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EFFECT OF MULTIDRUG RESISTANCE MODULATORS ON ACTIVITY AGAINST *HAEMONCHUS CONTORTUS* AND PHARMACOKINETICS OF IVERMECTIN AND MOXIDECTIN IN SHEEP

By

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Suggested short title:

MODULATION OF ACTIVITY AND PHARMACOKINETICS OF THE MACROCYCLIC LACTONE ANTHELMINTICS

With all my gratitude I would like to dedicate this thesis to my parents Evelásio and Rosa Branca Beltrão Molento. Their strength and invaluable examples of persistence, and truthfulness has shaped my every day life.
With love to Carla and Dante Rafael.

Com toda a minha gratidão, dedico esta tese aos meus pais Evelásio and Rosa Branca Beltrão Molento. Sua força e os incontáveis exemplos de persistência e o apoio incondicional fazem parte do meu dia-dia.
Para Carla e Dante Rafael com amor.

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LIST OF ABBREVIATIONS

Drugs and chemicals

AVM: avermectins

MBM: milbemycins

NMD: nemadectins

IVM: ivermectin

MOX: moxidectin

VRP: verapamil

CyA: cyclosporin A

DMSO: dimethyl sulphoxide

PG: propylene glycol

Pharmacokinetic parameters (units)

T ½ ab: absorption half-life

*C*max: peak plasma concentration

*T*max: time to peak plasma concentration

*T*½ d: distribution half-life

AUC$_{total}$: area under the concentration vs time curve extrapolated to infinity

AUMC$_{total}$: area under the first moment of the curve

MRT: mean residence time

*T*½ el: elimination half-life

Miscellaneous

GABA: gamma aminobutyric acid

HPLC: high performance liquid chromatography

PCR: polymerase chain reaction

mg/kg: milligrams per kilogram of body weight

GI: gastrointestinal

SC: subcutaneously

PO: per os (oral)

P-450: cytochrome P-450 system

CYP3A4: cytochrome P450 3A4

CO_2: carbon dioxide

SEM: standard error for the mean

SD: standard deviation

P-gp: phosphoglycoprotein, P-glycoprotein

MDR: multidrug resistance

MAR: multiple anthelmintic resistance

PF14: susceptible isolate passage over 14 generations / PF17

IVF14: ivermectin selected isolate passage over 14 generations / IVF17

MOF14: moxidectin-selected isolate passage over 14 generations / MOF17

FECRT: faecal egg count reduction test / FEC

L_1: first stage larvae / L_2, L_3, L_4

C: degree celcius

ml: milliliter

min: minutes

h: hour / hours

PT: post-treatment

TWC: total worm count

Mc: arithmetic mean of worms counted in the control group

Mtr: arithmetic mean of worms counted in the treated group

T test: Student's *T* test

CO_2: carbon dioxide

P: probability

µl: microlitre

n: number of observations, sample size

LD50: lethal dose to eliminate 50% of the worms

PREFACE

Although drug treatment is regarded as the most efficacious way of controlling parasitic infections, the reality is that drug resistance to all the anthelmintic groups is spreading all over the world. It is common sense that many animal producing entrepreneurs are confronting a very difficult situation regarding their production loss caused by nematode parasite infection. In the short term, attached to this unprofitable situation, lies the lack of new compounds that would alleviate the pressure on the available drugs. Therefore, the need to develop and apply new control strategies with the objective of combatting resistant gastrointestinal parasites is long overdue.

This research project deals with the introduction of the concept that has been used in cancer research for the treatment of resistant tumour cells. Considering the urgency and the promising initial results from single worm DNA samples, we were interested to test, using different models, the use of the endectocides anthelmintics ivermectin and moxidectin in combination with multidrug resistance modulators.

This dissertation has a General Introduction followed by 7 Chapters which include articles that were published or are being reviewed in peer reviewed journals. The articles were included as they were accepted, including abstract and a list of references at the end.

The experiments carried out for this research project comprehend the body of this thesis and the manuscripts have the **contribution of authors** of my supervisor Dr. Roger Prichard, who has provided financial resource for the laboratory work and guidance in the study designs and data interpretation. Dr. G. T. Wang from Fort Dodge Animal Health is a co-author in the first manuscript (Chapter 2) and was responsible for the drug selection of the various parasite strains. It is relevant to mention the co-authorship on manuscript 2 (Chapter 3) which was done in collaboration with Dr. M. Xu, a former post-doctoral fellow at the Institute of Parasitology, who contributed to this work, doing much of the molecular

biology experiments with the assistance from the other co-authors, Dr. William Blackhall, Dr. Paula Ribeiro, Dr. Robin Beech while I participated in the experimental design and execution of the *in vivo* work. Manuscript 5 (Chapter 6) has the co-authorship of Mr. Adrian Lifschitz, Mr. Juan Sallovitz, and Dr. Carlos Lanusse; the latter contributing to the resources required for the realization of this work, which took place in his laboratory at the Universidad Nacional del Centro in Tandil, Argentina, as well as contributing to the experimental design and analysis of the results. However, the hypothesis tested in this experiment was mine, with advice from my supervisor, and I was responsible for the overall conduct of this experiment. Mr. Lifschitz and Mr. Sallovitz assisted with pharmacokinetic sampling and the HPLC assays.

A series of appendices are included at the end of the thesis. Appendix I lists a number of protocols used for the *in vivo* experiments. Appendix II, shows the results from a parallel experiment carried out at the Macdonald Campus Farm utilizing the anthelmintic doramectin in calves, sponsored by Pfizer Animal Health Inc. Appendix III brings a list of publications, conference presentations and awards received by the author.

M. B. Molento
February, 2000

STATEMENT OF ORIGINALITY

To the best of the author's knowledge, the following information contained in this thesis constitute an original contribution to the scientific literature.

1. The determination of reduced efficacy of ivermectin and moxidectin against ivermectin- and moxidectin-selected *Haemonchus contortus* strains *in vivo* utilizing jirds and *in vitro* using a larvae migration assay. These findings have demonstrated for the first time side-resistance of ivermectin to the moxidectin-selected strain without prior exposure to ivermectin.

2. Demonstration of the increase in efficacy of ivermectin and moxidectin in association with verapamil and CL347,099, a verapamil analog, against selected strains of *Haemonchus contortus* in jirds.

3. Demonstration of the effect of verapamil and CL347,099, alone, against selected and unselected strains of *Haemonchus contortus in vivo* and *in vitro*.

4. The determination of side-effects after verapamil and CL 347,099 treatment in jirds and verapamil in sheep.

5. The increase in ivermectin and moxidectin efficacy when in combination with the multidrug resistance modulators verapamil, CL347,099, and cyclosporin A utilizing an *in vitro* larval migration assay.

6. The modification of the pharmacokinetic profile of orally administered ivermectin and moxidectin in association with verapamil in sheep.

ABSTRACT

Resistance to the avermectin/milbemycin class of anthelmintics in nematodes has become a serious problem worldwide due to their unrestricted usage. Resistance to these compounds is attributed to the over-expression of the transport protein, P-glycoprotein (P-gp). P-gp acts by pumping drug molecules out from the cell or organism. P-gp efflux activity can be blocked using multidrug resistance (MDR) modulators associated with chemotherapy to enhance their therapeutic effect. A series of experiments was undertaken to determine if the association of the anthelmintics, ivermectin (IVM) and moxidectin (MOX), and MDR modulators would increase the anthelmintics' efficacy against resistant parasites. Using an *in vitro* migration assay, IVM and MOX in the presence or absence of verapamil (VRP), CL347,099 and cyclosporin A (CyA) were used against IVM- and MOX-selected strains of *H. contortus*. The modulators alone had no effect on reducing the number of migrating larvae. IVM and MOX had a significant increase in efficacy of 52.7 and 58.3% respectively, when used in association with VRP, above that obtained with the anthelmintics alone. CL347,099 was also able to significantly increase the IVM and MOX efficacy by 24.2 and 38.9 %, respectively. The effect of IVM and MOX in combination with VRP and CL347,099 was determined in jirds infected with selected strains of *H. contortus*. The combinations of VRP with either IVM or MOX significantly reduced worm counts of the selected strains compared with the untreated controls, whereas IVM or MOX alone did not. CL347,099 plus MOX combination was significantly more efficacious than moxidectin alone against the selected strains. To evaluate the effect of VRP on the pharmacokinetic behaviour of the anthelmintics IVM and MOX, the drug combination was given to sheep. The IVM plus VRP treatment resulted in an increase of the pharmacokinetic parameters of IVM. The peak concentration (83%) and area under the curve (54%) were significantly different from the IVM treatment alone. VRP had no effect on the kinetics of MOX. Our hypothesis is that VRP blocked P-gp activity, inhibiting the transport of IVM at the biliary route.

ABRÉGÉ

À cause de l'utilisation non-réglementée des groupes d'anthelmintiques avermectine et milbemycine, la résistance des nématodes parasitaires est devenu un grave problème mondial. La résistance à ces médicaments est attribuée à une sur-expression de la protéine de transport, la P-glycoprotéine (P-gp). Cette protéine pompe les molécules de médicaments à l'extérieur des cellules et de l'organisme. Son activité peut être bloquée par l'utilisation de modulateurs MDR qui associés à la chimiothérapie augmente leur action thérapeutique. Une série d'expériences a été mise sur pied pour déterminer si l'association d'anthelmintiques, tel que ivermectine (IVM) et moxidectine (MOX), avec certains modulateurs MDR augmenterait l'élimination de parasites résistants. À l'aide d'un test *in vitro* de migration de larves, les souches d'*Haemonchus contortus* résistantes à l'IVM et MOX ont été testées en présence des anthelmintiques IVM et MOX et des modulateurs vérapamile (VRP), CL347,099 et cyclosporine A (CyA) en association avec IVM et MOX. Les modulateurs MDR, utilisés seuls, n'ont en aucune façon réduit le nombre de larves en mouvement. Par contre, l'association de IVM et MOX avec VRP a augmenté leur efficacité de 52,5 % et 58,3 % respectivement. CL347,099 a aussi augmenté l'efficacité de IVM et MOX de 24,2 % et 38,9 % respectivement. L'efficacité de IVM et MOX administré avec VRP ou CL347,099 a été déterminée chez les gerboises infectées de souches résistantes d' *H. contortus*. La combinaison de VRP avec soit IVM ou MOX, a réduit de façon significative le nombre de vers des souches résistantes en comparaison aux groupes témoins, tandis que administré seul, IVM et MOX n'ont eu aucun effet. L'association CL347,099 avec MOX a été plus efficace que l'emploi de MOX seul sur les souches résistantes. Pour évaluer l'impact de VRP sur le profile pharmacocinétique de IVM et MOX, les différentes combinaisons de médicaments ont été administrées à desmoutons. Le traitement IVM plus VRP a augmenté les paramètres pharmacocinetques de l'anthelmintique. La plus haute concentration (83 %) et l'aire sous la ourbe (54%) étaient statistiquement différentes des résultats obtenus de l'admnistration de IVM sans VRP. VRP n'a eu aucun effet sur la cinétique de MOX. Ces ésultats suggèrent que VRP bloque l'activité de P-gp et empêche ainsi le transport de IVM par la route biliaire.

ACKNOWLEDGEMENTS

As almost everything starts as a dream, I would like to thank my wife Carla for sharing all the plans of coming to Montreal. Such a big decision that ended up being a great experience in my life. Her contribution was also "quite" important in giving me the joy of being Daddy, bringing our son Dante Rafael. This little person that we have loved so much since his first moments. Her participation as a professional was also important in many informal discussions. After I was committed to pursue with graduate studies, the participation of Élida G. Campos was crucial. She was the one who made the first contacts for me at the Institute helping me to take off. My "godmother" showed me that life in a laboratory can be very exciting.

I was fortunate to have the incentive and excellent guidance of my research supervisor Dr. Roger K. Prichard. It never mattered if he was in Switzerland, Japan or Trinidad-Tobago, he always had a clear idea of what was going on and where to go with my research project. He also had influenced me to adventure other lands and to make it professionally rewarding, showing me how our research field is invaluable in today's world. At the Institute of Parasitology, I would like to acknowledge the essential help from Dr. Jim Smith, showing me how to use different computer packages and the HPLC. Also, to the Professors Marilyn Scott, and Paula Ribeiro for helpful advice. I would like to thank Dr. Siva Ranjan for her guidance at the beginning of my studies. In the laboratory, most of my work had the capable assistance of Ms. Christiane Trudeau. Merci Chris! I would like to extend my thanks to Mr. Gordon Bingham from the Institute Animal Facilities. The experience of working at the Institute of Parasitology is one of a kind, bringing people from all backgrounds and cultures and that itself made a great contribution to my years at Macdonald Campus of McGill University.

Part of my experiments were done in Argentina and the participation of Dr. Carlos Lanusse is appreciated. As well as, the great people in his lab. who helped me with sample collection, analysis, and some unforgettable Argentinian

barbecues. I would like to thank Fort Dodge Animal Health in the person of Dr. G. T. Wang for supplying the *Haemonchus contortus* larvae and for partially funding this project. The scholarship program between Brazil and the Quebec Government through Mr. Alain Proulx from the Ministère des Affaires Internationales, the Blair State Fellowship award and the Total Fee Waiver from McGill University, significantly helped me financially during these years.

To acknowledge my respect to the animals used in this project I would like to quote a phrase by Mahatma Gandhi; "The greatness of an nation and its moral progress can be measured by the way its animals are treated". Although I was always trying to eliminate them, I think of the parasites as singular creatures capable of outsmart us in so many ways, and for that I have only one thing to say; The fight is not over!!!

Herewith goes a warm thank you to many of my friends whom helped me in one way or another sharing some good moments. Thank you; Roberto, Élida Campos, Peter Gatongi, Christiane Trudeau, Bill Blackhall, Yi Yu, Mike, Susanne Vogelgsang & Denis Nobert, Patrick Galois, to many Brazilian friends; Wando Amorin, Marcelo Bittencourt, Ana Paula Góes and many others, to the Disband Group; Tom & Val Meredith, Gordon, and Ron, and to all friends from the Ste. Anne Renaissance Singers Group. Thank you to all my friends and family in Brazil cheering every time we have talked.

God was, is and will always be very present in my life!

THESIS OFFICE STATEMENT

In accordance with the regulation of the Faculty of Graduate Studies and Research of McGill University, the following is included in this thesis.

Candidates have the option, subject to the approval of their Department, of including as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If that option is chosen, connecting text that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that is more than a mere collection of manuscripts, in other words, results of a series of papers must be integrated.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include: a table of contents; an abstract in English and French; an introduction which clearly states the rational and objectives of the research; a comprehensive review of the literature, and a final conclusion and summary.

An additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and other, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

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CHAPTER 1

GENERAL INTRODUCTION

Nematode parasites such as *Haemonchus spp*, *Ostertagia spp.*, *Trichostrongylus spp.* or *Cooperia spp.* are responsible for immense decreases in animal performance by reducing the overall production and increasing mortality of young domestic ruminants. In the search to minimize this situation, producers frequently use chemicals for prophylactic treatment of parasitic infections. Anthelmintics drugs are regarded as the sole salvation for a heavily infected flock or herd and sometimes are used without taking into consideration important aspects such as frequency of treatment, parasite specificity, formulation, or route of administration. As a consequence, anthelmintic resistant individuals may be selected, who can pass their resistance on to future generations of parasites. Soon after the introduction of the benzimidazoles in the mid 60's, drug failure was observed, and the discovery of the avermectin and milbemycin class of anthelmintics in the early 80's provided a new alternative for the control of parasites in domestic ruminants. Unfortunately, by the end of that decade, parasites had acquired resistance to ivermectin, and more recently, resistance to moxidectin has been detected. It has also being demonstrated that anthelmintic resistance is not reversible even after many years of not exposing a parasite population to a specific compound.

The urgent need for new methods to control multidrug resistant (MDR) tumor cells, led to the use of an array of unrelated drugs that when associated with chemotherapy would increase the efficacy of the chemotherapy by blocking the activity of the transport protein, P-glycoprotein. It was also demonstrated that this drug-drug interaction could alter the pharmacokinetic parameters of the chemotherapeutic drugs in the patient. Such classes of the so called MDR

modulators include; calcium channel blockers, hormones, antibiotics, and immunossuppressors. Recent data has suggested that the nematode *Haemonchus contortus*, resistant to ivermectin and moxidectin, express a homolog P-glycoprotein gene to drug resistance cancer cells. Arising from this, one can hypothesize that resistance to macrocyclic lactones in *H. contortus* may be partially overcame by the use of ivermectin or moxidectin in association with MDR modulators, enhancing the efficacy of the anthelmintic drugs and possibly altering the pharmacokinetics of ivermectin and moxidectin in the host. The mechanism by which the drug combination may result in an increase efficacy of the anthelmintic drugs is hypothesized to be by blocking the anthelmintic efflux activity of P-glycoprotein by the MDR inhibitors. In the host, the activity of the MDR modulators would be measured by altering the pharmacokinetics of ivermectin and moxidectin, reducing the transport of these drugs by P-glycoprotein present at the hepatocytes to bile. A series of experiments included in this thesis were designed and carried out to test the hypothesis that the MDR reversing agents may increase anthelmintic efficacy against resistant worms, using different *in vitro* and *in vivo* models and the kinetics of ivermectin and moxidectin in the host. The objectives were;

1. To establish the appropriate LD50 for ivermectin and moxidectin against susceptible and resistant strains of *H. contortus* in jirds.

2. To determine whether verapamil and CL 347,099 alone have any effect against susceptible and resistant strains of *H. contortus* in jirds.

3. To determine the level of toxicity of verapamil and CL 347,099 alone and in combination with the anthelmintics in jirds and to determine the level of toxicity of verapamil alone or in combination with the macrocyclic lactones used at their therapeutic dosage in sheep.

4. To demonstrate if the efficacy of the macrocyclic lactones can be increased by their combination with verapamil or CL 347,099 against susceptible and resistant strains of *H. contortus* in jirds.

5. To determine the enhancing capacity of the MDR modulators verapamil, CL347,099, and cyclosporin A in combination with ivermectin and moxidectin against susceptible and resistant larvae of *H. contortus in vitro*.

6. To determine if verapamil is able to alter the pharmacokinetics of ivermectin and moxidectin used at their therapeutic dosage in sheep.

LITERATURE REVIEW

1.1 ORDER STRONGYLIDA

The order Strongylida is composed of the Superfamilies Trichostrongyloidea, Strongyloidea, Ancylostomatoidea, and Metastrongyloidea. These organisms are helminth parasites of the kingdom Animalia, phylum Nematoda (round worms) that belong to the superfamily Trichostrongyloidea, also described as the abomasal and small intestinal "hairworms" of ruminants (Georgi and Georgi, 1990).

1.1.1 Structure and Function

Most nematodes have a cylindrical form in cross-section, tapering at either end, and are covered by a colourless, somewhat translucent, cuticle. This cuticle is secreted by the underlying hypodermis, which projects into the body cavity forming two lateral cords, which carry the excretory canals, and a dorsal and ventral nerve cord. The muscle cells, arranged longitudinally, lie between the hypodermis and the body cavity. The latter contains fluids at a high pressure which maintains the turgidity and shape of the body. Locomotion is effected by undulating waves of muscle contraction and relaxation which alternate on the dorsal and ventral aspects of the worm. Most of the internal organs are filamentous and are suspended in the fluid-filled body cavity (Urquhart *et al.*, 1987, and Georgi and Georgi, 1990).

The nematodes that commonly affect domestic ruminant animals such as cattle, sheep or goats can be found in the abomasum: *Haemonchus contortus*, *H. placei*, *H. similis*, *Ostertagia ostertagi*, *O. circumcincta*, *Teladorsagia circumcincta*, and *Trichostrongylus axei*, in the small intestine; *Trichostrongylus colubriformis*, *Cooperia oncophora*, *C. pectinata*, *C. punctata*, *Nematodirus helvetianus*, *N. battus*, *Bunostomun phlebotomum*, *Strongyloides papillosus*, in the large intestine;

Oesophagostomum venulosum, *O. columbianum*, *O. radiatum*, *Chabertia ovina*, *Trichuris discolor*, and *T. globulosa*, and *Dictyocaulus viviparus* in cattle, and *D. filaria* in sheep and goats found in the lungs (Block, 1989, Georgi and Georgi, 1990).

The importance of all these parasites to the domestic animal industry is clear, but the following study will focus on *H. contortus*. This parasite has been the subject of intensive research and is one of the most economically important nematodes in which resistance has been detected worldwide.

1.1.2 Life Cycle

The life cycle of the superfamily Trichostrongyloidea, with the exception of Metastrongyloidea which have an intermediate host, is typically direct. They also have a free-living microbivorous first and second larval stages (L_1 and L_2) and an infective third larval stage (L_3). In the Nematoda, the sexes are distinct where the males are generally smaller than the females. Commonly, after reproduction inside the host, strongylid eggs are shed in the faeces in the morula stage of development. The secretion of chitinase and protease enzymes by the larvae will then digest the eggshell (Olsen, 1974). First stage larvae develop and hatch in a day or two and feed on microorganisms in the faeces. They shed the cuticular covering, molting into L_2. At completion of the L_2, a second molt is started but not completed in the external environment, so the infective L_3 larvae remains encased in the cuticle of the second stage. Infective L_3 larvae migrate out of the faeces and after 24 hours (h) more than 90% of L_3 are found within 10 centimetres (cm) of the faeces and can survive at temperatures of 4° Celsius (C) for several months. Animals (hosts) get infected by swallowing L_3 with the forage. The sheath is cast off in the rumen due to its pH and carbon dioxide (CO_2) concentrations, and within 12 h after being swallowed, the exsheathed L_3larvae appear on the surface of the abomasal mucosa ready to penetrate. By the end of the first day, nearly all of the worms are in the

gastric pits of the mucosa . L_3 larvae undergo the third molt to the fourth stage (L_4) in the abomasal mucosa. The L_4 stage molts to the fifth stage (L_5 or pre-adult) depending on whether it enters a period of arrested development (hypobiosis*) in the gastric glands. This usually occurs in the autumn and winter, or in the wet season in cool temperate climates.

The two most important components of the external environment affecting parasite survival are temperature (18 to 26° C, optimal) and humidity (90-100%). Blood-sucking parasites develop a piercing lancet before the final molt, enabling them to obtain blood from the mucosal vessels. The parasites become sexually mature in 18 days depending on seasonal conditions after entering the host. Following copulation, a further life cycle is initiated. Individual females of *H. contortus* can lay up to 5,000 eggs per day for several months under optimal climate conditions. The longevity of the worms in the host is uncertain (Olsen, 1974, Cheng, 1986, Urquhart *et al.*, 1987, Kimberling, 1988, Radostis *et al.*, 1989, and Smyth, 1990).

* Hypobiosis: Slowing down of life processes (Gibbs, 1986). A resting stage at some point in the parasite development (larval stages) enabling them to survive adverse conditions.

1.2 *HAEMONCHUS spp.*

1.2.1 Etiology

H. contortus is a blood-sucking abomasal nematode responsible for extensive losses in domestic ruminant animal production worldwide; exerting its greatest economic effect in animals in tropical and sub-tropical countries, where there is a good summer rainfall (Radostis *et al.*, 1989, and Georgi and Georgi, 1990).

The majority of the Trichostrongyloide and Strongyloide nematode infection

occurs when the host ingests the free-living L_3 larvae. Gastrointestinal nematode populations generally follow a yearly rise and fall on the pasture. Larvae available to grazing animals in the spring are either those that survived the winter, or dry season depending on the region or pasture and would only survive as long as their energy stores last, or are the progeny of hypobiotic larvae which have resumed development. The second population will be the offspring of the population acquired in the spring, or wet season. Susceptibility of the host population and environmental conditions will determine the level of available larvae. An *haemonchosis* outbreak can occur when the mean maximum temperature is 18° C with rainfall over 5.25 cm (Radostis *et al.*, 1989, and Urquhart *et al.*, 1987).

1.2.2 Clinical and Pathological Findings

Affected animals are often noticed for the first time when they lag behind, breath faster, have a staggering gait, and often go down. Grazing animals lie down a good deal of the time, often around the water troughs; the energy needed to walk and eat appears to be lacking. In most cases constipation rather than diarrhea is the main symptom. Chronic *haemonchosis* leads to extreme weight loss, followed by lethargy, pallor of the mucosa and conjunctiva, and the presence of submandibular and ventral abdominal edema (anasarca) rather than marked anaemia, these symptoms occur especially during the dry season when the larval uptake and reinfection are negligible, (Urquhart *et al.*, 1987, Radostis *et al.*, 1989, and Georgi and Georgi, 1990) .

The pathogenesis of *haemonchosis* is that of an acute haemorrhagic anaemia due to the blood-sucking abilities of both L_4 and adult worms. The migration of the larvae into the pits of the gastric glands in the abomasal wall, and the injury caused to the mucosa by the attachment of *H. contortus* adult worms cause abomasitis. The presence of *H. contortus* in the abomasum appears to interfere with the digestibility and absorption of proteins, calcium, and phosphorus,

increasing the host manifestation to the infection. The pH rises significantly, soon after infection, due to lower gastric acidity, and plasma pepsinogen levels rise at the same time (Radostis *et al.*, 1989). In an acute haemonchosis, anaemia, edema, lethargy, and dark-coloured faeces becomes apparent about two weeks after infection. Although diarrhea is not generally a feature, young animals are more likely to have it than adults (Kimberling, 1988). Hyperacute haemonchosis is when an apparently healthy sheep dies suddenly from a severe haemorrhagic gastritis (Radostis *et al.*, 1989). Poor nutrition will also exacerbate the infection, causing serious hypoproteinemia due to blood loss caused by the worm (Georgi and Georgi, 1990).

1.2.3 Diagnosis

In many situations farmers do not treat their animals without having observed the first sick animal. Therefore, signs of a subclinical infection will be obscured, and losses will be underestimated. Although assessing the impact of such parasitism under varying climatic and geographical conditions is difficult, epidemiological data reveals that sub-clinical parasitism among cattle is prevalent (Block, 1989). The history of the infection and clinical signs are often sufficient for the diagnosis of an acute syndrome especially if supported by faecal egg counts (FEC), but it must be remembered that low FEC may be encountered in gross *haemonchosis* when the bulk of the pathogenic worms are in the larval stage (Urquhart *et al.*, 1987). The faecal egg output may follow a standard pattern that has an important implication to epidemiology, particularly when correlated with other parasitological measurements (Block and Takagi, 1986). Roberts and Swan (1981) detected a strong relationship between FEC and total worm count (TWC) of *H. contortus* in sheep in Australia.

In the case of detecting anthelmintic resistance a simple and sensitive diagnostic tests would clearly be of great benefit (Le Jambre, 1992). For FEC,

faeces must be collected at least 7 to 10 days post-treatment to evaluate drug resistance (Scott *et al.*, 1991). However, Conder and Campbell (1995) suggested that this period has to be reviewed, because some drugs suppress parasite oviposition for longer than 10 days. At necropsy, one has to pay attention to changes in both the abomasum and the marrow of long bones. Other major findings are severe anaemia, gelatinization of fat deposits, anasarca, and the presence of *H. contortus* in the abomasum (Urquhart *et al.*, 1987).

1.3 PARASITE CONTROL STRATEGIES

The primary information required by producers when having to deal with worm infection is which anthelmintic should be used, when, and at what cost. Facing this situation, the effectiveness of any anthelmintic has to be evaluated, and only those drugs which are known to be effective against the target parasites should be used. Modern anthelmintics are efficient and comparatively nontoxic and because parasitism is so prevalent in some countries, efficient livestock production is virtually impossible without them. After deciding which drug to use, some factors have to be taken into consideration such as the route of administration and the animal's weight. A proper delivery system has to be followed if the commercial drug is to be used orally, sub-cutaneously, intra-abomasum, or intra-muscularly. In the case of oral treatments for ruminants, the importance of the oesophageal groove closure was reported to have altered the pharmacokinetics of oxfendazole, with 42% of the animals showing a complete rumen bypass which decreased drug efficacy and perhaps increasing selection for drug resistance (Prichard and Hennessy, 1981). On economic grounds, and to delay the onset of resistance to anthelmintics, the minimum number of anthelmintics treatments that would give adequate control is desirable (Copeman and Hutchinson, 1980). An adequate dosage is imperative, because if some animals receive underdosed treatments, parasites which are heterozygous for resistance may well survive (Coles, 1988).

