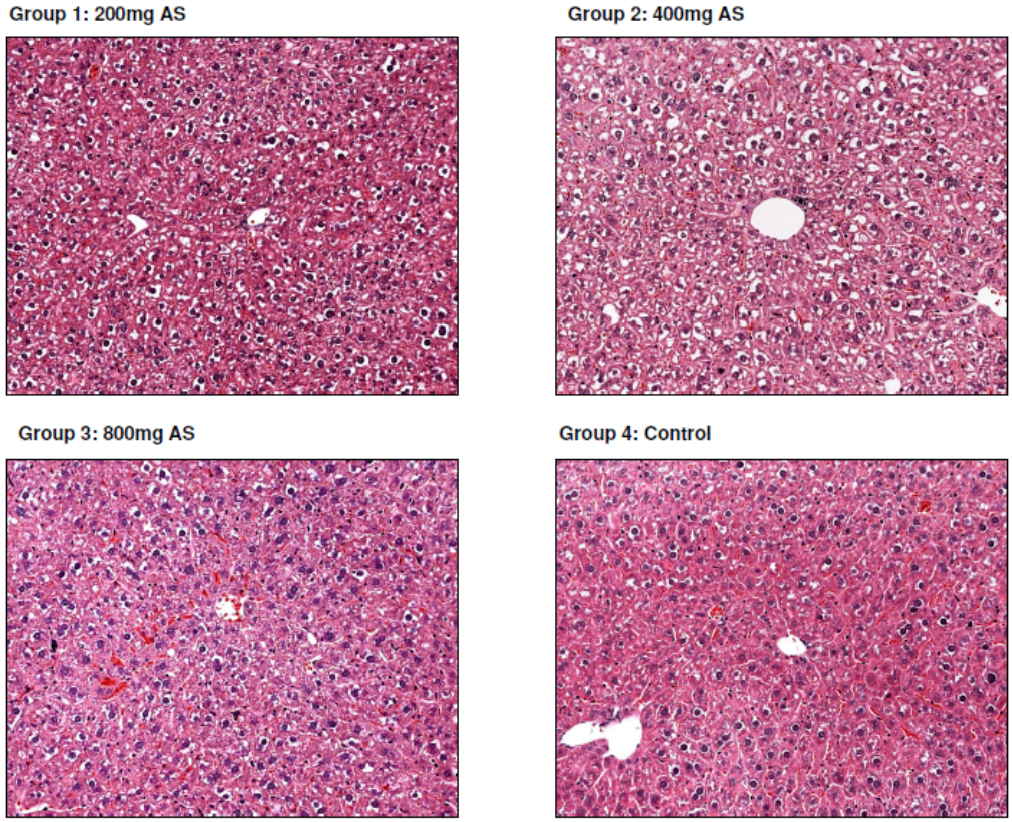


Figure 7



General Discussion and Conclusion

OZ78 and MT04 fasciocidal drug candidates

General Discussion

Rationale, Objectives and Aim of the Present Thesis

This PhD thesis describes the preclinical development of new promising synthetic peroxides gains fascioliasis.

The worldwide incidence of fascioliasis in livestock (sheep and cattle) amounts to about 300 million cases [1, 2]. Mortality and morbidity in livestock lead to high economic loss [3]. In addition, many humans are infected with this parasite. The global burden of fascioliasis is estimated to be 35'206 DALY's [4]. Though fascioliasis is a disease associated with low-income countries, it is often neglected that the disease is also a problem in European countries [5]. In Switzerland, the prevalence of bovine fascioliasis is estimated to be higher than 18% [6]. The importance of this disease has been underestimated for a long time and to date triclabendazole (TBZ) is the only available drug active against acute and chronic fascioliasis [7, 8]. The pipeline of new drugs against *F. hepatica* is empty and no new compound has been marketed since the 1980's [8].

The main goal of this research project was to study the promising lead candidates OZ78 and MT04 with regard to activity against *F. hepatica* in different *in vitro* models, to define the *in vivo* efficacy (in rat and in sheep) and to elucidate the PK-parameters (in rat and in sheep). In a further step, the toxic effects of OZ78 and MT04 were identified on a liver cell line (HepG2). To date, the mechanism of action of these synthetic peroxidic substances in killing *F. hepatica* is poorly understood [9]. Since the use of drugs can be optimized by understanding the drug mechanism, the deep knowledge of pharmacokinetics/pharmacodynamics is mandatory in the drug discovery [8].

OZ78 is a secondary ozonide (OZ), which is easy to synthesize, exhibits structural simplicity and has improved biopharmaceutical profile compared to first generation peroxides (e.g. artesunate, artemether) [10]. The OZs were basically developed for malaria drug discovery [10]. The common feature of *P. falciparum* and *F. hepatica* is that both parasites feed on blood [11-13]. Therefore it was interesting and worthwhile to test these malaria drug candidates also against fascioliasis. OZ78 is a promising drug

candidate in treating fascioliasis. Previous studies have shown that after 72 hours *in vitro* incubation time applying a concentration of 100 µg/ml OZ78, all *F. hepatica* worms were dead [14]. *In vivo* studies in rats showed a cure rate (worm burden reduction) of 100% against adult and juvenile worms with an orally applied dose of 100 mg/kg. OZ78 was also active against a TBZ-resistant strain (Oberon isolates) in rats [15].
This was the starting knowledge for this PhD thesis before I embarked on the project.

The thesis is divided into three main parts (*chapter 2, chapter 3-5,* and *chapter 6*). First, promising candidates against fascioliasis were studied in more detail. The aim was to identify other lead candidates (*chapter 2*). ST28, ST16 (1,2,4-trioxanes) and MT14, MT04 (1,2,4,5-tetraoxanes) were shown to be promising compounds against fascioliasis, complete worm burden reductions were achieved with all four substances at a dose of 100 mg/kg in rats. Therefore we studied the *in vitro* activity of these compounds on juvenile and adult worms. In addition, we determined dose-response relationships against adult and juvenile infection in rats. According to these findings, MT04 was the best candidate. After 72 hours *in vitro* incubation all adult and juvenile worms were dead at a concentration of 50 µg/ml and 100 µg/ml, respectively. MT04 showed an cure rate of 100% against adult and juvenile infection in rats with a dose of 50 and 100 mg/kg, respectively. SEM pictures with worms collected from MT04 treated rats revealed that the tegument was disrupted and showed severe damage. For the first time, the exact *in vitro* death time could be determined from treated adult *F. hepatica* flukes with calorimetric measurement. The heat flow production of control and treated flukes was measured over time.
In parallel, studies with OZ78 were carried out. After promising results in rat studies, the cure rate in a different species (non-rodent host) needed to be determined. OZ78 was applied to sheep at a dose of 50 mg/kg orally and subcutaneously. Unexpectedly, a big difference in the treatment outcome in sheep infected with *F. hepatica* was found compared to rat studies. Orally and subcutaneously applied OZ78 failed to cure infected sheep at a dose of 50 mg/kg (*chapter 4*). In order to explain these results, it was mandatory to determine PK parameters of OZ78 in sheep.
An appropriate analytical method for OZ78 measurement, in our case a liquid chromatography mass spectrometry method (LC/MS), had to be developed and validated, since drug concentrations have to be quantified simultaneously, accurately, precisely, and selectively in sheep plasma for PK parameters determination (*chapter 3*).

In a next step, we aimed to define the PK parameters in rats because of the variable activity obtained. Therefore we modified the OZ78 LC/MS sheep method for rat plasma and measured PK parameters in rats. Additionally, we adapted the OZ78 LC/MS method for MT04 measurement and measured PK parameters in rats likewise (*chapter 5*).
In a final step, insight into the toxic activity of MT04 and OZ78 was gained. A series of cell studies were conducted with MT04 and OZ78 in combination with different iron-containing solutions in order to get an insight into the reactitvity and toxicity of these substances. Since the reactivity of MT04 and OZ78 seemed to be haemin dependent, a radical formation is the most probable mechanism [9]. As controls, two established drugs (Artesunate (AS) and Dihydroartemisinin (DHA)) were used in these assays (*chapter 6*).

In this next part, I would like to discuss four points, which have not been discussed in such great detail in the individual chapters.

The following topics have been chosen for a more detailed discussion:

- Hit candidates and their activity against *F. hepatica*
- LC/MS methods for synthetic peroxide quantification
- Treatment outcome in sheep and rat
- Toxicity

1. Hit candidates and their activity against *F. hepatica (Chapter 2)*

As described in *chapter 2*, promising hit candidates were studied in more detail against *F. hepatica in vitro* and *in vivo* (rat) and against *E. caproni in vivo* (mice).

E. caproni is an intestinal fluke from the group of food-borne trematodes [16]. It is known that *E. caproni* and juvenile *Fasciola* worms are not big blood feeders [17], whereas the adult *Fasciola* worm is dependent on haemoglobin as a source of nutrition [13]. Therefore, we speculated that substances active against *E. caproni* were as well active against juvenile *F. hepatica* flukes. Blood (haemin) is proposed to play an important role in the activation of synthetic peroxides.

As mentioned before, MT04 was the best candidate out of four (ST16, ST28, MT14, and MT04) substances according to juvenile and adult fasciocidal activity in rats, but failed to cure *E. caproni* infection. MT14 was not as effective as MT04 against *Fasciola* in rats in neither adult nor in the juvenile model. However, in contrast to our hypothesis described above, MT14 showed best cure rates against *E. caproni* infection; a worm burden reduction of 100% was achieved in mice in a dose of 300 mg/kg. Only the trioxanes (ST16 and ST28) supported the hypothesis, both compounds showed the most promising cure rates against adult *F. hepatica* infection, even at a dose of 25 mg/kg and 50 mg/kg, a worm burden reduction of 87.5% and 100% was achieved, respectively. Nevertheless, both compounds failed to cure juvenile *F. hepatica* infection and *E. caproni* infection in mice.

Overall, a correlation of activity and feeding behaviour could not be confirmed.

A new lead drug candidate against fascioliasis needs to be active against juvenile and adult infection, as is the standard drug, TBZ [8, 18]. Obviously, the juvenile activity seems to be the essential factor for a new substance to be a good drug candidate, since juvenile flukes are responsible for most of the damage of fascioliasis [19, 20]. Drugs revealing a promising adult *Fasciola* worm burden reduction do not necessarily show the same effect on juvenile *F. hepatica*. Of course, it is not predictable, if a drug candidate shows good activity against adult and juvenile worms, since mechanism of action, PK and PD parameteres are the most commonly not known.