Thus, inadequately drenching animals might precipitate selection for resistance in the field. Charles *et al.*, (1989) reported that therapeutic dosages of levamisole, albendazole, and parbendazole that are commercially available for sheep and prescribed goats were not effective against parasites infecting goats, suggesting that the animals were being underdosed. The World Association for the Advancement of Veterinary Parasitology (WAAVP) officially recommends that highly effective anthelmintics must eliminate over 98% of the target parasites. An anthelmintic is considered effective when it eliminates 90-100% of the target parasites, 80-90% is considered moderately effective, and less than 80%, insufficiently active (Wood *et al.*, 1995).

Where the parasite survival mechanism during periods of adverse weather is larval hypobiosis, it is imperative that treatment with products effective against hypobiotic larvae is carried out during this period. Treatment should be directed toward preventing pasture contamination associated with activation of arrested larvae, which may accompany periparturient relaxation of immunity. Treating livestock at such a time, so as to preclude the spring pasture rise of larvae would effectively lower pasture contamination (Herd *et al.*, 1985). Gatongi and colleagues (1998) have demonstrated that treatment before the long rains would reduce the adult worm and hypobiotic larval populations and therefore lead to low pasture contamination during the following wet season in Kenya.

In small ruminants, treatments may be determined on the basis of faecal egg count (FEC) determination, as there is a direct relationship between faecal egg counts and population size of important nematodes (Roberts and Swan, 1981). Although this relationship has been proved to be only moderate, a tactical treatment based on FEC is more rational than treating every 3 or 4 weeks as practised by many producers (Waller *et al.*, 1993), and as is commonly done by sheep producers in the southern state of Paraná, Brazil (author's personal observations).

The effectiveness of alternating anthelmintics each treatment is still not satisfactorily answered. Some have advocated the strategy of using a single drug

until it is no longer effective, then changing it (Le Jambre *et al.*, 1978). Rapid rotation of anthelmintics has been largely discredited, as it selects for resistance to all of the drugs that are used in the rotation (Barnes and Dobson, 1990). Slow rotation (once a year) of anthelmintics with different modes of action has been shown to be quite effective (Prichard *et al.*, 1980). At the end of the first year, the surviving worms may have developed only a low level of resistance to the utilized anthelmintic, and should have no resistance to the new class then introduced. In practice, the slow rotation of anthelmintics has proved to be most effective in reducing the rate of selection for anthelmintic resistance. However, to be effective it must be practised with two or three classes of anthelmintic that are fully effective. Once resistance is present in a class of anthelmintic a slow rotation program will be compromised in some years. The model suggested by Smith (1990) is that of simultaneous use of two different anthelmintics. This strategy delays but does not prevent the development of resistance relative to, for example, drug rotation. If this strategy is contemplated, it is imperative that all of the anthelmintics used are effective at the start of the program. If worms already have a level of resistance to one of the products, this resistance will be intensified, and selection will occur to the other product (Sivaraj *et al.*, 1993). Treatment with a combination of different classes of anthelmintic has not received widespread acceptance as it effectively doubles the cost of the treatment, may cause toxicity to the host, and is disliked by drug registration authorities in most countries. Usually, helminth populations are overdispersed among hosts, so, most individuals are lightly infected and a few hosts harbor many parasites (Medley, 1994). thus, selective deworming heavily infected individuals is a strategy that may prove to be of great value and which should not put very much selective pressure on all of the worms in a population (Duncan and Love, 1991).

Successful worm treatment relies not only on drug treatment but on an effective combination of farm management practices. These strategies are employed mainly to reduce animal and pasture contamination, which would then

reduced considerably the selection pressure towards parasite resistance. The traditional approach of pasture hygiene for equines has been modernized. To some degree, the removal of faeces from pastures not only reduces the level of infective larvae, but it also increases the amount of grazable pasture (Herd, 1986). The practice of alternate grazing of species like ruminants/non-ruminants, or sheep/cattle may be useful in controlling nematodes. Grazing pastures with livestock that have acquired resistance to parasites before allowing susceptible populations to graze may also slow down the onset of anthelmintic resistance (Eysker *et al.*, 1986). The treat-and-move principle, where animals are moved from contaminated to safe or clean pastures, may reduce the need for frequent anthelmintic treatment in susceptible host populations (Waller *et al.*, 1989). On the other hand, when treated animals are moved to clean pastures, the only parasites available to contaminate the clean pastures will be those that have survived treatment, i.e., those that are resistant. Therefore, the practice of treat-and-move to clean pasture may act as a strong stimulus for resistance to develop. Echevarria *et al.*, (1993a) reported that pastures that were re-seeded after a crop of soya beans showed little immediate risk of nematode infection to sheep and cattle. Re-introducing a susceptible strain of a parasite would also decrease the frequency of a resistant allele in the population (van Wyk and van Schalkwyk, 1990).

Genetic selection of hosts for resistance against specific parasites may also be a useful alternative. Host-resistance against nematode parasites probably operates in two ways; through immunologic reactions, or by resilience to infection, which means a superior ability to compensate for parasite-induced damage. Genetic selection for immunologic enhancement will not only protect those individuals against the effects of worms but will lessen environmental exposure from and dependence on anthelmintics (Woolaston *et al.*, 1990). The major limiting factor is a method of selection of livestock resistant to parasites before exposing them to what could be fatal levels of infection (Craig, 1993). Genetic markers (haemoglobin, anaemia, MHC type, FEC) for resistance to gastrointestinal parasite infection in

sheep are also being developed (Beh and Maddox, 1996). A futuristic approach was propose by Roos (1997), where there is a need to find molecular markers that are linked with resistance in sheep. These genes could be used to inject sheep embryos to make transgenic resistant sheep. Biological control is also getting more public attention especially from the increasing awareness of the possible environmental hazards in using chemicals in the food chain, using agents such as nematode destroying fungi, viruses, bacteria, or protozoa to control nematodes. This approach has been defined as 'an ecological method designed by man to keep parasite populations at a non-harmful level using natural living antagonist' (Gronvold *et al.* 1996). Another very promising field is the development of specific or broad-spectrum vaccines against parasites (Emery, 1996). The most probable time to commercial availability of a vaccine against *H. contortus* based on concealed antigen was judged to be 5 years, and in excess of 10 years for vaccines against other nematode species (Barger, 1996). The only vaccines to be successfully developed against helminth parasites in veterinary medicine were irradiated larval vaccines against *Dictyocaulus viviparus* (Urquhart, 1985), *D. filaria* (Sharma *et al.*, 1981) and *Ancylostomum caninum* (Miller, 1978). Regardless of whether chemical or non-chemical approaches are used, monitoring control programs is very important. Medley (1994) suggests, that one important measurement in determining successful control programs is host morbidity reduction, which can be correlated with the number of heavily infected individuals.

Although the use of anthelmintic drugs to treat nematode infection is by far the simplest method to control parasites. We are aware of the rapid selection for strains that are resistant to all the available drugs. The observation of the failure of all antiparasitic drugs in the field in some parts of the world confirms that the situation is indeed very critical. Lately, the market has been inflated with large quantities of generic compounds, which belong to the same chemical groups as the ones already in use. As a consequence, the process of worm selection is being worsen because animal producers have the objective of lowering the farming cost.

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That is why researchers are testing different alternative means of parasite control, as well as the combinations of different management strategies. In Europe, drug-free practices are being encouraged by market pressure where people are looking for a more "ecological" or "nonchemical products".

1.4 ECONOMIC IMPACT OF PARASITISM

The literature is replete with observations of the economic significance of parasitic diseases and their impact in production animals. Parasitic infections can affect feed ingestion, feed digestibility and a variety of physiological processes, which can manifest in many ways. These include premature death, a change in the value of animals and their products at slaughter, reduced live-weight gain, reduced yield and quality of products such as milk, reduced capacity of work, altered production of dung for fuel and fertiliser, and altered feed conversion efficiency. These in turn can have effects on herd productivity, on the capacity to maintain and improve a herd, on human nutrition, on community development and on cultural issues relating to the use of livestock (Perry *et al.*, 1999). Some of these effects may be difficult to document especially when sub-clinical infections are present (Hawkins, 1993). The main problem is that controlled trials are difficult to conduct within the framework of normal management procedures (Copeman and Hutchinson, 1980). The estimation of total production losses has been widely applied, in both developed and developing country contexts, with possibly a recent increase in estimations in the developing countries, where dwindling budgets are demanding greater evidence of the need to invest scarce resources.

A survey in New England and the Saint Laurence valley showed that 98-99% of the herds, respectively, were subclinically infected (Block, 1986). Grisi and Todd (1978) investigated parasitism among 877 milking cows in the northern USA and found that 80.1, 60 and 48.8% of cattle examined in Pennsylvania, North Carolina, and Wisconsin, respectively, were sub-clinically or clinically infected, predominantly

with *Haemonchus* and *Cooperia spp.* An estimated loss of US$350 million/year was calculated as the loss in North America caused by parasitic gastroenteritis (subclinical and clinical) (Block and Takagi, 1986). Exercising a cost-benefit computer model, McLeod (1995), estimated that sheep round worm infection could cost an amount over AUS$ 222 million/year to the Australian grazing industry.

Herd *et al.* (1987) studied the effects of two strategic treatments with ivermectin at 0.2 mg/kg in spring and summer with 35 days interval. Heifers were evaluated during their first grazing season, and the major benefit of this strategy was derived from its effect in preventing a serious rise in pasture infectivity in the second half of the grazing season. As a consequence, the treated heifers grew faster and reached optimal breeding size as much as three months earlier than the untreated group, saving approximately 40 to 140 US$ per heifer. By the end of this study in northern USA, the weight gain was 62.3 kg for the treated group and 38.5 kg for the control group. A significant average increase of 42 kg of milk per lactation (305 days) for treated cows, was determined in a cooperative study utilizing a total of 9,000 lactations on 120 different farms in England, Scotland, and Wales (Michel *et al.*, 1982). The effect of anthelmintic treatment in dairy cattle was also evaluated by Block and Gadbois (1986) in Quebec, Canada, using morantel tartrate. The treated group (40 herds) produced 323 kg/animal more milk than the control group (40 herds), which represents an average increase of 4.8 % in milk production. Copeman and Hutchinson (1980) looking for the economic significance of bovine gastrointestinal nematode parasitism in North Queensland, Australia, estimated that animals treated every three weeks for the first five to eight months after weaning produced up to 50 kg of meat more than untreated animals. Also, on the west coast of Australia, up to 6.5% of untreated steers died or became chronically emaciated from nematode parasitism, suggesting that control procedures are justified in coastal, irrigated or swampy areas.

1.5 AVERMECTIN AND MILBEMYCIN

The microorganism that produces the avermectins was isolated from a soil sample at Kawana, Ito City, Japan by Dr. Satoshi Omura and his group in 1975. The avermectins are a family of oleandrose disaccharide derivatives of pentacyclic lactones. The drugs are produced by fermentation of the soil mold *Streptomyces avermitilis* (MA-4680) (Burg *et al.*, 1979). The avermectins have a very broad spectrum of activity against nematodes and are highly potent compounds controlling internal (all stages), and external parasites (Bogan and Armour, 1987, and Conder and Campbell, 1995). The milbemycins, fermentation products of *S. hydroscopicus aureolacrimosus*, were discovered in 1973 as acaricidal and insecticidal compounds for crop protection by Sankyo scientists, latter they were developed to be used in animals. The nemadectins, are produced by the chemical modification of a natural fermentation product from the actinomicete *S. cyaneogriseus noncyanogenus*, being also a broad spectrum drug for parasite control (Carter *et al.*, 1987,and Zulalian *et al.*, 1994). The avermectins and milbemycins molecules are semisynthetic, 16-membered macrocyclic lactones, and their structures are closely related. The milbemycins differ from the avermectins in that they have no sugar moiety at the C^{13} position and have the unsaturated side chain at the C^{25} position (Carter *et al.*, 1987, Zulalian *et al.*, 1994, and Conder and Campbell, 1995). The nemadectins also lack the disaccharide at C^{13}, but the nemadectins differ from the milbemycins proper in that they contain a trisubstituted double bond at C^{26} in their side chain. The ability of the avermectins and milbemycins to kill both endo- and ectoparasites gave rise to the name endectocides. They also cause reduced oviposition in ticks, abnormal egg formation in nematodes of ruminants and sterility in both males and females filarial nematodes.

Ivermectin (870 m. w., Fig. 1), a synthetic derivative of avermectins, was the first derivative compound to be selected for commercial development. This drug is

a mixture of not less than 80% of 22, 23-dihydro avermectin B_{1a} and not more than 20% of 22, 23-dihydro avermectin B_{1b} components. Ivermectin retains excellent potency and range against arthropods and nematode parasites of wild and domestic animals and humans with great safety (Fisher and Mrozik, 1989). Capable of such activity against a large spectrum of parasites, ivermectin caused a revolution in the animal production industry. Moxidectin (639.8 m. w., Fig.1) is produced by chemical modification of nemadectins, similar to the milbemycins and is called a second generation compound. Unlike ivermectin, moxidectin is essentially a single compound rather than a mixture of two closely related compounds (Adams, 1995). Moxidectin is also a broad spectrum drug against a similar range of parasites as ivermectin. Ivermectin and moxidectin are marketed for control of internal and external parasites of various ruminants, swine, dogs, chicken, and horses in many different formulations and mode of applications in all continents (Egerton *et al.* 1984, Fisher and Mrozik, 1992, and Conder and Campbell 1995). Other structurally similar compounds include abamectin, the naturally occurring precursor of and similar to ivermectin, doramectin, eprinomectin, and selamectin.

1.5.1 Mechanism of Action

A definitive description of the mode of action of the avermectins and milbemycins remains unclear. Several studies have suggested the existence of multiple sites of action for the avermectins, one of which was independent of the Gamma-aminobutyric acid (GABA) receptor-chloride (Cl^-) ion channel complex (Shaeffer and Haines, 1989). In an *in vitro* experiment, the authors demonstrated for the first time the binding of [^{3}H] ivermectin to membranes isolated from *Caenorhabditis elegans*, a free living soil nematode which is extremely sensitive to ivermectin. The researchers examined the effect of GABA on a specific ivermectin binding site and found no effect on [^{3}H] ivermectin binding concentrations up to 10

micromolar (μM) GABA. Specific binding was calculated by subtracting nonspecific from total binding. Analysis of the binding showed that the reaction proceeded by a two- step mechanism. Initially, a rapid unstable complex was formed, and after additional incubation this complex was transformed to a much more stable complex. The optimum pH for binding was between 7.1 and 7.6 where lower pH values resulted in sharply decreased [^{3}H] ivermectin binding. The binding reaction proceeded without a lag and reached 50% saturation in 2-10 minutes.

Glutamate-gated chloride channels have only been reported in invertebrates and have been expressed in oocytes injected with insect muscle poly $(A)^+$ RNA, which may explain the selective effects of the avermectins and milbemycins in invertebrates. Arena *et al.* (1991, and 1992), using *Xenopus laevis* oocytes (reptile model) transfected with mRNA from *C. elegans*, concluded that ivermectin directly opens an ivermectin-sensitive chloride current, and that the glutamate and avermectin-sensitive currents are mediated via the same channel. *X. laevis* oocytes injected with *C. elegans* poly $(A)^+$ RNA exhibited a rapid, activating glutamate-reversible and -irreversible ivermectin 4"-O-phosphate, ($IVMPO_4$) -sensitive current. Glutamate- and ivermectinPO_4-sensitive currents were observed after *in vitro* injection of RNA from a pool of 5,000 cDNAs. Subfractionation yielded two cDNA clones, glutamate-gated chloride channel -α (pGluCl-α) and -ß (pGluCl-ß) sub-units. These clones expressed functional homomeric Cl^- channels which were selectively responsive to ivermectinPO_4 or glutamate, respectively (Cully *et al.*, 1994). Arena *et al.* (1995) reported that avermectins and milbemycins have a common receptor which mediates their nematocidal effect: the glutamate-gated chloride channel. Four avermectin analogues, with different nematocidal activities in *C. elegans,* were tested on oocytes expressing GluCl-α and -ß, suggesting that these channels represent a target of avermectins in nematodes and may mediate the paralytic action of avermectins in other organisms (Cully *et al.*, 1994). Comparison of the GluCl-α and -ß proteins showed 45% amino-acid identity and 63% similarity with 1,383 and 1,302 base pairs, respectively. These proteins are

members of the ligand-gated ion channel superfamily, containing four transmembrane domains, denoted M_1 to M_4. The predicted protein of GluCl-α determined by the Sequence Analysis Software Package, contained a strong consensus sequence for a protein kinase C phosphorylation site located between the putative membrane-spanning domains M_3 and M_4 (Cully *et al.*, 1994). Dent and colleagues (1997), showed that *C. elegans* mutated at the avr-15 gene, lack neurotransmission by the M_3 in the pharyngeal motor neuron. They isolated a cDNA encoding the avr-15 gene and found the predicted protein was 85% identical to GluCl-α at the amino acid level. The gene was then designated GluCl-α2, and when it was replaced into avr-15 worms, GluCl-α2 restored M_3 neurotransmission. The researchers also showed that the ivermectin effect on the pharyngeal muscle, mediated by AVR-15/GluCl-α2, is sufficient to kill *C. elegans*. The pharyngeal muscle is required for feeding, and is known to receive an inhibitory motor neurone M_3, that is not likely to be GABA-ergic but glutamatergic (Laughton *et al.*, 1995 and Avery, 1993). A putative Glu-Cl subunit (HG4) was cloned from *H. contortus*, and isolated using RT-PCR, which has a 82% amino-acid identity with the *C. elegans* Glu-Cl β-subunit. No expression of HG4 on pharyngeal muscle could be detected (Delany *et al.*, 1998). Forrester *et al.* (1999) have cloned two full length Glu-Cl putative α-subunit cDNA's from *H. contortus* that exhibit different sized ligand binding domains forming a distinct sub-branch within this family.

Larval membranes from both ivermectin-sensitive and -resistant L_3 strains of *H. contortus* were examined by Rohrer *et al.* (1994) for the presence of high affinity [^{3}H] ivermectin binding sites. The *H. contortus* ivermectin binding site appeared to be similar to the *C. elegans* ivermectin binding site with respect to affinity for ivermectin and receptor density. Both tissue preparations displayed high affinity drug binding sites (Kd=0.13 nM). Receptor density (B_{max}=0.4 pmol/mg) was the same in both sensitive and resistant nematodes. However, Paiement *et al.*, (1999) suggests that modification in glutamate binding is involved in ivermectin-resistance in *H. contortus*.

Ivermectin binds specifically to Cl^- channel proteins, but its binding site is distinct from that of all other known effector molecules of the Cl^- channel. In the nerve cells of the parasite, ivermectin is believed to act by opening the Cl^- channel and increasing conductance after the influx of Cl^- ions, lowering membrane resistance and causing hyperpolarization of the resting potential of the postsynaptic cells (Fig. 2). This causes a flacid paralysis of the affected parasite followed by their death and expulsion. About 50% of the effect of ivermectin and moxidectin could be reversed on a shore crab muscle membrane with picrotoxin, a GABA antagonist active on Cl^- channels (Conder *et al.*, 1993). Other studies have also shown that the GluCl channels are an important and safe target for avermectins and for future antiparasitic drug development. Suggesting that there may be several classes of the GluCl channel with multiple functions. The exact sequence of events will only be determined when the ivermectin binding site is isolated from a variety of species using genetic tools that are available. It then might be possible to explain why ivermectin is highly active against such a wide spectrum of nematodes and arthropods and why it eliminates *O. volvulus* larvae but not adults (Fisher and Mrozik, 1992, Campbell, 1989). In addition, the presence of multiple target genes in the GluCl family will impact on the degree of resistance observed in the field (Cully *et al.*, 1996).

1.5.2 Pharmacokinetics

Understanding the pharmacokinetics and metabolic behaviour of broad-spectrum anthemintics in the host, and factors modulating that behaviour, is highly important for maximizing anthelmintic efficacy. The pharmacokinetics of the macrocyclic lactones is affected by the specific formulation used, the route of administration, and the animals species to which it is administered (Lanusse and Prichard, 1993). Ivermectin is a highly lipophilic substance and dissolves in most organic solvents, but is poorly soluble in water. It is stable at room temperature in

nonacid solutions and is degraded by UV light. The biological half-life (*T½*) of ivermectin in plasma following IV administration of 300 µg/kg to cattle and sheep is 2.8 and 2.7 days, respectively. In cattle, a subcutaneous injection of ivermectin at a dose of 0.2 mg/kg body weight attains a peak plasma concentration (C_p) of 44 ng/ml within 2 days, with a 2 week persistence of anthelmintic efficacy. Treatment of ivermectin in sheep results in a *T½* of 3-5 days and T_p within 24 h. The parent drug, 24-hydroxymethyl-H_2 B_{1a}, is the major liver residue 5 and 14 days PT in sheep and cattle, respectively, which is then conjugated to nonpolar fatty acids. Ivermectin's principal route of metabolism is hydroxylation, by the liver in cattle, sheep, and rat. It is then, conjugated to fatty acids as esters and deposited in the fat tissue (Chiu *et al.*, 1990). Zeng *et al.*, (1998) have reported that the enzyme, CYP3A4 is responsible for ivermectin metabolism. Studying the residual tissue concentration of ivermectin, Fisher and Mrozik (1992) used radiolabeled drugs to examine tissue levels from 4 steers slaughtered weekly for 4 weeks after receiving a sub-cutaneous dose of ivermectin at 0.3 mg/kg. The concentration of ivermectin 28 days post-treatment, in the liver and fat, was 11 and 29 ppb, respectively. Depletion half-life of ivermectin is 4.8 and 7.6 days for liver and fat respectively, in cattle. The low residues and comparative safety of ivermectin in mammals indicate no human health hazard from residues in meat.

Moxidectin is completely absorbed and transported in the serum and is not associated with any cellular components of the blood. After 24 h moxidectin accounts for approximately 60% of the total [^{14}C] moxidectin in the serum. The total radioactive residues showed half-lives from 9 (muscle) to 10 (fat) days (Zulalian *et al.*, 1994). Moxidectin is even more lipophilic and hydrophobic than ivermectin, and as a result, therapeutically effective tissue levels persist somewhat longer. The primary sites for the hydroxylation of moxidectin are the methyl groups attached to C^{14} and C^{18}.

1.5.3 Toxicity

Ivermectin and moxidectin are not toxic in any studied species of mammals, including humans, when used at therapeutic doses, but ivermectin is not recommended for dogs of the collie breed which can show signs of susceptibility to ivermectin when treated at 1.0 mg/kg or higher (Pulliam *et al.* 1985). These animals tolerated moxidectin at 15 µg/kg, five times the recommended dose for heart worm prophylaxis (Paul *et al.*, 1992). Ivermectin was used in monkeys without any toxic effect and has been used extensively in humans at an oral dose of 0.15 mg/kg for the treatment of onchocerciasis without any adverse drug-related effects (Greene *et al.*, 1985, and Campbell, 1989). Ivermectin was shown to be safe in the control of lymphatic filariasis in humans (Moulia-Pelat *et al.,* 1993). When ivermectin was used at an annual single dose of 0.4 mg/kg in humans, it reduced more than 80% of infections of *Wuchereria bancrofi* from the population (Moulia-Pelat *et al.*, 1995).

When given the normal dose of ivermectin (0.2 mg/kg), calves have shown an increase of erythrocyte indices. Using twice the normal dose, no sign of reproductive performance problems was observed in cows and bulls (e.g. decrease fertility, teratogenic problems, or lower semen quality). The lack of mutagenic activity of ivermectin in a variety of bacterial and mammalian genetic toxicity assays supports the conclusion that ivermectin should also be a noncarcinogenic drug (Campbell and Benz, 1984). Cattle treated six times a year, at four times the regular dose, showed ataxia, mydriasis, increased levels of glucose, urea nitrogen, sodium and potassium, and haematocrit-haemoglobin. The primary clinical pathologic change was the decrease in serum iron values; death was rare. Given oral doses equal to 20 times and SC doses of 40 times the therapeutic dose, ivermectin caused an acute toxic syndrome consisting of central nervous system (CNS) signs of depression, ataxia and possible death. An acute toxic reaction was observed at 1.0 mg/kg, 5 times the regular dose (Campbell, 1989). This syndrome is rarely seen in sheep, where this animal can be treated orally with doses of up to 4 mg/kg

without any adverse reactions.

Rae *et al.* (1994) reported that moxidectin (1% - injectable) used at 3 times the therapeutic dose in cows did not have deleterious effects on reproductive performance (e.g. problems with folliculogenesis, ovulation, and early embryonic phase of development). Although moxidectin cause no death to *Bos taurus* nor to *B. indicus* when given SC at up to 10 times the recommended dose, extra care has to be taken in calves under 100 kg of body weight due to their susceptibility to overdosing.

1.5.4 Excretion and Environment

Ivermectin is safe for humans, domestic and wild animals, and aquatic organisms, but can affect some dung fauna such as the larval stages of Diptera and Coleoptera (Campbell, 1989). The macrocyclic lactone drugs enter the environment by excretion from animals where more than 98% of both ivermectin or moxidectin is eliminated via faeces and only 2-3% of the dose is eliminated in the urine (Chiu *et al.*, 1990, Fisher and Mrozik, 1992, and Zulalian *et al.*, 1994). However, excretion of ivermectin can be detected in the mammary glands of lactating cows. Toutain *et al.* (1988) found that ivermectin could be detected in the milk for up to 17 days after treatment, recovering approximately 5% of the total dose. The use of ivermectin and moxidectin is currently not approved for lactating dairy cattle (Heller and Schenck, 1993, and Anonymous, 1995).

Because ivermectin and its metabolites are mainly excreted in bile, residues continue to appear in feces for substantially longer following subcutaneous injection than after oral dosing. The binding of avermectins to digesta particulates during gut transit may potentially lower drug bioavailability and also contribute to fecal residues (Steel, 1993). A more aqueous based injectable formulation in cattle may therefore reduce the impact of ivermectin treatment on dung fauna. When manure contains 10 to 20 ppb of ivermectin, the normal concentration level in the soil will

be 0.01 to 0.02 ppb or 0.1%, having no antifungal or antibacterial properties when present at 200 ppm in the soil. Ivermectin has a half-life of only 3 h when the manure is exposed to sunlight, but can stay in the soil for 91 to 217 days during the winter and 7 to 14 days during the summer. Although immobile in soil, ivermectin can penetrate to a depth of 5 cm, but it will not readily translocate into ground water. Aquatic organisms are sensitive to a level of 3 ppb of ivermectin and some fish to 5 ppb. The concerns raised about the possible adverse environmental impact of ivermectin might be less applicable, or non-applicable, in the case of moxidectin (Conder and Campbell, 1995).

1.6 IVERMECTIN AND MOXIDECTIN EFFICACY TRIALS

The uniqueness of the macrocyclic lactone endectocides is that only an extremely small quantity is necessary for anthelmintic activity by any route of administration. Ivermectin and moxidectin have been tested in a number of studies to evaluate their anthelmintic efficacies against all major gastrointestinal and lung nematodes and certain ectoparasites of domestic animals (Anderson and Robertson, 1982, and Booth and McDonald, 1988), in humans (Darge and Bütter, 1995), and in wild animals, as ivermectin is frequently used in Zoological Parks in worm control programs (Hill *et al*, 1993).

In Africa, ivermectin has been extensively used in humans against *Onchocerca volvulus*, an organism responsible for a disease that causes eye and skin lesions. More than 70 thousand people were each treated with a single yearly dose of 0.2 mg/kg (Campbell, 1989). The Onchocerciasis Control Programme in West Africa, where this disease is still a major cause of the river blindness, is aiming at treating millions of people (UNDP/World Bank/WHO, 1994). Ivermectin was also elected the treatment for the less appreciated but more widespread onchocercal skin lesions (hyperreactive onchodermatitis or sowda) by the World Health Organization (WHO, 1995). Darge and Bütter (1995) reported that 1-2

months after ivermectin treatment, the prevalence of microfilaria carriers had decreased from 100 to 19% (n=56).

Scott *et al.* (1991) investigated the changes in egg production of an ivermectin-resistant strain of *H. contortus* immediately after exposure to ivermectin in lambs. There was a rapid reduction in FEC to 800 EPG at 48 h after treatment compared with pre-treatment levels of 20,900 EPG. Eggs collected from female parasites treated with ivermectin failed to hatch after incubation, suggesting that ivermectin may have some action on the development or hatching of eggs from ivermectin-resistant females of *H. contortus*. Marchiondo and Szanto (1987), studying the efficacy of ivermectin in swine in Ohio, USA, treated infected animals (0.3 mg/kg) and observed an efficacy of 98.7, 53.9, and 87.6% against *Ascaris suum, Trichuris suis*, and *Oesophagostomum dentatum*, respectively, concluding that the efficacy of ivermectin was lower than expected. Uribe *et al.* (1987) reported the efficacy and persistency of a topical formulation of ivermectin against a natural infestation of *Dermatobia hominis* larvae in cattle in Colombia and Brazil. The efficacy was more than 99%, and the number of *D. hominis* were still significantly less in the treated group after 43 to 48 days post-treatment.

Williams *et al.* (1992) demonstrated that the efficacy of moxidectin against abomasal nematodes in cattle was 99.9% and against intestinal parasites was 99.4%, using two different doses (0.2 or 0.3 mg/kg) of moxidectin ($P<0.05$). Moxidectin also eliminates inhibited larvae of *O. ostertagi*. This study showed that during the 13 day holding period before slaughter, moxidectin-treated calves gained significantly more weight (12.7 kg) than control calves (6.4 kg) ($P<0.01$). Taylor *et al.* (1993) reported that injectable and topical moxidectin were effective for the reduction of nematode FEC in cattle. Whang *et al.* (1994) found that the efficacy of "injectable" and "pour on" moxidectin against gastrointestinal nematode infections in cattle, using egg-count reduction, was respectively 95.4 and 91.5% on day 7, and 92.9 and 84.8% on day 14 after treatment. These results indicate a long half-life for moxidectin (Zulalian *et al.*, 1994). Scholl *et al.* (1992) studied the effect of

moxidectin against cattle grubs and trichostrongyle nematodes. They found a 90-100% efficacy against trichostrongyle nematode egg production for three levels of subcutaneous administration (0.1, 0.2, 0.4 mg/kg). Ranjan *et al.* (1992) reported the efficacy of moxidectin against seven genera of nematodes in cattle two weeks after treatment; the mean FEC of both groups (0.2 and 0.3 mg/kg dose) were reduced by 98 -100%. Samson *et al.* (1992) detected an efficacy of 99.6 to 100% using injectable moxidectin at 0.2 mg/kg against internal parasites (abomasum, small and large intestine) in cattle in New Zealand. Losson and Lonneux (1993) tested the field efficacy of moxidectin (0.2 mg/kg) in cattle naturally infected with the ectoparasites *Chorioptes bovis* and *Sarcoptes scarbiei*. The parasite reduction from skin samples on days 7, 14, 21, and 28, were 69 and 93, 87 and 100, 99 and 100, and 98 and 100% for *C. bovis* and for *S. scarbiei* respectively.

The antiparasitic activity of moxidectin was evaluated in horses using different dose rates, (0.2, 0.3, or 0.4 mg/kg), formulations (injectable or gel), batches, and routes of administration. All groups tested showed a 100% removal of *Gasterophilus nasalis*, *Parascaris equorum*, *Strongylus vulgaris* and *S. edentatus*. The activity of moxidectin against *G. intestinalis* was inconsistent (varying from 89 to 100%). The removal of *Oxyuris equi* was excellent (100%), except for the animals treated with the gel formulation given 0.3 mg/kg (27%). Activity against migrating *S. vulgaris* was good, and killing of migrating *S. edentatus* in the ventral abdominal wall was excellent (100%). Moxidectin is also highly effective against *Habronema muscae* but is probably inactive against *Anoplocephala perfoliata* and *Thelazia lacrymalis* (Lyons *et al.*, 1992).

1.7 ANTHELMINTIC RESISTANCE (AR)

Anthelmintics have been used as the primary method for controlling parasitic gastrointestinal nematodes of domestic ruminants for many years. Unfortunately, when these anthelmintics are used frequently, resistance can develop, as in the

case of the producers in Texas or Paraguay who treated their flocks with ivermectin every 3-4 weeks during the spring and summer (Craig and Miller, 1990, DeVaney *et al.*, 1992, and Maciel *et al.*, 1996). Resistance to *H. contortus* was reported worldwide for ivermectin (Carmichael *et al.*, 1987, Van Wyk and Malan, 1988, Echevarria and Trindade, 1989, and Coles *et al.*, 1996) and moxidectin failed to control ivermectin-resistant *Ostertagia* species in goats (Leathwick, 1995).

The phenomenon of drug resistance was described by Conder and Campbell (1995) as "a heritable reduction in the sensitivity of a parasite population to the action of a drug, the reduction being expressed as a decrease in the frequency of individual parasites affected by the exposure to the drug in comparison to the frequency observed in the same population upon initial or prior exposure". Waller (1993) suggested that it is impossible for any drug to be 100% efficacious, against 100% of parasites species, 100% of the time. Therefore, on each occasion when an anthelmintic is used some survivors which carry the genes for resistance will likely persist. Prichard (1990) explained that genetic mutations take place constantly, and mutation is the basis for the biological diversity found in a species, where mutation in a single gene rather than polygenes may be responsible for the development of resistance after continuous drug treatment. This type of resistance will usually develop after the homozygous resistant gene is disseminated through the parasite population (Fig. 3). Genotypes that provide the species with characteristics which benefit, or do not greatly disadvantage the community, will be reproduced. Periodically the worms with the resistance genotype may have a survival advantage which contributes to the survival of the overall community. The genotype of the free-living population is therefore crucial in the development of significant anthelmintic resistance. If a high proportion of a population of parasites carry the resistant genes, progress towards resistance will be rapid, and conversely, if a high proportion carry the susceptible genes then process will be slow (Echevarria *et al.*, 1993b). Echevarria *et al.* (1993c) using a computer model to study *H. contortus* infection in sheep, found that the nematode control used by

farmers increased the frequency of genes which confer resistance from approximately 3 to 14% over a 20 year period.

Because resistance is a heritable characteristic, logic indicates that if the resistant population of worms is no longer exposed to that anthelmintic, within several generations it may revert to susceptibility and the anthelmintic will once again be effective (Craig, 1993); assuming that resistance is maladaptive in the absence of anthelmintic (Scott and Armour, 1991). However, observations of worm populations resistant to benzimidazoles indicate no reversion to susceptibility, and furthermore, that benzimidazole resistance may be associated with increased pathogenicity (Mair and Cripps, 1991). Therefore, resistance may reappear quickly following resumed use of anthelmintic due to the persistency of genes for resistance which may still be present in the worm population.

Before concluding that resistance occurs due to treatment failure, alternative causes of poor efficacy such as drug storage conditions, expiring dates, drenching techniques, and pasture conditions should be examined (Dorny and Vercruysse, 1993). Le Jambre (1990), emphasised that unless a drug treatment is 100% effective against an organism, there is the possibility that the treatment is selecting for resistance. Parasite selection can be originated on the farm after a few generations or when recently acquired animals are introduced into the flock without proper care, disseminating eggs and larvae. In Brazil, a resistant *H. contortus* strain was introduced after a large number of sheep were purchased from the south of the country and shipped to the northeast (Vieira *et al.*, 1992). The relatively rapid evolution of resistance to ivermectin may be a consequence of its high efficacy and therefore high selection pressure on the parasites. There is a direct relationship between the frequency of treatment and the selection of resistant individuals. When ivermectin is administered at intervals of 21 days, it allows susceptible *H. contortus* no opportunity to produce ova and thus propagate susceptible individuals in the population.

The development of ivermectin or moxidectin resistance in *H. contortus* is

still not fully understood, and it appears to have a multiple mechanism. Gill *et al.* (1998) reported that two strains of *H. contortus* that were selected under laboratory conditions had different responses when treated with ivermectin using *in vitro* assays compared to one selected-strain collected from the field. Xu and colleagues (1998) reported that the over-expression of the transport protein, P-glycoprotein in ivermectin-resistant strains of *H. contortus* may be linked with the mechanism of resistance. It is believed that P-glycoprotein would decrease ivermectin's concentration in the parasite nerve cells by pumping the drug out of the cell in an efflux mechanism. A distinct mechanism was reported by Blackhall *et al.* (1998) where different alleles of the α-subunit gene of the glutamate-gated chloride channel were detected among susceptible and ivermectin- and moxidectin-resistant strains of *H. contortus*. Paiement *et al.* (1999) characterized a glutamate binding site in *H. contortus*. Treatment of adult worms with ivermectin decrease the B_{max} value for glutamate binding in the susceptible strain but not in the resistant parasites; suggesting that the glutamate binding site may be involved in the phenomenon of drug resistance. Thus, these data strongly suggest that the mechanism of resistance to the macrolactones could be a result of more than one process, separated or associated.

1.8 DETECTION OF ANTHELMINTIC RESISTANCE

The early detection of emerging resistance is vital for the conservation of effective anthelmintics and for parasite control. Unfortunately, resistance is generally not recognized until it becomes a problem, due to our inability to assess subclinical resistance in the field (Conder and Campbell, 1995), and farmers are the ones who first point out that a certain drug is "failing to clean" the animals. Resistance should be suspected when FEC remains high or clinical signs persist following anthelmintic treatment. The veterinarian can make a strong presumptive diagnosis of resistance in a herd by a simple, although some times very expensive,

anthelmintic trial. But as described before, one must also rule out other possibilities. The most effective method for determining the existence of anthelmintic resistance problem is that of post-mortem evaluation of treated and untreated animals. Beveridge *et al.*, (1990) explained that this enables the investigator to determine unequivocally the species and stage of development of worms which are either susceptible or resistant to the tested compound. The use of laboratory animals rather than natural hosts has the advantage of being less costly, thus facilitating the use of a larger sample size. Although restricted in terms of parasite species and rate of parasite establishment, the use of laboratory animals as models for testing drug efficacy has been improved.

Several *in vitro* tests have been developed to determine if resistant populations of worms are present. Egg hatch assays, larval motility tests, larval development tests and tubulin binding assays have been developed and are simple and useful for different classes of anthelmintics based on their mode of action under stringent environmental conditions (Lacey and Prichard, 1986, Folz *et al.* 1987, Taylor, 1990, Hubert and Kerboeuf, 1992, Small and Coles, 1993, Rothwell and Sangster, 1993, and d'Assonville *et al.*, 1996). A polymerase chain reaction (PCR) technique has been developed for the diagnostic of *Teladorsagia circumcincta* resistant to benzimidazole (Elard *et al.*, 1999).

In the near future, techniques from molecular biology will enable us to detect anthelmintic resistance. DNA-based techniques should be able to determine the numbers of genes involved in resistance, the identification of DNA polymorphism closely linked to it, the level of resistance among certain populations, to which anthelmintic, and whether immediate measures have to be taken, depending on whether the level of resistance is high or low. The connection between resistance and a polymorphic marker can be accomplished without prior knowledge of the mode of action or target of the anthelmintic.

1.9 SIDE - RESISTANCE

The phenomenon of side-resistance is defined where the resistance to a compound is the result of selection by another compound with a similar mode of action (Prichard *et al.*, 1980). Some researchers have suggested that ivermectin and moxidectin have the same mechanism of action (Shoop *et al.* 1993, Conder *et al.*, 1993, and Watson *et al.*, 1994). Therefore, once a resistant strain is discovered for one of these drugs, the use of the other as a strategy of control should be discontinued (Shoop, 1992).

Conder *et al.* (1993) found that the efficacy of moxidectin at a dose which clears 98% of ivermectin susceptible *H. contortus* was only 47.2% against an ivermectin resistant strain in jirds. This model has been successfully used to predict the efficacy of anthelmintics for ruminants in the field. Shoop *et al.* (1993) reported that *O. circumcincta* and *T. colubriformis* resistant to ivermectin were also resistant to moxidectin. The effective dosage (ED 95) value for moxidectin was 0.148 mg/kg against *O. circumcincta* and 0.127 mg/kg for *T. colubriformis* in sheep. These ED 95 values are below the manufacturer's recommended dosage of 0.2 mg/kg, but they represent 31 (0.0047 mg/kg) and 9 times (0.014 mg/kg), respectively, the dosages of moxidectin needed to kill the susceptible isolates. The results of this study suggest a mutual resistance to ivermectin and moxidectin, and confirms that side resistance to moxidectin may occur spontaneously where resistance to ivermectin has been established. Leathwick (1995) reported a case of moxidectin failing to control ivermectin resistant *Ostertagia* species in goats.

However, some reports have suggested that organisms that are resistant to ivermectin are not necessarily resistant to moxidectin (Pankavich *et al.*, 1992, Craig *et al.* 1992, and Pomroy and Wheland, 1993). Craig *et al.* (1992) studied the efficacy of moxidectin against susceptible and ivermectin-resistant strains of *H. contortus* in lambs in Texas. Both moxidectin and ivermectin were effective in removing susceptible *H. contortus* with an efficacy of 100 and 99.7%, respectively.

The efficacy of moxidectin against the ivermectin-resistant strain was 99.9 and 100% at 0.2 mg/kg and 0.4 mg/kg, respectively, but the efficacy was only 38.8 and 53.1% in the lambs treated with ivermectin at 0.4 mg/kg and 0.8 mg/kg (2x and 4x the recommended dose rate for ivermectin), respectively. Pankavich *et al.* (1992), using moxidectin and ivermectin at 0.2 mg/kg oral drench in lambs against susceptible and ivermectin-resistant strains of *H. contortus,* reported 100% reduction in worm burdens when either moxidectin or ivermectin were used against the susceptible strain and 100 and 63.6% against the ivermectin-resistant strain, respectively. These results suggested that moxidectin is highly effective against this ivermectin-resistant isolate of *H. contortus*. Pomroy and Whelan (1993) tested the efficacy of ivermectin and moxidectin against an ivermectin-resistant strain of *O. circumcincta* in young sheep. Ivermectin reduced worm numbers by 51.1% whereas moxidectin was 94.8% effective.

Kieran (1994) suggested that the greater potency of moxidectin compared to ivermectin may involve differences in the modes of action of these two macrocyclic lactone endectocides, and by using moxidectin as the preferred endectocide in programmes of drug rotation, the risk of development of resistance could be reduced. However, Le Jambre *et al.* (1995) reported that the efficacy of ivermectin (0.4 mg/kg) and moxidectin (0.2 mg/kg) against the CAVR strain of *H. contortus* was 16 and 96%, respectively, the study indicated an emerging resistance to the latter anthelmintic. Our approach was to test ivermectin and moxidectin against a moxidectin-selected strain of *H. contortus* (Chapter 2). The results reveal a decrease in moxidectin as well as ivermectin efficacy suggesting that side-resistance is happening in this strain.

1.10 CROSS-RESISTANCE

Cross resistance, or as a more modern term, multiple anthelmintic resistance (MAR), occurs when drugs from different chemical groups fail to eliminate those

individuals from those populations to which resistance has already manifest (Prichard *et al.*, 1980). Resistance to more than two anthelmintic groups is already common and multiple anthelmintic resistance to gastrointestinal parasites was reported in New Zealand (Vermunt *et al.*, 1995), India (Yadav *et al.*, 1995), Brazil (Soccol *et al.*, 1996), the UK (Coles *et al.*, 1996), and in Kenya (Mwamachi *et al.*, 1995, and Waruiru *et al.*, 1997).

Bisset *et al.* (1992) evaluated the efficacy of moxidectin at 0.2 mg/kg (oral) against mixed anthelmintic-resistant (BZD and LEV, not to MOX) and -susceptible strains of nematodes in lambs by measuring FEC before and after treatments and worm counts 10 days after treatment. Moxidectin had 99.9% efficacy at post-mortem against *H. contortus, T. colubriformis, Nematodirus spathiger, O. circumcincta*, and *Cooperia curticei* for both strains, suggesting that moxidectin should provide a highly effective alternative to currently available products for control of nematodes in sheep. The same happening for ivermectin.

1.11 MULTIDRUG RESISTANCE (MDR)

Resistance in human cancer cells to structurally unrelated drugs has been called multidrug resistance (MDR). Many different biochemical mechanisms may characterize drug resistance in tumor cells, including decrease intracellular drug accumulation, defective transport, defective drug activation, altered DNA repair, gene amplification, and altered target proteins (Curt *et al.*, 1984). Although other mechanism of resistance have been investigated (Schneider *et al.*, 1990), the focus of this thesis will be on P-glycoprotein.

Increased expression of a cell surface P-glycoprotein (P-gp), a membrane transport glycoprotein, has been shown to be encoded by the *mdr* gene. P-gp is a molecule of 170 kDa consisting of 1280 amino acids (human MDR1) arranged as two homologous halves, each containing six transmembrane regions and a nucleotide (adenosine triphosphate-ATP) binding site (Fig. 4) (Gros *et al.*, 1986a,

Chen *et al.*, 1986, and Gottesman and Pastan, 1993). The MDR gene family is itself part of a large superfamily of ATP-binding cassette (ABC) genes. Ruetz and Gros (1994) stated that P-gp recognizes drug molecules within the cell lipid-bilayer through interaction with transmembrane domains and that both nucleotide-binding sites participate in ATP binding and/or hydrolysis, which energizes drug transport, perhaps by signal transduction to the transmembrane domains.

Lincke *et al.* (1992) had cloned genomic and complementary DNA sequences of four P-gp gene homologues of *C. elegans*, which were termed *pgp-1*, *pgp-2*, *pgp-3* and *pgp-4*. Detailed analyses of *pgp-1* and *pgp-3* showed that they encoded ATP-binding, membrane-spanning proteins, with characteristic features shared by most P-gp already described: MDR1 and MDR2 in humans (Lincke *et al.*, 1991); mdr1/mdr1b, mdr2, and mdr3/mdr1a in rodents (Gros *et al.*, 1986b); and pfmdr1 in *Plasmodium falciparum* (Foote *et al.*, 1989). The MDR homologue in *P. falciparum*, pfmdr1, may mediate resistance in the chloroquine (CQ) resistant organisms. The pfmdr1 is amplified in some CQ-resistant parasites but not in any of the sensitive isolates examined. At least 10-fold more pfmdr1 transcript was detectable in the resistant isolates (FAC8) than in the susceptible isolates (3D7) (Foote *et al.*, 1989).

1.12 PHYSIOLOGY OF P-GLYCOPROTEIN IN NORMAL TISSUES

The normal physiological function of P-gp is still unclear, but it is believed that P-gp can act by; (a) blocking toxic substances normally present in the environment to the entry of these compounds into the body, (b) removing drugs from the circulation once they have entered, and (c) protecting organs against deleterious actions from drugs that are in the circulation, sensitive to their toxicity (Gatmaitan and Arias, 1993, and Ambudkar *et al.*, 1999). The first hint as to what function P-gp might have came from studies in which monoclonal antibodies to P-gp were used to localize the protein in frozen sections of human tissues. All positive

tissues show plasma membrane localization of positive cell types allowing mapping of the distribution of P-gp expression (Table 1). Other techniques such as immunohistochemistry and Western blotting have also been used to detect P-gp.

P-gp is highly expressed in the capillaries of endothelial cells, and its expression in the luminal membrane of the blood brain barrier (BBB) and other blood-tissue barriers such as testes, suggest that it may be involved in regulating the entry of certain substances at these sites, protecting drug permeability (Sakata *et al.*, 1994). The BBB present in vertebrates consists of brain-capillary endothelial cells connected with tight junctions which normally function to limit the passage of protein and soluble polar compounds into the brain parenchyma, maintaining the brain homeostasis (Guyton, 1991). BBB drug permeability is usually correlated with drug lipophilicity.

The use of transgenic animals where the MDR genes were ablated gave some information about the normal function of P-gp at the BBB. Schinkel *et al.* (1995), have reported that in an accidental situation mice with a mutant *mdr*1 type P-gp gene were 50- to 100-fold more susceptible to ivermectin than normal mice showing neurotoxic symptoms that resulted in death. This indicates that completely blocking P-gp protection role leaves the BBB vulnerable to toxic effects. Pulliam *et al.* (1985), investigating post-treatment ivermectin toxicity in dogs, found that ivermectin was present in high concentrations in the CNS in collies. In that study, 1/200 of the lethal dose for beagles was lethal for collies; yet no data suggests a P-gp deficiency in that breed. The use of P-gp inhibitors to modulate drug resistance in P-gp-positive tumors *in vivo* may also have deleterious effects on the normal physiological events controlled by P-gp in the CNS (Shustik *et al.*, 1995).

In the liver, expression of MDR gene (MDR2 is strongly expressed in liver) products has been shown to occur primarily on the canalicular membranes of the biliary tract. Indirect evidence for the role of P-gp in biliary drug excretion comes from *in vitro* experiments, which have shown that daunorubicin transport across canalicular membranes is an unidirectional process that may be inhibited by

classical MDR modulators such as verapamil and quinidine (Gatmaitan and Arias, 1993). Speeg *et al.* (1992), have indicated in rats that biliary clearance of colchicine is blocked by cyclosporin A, and that biliary clearance of vinblastine is blocked by verapamil (Schrenk *et al.*, 1993). Thus, these data confirms that P-gp is expressed in the biliary canaliculi, and indicate that the use of MDR modulators is likely to impede drug transport function (Lum and Gosland, 1995). Therefore, the tissue distribution of P-gp is consistent with its postulated role as a transport protein and almost certainly has a normal physiological function in human tissues related to secretion and protection from naturally occuring toxins. It is interesting to note that only the human *mdr*3 (class I) and *mdr*1 (class II) P-gp isoforms confer the MDR phenotype *in vitro*, while the *mdr*2 (class III) isoform apparently does not (Georges *et al.*, 1990).

1.13 MULTIDRUG RESISTANCE MODULATORS

The accumulating evidence for the over-expression of P-gp in tumor cells with intrinsic or acquired resistance to chemotherapy led to the early investigation of their combination with non-cytotoxic agents known as chemosensitizers, MDR reversing agents or as commonly called, MDR modulators. A number of these compounds have demonstrated ability to inhibit P-gp transport function, restore accumulating defects, and reverse resistance *in vitro* and *in vivo*. Various classes of drugs, including a wide variety of pharmacologically and chemically dissimilar entities, such as the calcium channel blockers, detergents, immunosuppressants, and hormones (Table 2) have been used to overcome clinical MDR. The increase of cytotoxic drug dosages, the use of new chemotherapeutic agents; and the inhibition of the MDR1 gene product are some of the other alternatives used to combat clinical MDR (Shustik *et al.*, 1995). Lipophilicity is a common denominator of the chemosensitizors and the effectiveness of P-gp reversing agents in chemosensitized MDR cancer cells has stimulated a serious effort to define a

common pharmacophore necessary to circumvent P-gp mediated MDR (Ambudkar *et al.*, 1999).

An aspect of modulation of MDR that remains difficult to explain is the absence of a structure-activity relationship among modulators. An alternative possibility of drug reversing action was suggested by Beck (1987), where P-gp may alter the direct membrane turnover or vesicular trafficking and not work as a drug transporter only. Because verapamil disrupts lysosomal functions, it may interfere with the ability of vesicles to transport drug out of the cells, therefore increasing the concentrations of drug reaching specific targets. Wadkins and Houghton (1993) reported that verapamil proved to be intermediate in both its ability to enhance antitumor drug accumulation interacting with P-gp and its membrane perturbing activity reducing the amount of rhodamine 6G bound to membranes by > 50% in the absence of any P-gp or other proteins.

Verapamil (m.w. 454.59) (see Chapter 2 for chemical structure of the MDR modulators), a calcium channel blocker characterized by its high effectiveness, high plasma protein binding, and its fast metabolism, was the first drug to be tested as a MDR modulator of drug resistance in a clinical setting (Tsuruo *et al.*, 1981, and 1982). The half-life of verapamil is approximately 7 hours in humans, and after oral administration its bioavailability is only about 20%. Therapeutically, verapamil is used mainly in patients with cardiac conditions as an anti-anginal and anti-arrythimic therapy. Excitation-contraction coupling in all cardiac cells requires calcium influx, so verapamil reduces abnormal cardiac contractility and cardiac output, due to its mechanism of action (Katzung, 1995). Lonsberry *et al.* (1994) have reported the effect of high-dose verapamil administration on the Ca^{2+} channel density in rat cardiac tissue. Animals exhibited external symptoms to verapamil treatment in a dose-dependent manner.

Verapamil has been extensively used in *in vitro* and *in vivo* studies as a MDR modulator (Martin *et al.*, 1987, Yusa and Tsuruo, 1989, Katzung, 1995, and Watanabe *et al.*, 1995). Tanabe *et al.* (1990) indicated that verapamil reversed CQ

resistance in a resistant line of *Plasmodium chabaudi* in mice in a dose-dependent fashion (5.0 - 50.0 mg/kg) when accompained by daily injection of CQ (2.0 to 3.0 mg/kg), at which doses resistant parasites grew steadily in the absence of verapamil. Miki *et al.* (1992), using a CQ-resistant strain of *P. falciparum*, reported that the co-administration of CQ (6 mg/kg) and verapamil (50 mg/kg) reversed the resistance in this strain and inhibited the growth of the resistant parasites in an *in vivo* study. Valecha *et al.* (1992) reported that when verapamil was used subcutaneously in mice at a dose of 10-50 mg/kg daily for 4 days, combined with 3 mg/kg of CQ diphosphate, the growth of CQ-resistant *P. berghei* was severely suppressed, but the reversal was not complete. Verapamil by itself did not affect the growth of the CQ-susceptible parasites at the same dose range. Kyle *et al.* (1993), working with an Aotus - *P. falciparum* nonhuman-primate model, also determined that CQ-resistance can be reversed *in vivo* when associated with verapamil. Agbe and Yielding (1993), looking at the effect of verapamil on the antitrypanosomal activity of ethidium bromide or Berenil in mice, reported that verapamil enhanced the survival of mice infected with *Trypanosoma brucei brucei* when treated with VRP and either drug.

The MDR modulator most studied in recent trials is the fungus metabolite, cyclosporin A ($C_{62}H_{111}N_{11}O_{12}$), an agent that *in vitro* data indicated was perhaps the most potent reversing agent commercially available. Borel *et al.* (1976), reported that cyclosporin A inhibits humoral immunity as shown by the reduction of plaque-forming cells and of haemaglutinin titres in mice. It also suppresses cell-mediated immunity being highly active in the treatment of chronic inflamatory reactions, but exert only weak myelotoxicity. The precise mechanism of action of cyclosporin A on T cells remains unclear. As mentioned before, cyclosporin A has been demonstrated to have MDR properties when associated with cytotoxic agents *in vitro* and *in vivo* (Lum *et al.*, 1993; Watanabe *et* al., 1995). The new MDR candidate selected for these studies is the so called CL347,099 (C_{33} H_{40} N_2 O_4 S. HCl. C_2H_6 O, m. w. 643.3) which has being recently developed by Wyest-Ayerst to target P-gp.

This drug is an analog of verapamil having 70 fold less calcium channel blocker activity than its precursor. Greenberger *et al.* (1996) have demonstrated that a similar compound, CL 329,753, has improved MDR biological properties compared with verapamil and cyclosporin A.