In studies with schistosomes a so-called stage-specific susceptibility was shown for many drugs: using the same dose schedule the activity of a compound can be different

against various stages of a parasite [21]. The therapy failure of ST16 and ST28 against juvenile flukes could therefore also be a consequence of this stage-specific susceptibility. The response of an higher dose than 100 mg/kg against juvenile flukes has not been tested.

In our studies, only OZs with a good adult activity have been tested against juvenile infection *in vivo*. Therefore, no general conclusion about the structure-activity relationship (SAR) of OZs in juvenile worms is possible to date.

Overall, based on this knowledge I suggest a broader SAR study with the OZs against juvenile infection to determine which functional groups show the highest activity against this stage.

From the SAR study against adult infection, the best structure in killing adult fascioliasis was identified. In the next paragraph the substructures of OZs will be discussed in more detail.

Originally, the new synthetic OZs were developed for malaria treatment [10]. However, OZ78 was not active against *Plasmodia*, the carbonic acid side chain seemed to be the critical factor for this loss of activity [22, 23]. OZ78 and MT04 both have a carbonic acid side chain. OZ78 is deprotonated under physiological conditions and can not enter the erythrocytes where the malaria parasite is located [24]. On the other hand, *F. hepatica* are settled in the liver, gallbladder, and bile ducts and drug can reach the parasite via the blood or bile [16, 25].

The trematode gut of *F. hepatica* has a low pH of 5.5 at which the substance is only partially unionized [9, 13, 26]. Transmission electron microscopy (TEM) studies with OZ78 and *F. hepatica* revealed that the worm is affected when taking up the active substance [27]. On the contrary, SEM studies with *F. hepatica* revealed disruption of the tegument after treatment with MT04 and OZ78 *in vivo* [28]. Because of the higher pH in blood (pH 7.4) or in bile (pH 7.4-8.5), it is supposed that the ionized substance is accountable for this tegument damage [25, 30, 31]. However, whether this carbonic acid chain directly contributes to the fasciocidal activity is also not yet defined. It is more probable that the acidic side chain is responsible for reaching the target site rather than playing a main role in the fasciocidal activity. An alkyl group linked to a carbocylic acid functional group was the optimal structure length for activity. Molecules with a longer or shorter side chain were not as active as MT04 or OZ78 [9], which is an indication for the latter hypothesis. A follow up SAR study *in vitro* might be able to answer this

question, since substances with and without a carboxylic side chain should result in the same activity *in vitro*, if latter hypothesis is true.

The better activity of MT04 compared to OZ78 might be explained by the double peroxide bridge: the tetraoxane substructure (MT04) has been shown to be superior to the trioxolanes (OZ78) with respect to the anti-fasciola activity [12]. We suppose that this substructure is the significant factor for fasciocidal activity. Our proposed mechanism is as follows: MT04 or OZ78 are bioactivated from Fe^{2+} (from blood), which results in C-centered radicals, next ROS are produced and attack the worm as shown in Fig. 1 [11, 29-31]. A specific binding to a protein target is rather improbable for these synthetic peroxides in line as proposed for first generation peroxides [32], rather oxidative stress seems to be essential in killing flukes.

Clarification about the mechanism of MT04 and OZ78 would be an important addition of further studies and in finding a good lead candidate. To date, it could be clarified which peroxide structures are to best according to adult fasciocidal activity. However it is not known why the discussed structures are important in killing *Fasciola* and which structures are the best for juvenile activity.