1.14 DRUG-DRUG INTERACTION

The effects of the MDR modulators, verapamil and cyclosporin A (and its analog PSC 833) on cytotoxin pharmacokinetics was studied in controlled clinical trials. Kerr *et al.* (1986) observed that following treatment with a cytotoxin alone and during administration of verapamil, there was a marked increase in doxorubicin AUC, T½, and volume of distribution at steady state compared with doxorubicin treatment alone. Escalating doses of cyclosporin A were administered IV in patients in a variety of tumor types in association with etoposite. Cyclosporin A produced an increase in etoposite AUC of 80%. Also, cyclosporin A resulted in a 38% decrease in renal clearance and a 52% decrease in nonrenal clearence (Lum *et al.*, 1992). Sikic and collegues (1997) reported the use of cyclosporin A and PSC 833 in clinical trials in association with different anticancer agents. The pharmacokinetic interaction resulted in significant changes in the bioavailability of these drugs, with the decrease in the clearance of these cytotoxic drugs consistent with P-gp modulation at the biliary lumen, blocking excretion of drugs into bile.

Towards the goal of rendering P-gp-dependent MDR cells sensitive to anticancer drugs, one must take into consideration not only the toxicity of the chemosensitizer itself, but in addition, its ability to increase the accumulation of the anticancer drugs in tissues that are not the target (Drori *et al.*, 1995). These factors represent the basis for the potentiating toxicity that the combination of MDR modulators with MDR-related anti-cancer drugs can induce in healthy tissues (Watanabe *et al.*, 1995, and Drori *et al.*, 1995). Relling (1996), suggested that a perfect modulator would be the one that shifts up the dose-efficacy curve of a P-gp

substrate drug with no effect on systemic pharmacokinetics and no effect on the dose-toxicity curve. Toxic effects of verapamil have been rare in clinical use and are known to be dose-related (Katzung, 1995). Chikhale *et al.* (1995), using a rat-brain model reported that verapamil acted at the BBB by binding to the P-gp, inhibiting its normal function and increasing brain permeability.

1.15 P-GLYCOPROTEIN AND *HAEMONCHUS CONTORTUS*

Sangster (1993) indicated that at least three P-gp genes occur in adult *H. contortus* with a fourth in eggs. More recently, Xu and colleagues (1998) detected, using Northern blot analysis, that the mRNA levels of P-gp were higher in eggs of *H. contortus* ivermectin-resistant strains (2 strains) compared with their respective ivermectin-susceptible precursor strains (2 strains). Similar results have also been obtained in adults. Digestion of genomic DNA from adult worms with the restriction enzime Pvu II showed different patterns between the ivermectin-resistant and -susceptible strains. A Dde I digest of a P-gp fragment from adult worms also showed differing patterns between ivermectin-resistant and -susceptible strains, indicating a genetic difference between the strains at, or near, this gene (Blackhall *et al.*, 1998). Ivermectin was also found to be a good P-gp substrate (Pouliot *et al.* 1996). Sangster *et al.*, (1999) reported, using Southern blots of *H. contortus* drug-resistant isolates with an internucleotide binding domain probe, a pattern consistent with the involvement of P-gp in resistance ivermectin and moxidectin. The data from the drug combination experiments included in this research (Chapter 3, 4, and 5) may suggest the active involvement of P-gp in conferring resistance to *H. contortus*; giving strong evidence that P-gp may be involved in the mechanism of resistance in this parasite.

Alvinerie *et al.*, (1999) have determined that verapamil increased ivermectin absorption after the anthelmintic was used as a pour-on formulation in rats. The drug combination strategy increased C_{max} and AUC of ivermectin by 40%.

Verapamil has been used *in vitro*, associated with benzimidazole, to partially increase activity against benzimidazole-resistant strain of *H. contortus* (Beugnet *et al.*, 1997). However, Kotze (1998), has demonstrated that verapamil did not reduce the level of tolerance in an ivermectin-resistant larvae treated with ivermectin. Although very promising, this area of research is only at the beginning and there is a long way to be covered before a new control strategy, based on combination treatment, is introduced.

1.16 METABOLIC DRUG INTERATION

Recently, CYP3A4 was found to be the main isoform of the P450 enzyme responsible for ivermectin metabolism (Zeng *et al.*, 1998). This enzyme metabolizes many drugs and is identically expressed in the liver and in the intestine (Lown *et al.*, 1993, and Thummel *et al.*, 1996). In the duodenum and jejunum, CYP3A4 plays an important role in the first-pass metabolism after oral administration of its substrates in humans (De Waziers *et al.*, 1990). Kroemer *et al.* (1993) revealed the overlapping substrate/inhibitor specificity of CYP3A4 and P-gp for verapamil (Wacher *et al.*, 1995). The possibility of common regulatory factors for CYP3A4 and P-gp proteins has been demonstrated by Schuetz *et al.* (1996), who suggested that verapamil was a good inducer for P-gp and CYP3A4. The synergistic effects of CYP3A4-mediated metabolism and P-gp-mediated efflux in the gut epithelium may result in an unexpectedly high first-pass effect in the gut after oral administration. Thus, the inhibition or induction of CYP3A4 and/or P-gp caused by drug-drug interactions may affect the first-pass effect in the gut (Benet, 1995). More importantly, verapamil and ivermectin pass through and are metabolised by the liver hepatocytes.

Although resistance has not been reported yet, ivermectin has been extensively used in humans against *Onchocerca volvulus*, an organism responsible for a disease that causes serious eye lesions. Thus, although an extensive amount

of data has to be collected before attempting to safely confirm the positive results from the drug combination strategy, the data using large mammals, as described in Chapter 5 of this thesis, is a step forward to the careful identification of drug combinations that would be appropriate for human trials in the future.

1.17 ANIMAL MODEL

Conder *et al.* (1990, and 1992) described an experimental *in vivo* model for the ruminant helminth *H. contortus* utilizing jirds (*Meriones unguiculatus*). The animals are infected with L_3 of *H. contortus* to model what happens in natural hosts. The advantages of this model are that: (1) the site of establishment of the L_3 larval stage of *H. contortus* is the glandular portion of the stomach of jirds, which is similar to its predilection site in the abomasum of sheep, (2) the parasite that can be used is the same as that which infects sheep in the field, (3) the drug-efficacy results are highly reproducible, (4) jirds maintain a relatively stable parasite population through day 14 postinfection, (5) L_3 larvae molt twice during this period of 14 days (from L_3 to L_4, and to early adult), and (6) the cellular immune response is similar between jirds and sheep (Conder *et al.*, 1990, and 1992). Jirds, however, have to be lightly immunosupressed by adding 0.02% hydrocortisone, a short-duration synthetic corticosteroid, to their diets. This is necessary for the exsheated L_3 larvae to establish in the non-specific host. This treatment gives a marginal level of immunosuppression to the animals. Conder *et al.* (1992) reported that the mean number of *H. contortus* recovered from jirds at necropsy (day 13) was 108.1 from medicated animals (hydrocortisone) and 33.4 from the unmedicated group. Jirds were also used to test the efficacy of avermectins against six-day-old *T. colubriformis*. Avermectin B_{1a} and B_{2a} removed 100% of the worms at 0.0312 mg/kg (Ostlind and Cifelli, 1981).

Although jirds need the immunosuppressive diet to maintain the parasites, the effect of artificial immunosuppression in the treatment of parasites is of some

importance. Todd and Lepp (1972), treating rats infected with *Eimeria vermiformis* with 0.5 mg/kg of dexamethasone (Dx) daily, detected oocysts in the feces up to 18 days post-treatment in treated rats compared with no oocysts in the untreated group. Adams and Davies (1982) reported that sheep infected with *H. contortus* and treated with Dx (0.5mg/kg) had a worm recovery of 550 adult worms compared to 1658 worms in the untreated group, showing the toxic effect of Dx to ensheathed L_3 larvae. Presson *et al.* (1988) studied the effect of genetically resistant Merino sheep immunosuppressed with Dx (0.5 mg/kg, three treatments) on infections of *H. contortus*. In resistant and susceptible sheep without treatment, and resistant and susceptible treated groups, the average number of globular leukocytes present in the abomasum were 154.6, 7.2, 0, and 0 respectively. The treatment with Dx abolished differences between resistant and susceptible genotypes. Therefore, the high level of resistance to infection in resistant genotypes may be based on an immunological response.

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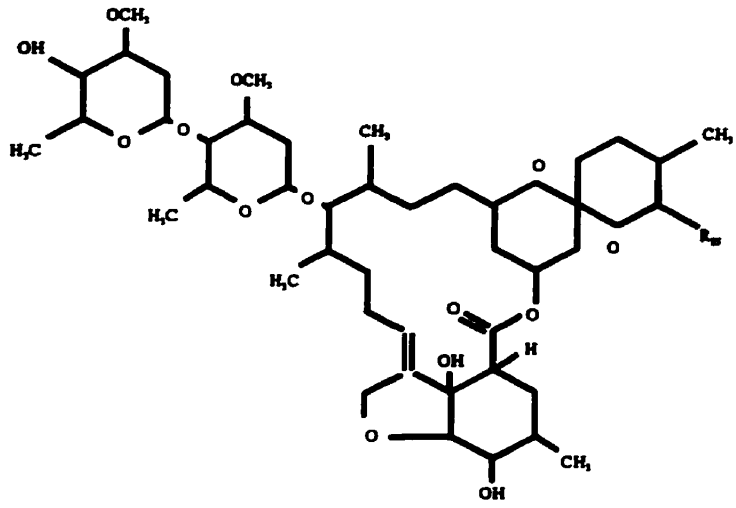
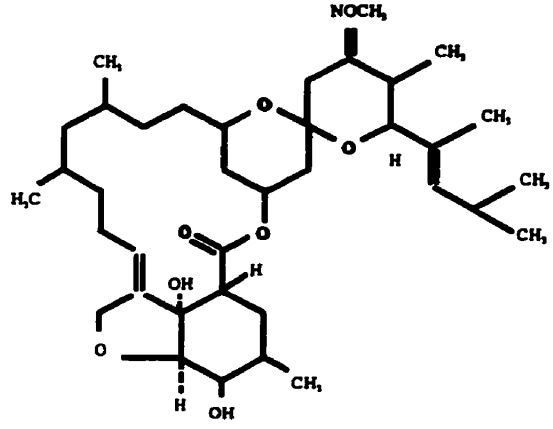


IVERMECTIN



MOXIDECTIN

Figure 1. Chemical structure of the macrocyclic lactones, ivermectin and moxidectin. Ivermectin: R_{25}= CH (CH_3) CH_2 CH_3 and CH (CH_3)$_2$

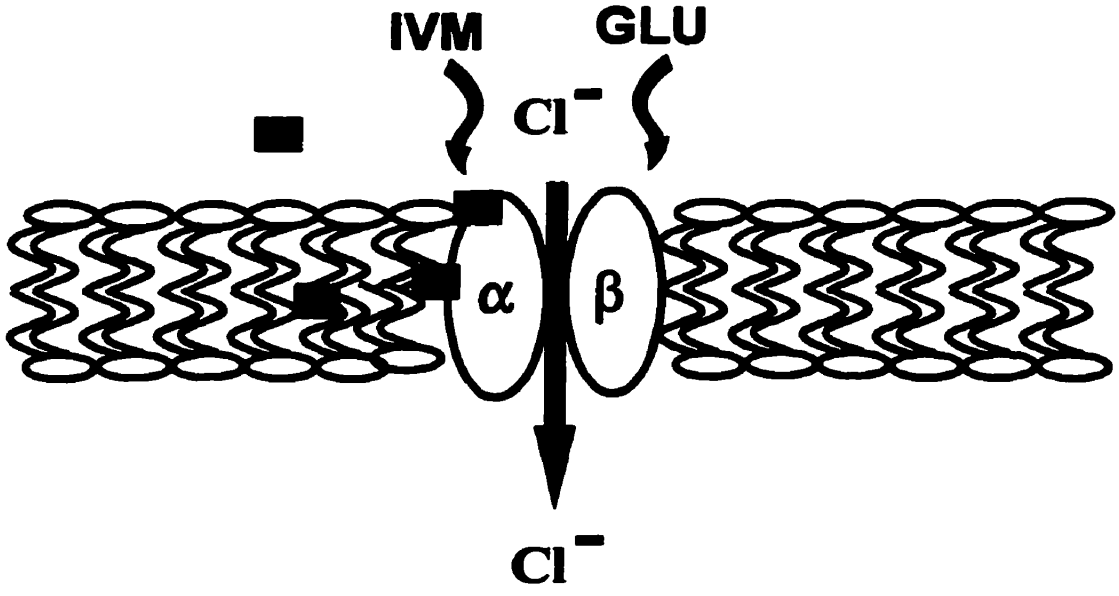


Figure 2. Mechanism of action of ivermectin. The figure shows the ivermectin drug molecule binding to the α sub-unit of the glutamate-gated chloride channel, opening it to the influx of chloride ions. Based on Arena *et al.* (1991, and 1992).

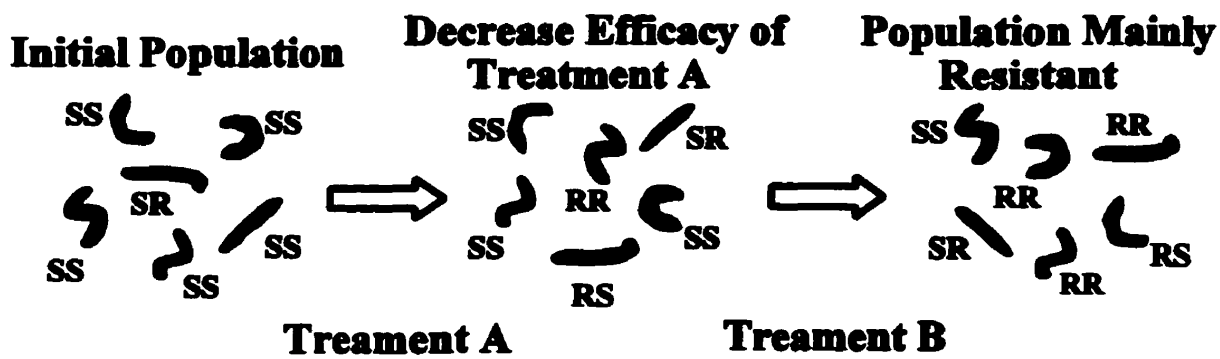


Figure 3. Hypothetical selection for nematode resistance. SS, homozygous susceptible; RR, homozygous resistant; SR/RS, heterozygous. Note that an heterozygous gene is already present when the parasites are exposed to the first treatment.

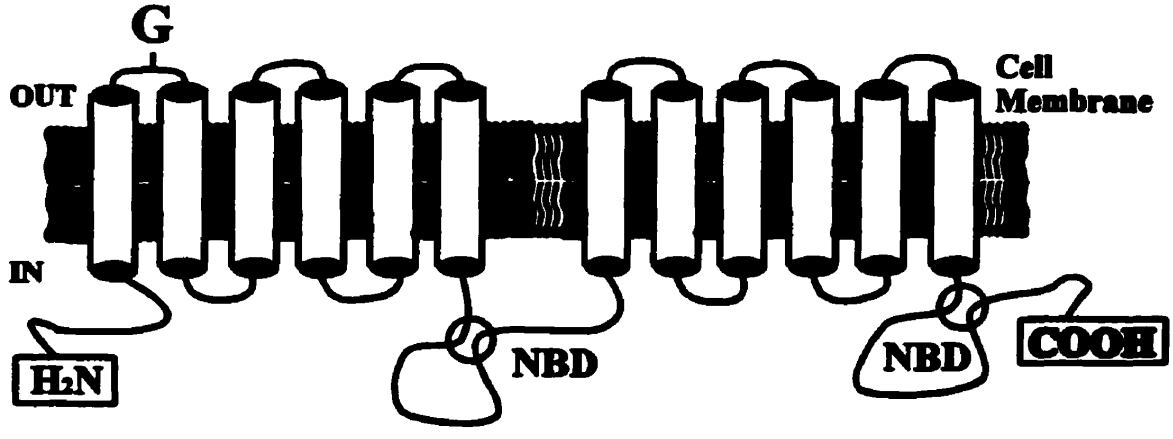


Figure 4. Structure of P-glycoprotein. The figure shows the 12 transmembrane domains, (NBC) nucleotide binding domain, and (G) glycosilation site (Gottesman and Pastan, 1993).

Table 1. Human tissues with high levels of expression of the MDR1/*mdr*1 gene. *Expression of human MDR2 (Thiebaut *et al.*, 1987, and Lum and Gosland, 1995).

ORGAN	TISSUE
Adrenal gland	Cortical cells
Brain	Endothelial cells
Gastrointestinal tract	Small intestines (luminal surfaces) Large intestines (luminal surface)
Hematopoietic cells	Natural killer cells (CD16+) T lymphocytes (CD8+ and CD4+) B lymphocytes (CD20+) Monocytes (CD14+) Myeloid progenitor cells (CD33+) Pluripotent stem cells (CD34+)
Kidney	Brush border of proximal tubules
Liver	Hepatocytes (luminal surfaces) Biliary ducts (luminal and apical surfaces)*
Lung	Bronchial cells (luminal surfaces)
Reproductive system	Breast (intermediate levels) Uterus (intermediate levels) Placenta Prostate
Skin	Sweat glands (luminal surface) Acrosurgieum (luminal surface)
Pancreas	Small ductules (luminal surface)

Table 2. Classes of agents that inhibit P-glycoprotein function (Georges *et al.*, 1990, and Gottesman and Pastan, 1993).

Chemical class	Example
Calcium channel blockers	Verapamil, azidopine, diltiazen, nifedipine, nicardapine, prenylamine, bepridil, nicardapine
Calmodulin antagonists	Trifluoperazine, chlorpromazine, trifluopromazine, fluphenazine, *cis* and *trans*-clopenthixol, W-12
Anti-arrhythmics	Quinidine, amiodarone
Antihypertensives	Reserpine
Antibiotics	Hydrophobic cephalosporin
Immunosuppressants	Cyclosporin A, FK506, rapamycin PSC 833
Hormones	Progesterone
Detergents	Tween-80
Other drugs	Terfenadine, tamoxifen, forskolin, vindoline, triton WR-1339, chloroquine, tioperidone

Introductory Statement

The first step in this research project was to determine the level of resistance of *H. contortus* to the macrocyclic lactones. An moxidectin-selected, and an unselected strain of parasites were used for this experiment. These strains were passaged in sheep for fourteen generations. We used jirds as an artificial host-model for *H. contortus* to compare responses of the anthelmintic-selected and unselected strains to ivermectin and moxidectin.

CHAPTER 2

Manuscript 1

DECREASE OF IVERMECTIN AND MOXIDECTIN SENSITIVITY IN *Haemonchus contortus* SELECTED WITH MOXIDECTIN OVER FOURTEEN GENERATIONS

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2.1 ABSTRACT

Ivermectin resistance in the nematode *Haemonchus contortus* has been reported in many parts of the world and many ivermectin resistant isolates have been found to have reduced sensitivity to moxidectin. However, it is unclear whether parasites that are selected with moxidectin would demonstrate reduced sensitivity to ivermectin. In this study, the effects of moxidectin and ivermectin on an unselected strain and a strain of *H. contortus* derived from the unselected strain but selected over fourteen generations with moxidectin, were compared in jirds. The recovery of adult worms and fourth stage (L_4) larvae following treatment were compared between strains and anthelmintics. Moxidectin-selected *H. contortus* showed reduced sensitivity to ivermectin as well as to moxidectin. Doses of 0.1 mg/kg of moxidectin and 0.4 mg/kg of ivermectin were necessary to obtain an efficacy of 95% or above against the moxidectin-selected strain of *H. contortus* compared with 0.025 mg/kg for moxidectin and 0.1 mg/kg for ivermectin required for a similar efficacy in the unselected strain.

2.2 INTRODUCTION

Ivermectin (IVM) and moxidectin (MOX) molecules are semisynthetic, 16-membered macrocyclic lactones derived from *Streptomyces spp.* (Burg *et al.*, 1979; Carter *et al.*, 1987). Resistance to IVM in the abomasal nematode *Haemonchus contortus*, was first reported in sheep (Carmichel *et al.*, 1987) and is now a serious problem worldwide. Leathwick (1995) reported that an IVM-resistance strain of *Ostertagia* spp. in goats in the field was also resistant to the normal use level of MOX. While IVM and MOX may have similar mechanisms of action, it is not clear that they have identical mechanisms of drug resistance. Conder *et al.* (1993) found that the efficacy of MOX at a dose which clears 98% of IVM susceptible *H. contortus* was only 47.2% effective against an IVM-resistant strain in jirds. Shoop

et al. (1993) reported that *Ostertagia circumcincta* and *Trichostrongylus colubriformis* resistant to IVM were also resistant to MOX. However, Craig *et al.* (1992) found that MOX against an IVM-resistant strain of *H. contortus* was 99.9 and 100% effective in lambs at 0.2 and 0.4 mg/kg, respectively, but the efficacy of IVM was only 38.8 and 53.1% at 0.4 and 0.8 mg/kg, respectively. The recommended dose rate for both anthelmintics is 0.2 mg/kg. Oosthuizen and Erasmus (1993) tested both compounds (0.2 mg/kg) against the multiple anthelmintic resistant "White River Krtz" strain of *H. contortus* and found the efficacy of MOX and IVM to be 99.9 and 29.1 %, respectively. To date all of these studies have looked at the effect of MOX against nematode isolates that have been selected with IVM. No studies have determined the effect of IVM against nematode isolates that have been selected with MOX.

The objective of this study was to determine whether IVM efficacy changes as a result of MOX selection and the relative potency of MOX and IVM against MOX-selected isolates, as well as unselected isolates of *H. contortus* in the jird (*Meriones unguiculatus*).

2.3 MATERIAL AND METHODS

Helminth-free female jirds (Charles River, Canada), 30-35 grams, were fed with commercial diet supplemented with 0.02% of hydrocortisone. This treatment has been found to increase the establishment of the exsheathed third stage (L_3) larvae in this nonspecific host (Conder *et al.*, 1990). The effect of hydrocortisone on establishment was assessed in this study. Two days after arrival, animals were placed on the special diet (day -5) and continued to receive it until the end of each trial. At day 0 all animals were infected orally with 1000 exsheathed L_3 larvae of *H. contortus* (total volume of 0.1 ml per animal). The vial that contained the larvae was maintained in a water bath (37°C) and stirred to prevent the larvae from clumping together thereby ensuring an homogeneous sample. The jirds were infected with

H. contortus that were either MOX-selected (MOF14) or unselected (PF14). These strains have been described elsewhere (Xu *et al,*. 1998). At day 10, jirds were allocated to 18 groups of 6 animals per group and were orally treated with anthelmintic at one of four dose rates (4 for IVM and 4 for MOX) or left untreated. Animals were observed throughout the experiment for any toxic effects. Jirds were killed 3 days after treatment by carbon dioxide inhalation. After the stomachs were removed from the animals, the stomachs were cut in half transversely, leaving the posterior portion (glandular part) to be processed (Conder *et al.*, 1992) and examined under a dissecting microscope. Jirds were housed in shoe-box cages (2 per cage). Water and food were available *ad libitum.*

Drug efficacy was based on arithmetic means and the total worm count (TWC) reduction test was determined according to Coles *et al.* (1992). Comparison of treatments with the control group and between strains under the same treatment was analyzed using ANOVA followed by pairwise multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

MOX drench solution was supplied by Fort Dodge Animal Health (Princeton, NJ), IVM (MSD/AGVET) was purchased from CDMV Inc. (St. Hyacinthe, Canada), and hydrocortisone from ICN Biomedical (Aurora, OH). Anthelmintic drugs were diluted in distilled water and were vortexed immediately prior to use.

2.4 RESULTS

The effect of the hydrocortisone treatment in the jird model was tested prior to the start of the anthelmintic titration phase. At necropsy, animals in the groups infected with the unselected and selected strains that received the commercial diet plus hydrocortisone had significantly higher mean worm counts, 10 days after infection, than animals not receiving hydrocortisone in the diet (Table 1).

When the anthelmintics were titrated, no toxic effects were observed on the jird. Multiple comparison revealed that all treatments with both anthelmintics were

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2.5 DISCUSS[illegible]

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significantly different from the control group for the same strain. Resistance was detected by comparing drug treatments against PF14 and MOF14 strains (Table 2). At low doses, both anthelmintics had a significantly different worm reduction response ($P<0.05$). Using a dose of 0.025 mg/kg against PF14 and MOF14 strains moxidectin resulted in an efficacy of 96 and 61% and IVM had an efficacy of 55 and 28%, respectively. To obtain an efficacy of ≥95%, moxidectin had to be given at 0.1 mg/kg against MOF14, and IVM had to be given at above 0.1 and 0.4 mg/kg for PF14 and MOF14, respectively (Table 2).

2.5 DISCUSSION

The jird model was shown to be useful for the evaluation of anthelmintic efficacy and parasites were found only in the posterior part of the stomach (glandular region). The results obtained at 0.1 mg/kg for MOX and IVM against the susceptible strain are in agreement with Conder *et al.* (1990, 1993) using an IVM-susceptible strain. After 14 generations of laboratory selection with MOX, the resistant ratio was approximately 2-4 for MOF14 compared with PF14, the parental strain.

Resistance to ivermectin from *H. contortus* is believed, in part, to be due to the over-expression of P-glycoprotein, a transport protein, which acts as an efflux pump in resistant organisms. The multidrug resistance (MDR) reversing agent verapamil enhanced IVM and MOX efficacy by 13 and 26%, respectively against MOX-selected strain (Xu *et al.*, 1998). The relative affinity of IVM or MOX for the nematode P-glycoprotein remains to be determined and may affect the rate of selection of resistance.

Although the MOF14 strain was selected using only MOX, MOF14 appeared to have a higher degree of resistance to IVM than to MOX. The data presented in this study demonstrates for the first time that when a nematode becomes resistant to MOX, it also shows side-resistance to IVM. These results have implications for

anthelmintic rotation programs of parasite control.

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Table 1. Addition of hydrocortisone to the diet of jirds on the establishment of *Haemonchus contortus*. Results show total worm count (TWC) ±SE (n=4)

Treatments	Unselected - PF14			Selected - MOF14		
	TWC	(SE)		TWC	(SE)	
Com. Diet + Hydrocortisone	63	(9)	a	50	(6)	a
Com. Diet	25	(2)	b	14	(7)	b

Different letters (a, b) indicate that values were significantly different ($P<0.01$).

Table 2. Total worm count - TWC (±SE) and percent efficacy (%) of moxidectin (MOX) or ivermectin (IVM) mg/kg against MOX-selected and -unselected strains of *Haemonchus contortus* in jirds (n=6)

	Unselected[a] - PF14			Selected[a] - MOF14		
Treatment/ dose	TWC	(SE)	(%)	TWC	(SE)	(%)
Placebo	80	(7)	—	123	(12)	—
MOX / 0.0125 [b]	19	(3)	76	85	(6)	31
MOX / 0.025[b]	4	(1)	96	48	(15)	61
MOX / 0.05	0.3	(0.2)	99	10	(3)	92
MOX / 0.1	0		100	2	(1)	98
IVM / 0.025 [b]	36	(7)	55	89	(14)	28
IVM / 0.1 [b]	7	(3)	91	53	(11)	57
IVM / 0.4	0		100	5	(2)	96
IVM / 1.6	0		100	1	(0.5)	99

a. All groups are statistically different from control group within strain. b. TWC are statistically different across strains ($P<0.05$).

Connecting Statement I.

Studies in the Chapter 3 report the increase of ivermectin and moxidectin efficacy against moxidectin-selected *Haemonchus contortus* parasites after their combination with the multidrug resistance modulator, verapamil. This paper reports the cloning and sequencing of P-glycoprotein (refer to the Preface for co-authorship acknowledge) suggesting its involvement in anthelmintic resistance.