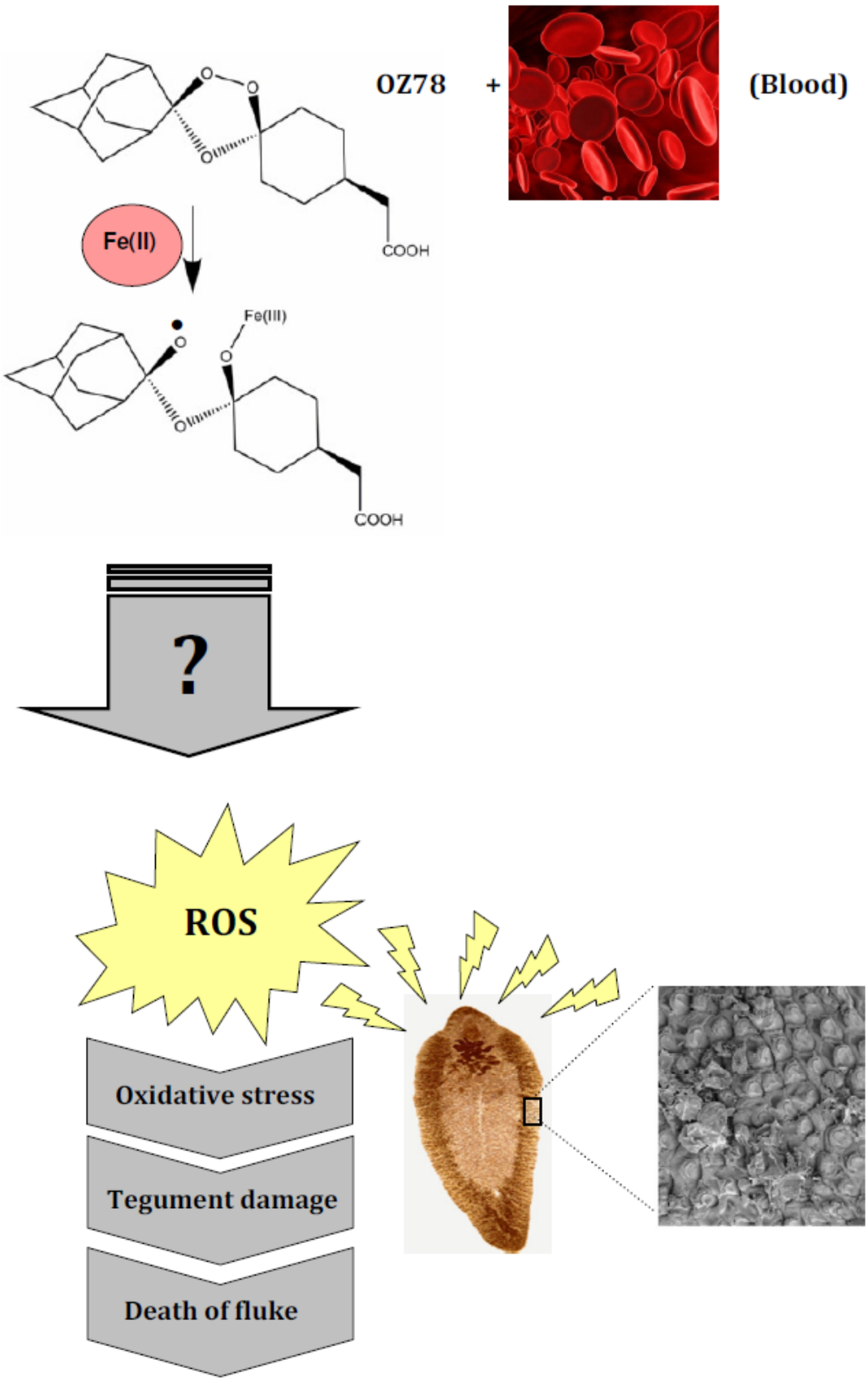


Figure 1: Proposed mechanism of action of OZ78.

A further aspect worth discussing is that a metabolite of MT04 or OZ78 may contribute to the activity. From SEM studies with AM and AS we know that the parent drugs (AS and AM) cause extensive tegument damages to *F. hepatica* after *in vitro* and *in vivo* studies, whereas the active metabolite DHA resulted in less damage. However, DHA treatment resulted in damage of intestine and seemed to attack the worm after ingestion [33]. To date, we do not know if a metabolite of OZ78 or MT04 contributes to fasciocidal activity, and if the damage within the worm was caused by the parent drugs or metabolites. Still, there is no clear insight into the exact mechanism and further investigations are needed in this field.

Different preliminary studies were done, since involvement of different CYP enzymes (Phase I) has been described for OZ277 and OZ439 [34, 35]. Therefore it was reasonable to determine the role of CYP enzymes in metabolism for OZ78 and MT04. OZ78 and MT04 were incubated at different concentrations (1, 10, and 100 µM) in microsome-solution and after different time-points samples were collected, the reaction was stopped by adding of ethanol and after centrifugation directly infused into the MS/MS system. But no plausible hydroxylated product could be detected in negative mode or in positive mode. More studies are needed to evaluate the full metabolism of these substances.

We also searched in sheep plasma samples for possible metabolites (Phase II). Sheep samples obtained 1 hour post-treatment in the framework of the PK study were processed as described in LC/MS part. However, instead of injection into the LC system, the solution was directly infused into the MS/MS system and scanned for metabolites. However, no explainable metabolite could be found.

The evident difference in molecule structure and activity of triclabendazole (TBZ, standard drug [36]) and OZ's does not allow any speculations about possible activity mechanisms. It is rather improbable that a common mechanism lies behind OZ's and TBZ according to fasciocidal activity [36]. Note that it has been shown that OZ78 was active against a triclabendazole resistant strain [15]. Hence, it is unlikely that the drugs attack the same target. The only similarity between the two drugs is that TBZ and OZ78 have both in common a low molecule mass 359.66 g/mol [36] and 322.4 g/mol, respectively. Furthermore, it has been shown that the tegument of the worms is attacked by both substances (TBZ and OZ78) [20, 28, 36]. The importance of these common features is not clear and seems not to be relevant at the moment.

2. LC/MS methods for synthetic peroxide quantification (*Chapter 3 and 5*)

The lack of activity of OZ78 against *F. hepatica* infection in sheep was a surprising result. Therefore, it was important to elucidate the PK parameters of OZ78 in sheep. An analytical method had to be established and validated according to USFDA guidelines (*chapter 3*) [37].

LC/MS is a sensitive and frequently used tool for PK sample measurement [38]. A triple API365 MS/MS was available for analytical measurements.

Insufficient analytical information has been published about these OZ's, especially the ones with an acidic functional group. Until present, only a hydrophilic interaction liquid chromatography-mass spectrometry (HILIC/MS) method for OZ277 has been developed and published [39]. The developed method in this project needed to be precise, accurate, and selective for OZ78 measurement in sheep plasma. However, the presented OZ277 method could not be used for OZ78 measurements. OZ78 is a molecule with a low molecule mass (322.4 g/mol), the molecule contains no nitrogen and no fragment was found to be specific for OZ78 (monitored by direct infusion of OZ78 solution into the MS/MS system). The compound had to be measured in negative mode (deprotonated parent molecule mass). Unfortunately, the negative mode does not allow the same sensitivity as the positive mode because of the creation of insignificant very low mass molecules [40]. The absence of a specific fragment pattern for OZ78 did not allow exploiting the complete MS/MS system. The use of MS/MS would have increased the selectivity [41]. For further studies with similar molecules, it might be an option to apply on-line solid phase extraction (SPE). On-line SPE allows the combination of extraction and analysis [41]. In addition, high precision, high sensitivity, and automation are the advantageous aspects of this technique [41].