CHAPTER 3

Manuscript 2

IVERMECTIN RESISTANCE IN NEMATODES MAY BE CAUSE BY ALTERATION OF P-GLYCOPROTEIN HOMOLOG

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3.1 ABSTRACT

Resistance to ivermectin and related drugs is an increasing problem for parasite control. The mechanism of ivermectin resistance in nematode parasites is currently unknown. Some P-glycoproteins and multidrug resistance protein have been found to act as membrane transporters which pump drugs from the cell. A disruption of the mdr1a gene, which encodes a P-glycoprotein in mouse, results in hypersensitivity to ivermectin. Genes encoding members of the P-glycoprotein family are known to exist in nematodes but the involvement of P-glycoprotein in nematode ivermectin-resistance in the sheep nematode has not been described. Our data suggest that a P-glycoprotein may play a role in ivermectin resistance in the sheep nematode parasite *Haemonchus contortus*. A full length P-glycoprotein cDNA from *H. contortus* has been cloned and sequenced. Analysis of the sequence showed 61-65% homology to other P-glycoprotein/multidrug resistant protein sequences, such as mice, human and *Caenorhabditis elegans*. Expression of P-glycoprotein mRNA was higher in ivermectin-selected than unselected strains of *H. contortus*. An alteration in the restriction pattern was also found for the genomic locus of P-glycoprotein derived from ivermectin-selected strains of *H. contortus* compared with unselected strains. P-glycoprotein gene structure and/or its transcription are altered in ivermectin-selected *H. contortus*. The multidrug resistance reversing agent, verapamil, increased the efficacy of ivermectin and moxidectin against a moxidectin-selected strain of this nematode in jirds (*Meriones unguiculatus*). These data indicate that P-glycoprotein may be involved in resistance to ivermectin and other macrocyclic lactones in *H.contortus*.

Key words: *Haemonchus contortus*; P-glycoprotein; Drug resistance

Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank data base with the accession number AF 003908

Abbreviations: Ivermectin (IVM); moxidectin (MOX); Multidrug resistance (MDR); P-glycoprotein (P-gp); verapamil (VRP); MOX-selected (MOF14); IVM-selected (IVF14) or unselected (PF14); polymerase chain reaction (PCR), unselected strain from MSD AgVet (MKIS); IVM-selected strain from MSD AgVet (MKIR); *Haemonchus contortus* (*H. contortus*); Lethal dose 95% (LD95); *Caenorhabditis elegans* (*C. elegans*).

3.2 INTRODUCTION

Resistance to ivermectin (IVM), a macrocyclic lactone anthelmintic, in nematode parasites of livestock has become a serious problem in many parts of the world (1, 2), and the increasing use of the anthelmintics in animals and humans is likely to increase the incidence of resistance. The mechanism underlying this resistance has not yet been established. Detection of field resistance is currently dependent on the analysis of drug efficacy involving *post mortem* worm counts, nematode egg-count reduction or the use of *in vitro* assays of the development of immature stages. These methods are insufficiently sensitive to monitor the development of resistance before it is overt. Understanding the molecular basis of resistance to IVM and that of other macrocyclic lactone anthelmintics should assist in the development of more sensitive methods for monitoring the development of resistance and possibly for reversing it.

IVM is thought to bind to an alpha subunit of a glutamate-gated chloride channel in nematode cell membranes (3, 4) resulting in the hyperpolarization of neuromuscular cells and worm paralysis. The possibility that IVM resistance is due to altered binding to its receptor has been investigated in IVM resistant and sensitive *Haemonchus contortus* (5). These workers concluded that resistance was not due to an alteration in its binding to the glutamate-gated chloride channel receptor.

P-glycoprotein have been considered to cause drug resistance in mammalian

tumor cells (6, 7). P-glycoprotein in *Leishmania* appear to be involved in drug resistance (8-11). In some drug-resistant strains of the malaria parasite *Plasmodium falciparum*, amplification of a P-glycoprotein gene (pfmdr1) is observed (12, 13). In the free living nematode *C. elegans*, a P-glycoprotein has been observed to provide protection against toxic compounds made by plants and microbes in the rhizosphere (14). The nematode *C. elegans* has multiple proteins, homologues of the mammalian proteins involved in cellular resistance to chemotherapeutic drugs, that protect the worms from the toxic effects of heavy metals (15). It has been observed than the disruption of the mdr1a P-glycoprotein (P-gp), a membrane transport glycoprotein (6), in the mouse leads to a marked increase in central nervous system sensitivity to IVM (16), and it has been shown that IVM is a potent P-gp ligand in mammalian cells (17, 18). In view of the mammalian evidence that IVM may bind to and be transported by P-gp, we investigated the possibility that a P-gp might mediate ivermectin resistance in this parasite.

3.3 METHODS

3.3.1 Parasites

Strains of *H. contortus* that were unselected (MKIS) or selected with IVM (MKIR) were reported elsewhere (5). In addition, a moxidectin (MOX)-selected (MOF14), an IVM-selected (IVF14), and an unselected (PF14) strain passaged over 14 generations, were obtained from Fort Dodge Animal Health (Princeton, NJ). These strains were derived from the same parent isolate, and the passages consisted of an artificial infection with the larvae from treatment survivors of the previous generation of *H. contortus* in naïve lambs with subsequent treatment with MOX or IVM in the case of the resistant strains or with no treatment (susceptible). At the 12th generation, the lethal dose to eliminate 95% (ED_{95}) of the parasites for

MOX, in the MOX-selected stain, and the ED_{95} for IVM, in the IVM-selected strain, were reported to have increased 5.3 and 9.7 fold, respectively, when compared with the unselected strain (19).

3.3.2 Anthelmintics

IVM (0.8 mg/ml solution for sheep, Merck, Sharp and Dohme) was purchased at CDMV Inc. (St. Hyacinthe, Canada). MOX (0.01% drench solution) was supplied by Fort Dodge Animal Health (Princeton, NJ), and VRP was purchased from Sigma (St. Louis, MI). MOX was used at 0.017 mg/kg, and IVM was used at 0.028 mg/kg. Anthelmintic drugs were administered orally. VRP was used subcutaneously concomitantly with the anthelmintics at 20 mg/kg. VRP dosage was determined based in our previous results (data not shown). All drugs were diluted in distilled water prior to the treatments.

3.3.3 Cloning PGP-A cDNA from H. contortus

Two degenerate primers 5'-ACNGTNGCNYTNGTNGG-3' (sense) and 5'-GCNSWNGTNGCYTCRTC-3' (antisense), based on the conserved ATP-binding domains of the P-gp sequences in the free-living nematode *Caenorhabditis elegans* (20), were constructed and used to generate a 432 bp P-gp fragment from a *H. contortus* cDNA library by the polymerase chain reaction (PCR). The same library was screened with this 432 bp fragment, labelled with ^{32}P (Random primed DNA labelling kit, Boehringer Mannheim Biochemica). A partial 2.7 kb cDNA lacking the 5' end of PGP-A was obtained and sequenced. A pair of primers, the nematode spliced leader sequence SL1 (5'-GGTTTAATTACCCAAGTTTGAGA-3') (20) and a specific reverse primer 5'-CGATGCGTACAATGACGGT-3', were constructed for identifying the 5' end of PGP-A. The 5' end of the PGP-A sequence was subsequently amplified by RT-PCR with denaturing at 94°C for 1 min, annealing at

55°C for 1 min and extension at 72°C for 2 min., for 25 cycles from mRNA of *H. contortus*. The PCR products were cloned and sequenced. A protein sequence was analysed to predict the possible hydrophobic transmembrane domains and the sequence was aligned with other MDR proteins by ClustalW protein analyses program (21).

3.3.4 Northern Blot Analysis

RNA was extracted from four separate batches of eggs of the unselected or IVM-selected strains of *H. contortus*. Eggs were isolated from the feces of sheep that had been given a mono-infection of one of the respective strains. The collected eggs were stored at 4 C. Storage varied from eggs used the same day as they were isolated, to those used at 4 weeks. Each pair of IVM selected and unselected samples were subjected to the same period of storage after eggs were collected from the faeces of sheep. Total RNA was extracted from the purified eggs using TRIzol Reagent (Gibco BRL). Total RNA was subjected to electrophoresis on a denaturing formaldehyde agarose gel (22) and transferred to a H-bond nylon membrane. The membrane was pre-hybridized at 65° C in 10% dextran disulfate, 1% SDS and 1.0M NaCl for 4 h. The 432 bp fragment, labelled with [^{32}P]d-CTP by random priming, was added and allowed to hybridize overnight at 65° C. The membrane was washed with 2X SSC, 0.1% SDS at 65° C for 30 min and with 0.5X SSC at 35 ° C for 1 h and then autoradiographed. A 1.6 kb mouse [^{32}P]-labelled actin DNA probe (kindly supplied by G. Matlashewski) was hybridized the same blot, as an internal control.

3.3.5 Southern Blot Analysis

Genomic DNA from both MKIS and MKIR strains of *H. contortus* was extracted from adult worms (23) and ≈ 20 µg DNA was digested with *ClaI*, *PstI*,

*Eco*RI or *Pvu*II. The digested DNA was run on a 1% agarose electrophoresis gel and blotted on a nylon membrane. The membrane was hybridized with the 432 bp probe, using the same protocol as in the Northern Blot Analysis.

3.3.6 *Assessment of the effect of the multidrug resistance reversing drug verapamil in vivo*

H. contortus infections were established in the jird (*Meriones unguiculatus*). Female jirds, 30-35 grams (Charles River, St-Constant, Canada) were maintained on a diet containing 0.02% hydrocortisone from 5 days prior to infection until necropsy (24). All animals were infected with 1000 exsheathed third-stage larvae (L_3) on day 0, and treated on day 10. Prior to the treatment, jirds were randomly divided into groups of six animals per treatment, and on day 13 after infection, were necropsied for worm-count determinations. Animals were housed in shoe-box cages containing wood shavings. Water and food was available *ad libitum*

3.4 RESULTS

3.4.1 *Cloning PGP-A cDNA from H. contortus*

A 432 bp PCR fragment of the ATP-binding region of a P-gp cDNA homolog was amplified by PCR, cloned (TA vector, Invitrogen) and sequenced. Sequence data showed that five of nine selected clones were identical. The result of a GenBank Blast search showed 85-94 % homology of this fragment to P-gp or multidrug resistance proteins from other sources. This fragment was labelled and used as a probe to screen the cDNA library and dozens of positive clones were identified. Only clones containing fragments larger than 2.0 kb were subjected to sequencing. In addition to the sequence reported here, a 2.8 and a 3.5 kb truncated cDNA of P-gp having high homology with the reported sequence were also found

(data not shown). These fragments represent the 3'-end of different P-gp homolog based on comparison with sequences in GenBank. In order to obtain the 5'-end sequences to the truncated P-gp clones, we designed a forward primer based on the nematode spliced leader sequence SL1. An antisense primer, based on the sequence of these partial *H. contortus* P-gp cDNAs, was also employed. One of the primers, based on a truncated 2.7 kb clone, generated a 1.8 kb 5'-end PCR fragment from mRNA of *H. contortus* using reverse transcriptase followed by PCR. The 1.8 kb fragment was sequenced, and 340 bp of the 3'-end were identified as corresponding exactly witht the 5'-end of the 2.7 kb truncated cDNA. The combined sequence of the 1.8 and 2.7 kb fragments was named PGP-A. The protein sequence was translated from the full length PGP-A cDNA (GenBank No. AF003908). There are two ATP binding cassette in PGP-A and the Kyte-Doolittle Hydrophobicity Plot showed that the protein has 12 transmembrane domains. An alignment (21) of the translated PGP-A with human mdr1, mouse mdr1 and *C. elegans* pgp-1 is shown (Fig.1). PGP-A is 61% homologous to *C. elegans* pgp-1, 64% homologous to mouse mdr1a and 65% homologous to human mdr1.

3.4.2 Northern Blot Analysis

RNA extracted from paired batches of eggs of IVM-selected and unselected strains was subjected to Northern blot analysis (Fig.2). Bands at approximately 4.2 kb represent P-gp homolog mRNA and bands at approximately 1.6 bp represent actin mRNA. The blots were subjected to densitometry, and the intensity of the P-gp RNA bands was corrected for the intensity of the actin band, to control for RNA loading. Each paired isolated eggs (S and R) were collected together, the eggs isolated and stored at 4°C prior to RNA extraction. The results represent four separate isolations with variation in the duration of storage prior to RNA isolation between pairs. Differences in the quantities of eggs collected, duration of storage and the loading of RNA could accont for variations in the density of bands between

different pairs of samples. In addition, MKIS/MKIR and PF14/IVF14 were subjected to different selection histories. However, in all cases, variations in RNA loading were corrected for the actin mRNA. The results showed that P-gp mRNA was consistently present in higher amounts in RNA samples from IVM-selected strains of *H. contortus*, in all paired comparisons of the IVM-selected with the unselected strains (Table 1).

3.4.3 Southern Blot Analysis

A restriction polymorphism was observed in the Southern blot. Althought Southern blot analysis, using the 432 bp probe, of genomic DNA from both unselected and IVM-selected *H. contortus* (MKIS and MKIR, respectively) showed that the intensity of restriction patterns obtained with the enzymes *ClaI, PstI* and *Eco*RI were different, these differences in intensity could be due to differences in loading DNA. However, the band pattern from PvuII digestion was qualitatively different between the strains (Fig.3A and B). Differences have been confirmed by restriction analysis following PCR using selected PGP-A primers with DNA extracted from individual worms from unselected or IVM- or MOX-selected strains of *H. contortus* (24).

3.4.4 The effect of verapamil in vivo

The effect of verapamil on anthelmintic efficacy in jirds was investigated. The *H. contortus*-jird model has previously been used to assess the efficacy of anthelmintics *in vivo* (25). A preliminary experiment was undertaken to identify doses of IVM and MOX that produced efficacies of 50 to 95% against the MOF14 and PF14 strain of *H. contortus* in the jird (data not shown). In addition, the effects of VRP, a classical MDR reversing agent and calcium channel blocker, on *H. contortus* in the jird, and the toxicity of VRP against the jird host were also assessed

(data not shown). MOX was used at 0.017 mg/kg and IVM was used at 0.028 mg/kg, orally. VRP was used subcutaneously at 20 mg/kg, concomitantly with the anthelmintic administration. VRP alone had no anthelmintic activity and, at 20 mg/kg, did not cause any mortality or pathology in the jirds. No significant differences between the VRP/anthelmintic combination compared with anthelmintic alone were observed against the unselected strain. However, coadministration of VRP significantly increased the efficacy of MOX and IVM against the MOX-selected strains of *H. contortus*. Against the MOF14 strain, the mean efficacies of MOX were 70 and 96%, and for IVM were 80 and 93% respectively in the absence and presence of VRP (Table 2).

3.5 DISCUSSION

A full length cDNA P-gp homolog from *H. contortus* was cloned and sequenced. Sangster (26) had suggested that *H. contortus* may possess four P-gp genes, although no sequence information was given. The sequencing data of our three P-gp clones indicate a high degree of identity at the DNA level. The predicted amino acid sequences of the three clones are very similar and they may reflect allelic variation of a single genetic locus. One of the cDNA clones has a 53 bp intron in its sequence, leading to stop codons in the reading frame. It is possible that incorrect mRNA splicing ocurred during gene expression. The *PvuII* restriction enzyme analysis has shown that a polymorphism exists in this P-gp between IVM-selected and unselected nematodes. This phenomenon could be a marker for drug resistance. Furthermore, Northern blot analysis (Fig. 2) has shown a higher level of P-gp mRNA in drug-selected strains than in unselected strains. The eggs were isolated from sheep faeces collected over a 24 h period from each pair of strain comparisons. The purified eggs were stored for different lengths of time at 4 C prior to RNA extraction. At this temperature, the eggs may slowly continue their development without hatching. Each sample of eggs would contain a variable

mixture of different egg development stages. The signal strength of mRNA expression of P-glycoprotein may vary with the development and lead to the variations between the different sample preparation seen in Fig. 2. Nevertheless, estimates of P-gp mRNA were in all cases corrected for actin mRNA (Table 1). These data taken together suggest that an alteration in P-gp gene structure and/or its transcription may be involved in resistance to macrocyclic lactone anthelmintics such as the avermectins and milbemycins. Our results indicated that resistance to ivermectin is, in part, due to changes in parasite P-gp, resulting in overexpression of the P-gp. IVM is a substrate for P-gp transport (17, 18). Overexpression of P-gp in resistant nematodes may modulate drug concentration at the site of action in the parasite. Such a mechanism may be somewhat analogous to the P-gp-dependent tolerance of IVM by the central nervous systems of mammals (16). Drug resistance in parasites may have multiple mechanisms (27), and the role of P-gp in drug resistance may vary depending on the chemistry of the antiparasitic drug, the subtypes of P-gp genes found in different species and different isolates and the history of drug selection (14, 15, 28, 29). In *C. elegans,* mutation in pgp-3 but not pgp-1 confers sensitivity to both colchicine and chloroquine (14), but mrp-1 and pgp-1 deletion both cause hypersensitivity to heavy metals (15). In amebiasis, emetine-resistance could be conferred by a P-gp gene (30).

The sequences of three P-gp cDNA clones, isolated from a *H. contortus* cDNA library, were similar and they may represent alleles of the same gene. Nevertheless, considerable genetic variation has been observed at the PGP-A locus in *H. contortus* (24). There is evidence that mutations of a downstream protein-binding sequence within a P-glycoprotein promoter region may alter P-gp transcription (31). Differences between the DNA sequences of IVM-selected and unselected worms could occur at a promoter region of P-gp, which could contribute to the development of P-gp mRNA in IVM-selected *H. contortus.*

Given the evidence that IVM resistance may be similar to MDR in tumor cells, it was of interest to determine whether a MDR reversing agent could increase

the efficacy of macrocyclic lactones against strains of parasites that have been selected with these anthelmintics. The MDR reversing activity of VRP has been characterized in vivo (32, 33). The jird model has been successfully used for assessing anthelmintic efficacy against drug sensitive and resistant strains of *H. contortus* (34). It is significant that the coadministration of VRP increased the efficacy of the macrocyclic anthelmintics against drug-selected parasites in the jird model. This provides further evidence that an alteration in P-glycoprotein in drug selected strains may contribute to IVM resistance *in vivo*.

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Figure 1. P-glycoprotein ClustalW Formatted Alignments. The putative protein sequence of PGP-A from *H. contortus* aligned against those of *C. elegans* pgp-1 (accession number X65054) (20), human MDR1 (accession number M14758) (35) and mouse mdr1a (accession number M33581) (36) which are involved with multidrug resistance.

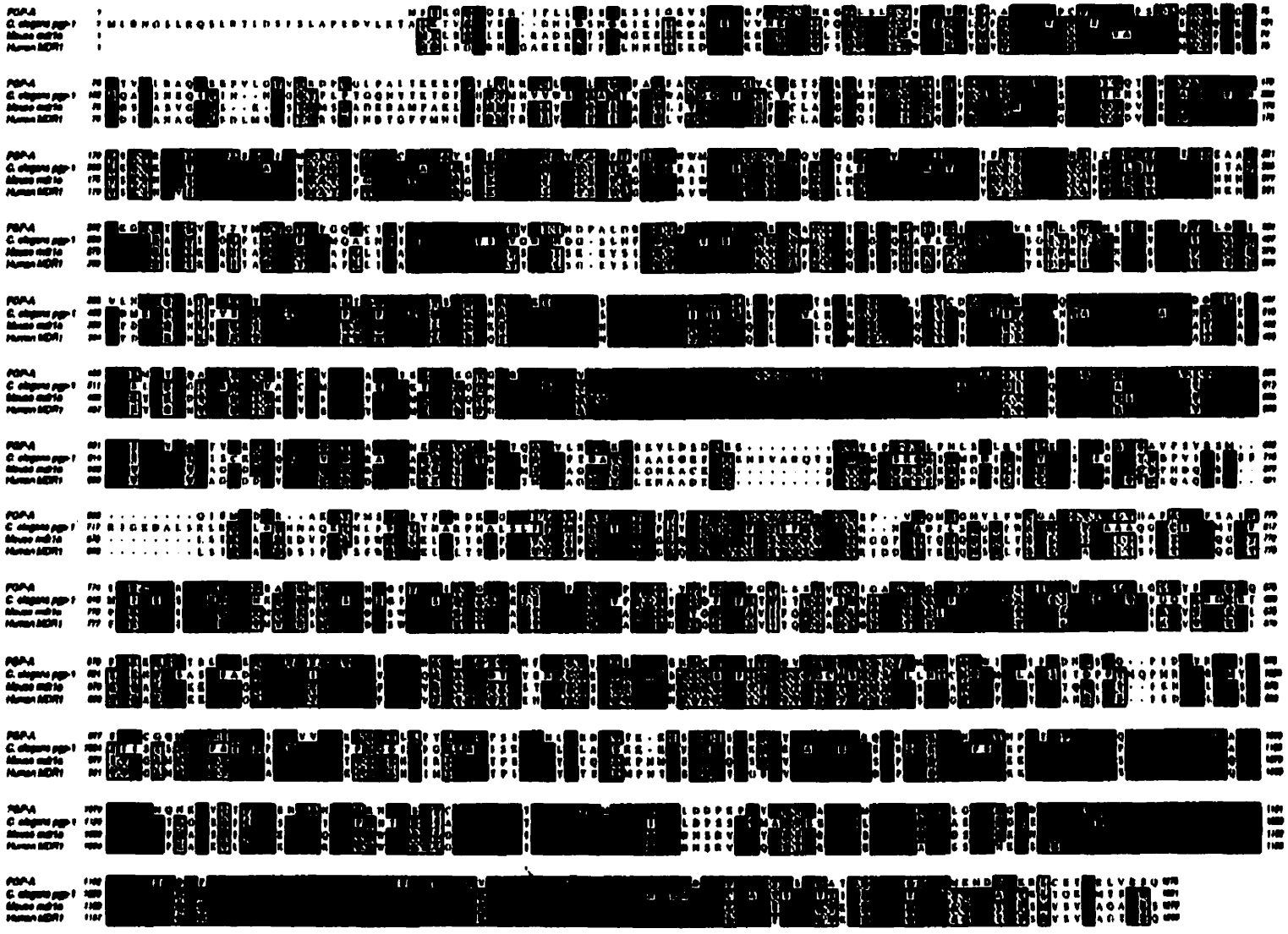

Figure 2. Northern blot analysis of mRNA extracted from four separate paired isolations of eggs, each pair being an isolation from an unselected and an IVM-selected strains of *H. contortus*. The strains were designated MKIS (unselected) and MKIR (IVM-selected) (5), and PF14 (unselected), and IVF14 (IVM-selected), respectively. Following electrophoresis the RNA was probed with the 432 bp P-gp fragment obtained from the *H. contortus* cDNA library and the mouse actin probe.

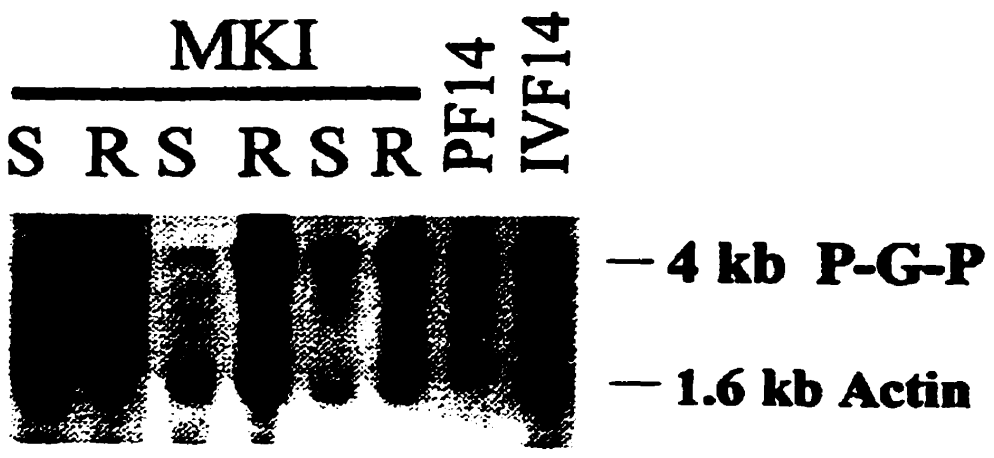
MKI
S R S R S R
PF14
IVF14
— 4 kb P-G-P
— 1.6 kb Actin

Figure 3. Southern blot analysis of genomic DNA from both MKIS and MKIR strains of *H. contortus*. (A) Genomic DNA was digested with either *Cla*I, *Pst*I, *Eco*RI or *Pvu*II. Following electrophoresis, the DNA was probed with the 432 bp P-gp fragment obtained from the *H. contortus* cDNA library. (B) Replicate genomic DNA samples from the same strains digested with *Pvu*II.


(A)
EcoRI
ClaI
PvuII
PstI
S R S R S R S R
kb
12
7.2
4.8
2.9
1.4
0.7
(B)
PvuII
S R

Table 1. Four separate comparisons of mRNA levels for P-glycoprotein in unselected and IVM selected strains of *H. contortus* eggs.

Comparison	Strains (mRNA)	P-gp mRNA corrected for actin mRNA (R/S)
1	MKIR/MKIS	12.6
2	MKIR/MKIS	11.0
3	MKIR/MKIS	3.5
4	IVF14/PF14	1.5

Image analysis of gel autoradiographs (Fig. 2) was undertaken for quantitation of mRNA expression. The data in the table was normalized following gel densitometry to the same level of actin mRNA. MKIS, unselected; MKIR, IVM-selected strains; PF14, unselected; IVF14, IVM-selected strain.

Table 2. Effect of MOX and IVM treatment in the presence and in the absence of VRP against the MOX-selected (MOF14) strain of *H. contortus* in the jird.

Treatment	Worm count ± SE		Efficacy (%)
Control	46	7	0
VRP	80	9	0
MOX	14	3	70
MOX+VRP	2	1	96
IVM	9	1	80
IVM+VRP	3	1	93

The combination of VRP with MOX was significantly different from the MOX-treated group ($P<0.012$), and the IVM plus VRP-treatment group was significantly different from IVM alone ($P<0.02$).

Connecting Statement II.

The data included in Chapter 4 demonstrates the positive effect of the multidrug reversing agents, verapamil and CL 347,099 in jirds. The objective was to compare a MDR modulator, without calcium channel blocking activity, with verapamil against selected strains of *H. contortus in vivo*. This study also reports the side-effects produced after verapamil alone treatment.

CHAPTER 4

Manuscript 3

EFFECTS OF THE MULTIDRUG-RESISTANCE-REVERSING AGENTS VERAPAMIL AND CL 347,099 ON THE EFFICACY OF IVERMECTIN OR MOXIDECTIN AGAINST UNSELECTED AND DRUG-SELECTED STRAINS OF *HAEMONCHUS CONTORTUS* IN JIRDS (*Meriones unguiculatus*)

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4.1 ABSTRACT

The development of anthelmintic resistance is making parasite control in small ruminants problematic. Following the discovery that the drug transporter, P-glycoprotein may be involved in macrocyclic lactone resistance in *Haemonchus contortus*, we have determined the effect of two multidrug resistant modulators, verapamil and CL347,099 on the efficacy of ivermectin and moxidectin against unselected and drug-selected strains of *H. contortus*. CL347,099 is an analog of verapamil with multidrug resistance properties, but with less calcium channel blocking activity. The combinations of verapamil with either ivermectin or moxidectin significantly reduced worm counts of the selected strains compared with the untreated controls, whereas ivermectin or moxidectin alone did not significantly reduce worm counts compared with the untreated controls. CL347,099 plus moxidectin combination was significantly more efficacious than moxidectin alone against the ivermectin selected strain. The drug combination regimes were without adverse effects on the jirds. However, higher levels of verapamil (40 mg/kg and above) produced some toxicity.