The presented and used OZ78 method has its limitations. The sensitivity of the method was dependent on the batch of plasma used. In some plasma samples interfering peaks were observed and the measurements needed to be repeated with another plasma batch. Furthermore, the run time of about 15 minutes as well as sample preparation were time-consuming. However, the method could be used to measure the generated samples and to elucidate important PK parameters such as C_{max}, T_{max}, AUC, and $T_{1/2}$. For future measurement, a shorter method would be desirable, for higher sample through-

put and to lower costs of consumables. Thus, for further studies it would be worthwhile to test on-line SPE to optimize all these negative aspects.

An advantage of the developed OZ78 method is the fast adaptation for MT04 measurements. The same method could be used for MT04 detection in sheep plasma, only the detected m/z ratio had to be modified for MT04. Additionally, the OZ78 and MT04 LC/MS sheep method could be used for rat plasma measurement. Rat plasma was diluted in sheep plasma and measured. A validation was done to confirm that the dilution of rat plasma in sheep plasma results in accurate and precise measurements.
For further studies, in order to achieve a better sensitivity, a dilution of rat plasma in a mobile phase could be done.

To date it is not possible to measure matrices other than plasma with the presented OZ78 or MT04 LC/MS method. For measurement of urine or bile samples the method needs to be optimized. The application of on-line SPE might also be useful for these matrices.
Preliminary studies with off-line SPE were done. Spiked bile samples were processed with different cartridges (product series: strata, phenomenex) and solvents. The results and measured peak were not reproducible and the sensitivity was not satisfactory. Automation could solve this problem and yieald reproducible results.

In conclusion, for further studies it would be worthwhile to try to optimize the method for OZ78 as well as for MT04 for a faster sample through-put and for a better sensitivity. In addition, the method should be expanded out to screen other matrices as urine or bile.

3. Treatment outcome in sheep and rat (*Chapter 4 and 5*)

As mentioned above, the lack of activity of OZ78 in sheep was a surprising result (*chapter 4*). OZ78 is a promising drug candidate against fascioliasis because after 72 hours *in vitro* post incubation all flukes were dead using a concentration of 100 µg/ml. A cure rate of 100% was obtained with an orally applied dose of 100 mg/kg against adult and juvenile flukes in rats. However surprisingly, OZ78 failed to cure *F. hepatica* infection in sheep after subcutaneous or oral application of 50 mg/kg.

First, it was supposed that the drug was not absorbed. A possible reason might be the difference between the species concerning their digestion [42].

However, high concentrations were measured after subcutaneous as well as oral application of 50 mg/kg OZ78. T_{max} was defined to be about 1 hour for both treatments and C_{max} were 45.8 µg/ml after oral and 13.7 µg/ml after subcutaneous administration. Hence to answer the question why we had a therapy failure in sheep, we wanted to compare the PK parameters of OZ78 between treated rats and sheep.

Hence in a next step, a PK study was conducted in healthy rats with OZ78 and MT04 applied at a dose of 50 mg/kg. A time profile of 24 hours was covered for PK parameter characterization. PK parameter calculation was done with a non compartment model. MT04 reached a C_{max} of 49.8±9.1 µg/ml after 2.7±1.2 h (T_{max}), whereas for OZ78 a mean peak concentration of 70.1±19.1 µg/ml (C_{max}) was reached after 1.6±1.6 h (T_{max}). The mean AUCs were 31'258.8±6'232.7 and 29'794.1±3'990.6 µg min/ml for MT04 and OZ78 and the assessed half lives ($T_{1/2}$) of MT04 and OZ78 were 6.4±5.7 and 2.5±1.5 hours, respectively. We observed high inter-individual variations between rats according to PK parameters and concentration-time profiles, which explain the high SDs.

A clear difference between sheep and rat PK parameters after OZ78 treatment was observed for the AUC. The AUC in sheep was, after oral as well as after subcutaneous treatment, clearly lower than in orally treated rats (estimated 4.3 times lower for oral and 2.3 times lower for subcutaneous treatment). The differences in $T_{1/2}$, $C_{max,}$ and T_{max} between sheep and rats were not as highly pronounced as for AUC.

Hence, it is possible that flukes in sheep were not as long in contact with the drug as in rats and could therefore not be killed. Therefore, the question raises, what will happen if another route of application would be used for example intramuscular treatment?

Efficacy and PK-studies with AS and AM showed that an intramuscular treatment reached promising worm burden reductions (>80%) against *F. hepatica* in sheep with doses of 40-60 mg/kg and 160 mg/kg, respectively [43, 44], whereas the oral treatment of AM 80 mg/kg failed to cure the infection [43]. An intramuscular treatment with OZ78 and MT04 in sheep might possibly result in a higher worm burden reduction, since the treatment outcome is dependent on the route of administration.
A reasonable explanation for the therapy failure after subcutaneous application might be precipitation of the substance. After the subcutaneous administration of OZ78 in sheep, swelling at the injection site was observed. This swelling could be caused by precipitation of the substance, which could explain the low AUC after subcutaneous administration and the therapy failure.