4.2 INTRODUCTION

The macrocyclic lactones, ivermectin and moxidectin, are potent anthelmintic drugs used against endo- and ectoparasites of animals and humans (Campbell, 1989, Ranjan *et al.*, 1992, and Darge and Butter, 1995). Resistance to these drugs has been reported worldwide (Leathwick, 1995, Coles *et al.*, 1998).

In human cancer cells, multidrug resistance (MDR) to chemotherapy is associated with the increased expression of P-glycoprotein, a membrane transport glycoprotein, encoded by the MDR1 gene (Chen *et al.*, 1986). P-glycoprotein functions as an ATP-driven pump for many structurally unrelated cytotoxic drugs, reducing intracellular drug concentration and increasing drug efflux (Gottesman and

Pastan, 1993). Xu *et al.* (1998) and Blackhall *et al.* (1998) have provided evidence that ivermectin and moxidectin select a specific allele of the P-glycoprotein gene, Pgp-A in *H. contortus* and Sangster *et al.* (1999) has confirmed selection on the Pgp-A gene and presented evidence for the involvement of another P-glycoprotein gene in ivermectin resistance in *H. contortus*.

In view of the evidence for the role of P-glycoprotein in macrocyclic lactone resistance in *H. contortus* a strategy to reverse resistance would be to co-administer MDR reversing agents with a macrocyclic lactone. These compounds may act to competitively inhibit P-glycoprotein drug binding domains reducing drug efflux and thus increasing drug activity. Verapamil, which is used clinically as a calcium channel blocker, has been used in several *in vitro* and *in vivo* studies as an MDR reversing agent with moderate effectiveness (Katzung, 1995, Watanabe *et al.*, 1995). Verapamil has been used with a benzimidazole to increase anthelmintic activity against a benzimidazole-resistant strain of *H. contortus* (Beugnet *et al.*, 1997). Xu and colleagues (1998) reported that verapamil was able to significantly increase the activities of ivermectin and moxidectin against a moxidectin-selected strain of *H. contortus* in jirds. Kerboeuf *et al.* (1999) reported that the fluorescent probe Rhodamine 123, used to detect resistant cancer cells over expressing P-glycoprotein, gave a significantly higher level of green fluorescence in resistant parasite eggs of *Haemonchus contortus*, when associated with verapamil, as compared with susceptible eggs.

CL 347,099 is a new modulator candidate believed to have MDR properties. CL 347,099 derives from verapamil and has 70 fold less calcium channel blocker activity (Greenberger *et al.*, 1996) and is believed to cause less host toxicity.

The objective of this study was to evaluate the effects of the MDR reversing agents verapamil and CL 347,099 on ivermectin and moxidectin efficacy against *H. contortus* in jirds. Prior to the drug combination trials, verapamil and CL 347,099 were tested in different concentrations in the jirds with the objective of assessing a safe dosage which could be used concomitantly with an anthelmintic. Adverse

effects of verapamil have been rare in clinical use and are known to be dose-related. The MDR candidates were also evaluated for their worm reducing activity.

4.3 MATERIAL AND METHODS

4.3.1 Parasite Strains

Three strains were selected over 17 passages, which consisted of infecting 4-6 months old lambs with the larvae from treatment survivors of the previous generation of *H. contortus* which had been treated with moxidectin or ivermectin at a dose rate which would remove 90-95% of the worms, or with no treatment in the case of the parental strain. The strains were denominated, moxidectin-selected (MOF17), ivermectin-selected (IVF17), and unselected (PF17).

4.3.2 Drugs

Ivermectin (0.8 mg/ml solution for sheep/ MSD AgVet) was obtained from CDMV Inc. (St. Hyacinthe, QC) and moxidectin (0.01% drench solution), and CL347,099 were supplied by Fort Dodge Animal Health. Verapamil was obtained from Sigma (St. Louis, MI).Verapamil and CL 347,099 were prepared in ethanol and propylene glycol (50:50) at a concentration of 50 mg/ml as a stock solution. The stock solutions were further diluted with distilled water to the desired dosage immediately before the drug combination treatment.

4.3.3 Experimental Animals

To investigate the drug combination efficacy an experimental *in vivo* model was used for the ruminant helminth *H. contortus*. For this, helminth-free, outbred, female jirds, 30-35 grams (Charles River, Canada) received commercial `Chow

5001` (Purina Mills Inc., Indiana) plus 0.02% of hydrocortisone (ICN, Ohio) mixed with their food. This treatment was needed to help *H. contortus* establish in this experimental host (Conder *et al.*, 1992). Water and food were available *ad libitum*, and temperature and humidity were monitored twice daily.

4.3.4 Experimental Design

Two days after arrival, animals were placed on the special diet (day -5) and continued to receive it until the end of each trial. At day 0, animals were divided, using a complete randomized block design, in groups of 6 animals per group and were orally infected with 1000 exsheathed third stage (L_3) larvae of MOF17, IVF17 or PF17 strains of *H. contortus*. Larvae were exsheathed by gently bubbling carbon dioxide into the aqueous suspension of larvae for 10 min.; then L_3 were placed in a shaker-waterbath for 1 h. At day 10 post-infection, all animals were treated orally with an anthelmintic and/or subcutaneously with the MDR modulator, or left untreated. For the biotolerability experiment, verapamil was given at 20, 40, 60, and 80 mg/kg, and CL 347,099 at 25, 50, 75 and at 100 mg/kg. For the drug combination trial, animals were treated with ivermectin at 0.0010 mg/kg against PF17, and given at 0.0031 mg/kg against the selected strains. Moxidectin was given at 0.0009 mg/kg against PF17 strain, and at 0.0030 mg/kg against the selected strains. The doses of ivermectin and moxidectin alone were previously established as eliminating approximately 50% of the parasites. One group received a placebo treatment. The animals were observed at 5, 15, 30, 45, 60, 90, 120 min. and 3, 4, 6, 8, 12, 16, and 24 h post-treatment (PT) and once daily for up to 14 days post-infection. All animals were euthanased 3 days PT by CO_2 inhalation, and the stomachs were recovered and cultured for subsequent total worm count (TWC, ± SE). Drug efficacy was determined according to the formula:

$$E = [(Mc - Mtr) \div Mc] \times 100$$

Where, E is the percentage reduction, Mc is the arithmetic mean of worms counted in the control group, and Mtr is the mean of worms count in a treated group. Comparison between treatments within each strain was made by one way analysis of variance followed by pairwise multiple comparisons made using the Bonferroni method. A value of $P<0.05$ was considered statistically significant.

4.4 RESULTS

4.4.1 Parasite Establishment

An initial assessment of parasite establishment was made in the jird. The average number of parasites at day 13 post-infection was 129 for PF17, 129 for IVF17, and 95 for MOF17 (n=3).

4.4.2 Effects of the MDR reversing agents alone

Verapamil alone had no effect on the number of worms counted after necropsy at any dose rate against the unselected (PF17) strain of *H. contortus* in jirds when given alone at 20, 40, 60, and 80 mg/kg. Against the MOF17 strain verapamil appeared to have a weak, but not significant activity when used in high doses, reducing the rate of worm counts by 7, 33, 39, and 36% when used at the same dose rates as above. At 20 mg/kg, the dosage that was selected to be used for subsequent experiments, verapamil had no effect on reducing larval counts compared to the control groups for all the strains (Table 1).

Neither the different parasite strains nor the ivermectin dosage rate produced any differences in the effect of verapamil on jird toxicity. Therefore, for the analysis of the effect of verapamil, data from parasite strains and ivermectin dose rate were combined, so that, verapamil alone and verapamil plus ivermectin treatment side-

effects could be accessed. Although ivermectin alone produced no toxicity in jirds when used at the approximately LD50 dosage (data not shown), verapamil alone was found to cause a reduced survival rate of 88% when given at 60 mg/kg, reaching 75% survival when used at 80 mg/kg compared with 100% for the placebo group (Fig. 1). The combination ivermectin plus verapamil also showed adverse effects at 40 mg/kg, reducing the survival rate of the animals by 20%. There was little or no difference in toxicity to the jirds when verapamil alone or verapamil, in combination with ivermectin, was used at 60 or 80 mg/kg. There were no signs of disturbances when jirds were treated with verapamil at 20 mg/kg in the presence or in the absence of ivermectin (Fig. 1). All the clinical symptoms caused by verapamil at ≥40 mg/kg appeared within the first 30 min PT and the most pronounced acute reactions observed in the animals were poor coordination, prostration, tremors, and hypothermia, death occurred within the first 2 - 3 h PT.

When the candidate agent CL 347,099 was tested alone at 25, 50, 75, and 100 mg/kg against the PF17, MOF17, and IVF17 strain it showed no significant difference in parasite elimination compared with the control group (data not shown). We also observed that CL 347,099 showed no adverse effects on the jirds when given subcutaneously at any dose rate. Based on these results a dosage of 100 mg/kg was chosen to be used in the following drug combination experiments.

4.4.3 Effect of the MDR reversing agents / anthelmintics combinations

Mean worm counts and calculated efficacy for all of the treatments tested are shown in Table 1. Differences in mean worm counts following various treatments were compared for significance (Table 2). At the dose rates used, neither ivermectin nor moxidectin produced significant reductions in worm counts for the ivermectin and moxidectin selected strains, compared with the untreated controls. However, the combinations of verapamil with either ivermectin or moxidectin, against the drug-selected strains did significantly reduce worm counts compared with the

controls.

CL 347,099 in combination with moxidectin, against the ivermectin-selected strain, also produced significant reduction in worm count compared with the untreated controls. Furthermore, this combination was significantly more efficacious than moxidectin alone against this strain.

4.5 DISCUSSION

Resistance in *H. contortus* has been shown to be related with the over expression of a P-glycoprotein gene. The use of MDR modulators are supposed to increase chemotherapeutic agents efficacy by blocking P-glycoprotein efflux activity. In this study, verapamil plus ivermectin or moxidectin produced significant reductions of worm counts of the drug selected strains, compared with the untreated controls; whereas ivermectin or moxidectin alone did not produce significant reductions. Similarly, CL 347,099 in combination with moxidectin significantly reduced worm counts of the IVF17 strain, compared with the controls, while moxidectin alone did not produce a significant reduction. Furthermore, the difference in IVF17 worm count between the CL 347,099 plus moxidectin and moxidectin alone was significant.

Although verapamil is one of the most studied chemoenhancer drugs used in the clinical setting, its precise mechanism of action as an MDR reversing agent is not well understood. Verapamil side effects are well described in the literature including dizziness, nausea, and constipation and the decrease of myocardial activity which can lead to cardiac arrest and death (Lonsberry *et al.*, 1994). Physiological adverse responses seem to be on extension of its vasodilatory action, causing hypertension and myocardial infarction (McTavish and Sorkin, 1989). In our experiments, when verapamil was used alone or in combination with ivermectin at 20 mg/kg it caused no apparent adverse effects in the jird. However, experimental animals showed increased signs of intoxication including mortality after verapamil

was used at higher concentrations. The side-effects of the MDR modulator alone were similar to those observed with the drug combination, suggesting that verapamil alone was responsible for the toxicity.

These results indicate that the drug combination strategy, involving a macrocyclic lactone and a MDR reversing agent, has some potential to increase anthelmintic efficacy against resistant nematodes. However, toxicological considerations will be important in the selection of MDR reversing agents to be used in combination with ivermectin or moxidectin.

Acknowledgements

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Table 1. Total worm count (TWC) ± SE after necropsy and percent efficacy of ivermectin (IVM) and moxidectin (MOX) alone or in the presence of verapamil (20 mg/kg) or CL 347,099 (100 mg/kg) against selected and unselected strains of *Haemonchus contortus* in jirds (n=6).

Treatment	PF17		MOF17		IVF17	
	TWC ± SE	(%)	TWC ± SE	(%)	TWC ± SE	(%)
Control	129 ± 14	—	87 ± 11	—	113 ± 5	–
Verapamil	98 ± 12	24	64 ± 6	26	106 ± 12	5
CL 347,099	136 ± 23	—	77 ± 9	11	107 ± 21	4
IVM	52 ± 8	60	79 ± 7	9	84 ± 5	26
IVM + Verapamil	42 ± 8	67	47 ± 7	46	59 ± 7	48
IVM + CL 347,099	54 ± 11	58	87 ± 10	0	97 ± 17	14
MOX	89 ± 12	31	61 ± 9	30	85 ± 7	25
MOX + Verapamil	60 ± 8	53	49 ± 7	43	55 ± 9	51
MOX + CL 347,099	86 ± 20	33	76 ± 5	13	45 ± 5	60

Table 2. Treatment groups* which are significantly different from each other (P<0.05), within each parasite strain. Analysis by ANOVA followed by Pairwise Multiple Comparison (Bonferroni's method).

Comparisons	Strains		
	PF17	MOF17	IVF17
Control vs. VRP	No	No	No
Control vs. CL	No	No	No
Control vs. IVM	Yes	No	No
Control vs. MOX	No	No	No
Control vs. IVM + VRP	Yes	Yes	Yes
Control vs. IVM + CL	Yes	No	No
Control vs. MOX + VRP	Yes	Yes	Yes
Control vs. MOX + CL	No	No	Yes
IVM vs. IVM + VRP	No	No	No
IVM vs. IVM + CL	No	No	No
MOX vs. MOX + VRP	No	No	No
MOX vs. MOX + CL	No	No	Yes

* Ivermectin (IVM), moxidectin (MOX), verapamil (VRP), and CL 347,099 (CL)

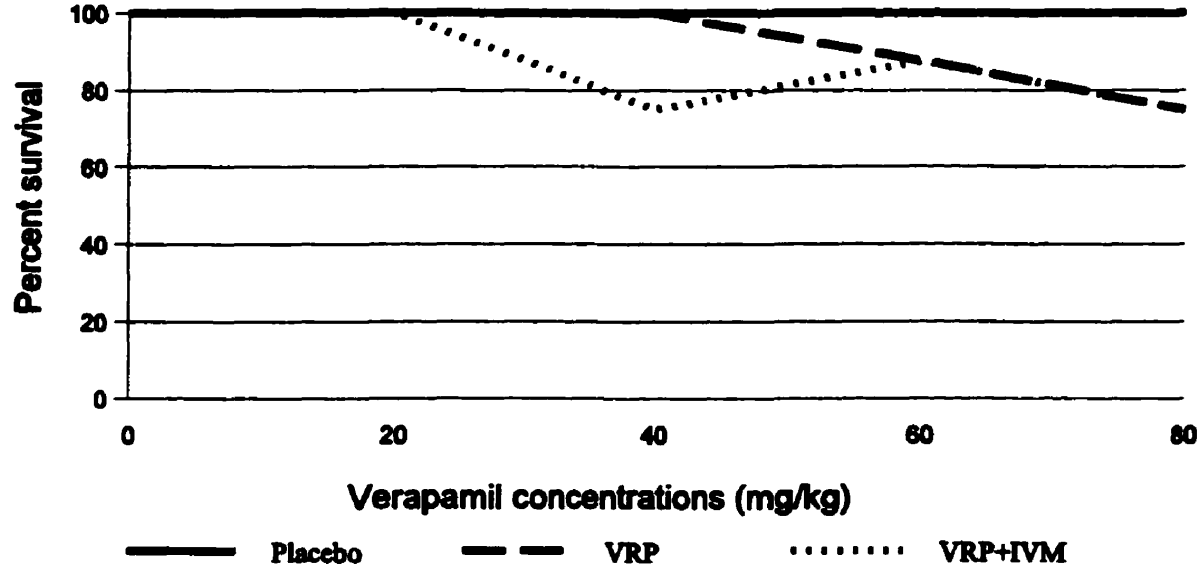

Figure 1. Percent survival of jirds treated with verapamil in the presence and in the absence of ivermectin (n=3 per group).

Connecting Statement III.

An *in vitro* migration assay was used with the intention to test the effects of different MDR modulators in a host-free system, following the positive results from the *in vivo* experiments. On this occasion, the combination anthelmintic/modulators was tested against the exsheathed third stage larvae of *H. contortus*.

CHAPTER 5

Manuscript 4

EFFECT OF MULTIDRUG RESISTANCE MODULATORS ON THE ACTIVITY OF IVERMECTIN AND MOXIDECTIN AGAINST LARVAE OF IVERMECTIN- OR MOXIDECTIN-SELECTED STRAINS OF *Haemonchus contortus*

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5.1 ABSTRACT

Nematode parasites have shown resistance to the anthelmintics, ivermectin and moxidectin, and there is evidence that the over-expression of P-glycoprotein may account, at least in part, for resistance to ivermectin. This study was set up to determine whether the multidrug resistance (MDR) modulators, verapamil, CL 347,099, an analog of verapamil, and cyclosporin A, would enhance the efficacy of ivermectin and moxidectin against ivermectin- and moxidectin-selected strains of *H. contortus* as determined in an *in vitro* larval migration assay. The modulators were used at concentrations at which, when used alone, they had no effects on the number of migrating larvae. Ivermectin and moxidectin showed an increase in efficacy when used in association with verapamil against the ivermectin- and moxidectin-selected strain. CL 347,099 also increased the ivermectin and moxidectin efficacy against both selected strains. At the concentrations tested cyclosporin A showed no increasing efficacy for the anthelmintics, only for ivermectin against the ivermectin-resistant strain.

5.2 INTRODUCTION

To control the negative effects of parasite infection in farm animals producers worldwide rely heavily on the use of anthelmintic drugs. Many strains of nematodes have evolved resistance to one or more of the different classes of anthelmintics. Finding means to overcome anthelmintic resistance and maintaining effective parasite control is becoming increasingly important. Ivermectin and moxidectin derive from *Streptomyces spp.* and have a broad spectrum of activity against nematode parasites in livestock and humans. Resistance of *H. contortus* to ivermectin was first reported in sheep (Carmichel *et* al., 1987), and resistance to moxidectin was reported in *Ostertagia* spp. in goats (Leathwick, 1995). Multiple anthelmintic resistance (MAR) involving ivermectin and several other anthelmintics

has also been reported (Waruiru, 1997).

Multidrug resistance (MDR) is a phenomenon associated with the over expression of the membrane transport protein P-glycoprotein (P-gp) in MDR cells (for reviews see Shustik *et al.*, 1995; Wadkins and Roepe, 1997). P-gp functions as an ATP-driven, active efflux pump for many unrelated cytotoxic drugs, reducing the intracellular drug concentration. In the soil nematode *Caenorhabditis elegans*, P-gp has been shown to be responsible for protection against some toxins (Broeks *et al.*, 1995). Pouliot *et al.* (1997), have proposed that ivermectin interacts with the P-gp drug-binding site in cancer cells, and ivermectin may be both a substrate and an inhibitor of P-gp (Didier and Loor, 1996). Recent findings showed that ivermectin-selected strains of *H. contortus* have higher levels of P-gp mRNA than do unselected strains (Xu *et al.*, 1998), leading us to suggest that the over expression of P-gp in this parasite may be a consequence of ivermectin selection. Furthermore, Blackhall *et al.* (1998), found that repeated ivermectin and moxidectin treatment selected for a specific allele of P-gp in *H. contortus*. Sangster *et al.* (1999) have independently found selection on P-gp genes in ivermectin resistant field strains of *H. contortus*.

P-gp antagonists, termed MDR modulators have been used to augment drug activity against MDR organisms. These drugs are purported to compete with structurally unrelated drugs at the P-gp-binding site (Watanabe *et al.*, 1995). The calcium channel blocker, verapamil, significantly increased the worm reduction effect of ivermectin and moxidectin against a moxidectin-selected strain of *H. contortus* in jirds (Xu *et* al., 1998). CL 347,099 has been proposed as an MDR modulator, having 70 fold less calcium channel blocker activity than verapamil (Greenberger *et al.*, 1996). Cyclosporin A is an immunosuppressor that has been demonstrated to have MDR modulating properties when administered with cytotoxic agents *in vitro* and *in vivo* (Lum *et al.*, 1993; Watanabe *et al.*, 1995) (Fig. 1).

A series of experiments were designed to determine differences in the effect of ivermectin or moxidectin in the presence and in the absence of MDR candidates

against larvae of unselected, ivermectin-, and moxidectin-selected strains of *H. contortus*. In order to assess the effects of these MDR candidates against the nematodes, without possible modulation of the effects on drug concentration by a host animal, and for reasons of economy and feasibility, a modified *in vitro* screening assay using third stage larvae of *H. contortus* was employed (d'Assonville *et al.*, 1996).

5.3 MATERIAL AND METHODS

5.3.1 Strains

Three strains were selected after 17 passages in sheep. The selection of the isolates consisted of an artificial infection with the larvae from treatment survivors of the previous generations of *H. contortus* in naïve 4-6 month lambs with subsequent treatment with moxidectin or ivermectin in the case of the resistant strains or with no treatment in the case of the parental strain. The strains of *H. contortus* were denominated moxidectin-selected (MOF17), ivermectin-selected (IVF17) and unselected (PF17), and were supplied by Fort Dodge Animal Health (Princeton, New Jersey). All the strains were tested at the 12th generation in sheep to determine the differences in anthelmintic parasite elimination. Wang *et al.* (1995) reported that moxidectin and ivermectin had a decrease on their efficacy by 5.3 and 9.7 fold, respectively, when compared with the unselected strain.

5.3.2 Drugs

Ivermectin (0.8 mg/ml solution for sheep) was purchased at CDMV Inc. (St. Hyacinthe, Quebec) and moxidectin (0.01% drench solution) was supplied by Fort Dodge Animal Health. Verapamil was purchased from Sigma (St. Louis, Missouri), and CL 347,099 was supplied by Fort Dodge Animal Health. Cyclosporin A was

kindly provided by Dr. E. Georges. Verapamil and CL 347,099 were diluted with dimethyl sulfoxide (DMSO) to a concentration of 20 mM and cyclosporin A was diluted in ethanol to a concentration of 10 mM. The standard solutions were further diluted with distilled water to the desired concentration before use.

5.3.3 In vitro migration assay

An assay modified from d'Assonville *et al.* (1996) was used. Third stage (L_3) larvae of *H. contortus* were collected from donor sheep feces and exsheathed using 0.073% of sodium hypochlorite for approximately 2 h. When > 90% of the motile larvae were exsheathed, they were washed 3 times in distilled water and subdivided in groups of 600. Larvae were placed in 0.5 ml of distilled water while drug treatments (0.5 ml) were prepared in separate vials. Drug solutions were then added to the water containing the L_3 larvae and the vials placed in an incubator at 27° C for 6 hr. After that, 1 ml of an agar solution (1.4%) at 45° C was added to the treated larvae. The agar/larvae solution was then immediately transferred to a previously prepared apparatus using a 5ml pipette. The apparatus consisted of a petri dish containing a plastic mesh on the base, a metal mesh and a plastic cylinder on the top. The apparatus was previously filled with 22 ml of distilled water and put into a freezer allowing the frozen water to cover the mesh, leaving sufficient space on the plastic cylinder to fit the final solution. The apparatus was placed in an incubator for 18 hr at 27° C under a 150 MHz light bulb after the solution was added. The exposure to light stimulates the larvae and allows the motile ones to migrate out of the agar portion. Following that, the liquid portion was poured into a 50-ml centrifuge tube and allowed to settle, the final volume analyzed was 10 ml disposing of the extra liquid. The tubes were then vortexed briefly and triplicate samples of 1 ml were taken, 10 µl of lugol iodine was added to each samples. Larval count was estimated under a magnifying microscope and the final result was multiplied by 10. Experiments were performed in triplicates.

5.3.4 Statistical analysis

Anthelmintic effect was determined according the formula:

$$E = [(Mc - Mtr) + Mc] \times 100$$

Where, E is the percentage of efficacy, Mc is the mean number of larvae counted in the control group, and Mtr is the mean number of larvae counted in the treated group. Percent increase was calculated by determining the initial percent efficacy of anthelmintic alone groups and comparing them with the drug combination groups (*e.g.* ivermectin 52% vs. ivermectin plus verapamil, 73%, result in an increase of 40% of ivermectin effect). Arithmetic means were used and comparison between treatments was analyzed by Student's "*t*" test. A value of $P<0.05$ was considered statistically significant. Comparison was made for each anthelmintic against its combination with the MDR modulators for each strain.

5.4 RESULTS

The MDR reversing agents were tested alone in various concentrations prior to the combination trials against all strains. The objective was to determine whether the MDR modulators alone had any effect on the migration of parasites and to select a dose that possessed no intrinsic effect, on its own, against the parasite. Verapamil, CL 347,099 and cyclosporin A had no effect at concentrations up to 100, 100mM, and 24μM respectively, when given alone (Fig. 2). The diluents for all compounds were also tested. Propylene glycol, ethanol and dimethyl sulfoxide showed no activity in reducing the number of migrating larvae when tested against the parasite isolates at the concentrations used to dilute the drugs(data not shown).

A preliminary drug concentration curve was necessary to establish the approximate concentration that reduced larval counts by 50% for the anthelmintics.

The IVF17 strain was shown to be 2 to 4-fold more resistant to ivermectin then the PF17 strain in the larval migration assay. From this preliminary data, concentrations of 1 and 4 µM for ivermectin and 8 and 16 µM for moxidectin (data not shown) were estimated as the approximate LD50 against the unselected and selected strains, respectively.

Moxidectin in combination with verapamil (2 mM) had an efficacy of 65% compared to 41% for moxidectin alone (P=0.018) against the MOF17 strain, leading to an increase in efficacy of 58.3%. Ivermectin in combination with verapamil also showed a significantly higher (P=0.048) effect (84%) when compared with ivermectin alone (55%) against the same strain (Fig. 3). When the combination was used against the IVF17 strain, verapamil significantly increased the efficacy of moxidectin but not of ivermectin from 59 (moxidectin alone) and 62% (ivermectin alone) to 74 (P=0.019) and 74%, respectively. Against the IVF17 strain, CL 347,099 (2 mM) significantly increased the efficacy of moxidectin from 59 to 82% (P=0.028) and against the MOF17 from 41 to 72%. The combination of ivermectin plus CL347,099 significantly increased the efficacy of ivermectin against the MOF17 strain from 55 to 77% (P=0.038) and against the IVF17 strain from 62 to 77% (P=0.035). CL 347,099 increased moxidectin's efficacy (P=0.049) against PF17. Cyclosporin A at 3 µM had no positive effect when combined with the endectocides against PF17 or MOF17 strain, but did increase efficacy of ivermectin against the IVF17 strain (P=0.029) (Fig. 3).

5.5 DISCUSSION

The *in vitro* migration assay is a rapid and cost effective tool for the determination of the effects of drugs that paralyze nematodes (d'Assonville *et al.*, 1995). The facility to harvest and continuously use L_3 larvae from donor animals and the ability to conduct the assessment of synergistic effects of the MDR reversing agents against the nematodes, without host modulation of drug

concentrations, are additional advantages of this model. In the host, the ingested L_3 larvae are affected by the antiparasitic drugs once they attach to the abomasal mucosa.

The mode of action of the selected chemosensitizers as MDR reversing agents has not been fully established. Yusa and Tsuro (1989), have showed that verapamil directly binds to P-gp, suggesting that it reverses MDR by competitively inhibiting drug transport. Verapamil and CL 347,099 have been shown to significantly increase the anthelmintic effect of ivermectin and moxidectin against selected strains of *H. contortus* in jirds (Xu *et al.*, 1998, Molento and Prichard, 1999). However, it is possible that these P-gp antagonist may exert their effects *in vivo* by altering host metabolism and disposition of the anthelmintics. In the current *in vitro* study this possible effect was not a complication. Verapamil but not CL 347,099 is a calcium channel blocker, despite the structural similarity of these compounds. Therefore, the calcium channel blocking activity of verapamil does not appear to be involved with the increase of the endectocide activity. This is further suggested by the lack of effects of these substances by themselves against the larvae. Armson *et al.* (1995), have found that cyclosporin A showed no synergistic effect when administered with thiabendazole against a murine strongyloidiasis infection. On the other hand, cyclosporin A considerably enhanced the cytotoxic potency of adriamycin in several MDR gastric cancer cells lines (Holm *et al.*, 1994). The present data have demonstrated no clear additive effect of the combination of cyclosporin A with the anthelmintics on larvae migration, at the concentration tested.