However, species differences and infection intensity might also play a role. From previous studies, we know that the response of treatment with semi-synthetic and synthetic substances varies among different animals. For example OZ78 has been tested against schistosome species in hamster and mice and a different response was obtained. OZ78 revealed a worm burden reduction >80% in hamster against juvenile and adult *S. mansoni* with an orally applied dose of 200 mg/kg, whereas OZ78 at a dose of 400 mg/kg was inactive against adult *S. mansoni* in mice. It seems that differences in drug disposition or different immune response could be an explanation for this discrepancy, however, no clear answer could be yet found [21, 45]. Another example of this species differences was shown for AM in rat and sheep. An oral application of AM at 200 mg/kg cured juvenile and adult *F. hepatica* infection in rat, whereas a low activity was observed in sheep after an orally applied dose of 80 kg/mg [46].
In conclusion, a correlation of rat to sheep activity after oral treatment is not possible. Hence, we can not predict the outcome in sheep after rat studies treated orally. However this does not exclude that an intramuscular treatment of MT04 or OZ78 could not cure the infection.
As mentioned, another aspect is the influence of infection intensity. A study conducted in Egypt with AM in humans showed that the response to a treatment against fascioliasis was infection dependent [47]: the stronger the infection, the lower the cure rate. This could also be a reason for the therapy failure of OZ78. OZ78 treated sheep were experimentally infected, whereas sheep used in the study with AS and AM were naturally infected, hence had a lower number of worms, similar to rats [43].

A last important point, *F. hepatica* are settled in the liver, bile ducts, and gallbladder of the host. Determination of bile concentrations might answer the question of why the oral application of OZ78 in sheep failed to cure the infection. To date, we do not know the concentration of drug in bile after oral or other treatment.

Unfortunately, therapy failure in sheep means a stop of the development of these drugs, since sheep and cattle are target animals. Triclabendazole the drug of choice in treating *F. hepatica* infection cured sheep and goat infected with juvenile and adult flukes at a dose of 5-10 mg/kg, and is well tolerated with a safety index of 20-40 depending on the applied dose [48, 49]. An alternative novel treatment should be comparable in activity with TBZ. Before the substances OZ78 and MT04 are discarded, however, intramuscular application in sheep is worthwhile to test.

4. OZ78 and MT04 safe drugs or toxic compounds? (*Chapter 6*)

During preclinical studies it is important to define the toxicity of substances. Vennerstrom et al. did already some toxicity studies with OZ78 and no mutagenic potential could be found [10]. These studies were done with respect to antiplasmodial activity.
But in the helminth field, higher concentrations than in malaria treatment were applied and therefore it is important to measure toxic effects in the micro-molar range.
Different information has been published about reaction way and possible toxic mechanism of the synthetic peroxides. It has been proposed that haemin-dependent iron plays an important role [24].
OZ78 is a haemin dependent substance according to activity against blood-feeding parasites [50]. Since *Fasciola* is a blood feeder, haemin most likely plays an important role in the drug action of OZ78 [13]. SEM pictures done with *Fasciola* and OZ78 or MT04 revealed that the reactivity of these substances was increased in presence of haemin. Therefore, we were interested in the toxicity of OZ78 and MT04 combined with different iron sources such as anorganic iron (Fe(II), Fe(III)), iron in haemin or no supplement. The assays were conducted with HepG2 cells, a liver cell line, since *Fasciola* is a liver fluke.
Different toxicity assays were applied such as adenylate kinase (AK) release, which reflects the loss of cell membrane integrity, ATP content, and at last ROS potential was measured (*chapter 6*).

A clear toxic signal was generated with MT04 (500 µM) combined with anorganic iron expressed in AK release, and a reduced ATP content was obtained with MT04 (500 and 100 µM) combined with haemin or iron, whereas OZ78 did only induce moderate cell-toxicity.
However, both MT04 and OZ78 generated a high ROS signal already after 90 min in combination with haemin. It is not surprising that the signal was higher with MT04 than with OZ78. As mentioned, MT04 has two peroxide-bridges.

With the gained knowledge, we suppose that a radical formation is the most probable mechanism. The generated radicals attack the flukes in liver, gallbladder, and bile. More

studies are needed to confirm this hypothesis. Control tests could be done with substances similar to MT04 or OZ78 but without peroxide bridges. They should not generate a ROS signal. The loss of activity of these compounds against *F. hepatica* infection *in vivo* was shown [9].

During our *in vivo* studies no rat died and they looked vital. However, additional *in vivo* studies on toxicity in animals are needed. Especially MT04 could be a "risky substance" because of the two peroxide bridges and hence higher potency than OZ78. Further studies elucidating the toxicity are needed. But for the moment, MT04 and OZ78 are promising substances which react by ROS generation.

Conclusion

In the framework of my PhD thesis, I have worked on different aspects of the drug development process ranging from *in vitro*/*in vivo* studies, analytics, pharmacokinetic, and toxicity with a focus on OZ78 and MT04 and derivates.

With OZ78 and MT04, two promising drug candidates against fascioliasis were studied in more detail. Both substances achieved good results *in vitro* and *in vivo* in rats. OZ78 was already tested in sheep, but failed to cure *F. hepatica* infection after oral or subcutaneous application.