Clinical trials using the drug combination strategy in humans (verapamil and adriamycin), and verapamil alone in mice (Lonsberry *et al.*, 1994) have demonstrated that the MDR modulators can exhibit toxic side effects. The combination of the anthelmintics and the MDR modulators have been studied in jirds (Molento and Prichard, 1999) and in sheep (unpublished results). Verapamil has shown a dose-dependent toxic effect in both animals regardless of the

presence or the absence of the anthelmintics.

These data are consistent with our previous results where the MDR modulators, verapamil and CL 347,099, augmented the antiparasitic effect of ivermectin and moxidectin against resistant strains of *H. contortus in vivo*. In the current *in vitro* study, in which modulation of host pharmacokinetics can be ruled out, the enhancing effects of verapamil and CL 347,099 are believed to be by means of blocking P-gp efflux activity in the nematode.

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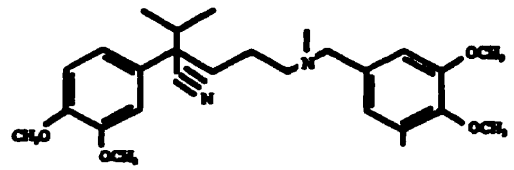
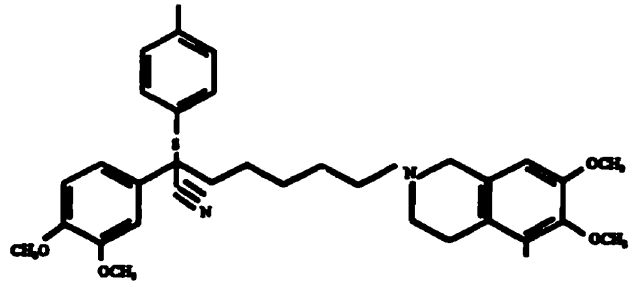
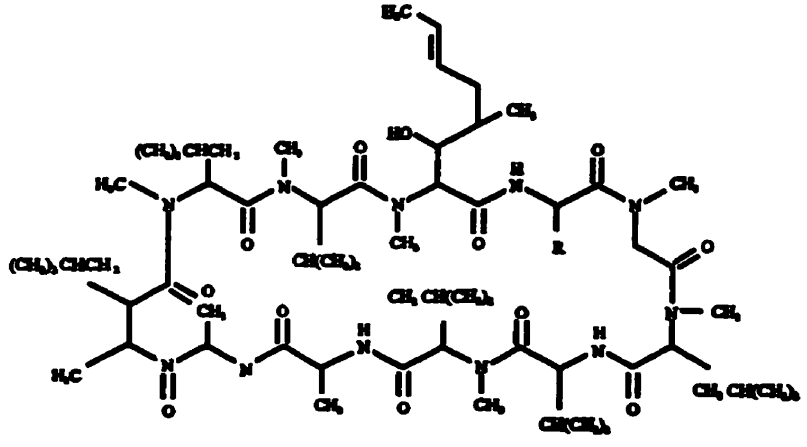


VERAPAMIL



CL 347,099



CYCLOSPORIN A

Figure 1. Chemical structures of the MDR reversing agents.

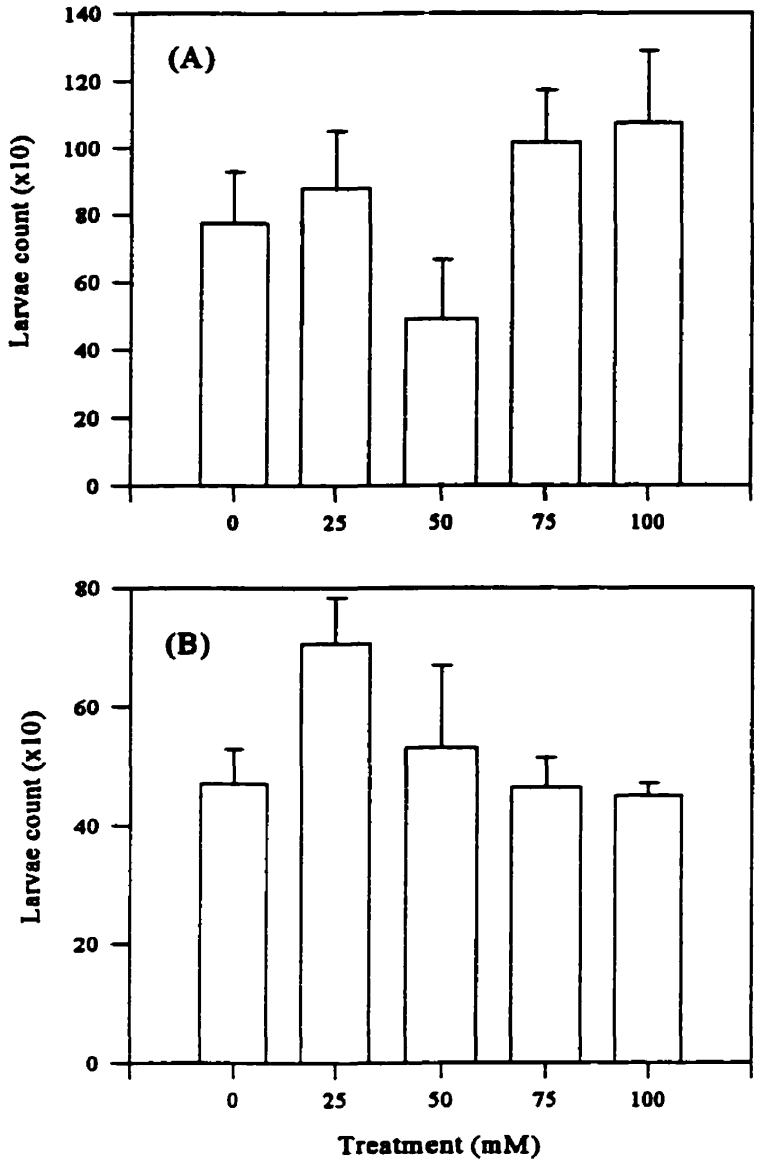


Figure 2. Efficacy (SEM) of CL 347,099 alone against (A) unselected and (B) ivermectin-selected strains of *H. contortus*.

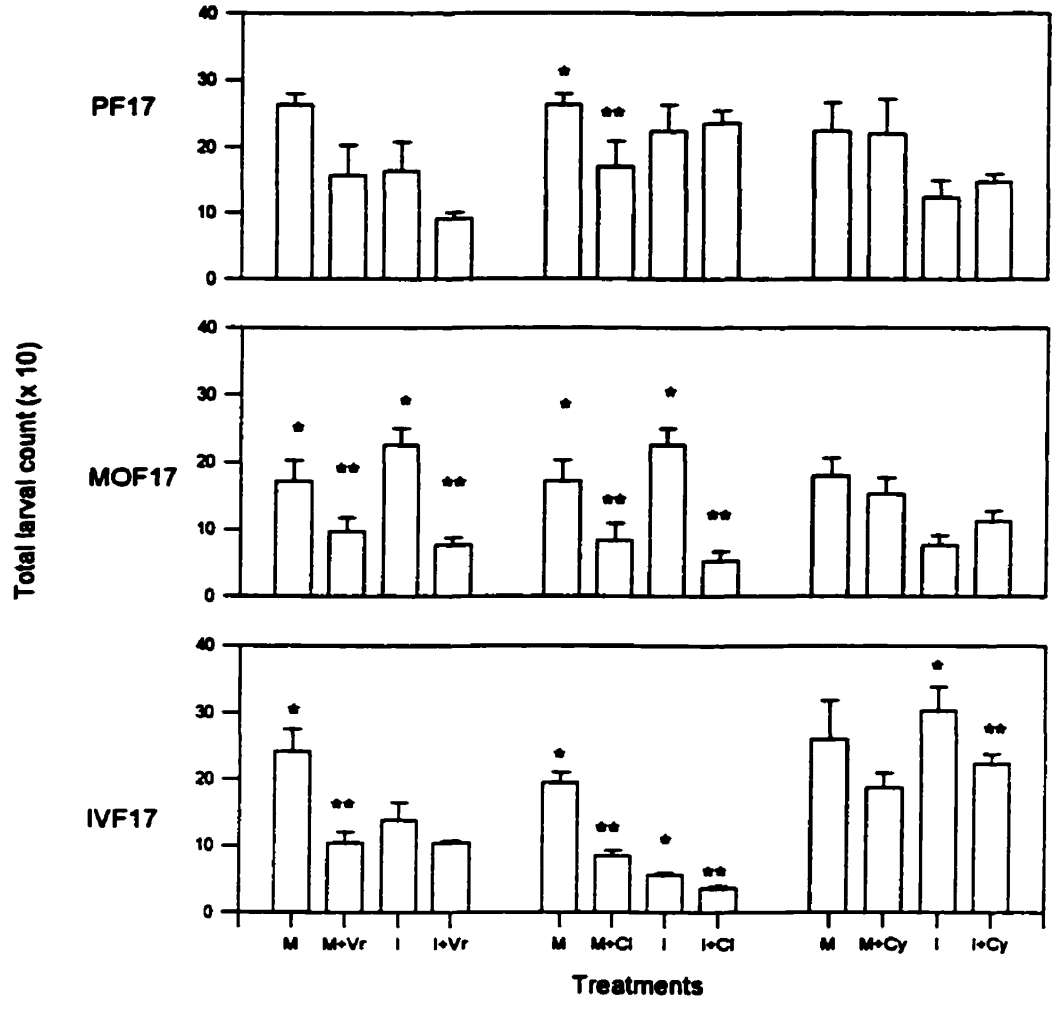


Figure 3. Comparative activity of moxidectin (M) or ivermectin (I) in the presence or absence of the MDR modulators verapamil (Vr), CL347,099 (Cl), and cyclosporin A (Cy) against unselected and selected strains of *H. contortus* (SEM). Moxidectin was used at 8 and 16 µM and ivermectin was used at 1 and 4 µM against PF17 and MOF17/IVF17 respectively. Verapamil and CL 347,099 were used at 2mM and cyclosporin A at 3 µM. *, ** Groups are significantly different ($P<0.05$). Results from triplicate experiments.

Connecting Statement IV.

In previous Chapters, it has been shown that MDR modulators could increase the activity of ivermectin and moxidectin against drug selected strains *in vivo* and against larvae *in vitro*. The *in vitro* effects on larval migration suggests a direct effect of the MDR modulators on the nematodes to enhance the activity of the anthelmintics. However, the *in vivo* effects could be due to either an effect of the MDR modulators on the parasite alone, an effect on the pharmacokinetic behaviour of the anthelmintics in the host or to a combination of these effect. The study in Chapter 6 deals with the ability of verapamil to influence pharmacokinetic changes of ivermectin and moxidectin in the host. The experimental design was influenced by previous data reporting the enhancing effects of MDR modulators on drug pharmacokinetics in human patients. In this study, we wanted to determine whether the MDR modulator, verapamil altered the pharmacokinetic behaviour of ivermectin and moxidectin and if so whether these endectocides responded similarly. Because of the known elimination of ivermectin and moxidectin in the feces, it was hypothesized than any effect of the MDR modulators on liver P-glycoprotein may influence the rate of biliary elimination and thus plasma pharmacokinetic disposition.

CHAPTER 6

Manuscript 5

INFLUENCE OF VERAPAMIL ON THE PHARMACOKINETICS OF THE ANTIPARASITIC DRUGS IVERMECTIN AND MOXIDECTIN IN SHEEP

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6.1 ABSTRACT

P-glycoprotein is a transport protein that participates in the mechanism of active secretion of different molecules from the bloodstream to the gastrointestinal tract. The aim of the current work was to evaluate the effect of verapamil, a P-glycoprotein substrate, on the pharmacokinetic behavior of the anthelmintics ivermectin and moxidectin in sheep. Thirty two sheep were divided into 4 groups and treated orally with either ivermectin or moxidectin alone at 200 µg/kg or one of the anthelmintics co-administered with verapamil at 3 mg/kg (3 times at 12 h intervals). Blood samples were collected for 30 days post-treatment and plasma was analyzed to determine anthelmintic concentration by HPLC. The ivermectin plus verapamil treatment resulted in higher ivermectin concentrations in plasma over the whole drug detection period of 12-15 days compared to ivermectin alone treatment. The ivermectin peak concentration (C_{max}) was significantly higher (83%) after ivermectin plus verapamil compared with ivermectin alone treatment. The total ivermectin area under the curve (AUC) for plasma was also significantly higher (54%) following co-administration. Ivermectin concentration at 12 days post-treatment was 57% higher following the ivermectin plus verapamil treatment. Verapamil had no effect on the kinetics of moxidectin. The results demonstrate that verapamil induced a significant alteration in the plasma disposition of ivermectin in sheep, probably due to an interference with a P-glycoprotein-mediated elimination mechanism, which could have an important impact on efficacy against resistant or rate limiting parasites and on the persistency of its antiparasitic activity.

6.2 INTRODUCTION

Ivermectin and moxidectin are 16-membered macrocyclic lactones that are produced by fermentation of soil molds of *Streptomyces* spp. (Burg *et al.*, 1979, and Carter *et al.*, 1987). Ivermectin is a member of the avermectin subgroup, having

oleandrose residues, whereas moxidectin is a milbemycin without sugar residues. These avermectin and milbemycin anthelmintic have a broad spectrum activity against internal and external parasites in animals (Bogan and Armour, 1987). Unfortunately, after just a few generations of parasites being exposed to ivermectin, resistance was reported (van Wyk and Malan, 1988) and has since become common worldwide. Ivermectin and moxidectin are believed to act by binding to the glutamate-gated chloride channels, resulting in a motor paralysis of the parasite (Arena *et al.* 1995). After treatment, these drugs are found mainly in liver, bile and fat (Chiu *et al.*, 1990, and Afzal *et al.*, 1994). In sheep, moxidectin has a terminal half-life of 21 days when given orally compared to 2.5 days for ivermectin (Marriner *et al.*, 1987, and Alvinerie *et al.*, 1998). This difference may be due to the higher lipophilic properties of moxidectin and/or to different rates of efflux and elimination. Ivermectin and moxidectin excretion is 97-98% via feces regardless of the route of administration, and from this 60-68% of ivermectin is eliminated as unmetabolized drug (Chiu *et al.*, 1989, and Fisher and Mrozik, 1992). The cytochrome P450 isoform CYP3A4 is responsible for the metabolism of the remaining 30-40% of ivermectin (Zeng *et al.*, 1998). For moxidectin, 90% of residues are found in fat and correspond to the unaltered parent compound (Afzal *et al.*, 1994).

Drug combinations are being tested in clinical trails to combat tumor cells that express the multidrug resistance (MDR) gene, characterized by the increase expression of a membrane transport monomeric protein, P-glycoprotein (Rosenberg *et al.*, 1997). P-glycoprotein is a molecule of 170 kDa arranged as two homologous halves, each containing six transmembrane regions and a nucleotide binding site (Chen *et al.*, 1986, and Gottesman and Pastan, 1993). In cancer cells, P-glycoprotein functions as an ATP-driven, active efflux pump for many unrelated cytotoxic drugs, reducing the intracellular drug concentration by increasing drug efflux (Sharom *et al.* 1995). P-glycoprotein also participates in the mechanism of active secretion of different molecules from the bloodstream to the gastrointestinal tract.

Gianni *et al.* (1997) have indicated that even small modifications to the dose of MDR modulators can have profound effects on the plasma disposition of chemotherapeutic drugs on cancer patients. In clinical trials, Kerr *et al.* (1986), reported a significant pharmacokinetic interaction when verapamil and adriamycin were used in combined treatment. Verapamil was able to increase adriamycin peak plasma levels with a longer terminal half-life, with no increase of adriamycin toxicity. Kantola *et al.* (1998), reported that verapamil considerably increased the mean peak concentration in serum of simvastatin by 2.6-fold and that the area under the serum concentration-time curve increased 4.6-fold. In a perfused rat liver model, the excretion of vincristine into the bile and its selective inhibition by verapamil provided indirect evidence for the contribution of P-glycoprotein to the biliary excretion of vincristine while the hepatic uptake was not altered (Watanabe *et al.*, 1992).

Xu *et al.*, (1998) and Molento and Prichard (1999) reported that verapamil could increase the efficacy of ivermectin and moxidectin against moxidectin resistant nematodes when administered in combination with anthelmintic. Pouliot and colleagues (1996) demonstrated that ivermectin is a substrate for P-glycoprotein. In view of the fact that verapamil increases the activity of these anthelmintics *in vivo* and that biliary P-glycoprotein may play a role in the elimination of the macrocyclic lactones, it was of interest to determine whether the MDR reversing agent verapamil, used in combination with ivermectin and moxidectin, may alter the pharmacokinetic profiles of these macrocyclic lactones in comparison with those of ivermectin and moxidectin given alone to sheep.

6.3 MATERIAL AND METHODS

6.3.1 Drugs

All animals were treated with ivermectin (Ivomec®, CDMV, Ste. Hyacinthe, PQ), or

moxidectin (Cydectin®, Fort Dodge Animal Health, BA, Argentina) alone or in combination with verapamil (Sigma, St. Louis, MI). The anthelmintics were administered orally (PO) and verapamil was diluted in saline water prior to subcutaneously (SC) treatment.

6.3.2 Animals

Thirty two, year old sheep weighting between 33 to 43 kg were selected. The animals were ear tagged and randomly divided in four groups of eight animals each and allocated to open wooden pens with concrete floor, prior to dosing until the end of the experiment. They were fed with hay plus a ration made of mixed ground grains given twice daily. Water was available *ad libitum*. The animals had their wool trimmed on the ventral part of the neck to facilitate blood collection. Control animals had temperatures of 38.5-39.5° C, and respiratory frequency of 20-40/min.

6.4 Experimental design

6.4.1 Verapamil bioavailability

The bioavailability of verapamil (5 mg/kg) was determined in 3 animals prior to the macrocyclic lactone experiments using a modification of the method of Yazan and Bozan (1995). Blood was collected at 0.2, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 9, 12, 18, 24, 48 and 72 h PT and analysed by reversed phase HPLC. Benzoquinoline was used as internal standard and the mobile phase was acetonitrile and orthophosphoric acid 85% (67:33). The HPLC used for verapamil analysis was a Beckman System Gold equipped with a Phenomenex reversed-phase column (Bondex 10 µl particle size, C_{18}, 300 X 3.9 mm I.D.). The detector was a Shimadzu RF-535 fluorometer. Stock solutions were prepared with increasing concentrations of verapamil added to blank plasma.

6.4.2 *Evaluation of verapamil side effects: dose adjustment*

With the objective of measuring external manifestations after verapamil treatment alone in sheep, healthy animals were randomly allocated using a 4x4 Latin square design, with washout periods of 10 days. All 4 animals per treatment period received a 4-ml SC injection in the ventral region of 0 (placebo), 0.2, 2, 3, 5, 10, 15, and 20 mg/kg of verapamil. Blood was collected in heparinized tubes after 0.5, 1, 2, 3, 4, 5, 6, 9, 12, and 24 h post-treatment (PT). Clinical observations were made before each sampling and for up to five days PT. Hay, minerals and water were available *ad libitum*.

6.4.3 *Effects of verapamil on ivermectin and moxidectin kinetics*

The pharmacokinetics of ivermectin or moxidectin alone at the therapeutic dosage of 200 μg kg^{-1} of body weight or concomitantly with verapamil at 3 mg/kg x 3 at 12 h intervals were investigated in sheep using a complete randomized block design. The objective was to assess whether the pharmacokinetic behaviour of ivermectin or moxidectin were changed following drug combination treatment. Marriner *et al.* (1987) have demonstrated that when ivermectin is given orally the peak plasma concentration is reached after 12-20h PT, and the MDR modulator verapamil has an extensive liver first-pass metabolism (MacTavish and Sorkin, 1989). Verapamil has a much shorter half life in the animals than either ivermectin or moxidectin so that it was decided to administer verapamil three times at 12 h interval in order to enhance the possibility of seeing any effect on the pharmacokinetics of the anthelmintics.

Blood was collected in heparinized tubes at 0, 1, 2, 3, 8, 12, 18, 24, and 36 h and at 2, 3, 4, 5, 7, 9, 12, 15, 20, 25, and 30 days PT. Plasma was immediately separated by centrifugation at 3000 g/min and stored at -20°C before analysis. Pharmacokinetic analysis of both macrocyclic lactones was performed using high

performance liquid chromatography (HPLC) with automated solid-phase extraction and fluorescence detection (Lanusse *et al.*, 1997). Animals were monitored daily for any external clinical symptoms.

Pharmacokinetic analysis of both macrocyclic lactones was performed using high performance liquid chromatography (HPLC) with automated solid-phase extraction and fluorescence detection (Alvinerie *et al.*, 1995, Lanusse *et al.*, 1997). Briefly, a 1 ml aliquot of plasma sample was combined with 100 µl of internal standard (abamectin 100 ng/ml) and then mixed with 1 ml of acetonitrile. After mixing, the solvent-sample mixture was centrifuge at 2000g and the supernatant obtained was placed on the appropriate rack of an Aspec XL autosampler (Supelco, Villiers le Bel, France) and automated solid-phase extraction was performed. The analytes were eluded with 1 ml of methanol and concentrated to dryness under a stream of nitrogen. Reconstitution was done using 100 µl of a solution of N-methylimidazole (Sigma Chemical, St. Louis, MO, USA) in acetonitrile (1:1). Derivatization was initiated by adding 150 µl trifluoroacetic anhydride (Sigma Chemical, Co., St. Louis, MO, USA) solution in acetonitrile (1:2). After completion of the reaction (<30s), an aliquot (100 µl) of this solution was injected directly into the chromatograph. Ivermectin and moxidectin plasma concentrations were determined using a Shimadzu 10 A HPLC system (Shimadzu Corporation, Kyoto, Japan). HPLC analysis was undertaken using a reverse phase C_{18}column (Supelco, 3 µm, 4.6 mm x 150 mm) kept in a column oven at 30° C (Shimadzu Corporation, Kyoto, Japan) and a fluorescence detector (Spectrofluorometric detectorRF-10, Shimadzu, Kyoto, Japan), reading at an excitation wavelength of 365 nm and emission wavelength of 475 nm.

6.4.4 Pharmacokinetics calculations

The plasma concentrations versus time curves obtained after each treatment in each individual animal were fitted with PK Solutions (Ashland, Ohio, USA)

computer software. Pharmacokinetic parameters were determined using a model-independent method. The peak plasma concentration (*C*max) and time to peak plasma concentration (*T*max) were read from the plotted concentration-time curve for each animal. The terminal (elimination) half-life (*T*½ el) and absorption half-life (*T*½ ab) were calculated as ln 2/β and $2/k_{ab}$, respectively, where β is the terminal slope (h^{-}) and k_{ab}, is the rapid slope obtained by feathering which represents the first order absorption rate constant (h^{-}). The area under the concentration vs time curves (AUC) were calculated by the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity by dividing the last experimental concentration by the terminal slope (β). Statistical moment theory was applied to calculate the mean residence time (MRT) for ivermectin and moxidectin as follows;

$$MRT = AUMC / AUC$$

where AUC is as defined previously and AUMC is the area under the curve of the product of time and drug concentration versus time from zero to infinity (Gibaldi and Perrier, 1982). Ivermectin plasma concentrations are presented as mean ± SD. The pharmacokinetic parameters are reported as mean ± SD. Treatment comparison were made using Student's " *t* " test and $P<0.05$ was considered to be statistically significant.

6.5 RESULTS

6.5.1 Verapamil bioavailability

The HPLC retention time for the internal standard and verapamil were 12.3 and 18.5 min. respectively, (data not shown). Verapamil was detected in the range of 5 to 400 ng/ml. Peak verapamil concentrations occurred at 1 h after administration of 5 mg/kg, and the MRT for verapamil, at this dose was 4 h.

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6.5.2 *Evaluation of verapamil side effects: dose adjustment*

Verapamil given to animals at 20 mg/kg induced external manifestations which started 1.5 h PT. Symptoms included increase in respiratory frequency (160/min), two step breathing, decrease in activity (lying down), loss of equilibrium (difficulty to walk), and loss of appetite. Temperature and ocular membrane colour were normal and the recovery of the animals to normal occurred 6 h PT. External manifestations started 1 h after treatment for animals that received verapamil at 15 and 10 mg/kg. The clinical symptoms detected in sheep varied from apathy, tremor, dizziness, and increased respiratory rate to up to 140/min. Animals had difficulty breathing and extended their necks to facilitate the process. Some manifestations deteriorated (respiratory rates) by 2 h PT, but animals returned to normal by 3.5 and 4 h PT after 10 and 15 mg/kg, respectively. The animals that received 5 mg/kg of verapamil showed some mild side-effects such as loss of appetite and apathy 1.5 h PT lasting for 30 min. The animals that received verapamil at 3, 2, and 0.2 mg/kg did not present any clinical manifestations. The dosage of verapamil chosen to be used for the following experiments was 3 mg/kg of body weight SC.

6.5.3 *Effects of verapamil on ivermectin and moxidectin kinetics*

The plasma concentrations after ivermectin and moxidectin treatment alone or ivermectin or moxidectin plus verapamil are shown in Figures 1 and 2. For all drug regimes the anthelmintics were detected in plasma at the first sampling point 1 h PT in sheep. Moxidectin exhibited a significantly higher C_{max} than ivermectin (17.2 versus 6.88 ng/ml), respectively. However, the time of peak concentration (0.79 and 0.86 h), respectively were similar. The MRT of moxidectin was 6.2 fold greater than for ivermectin and the AUC was 4.9 fold greater, respectively (Table 1).

The combination ivermectin/verapamil resulted in a substantial increase of all the pharmacokinetic parameters of ivermectin compared with ivermectin

treatment alone (Table 1). However, verapamil had no effect on altering the kinetics of moxidectin (Table 1). The ivermectin plus verapamil treatment resulted in higher ivermectin plasma concentrations over the whole drug detection period of 12-15 days compared with the ivermectin alone group. The AUC of ivermectin was significantly higher (54%) following the co-administration (P= 0.022) (Fig. 3). The C_{max} of ivermectin was 83% higher after the ivermectin plus verapamil compared with the ivermectin alone treatment (Fig. 4). The $AUMC_{total}$ was also higher (67%) for the drug combination. The higher value for MRT of ivermectin plus verapamil compared to the ivermectin alone group suggests a slower excretion process which indicates a longer retention of the drug in circulating plasma. Ivermectin concentration at 12 days PT was also 57% higher following the ivermectin plus verapamil treatment. The $T½_{el}$ for ivermectin was longer in the presence of verapamil. Although the differences in $T½_{ab}$, T_{max}, and $T½_{d}$ between the drug combination and the ivermectin alone treatment did not achieve statistical significance, they tended to be higher in the presence of verapamil.

6.6 DISCUSSION

Verapamil pharmacokinetic parameters determined were in agreement with Romanová *et al.* (1994). The pharmacokinetic parameters MRT and AUC for moxidectin after oral administration are in close agreement with those reported by Alvinerie *et al.* (1998). The values for Cmax, *T*max, and *T*½ el fall within the standard deviation range reported by Alvinerie *et al.* (1998). The values of C_{max}, T_{max}, AUC, and *T*½ el for ivermectin are very similar to those of Marriner *et al.* (1987). The differences in the MRT and AUC between moxidectin and ivermectin pharmacokinetic parameters reflect the persistence efficacy demonstrated by moxidectin in the field (Rendell and Callinan 1996).