With the developed analytical LC/MS methods for OZ78 and MT04 we could determine the PK parameters in rats and in sheep and concluded that flukes in sheep were exposed too shortly to the drug. Hence, another application route would be worthwhile to try. I would propose an i.m. treatment since treatment outcomes with related compounds AS and AM were promising after i.m. application. The etablished LC/MS method is a powerful basis to further development using different body fluids.

In order to answer the question of why the oral application failed to cure the infection in sheep, bile studies are needed. The presented analytical method needs to be adapted for bile so that concentrations in this body-fluid can be determined.

The toxicity of OZ78 and MT04 was tested against liver cells (HepG2). MT04 and OZ78 in combination with haemin generated a high ROS signal. We believe that these ROS were responsible for fasciocidal activity. In further studies it is important to test toxicity in animals, since toxicity in HepG2 cells has been shown for MT04 in the presence of iron as demonstrated by AK release and lower ATP content. The importance of these findings is not clear to date.

References

[1] Robinson MW, Dalton JP, Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos Trans R Soc Lond B Biol Sci 2009;364:2763-76.

[2] Mas-Coma S, Bargues MD, Valero MA, Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol 2005;35:1255-78.

[3] Schweizer G, Braun U, Deplazes P, Torgerson PR, Estimating the financial losses due to bovine fasciolosis in Switzerland. Vet Rec 2005;157:188-93.

[4] Furst T, Keiser J, Utzinger J, Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. Lancet Infect Dis 2011;

[5] Knubben-Schweizer G, Deplazes P, Torgerson PR, Rapsch C, Meli ML, Braun U, [Bovine fasciolosis in Switzerland: relevance and control]. Schweiz Arch Tierheilkd 2010;152:223-9.

[6] Rapsch C, Schweizer G, Grimm F, Kohler L, Bauer C, Deplazes P, et al., Estimating the true prevalence of Fasciola hepatica in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. Int J Parasitol 2006;36:1153-8.

[7] Keiser J, Utzinger J, Chemotherapy for major food-borne trematodes: a review. Expert Opin Pharmacother 2004;5:1711-26.

[8] Fairweather I, Boray JC, Fasciolicides: efficacy, actions, resistance and its management. Vet J 1999;158:81-112.

[9] Zhao Q, Vargas M, Dong Y, Zhou L, Wang X, Sriraghavan K, et al., Structure-activity relationship of an ozonide carboxylic acid (OZ78) against Fasciola hepatica. J Med Chem 2010;53:4223-33.

[10] Vennerstrom JL, Arbe-Barnes S, Brun R, Charman SA, Chiu FC, Chollet J, et al., Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 2004;430:900-4.

[11] Keiser J, Utzinger J, Artemisinins and synthetic trioxolanes in the treatment of helminth infections. Curr Opin Infect Dis 2007;20:605-12.

[12] Wang X, Zhao Q, Vargas M, Dong Y, Sriraghavan K, Keiser J, et al., The activity of dispiro peroxides against Fasciola hepatica. Bioorg Med Chem Lett 2011;21:5320-3.

[13] Lowther J, Robinson MW, Donnelly SM, Xu W, Stack CM, Matthews JM, et al., The importance of pH in regulating the function of the Fasciola hepatica cathepsin L1 cysteine protease. PLoS Negl Trop Dis 2009;3:e369.

[14] Keiser J, Utzinger J, Tanner M, Dong Y, Vennerstrom JL, The synthetic peroxide OZ78 is effective against Echinostoma caproni and Fasciola hepatica. J Antimicrob Chemother 2006;58:1193-7.

[15] Keiser J, Utzinger J, Vennerstrom JL, Dong Y, Brennan G, Fairweather I, Activity of artemether and OZ78 against triclabendazole-resistant Fasciola hepatica. Trans R Soc Trop Med Hyg 2007;101:1219-22.

[16] Toledo R, Esteban JG, Fried B, Current status of food-borne trematode infections. Eur J Clin Microbiol Infect Dis 2012;

[17] Dawes B, Juvenile stages of Fasciola hepatica in the liver of the mouse. Nature 1961;190:646-7.

[18] Keiser J, In vitro and in vivo trematode models for chemotherapeutic studies. Parasitology 2010;137:589-603.

[19] Saba R, Korkmaz M, Inan D, Mamikoglu L, Turhan O, Gunseren F, et al., Human fascioliasis. Clin Microbiol Infect 2004;10:385-7.
[20] Fairweather I, Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet Parasitol 2011;180:133-43.
[21] Xiao SH, Mei JY, Jiao PY, Schistosoma japonicum-infected hamsters (Mesocricetus auratus) used as a model in experimental chemotherapy with praziquantel, artemether, and OZ compounds. Parasitol Res 2011;108:431-7.
[22] Dong Y, Wittlin S, Sriraghavan K, Chollet J, Charman SA, Charman WN, et al., The structure-activity relationship of the antimalarial ozonide arterolane (OZ277). J Med Chem 53:481-91.
[23] Tang Y, Wittlin S, Charman SA, Chollet J, Chiu FC, Morizzi J, et al., The comparative antimalarial properties of weak base and neutral synthetic ozonides. Bioorg Med Chem Lett 2010;20:563-6.
[24] Creek DJ, Charman WN, Chiu FC, Prankerd RJ, Dong Y, Vennerstrom JL, et al., Relationship between antimalarial activity and heme alkylation for spiro- and dispiro-1,2,4-trioxolane antimalarials. Antimicrob Agents Chemother 2008;52:1291-6.
[25] Mas-Coma S, Epidemiology of fascioliasis in human endemic areas. J Helminthol 2005;79:207-16.
[26] Meunier B, Robert A, Heme as trigger and target for trioxane-containing antimalarial drugs. Acc Chem Res 2010;43:1444-51.
[27] Halferty L, O'Neill JF, Brennan GP, Keiser J, Fairweather I, Electron microscopical study to assess the in vitro effects of the synthetic trioxolane OZ78 against the liver fluke, Fasciola hepatica. Parasitology 2009;136:1325-37.
[28] Keiser J, Morson G, Fasciola hepatica: Surface tegumental responses to in vitro and in vivo treatment with the experimental fasciolicide OZ78. Exp Parasitol 2008;119:87-93.
[29] Mercer AE, Maggs JL, Sun XM, Cohen GM, Chadwick J, O'Neill PM, et al., Evidence for the involvement of carbon-centered radicals in the induction of apoptotic cell death by artemisinin compounds. J Biol Chem 2007;282:9372-82.
[30] Creek DJ, Charman WN, Chiu FC, Prankerd RJ, McCullough KJ, Dong Y, et al., Iron-mediated degradation kinetics of substituted dispiro-1,2,4-trioxolane antimalarials. J Pharm Sci 2007;96:2945-56.
[31] O'Neill PM, Posner GH, A medicinal chemistry perspective on artemisinin and related endoperoxides. J Med Chem 2004;47:2945-64.
[32] Wang J, Huang L, Li J, Fan Q, Long Y, Li Y, et al., Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. PLoS One 2010;5:e9582.
[33] Duthaler U, Huwyler J, Rinaldi L, Cringoli G, Keiser J, Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with Fasciola hepatica. Vet Parasitol 2011;
[34] Zhou L, Alker A, Ruf A, Wang X, Chiu FC, Morizzi J, et al., Characterization of the two major CYP450 metabolites of ozonide (1,2,4-trioxolane) OZ277. Bioorg Med Chem Lett 2008;18:1555-8.
[35] Charman SA, Arbe-Barnes S, Bathurst IC, Brun R, Campbell M, Charman WN, et al., Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria. Proc Natl Acad Sci U S A 2011;108:4400-5.
[36] Keiser J, Engels D, Buscher G, Utzinger J, Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin Investig Drugs 2005;14:1513-26.

[37] FDA guidance for industry bioanalytical method validation UDoHaHS, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). 2001.
[38] McMaster MC, *LC/MS a practical user's guide*. 2005, United States of America: Wiley.
[39] Bhamidipati RK, Morizzi J, Chiu FC, Shackleford DM, Charman SA, Simultaneous determination of OZ277, a synthetic 1,2,4-trioxolane antimalarial, and its polar metabolites in rat plasma using hydrophilic interaction chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:2989-95.
[40] de Hoffman E, Stroobant V, *Mass Spectrometry Principles and Applications*. Third ed. 2007: Wiley.
[41] Lee MS, Kerns EH, LC/MS applications in drug development. Mass Spectrom Rev 1999;18:187-279.
[42] Goodall ED, Kay RN, Digestion and Absorption in the Large Intestine of the Sheep. J Physiol 1965;176:12-23.
[43] Keiser J, Rinaldi L, Veneziano V, Mezzino L, Tanner M, Utzinger J, et al., Efficacy and safety of artemether against a natural Fasciola hepatica infection in sheep. Parasitol Res 2008;103:517-22.
[44] Keiser J, Veneziano V, Rinaldi L, Mezzino L, Duthaler U, Cringoli G, Anthelmintic activity of artesunate against Fasciola hepatica in naturally infected sheep. Res Vet Sci 2010;88:107-10.
[45] Xiao SH, Keiser J, Chollet J, Utzinger J, Dong Y, Endriss Y, et al., In vitro and in vivo activities of synthetic trioxolanes against major human schistosome species. Antimicrob Agents Chemother 2007;51:1440-5.
[46] Keiser J, Shu-Hua X, Tanner M, Utzinger J, Artesunate and artemether are effective fasciolicides in the rat model and in vitro. J Antimicrob Chemother 2006;57:1139-45.
[47] Keiser J, Sayed H, El-Ghanam M, Sabry H, Anani S, El-Wakeel A, et al., Efficacy and safety of artemether in the treatment of chronic fascioliasis in egypt: exploratory phase-2 trials. PLoS Negl Trop Dis 2011;5:e1285.
[48] Wolff K, Eckert J, Schneiter G, Lutz H, Efficacy of triclabendazole against Fasciola hepatica in sheep and goats. Vet Parasitol 1983;13:145-50.
[49] Boray JC, Crowfoot PD, Strong MB, Allison JR, Schellenbaum M, Von Orelli M, et al., Treatment of immature and mature Fasciola hepatica infections in sheep with triclabendazole. Vet Rec 1983;113:315-7.
[50] Xiao SH, Xue J, Mei JY, Jiao PY, Effectiveness of synthetic trioxolane OZ78 against Schistosoma japonicum in mice and rabbits. Parasitol Res 2011;