Expression of P-glycoprotein is tissue-specific with quite distinct levels of expression and function. The Class 3 gene (MDR2 in humans) is highly expressed

in the apical surface of the biliary canalicular membrane of hepatocytes facing an excretory compartment and functioning as a transport protein for many products into bile. Although not fully clarified, this MDR class, in opposition to Class 1 or 2 does not confer multidrug resistance but its function can be blocked by the use of MDR modulators, such as the calcium channel blocker, verapamil. The knowledge that P-glycoprotein is expressed in normal tissues and that the MDR modulators have access to it has raised many therapeutic possibilities. Thus, many sites for drug-drug interaction have been suggested, including absorption sites in the gastrointestinal tract, and the carrier-mediated transport across plasma membranes; e.g. hepatic or renal uptake, secretion, or metabolism (Ito *et al.*, 1998).

P-glycoprotein is expressed in liver, where it is found only at the apical pole of the canalicular membrane and it is believed to be involved with the transmembrane transport (Bushman *et al.*, 1992). Ruetz and Gros (1995), using yeast secretory vesicles stably expressing *mdr2*, tested the translocation of phosphatidylcholine into bile in the presence or absence of *mdr2* (-/-, +/+). The increasing *mdr2*-mediated translocation (+/+) was abrogated by verapamil, demonstrating that *mdr2* P-glycoprotein is a lipid transporter and functions as a flippase and translocase moving lipids from one leaflet of the membrane to the other (Ruetz and Gros, 1994). As mentioned above, ivermectin is a substrate of P-glycoprotein. Thus, the transport of ivermectin in and from the liver might be through *mdr2* P-glycoprotein facilitating the transport of ivermectin into the bile. If that is so, an explanation for our data is that verapamil is altering ivermectin pharmacokinetics by decreasing its enterohepatic circulation and consequently decreasing drug clearance. There are different possible explanations for the lack of effect of verapamil on moxidectin pharmacokinetics. One possibility is that moxidectin is more rapidly distributed to fat than is ivermectin from where it is slowly eliminated. The effects of verapamil would only be transient due to its much shorter half life. Another possibility is that moxidectin may be a weaker P-glycoprotein substrate than ivermectin (Pouliot, Prichard, and Georges, unpublished results).

This latter possibility would explain the lack of effect of verapamil on the elimination of moxidectin and may also account for the much longer residence time for moxidectin than ivermectin. Under normal circumstances, ivermectin may be actively excreted by the action of P-glycoprotein in the hepato-biliary system, whereas moxidectin may be little affected by this efflux mechanism, leading to its long residence time.

It is also unclear if the modulation of P-glycoprotein at the biliary surface would have any consequences for increasing host toxicity to similar drug combinations. In our studies, sheep only expressed signs of toxicity after verapamil was given alone at 5 mg/kg or above. This can be attributed to the vasodilatory action of verapamil. The combination of ivermectin or moxidectin with verapamil at 3 mg/kg x 3 at 12 h intervals produced no signs of toxicity.

Metabolic drug interactions have been reported and the alteration of ivermectin bioavailability might also have been dependent on alterations in the rate of ivermectin metabolism. Recently, CYP3A4 was found to be the main isoform of the P450 enzyme responsible for ivermectin metabolism (Zeng *et al.*, 1998). This enzyme is responsible for the metabolism of many drugs and is expressed in liver and in the intestine (Lown *et al.*, 1993, Thummel *et al.*, 1996). Other studies revealed the overlapping substrate/inhibitor specificity of CYP3A4 and P-glycoprotein for verapamil (Wacher *et al.*, 1995), suggesting that the formation of the metabolite, norverapamil is through CYP3A4 after incubation with verapamil (Kromer *et al.*, 1993). The possibility of common regulatory factors for CYP3A4 and P-glycoprotein proteins have been demonstrated by Schuetz *et al.* (1996), suggesting that verapamil is a good inhibitor for P-glycoprotein and for CYP3A4. Thus, the drug-drug interaction based on metabolic inhibition involving CYP3A4 cannot be neglected. However, in view of the fact that 60-68% of ivermectin is normally excreted unchanged, we believe that the principal cause of the verapamil mediated alteration of ivermectin pharmacokinetics is by inhibiting a P-glycoprotein transport mechanism.

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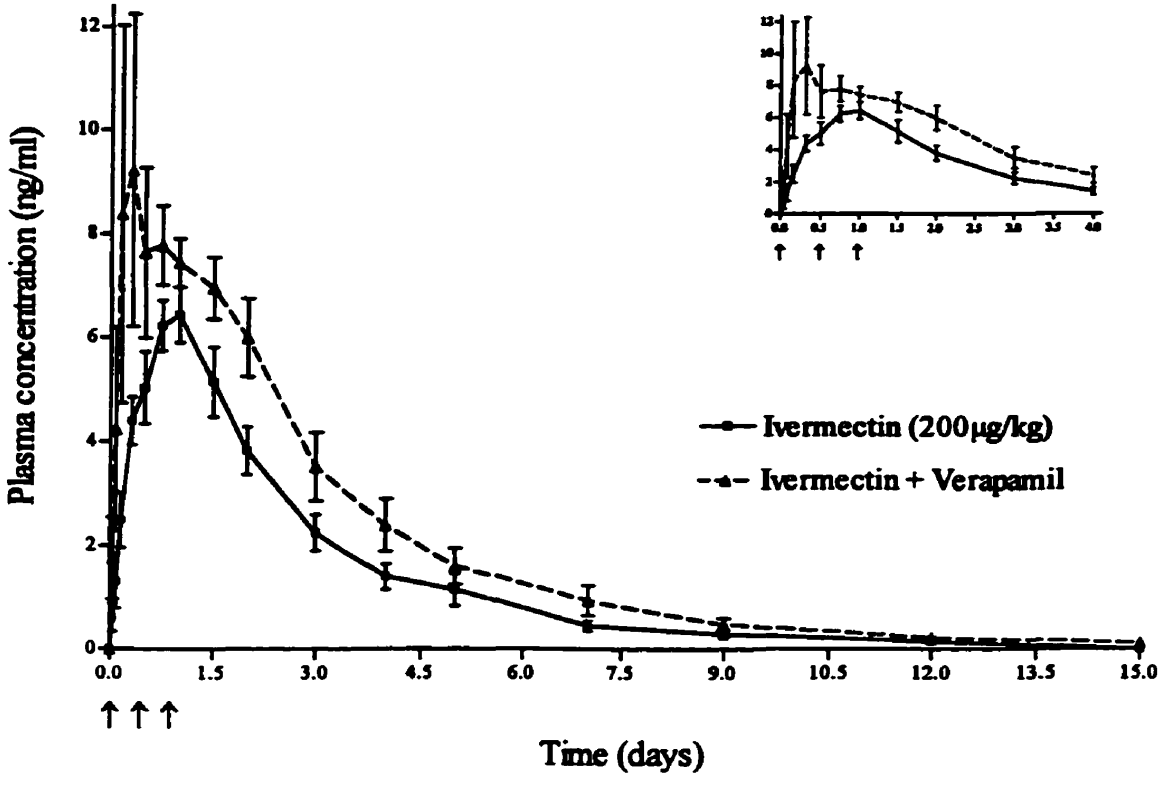

Figure 1. Mean (SEM) plasma concentration of ivermectin after administration of ivermectin or ivermectin plus verapamil in sheep (n=8 per group). Insert graph shows ivermectin concentration over a period of 4 days. ↑ Verapamil treatments.

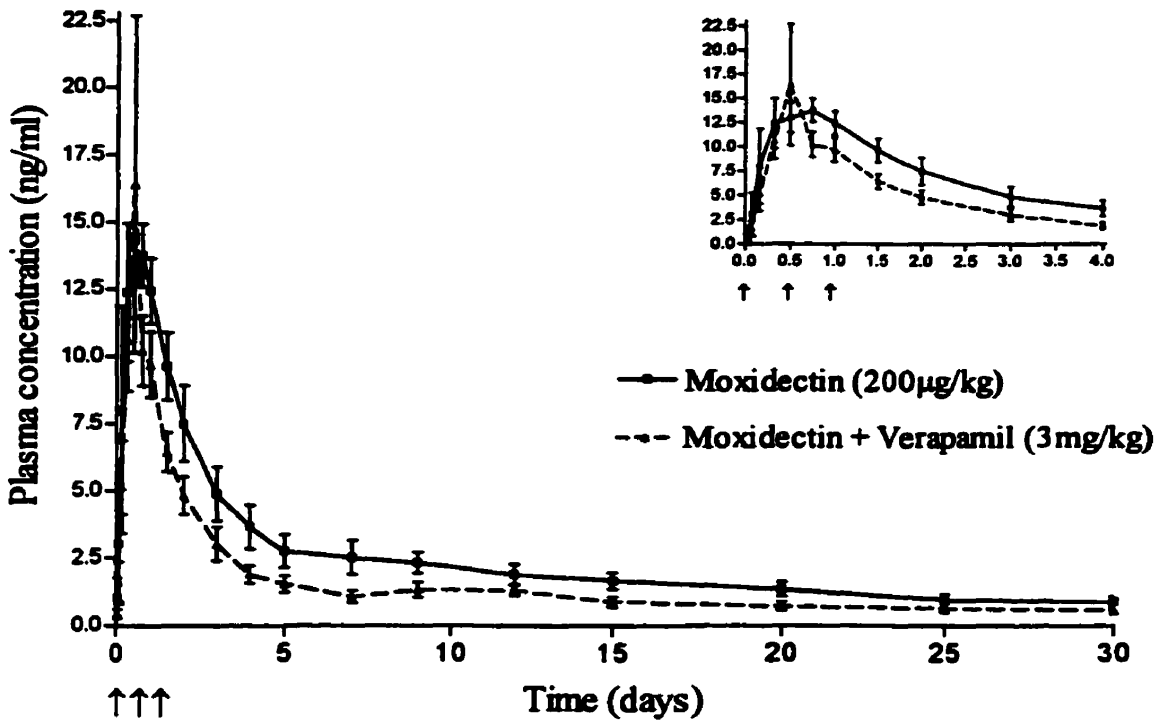

Figure 2. Mean (SEM) plasma concentration of moxidectin after administration of moxidectin or moxidectin plus verapamil in sheep (n=8 per group). Insert graph shows moxidectin concentration over a period of 4 days. ↑ Verapamil treatments.

Table 1. Pharmacokinetic parameters* (Mean ± SD) of ivermectin (IVM) and moxidectin (MOX) obtained after their administration either in the presence or in the abscence of verapamil (VRP) to sheep (n = 8 per group).

	Treatments			
Kinetic Parameters	IVM	IVM + VRP	MOX	MOX + VRP
T ½ ab (days)	0.29 (0.13)	0.34 (0.25)	0.2 (0.11)	0.17 (0.06)
Cmax (ng/ml)	6.88 (1.68)	12.6 (7.57)	17.2 (7.39)	12.3 (4.61)
Tmax (days)	0.86 (0.34)	0.91 (0.59)	0.79 (0.55)	0.53 (0.19)
T ½ d (days)	0.83 (0.48)	1.08 (0.38)	0.67 (0.18)	0.71 (0.16)
AUCtotal (ng.d/ml)	19.3 (6.03)	29.7 (10.5)	94.9 (46.0)	62.3 (28.9)
AUMCtotal(ng.d^2/ml)	57.7 (34.0)	96.5 (45.7)	1776 (1417)	1410 (1504)
MRT (days)	2.85 (0.92)	3.21 (0.57)	17.7 (7.26)	19.7 (12.1)
T ½ el (days)	2.14 (0.65)	2.90 (0.66)	15.4 (5.60)	16.2 (7.30)

* **T½ ab**: absorption half-life; **Cmax**: peak plasma concentration; **Tmax**: time to peak plasma concentration; **T½ d**: distribution half-life; **AUCtotal**: area under the concentration vs time curve extrapolated to infinity; **AUMCtotal**: area under the first moment of the curve; **MRT**: mean residence time; **T½ el**: elimination half-life. Ivermectin and moxidectin were given orally at 200µg/kg and verapamil was given subcutaneously at 3 mg/kg.

The pharmacokinetic parameters of **Cmax** and **AUCtotal** are significantly different between the ivermectin and ivermectin plus verapamil treatment groups ($P<0.05$).

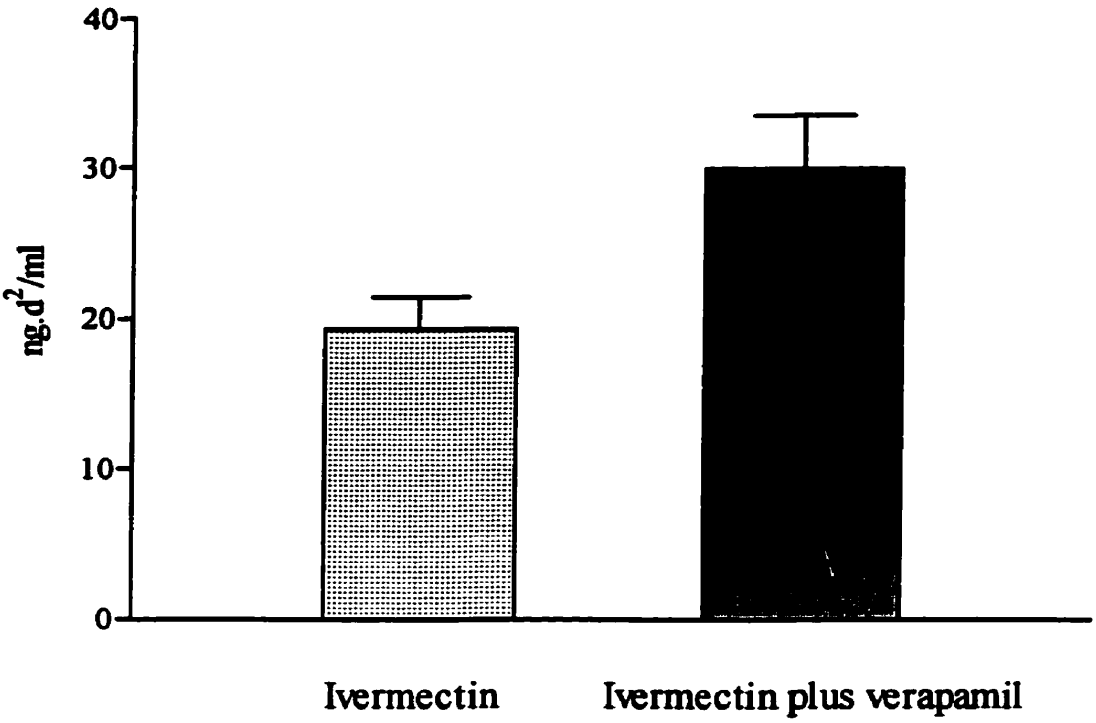

Figure 3. Mean (SEM) area under the concentration curve vs time curve (AUC) extrapolated to infinity for ivermectin after its administration either alone or co-administered with verpamil in sheep. Groups were significantly different (P=0.022).

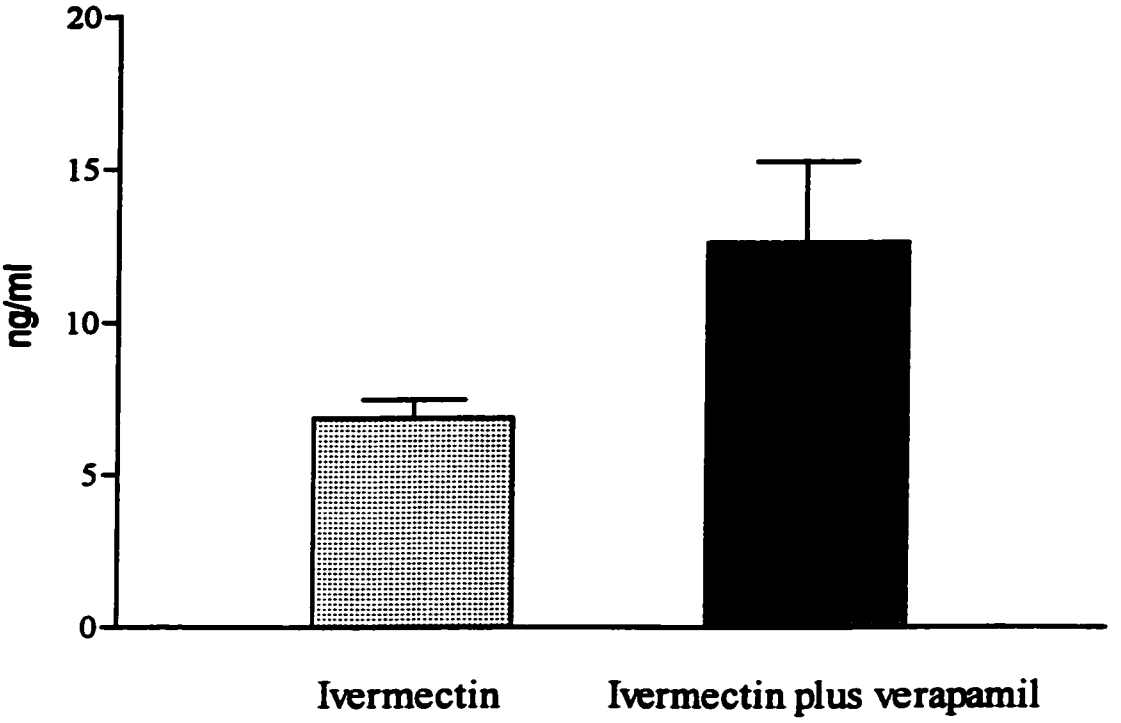

Figure 4. Mean (SEM) peak plasma concentration (Cmax) for ivermectin after its administration either alone or co-administered with verapamil in sheep. Groups were significantly different (P=0.048).

CHAPTER 7

GENERAL DISCUSSION

The control of domestic animal parasites using chemical compounds is economically advantageous with considerable benefits of increased productivity and sales. But it is doubtful that control programs which have anthelmintic treatment as the main component can avoid the increasing problem of anthelmintic resistance. Before the scientific community became aware of the problem of nematode resistance the future seemed secure, due to the availability of drugs such as the avermectins. Today the prognosis is obscure. Yadav and colleagues (1993) reported an outbreak of *H. contortus* infection in sheep associated with resistance to two of the anthelmintic groups, and this situation is now common in many parts of the world where intensive small ruminant enterprises depend on professionals to bring alternatives to the industry. In this context, it is also unrealistic to assume that the release of an alternative, highly effective anthelmintic drug will keep pace with resistance to existing drugs. To complicate things, anthelmintic resistance, as is the case of *H. contortus*, may have multiple mechanisms (Gill *et al.*, 1998, Xu *et al.*, 1998, and Blackhall *et al.*, 1998a, 1998b). Fortunately, new technology has come into play to help us understand the complexity of these organisms and to improve on the empirical way of drug development that was used in the past. It is possible today to envision what still has to be achieved. Hopefully knowing how to get there in terms of drug targets and vaccines, molecular epidemiology/diagnostics, control strategies, and the involvement of P-glycoprotein in resistant parasites will help us maintain control of parasites in farm animals.

Since its discovery in cancer cells (Juliano and Ling, 1976), P-glycoprotein has been associated with drug resistance. However, not until recently was it possible to determine the structure of P-glycoprotein using electron microscopy and

image analysis. When viewed from above the membrane plane of CH'B30 cells, the protein is toroidal, with a 6-fold symmetry and a diameter of about 10 nm. There is a large central pore of about 5 nm in diameter, which is closed at the inner (cytoplasmic) face of the membrane, forming an aqueous chamber within the membrane. An opening from this chamber to the lipid phase is present. The projection of the protein perpendicular to the membrane is roughly rectangular with a maximum depth of 8 nm and two 3 nm lobes exposed at the cytoplasmic face of the membrane, likely to correspond to the nucleotide binding domains (Rosenberg *et al.*, 1997). The presence of the P-glycoprotein was found to correlate with both the degree and the relative decrease in drug accumulation. Extensive studies of the biochemistry of the *mdr1* gene product support its role as an efflux pump of chemotherapeutic drugs from the cytoplasm (Hammond *et al.*, 1989). Although kinetic characterization is lacking; it is likely that substrates bind to specific domains of the protein, which subsequently undergo an energy-dependent conformational change, allowing the substrate to be released on the exterior side of the membrane (Ford, 1995) (Fig. 1). Unlike other carrier molecules, however, P-glycoprotein is less selective with regard to substrate, and it has been suggested that this protein contains multiple nonoverlapping drug-binding sites, each having different affinities for different drugs or classes of drugs (Loor *et al.*, 1992). As an example, Nare *et al.*, (1994) have demonstrated that human MDR cells selected for resistance against vinblastine (CEM/VBL) are cross-resistant to the anthelmintic benzimidazole after observing a reduction of benzimidazole accumulation in drug resistant cells compared to the susceptible ones and the capacity of verapamil to potentiate the accumulation of benzimidazole in drug-resistant cells.

In the areas of herd health and infectious diseases, the veterinary field has to recognize and implement discoveries that are coming from human medicine. Therefore, this research thesis was initiated to investigate the use of drug combination strategies for control of veterinary parasites, brought from the oncology and experimental pharmacology fields with the objective of lessening the negative

impact of anthelmintic resistance. The results included in this thesis and some more data from our group have determine the enhancing ability of MDR modulators associated with the macrocyclic lactones, the overexpression of P-glycoprotein in *H. contortus* selected with ivermectin and moxidectin (Xu *et al.*, 1998, and Molento and Prichard, 1999a) and also determine that ivermectin and moxidectin selected an allele (P-glycoprotein) that confers resistance in *Haemonchus contortus* (Blackhall *et al.*, 1998b). Sangster *et al.* (1999), have reported that the region between the nucleotide binding domains of P-glycoprotein, termed the internucleotide binding domains, reveals a pattern consistent with the involvement of P-glycoprotein in resistance to avermectins/milbemycins anthelmintics.

In the final section of this thesis, I would like to address some issues which demand further clarifications intended to support these findings.

7.1 *IN VIVO/IN VITRO* ANIMAL MODELS

The initial work has indicated that the macrocyclic lactones respond similarly (side-resistance) when a strain of *H. contortus* is selected with moxidectin as opposed to ivermectin (Molento *et al.*, 1999a); shifting the concentration/response curves for both anthelmintics. However, the very high lipophilicity of moxidectin may influence its relative efficiency compared with ivermectin. This data supports the principle that the two drugs act at the same site, as suggested in the literature (Conder *et al.*, 1993, and Shoop *et al.*, 1993). The relevance of this initial work can be determined when farmers are selecting a new compound for a drug rotation strategy. Chapters 3 and 4 report the enhancement of the efficacy of ivermectin and moxidectin when these anthelmintics were used in combination with the MDR modulators verapamil and CL347,099 using an *in vivo* jird model (Xu *et al.*, 1998 and Molento and Prichard, 1999a). Verapamil was elected for the experiments because it has been demonstrated to have relatively good reversal activity *in vitro* and in initial clinical trials in humans. CL 347,099 was also investigated because it is an analog of verapamil with putative MDR reversing properties but with

negligible Ca^{2+}-channel blocking activity. The findings that the two pharmacologic agents, verapamil and CL 347,099, when used in combination with ivermectin and moxidectin, could partially overcome resistance to the macrocyclic lactone anthelmintics indicate the involvement of the transport protein P-glycoprotein in the development of resistance to the macrocyclic lactone anthelmintics using the model described by Ford (1995) (Fig. 1). It also suggests the great potential of the drug combination strategy for clinical applications most importantly in farms where resistant parasites are the cause of decline profits.

The *in vitro* migration assay experiments (Molento and Prichard, 1999b, submitted), indicate that although conducted on a different stage of the parasite than the target in the host, it can be used to determine anthelmintic efficacy and gives comparable results to the jird model. This indicates that the *in vitro* model is a practical and reliable tool for studying drug combination strategies and for simulating *in vivo* drug responses. Furthermore, the *in vitro* assays allows the effects of the anthelmintic/MDR reversing agent combinations to be determined on the nematode without the intervention of the host to modulate the drug concentration. The data resulting from the larval migration assay clearly indicates a direct effect of the MDR modulator/macrocyclic lactone combination on the nematode.

7.2 VERAPAMIL SIDE-EFFECTS

The co-administration of drugs to a patient or host to combat resistant organisms raises a concern about side-effects. Umbenhauer *et al.*, (1997), have demonstrated that a subpopulation of the CF-1 mouse strain was very sensitive to the neurotoxicity induced by the avermectins. They described a restriction fragment length polymorphism that was able to predict which animals are P-glycoprotein deficient. The P-glycoprotein expression in the brain (primarily mdr1a and low mdr1b, mdr2) is dependent on the genotype, which also determines the susceptibility to the avermectin-induced neurotoxicity. Therefore, the ability to

segregate this strain into (-/-) and (+/+) animals may prove useful for examining the physiological role of P-glycoprotein in drug absorption, distribution and related toxicity in the host animal. Kwei *et al.* (1999), using the same strains of P-glycoprotein-deficient mice, reported that verapamil increased the brain concentration of ivermectin in (+/+) mice. The absence of P-glycoprotein in (-/-) mice resulted in higher blood concentration of ivermectin after oral dosing compared to the (+/+) mice.

The data reported in this thesis documents toxicity effects of verapamil when used in association with ivermectin in jirds and sheep. The results obtained in the jird experiments have demonstrated that these animals are quite resistant to the stress caused by high doses of verapamil (up to 40 mg/kg), whereas sheep were demonstrated to be much more susceptible (5 mg/kg and above being toxic) to its chemical effects (Molento and Prichard, 1999a, and Molento *et al.*, 1999b submitted). Clearly, the clinical findings noticed in these different mammals were caused by the calcium channel blocker activity of verapamil without ivermectin adding to this effect. Ivermectin was safe, even at high doses, when used alone in jirds in our experiments. Further studies are necessary for the identification of more specific, more potent, and less clinically toxic MDR agents for clinical use.

7.3 PHARMACOKINETIC OF THE MACROCYCLIC LACTONES

P-glycoprotein has been localized in the brush border membrane of mammalian proximal renal cells, at the biliary canaliculi of the hepatocytes and in many other organs. The physiological function of P-glycoprotein in different cells or organs remains unclear but a growing amount of information suggests that it can play an important role at the different steps of drug pharmacokinetics; *i.e.*, absorption, distribution, and elimination. The extent by which drug pharmacokinetics are altered by a modulator will depend on the fraction of drug that is normally eliminated by the P-glycoprotein efflux mechanism. The alterations in drug levels are also compatible with relative high expression levels of P-

glycoprotein in different tissues. Of note is the influence of the route of drug administration. Apparently, absorption of many drugs from the gut is normally largely inhibited by intestinal P-glycoprotein, while the role of P-glycoprotein in elimination of these drugs may be smaller.

Combination treatment of cytotoxins with MDR modulators was reported to have altered the pharmacokinetics of cytotoxins in clinical trials phase I and II in humans (van de Vrie *et al.*, 1998). Kerr *et al.* (1986), showed in five patients a 30% decrease of doxorubicin clearance associated with a prolonged half-life when verapamil was co-administrated. Alterations of etoposide kinetics by cyclosporin A in patients who were administered paired courses of etoposide and both drugs, have been reported by Lum *et al.* (1992). In ten patients, whose cyclosporin A levels were greater than 2 µg/ml, systemic exposure to etoposide increased AUC by 80% with a 46% decrease in total clearance and a 108% increase in plasma half-life. The authors concluded that this pharmacokinetic interaction was consistent with alterations of P-glycoprotein efflux function in normal tissues. A similar observation has been reported with doxorubicin where the addition of cyclosporin A resulted in a 55% increase of doxorubicin AUC and a 350% increase of the doxorubicinol metabolite (Erlichman *et al.*, 1993).

There are a few studies on plasma levels and AUC of drug combinations in animals. The AUC of drugs given as combinations can be elevated in animals which may be the result of blocking the P-glycoprotein activity altering intestinal, biliary or renal absorption and/or excretion. The modulator SDZ PSC-833, partially inhibited the active biliary excretion of colchicine in rats (Speeg *et al.*, 1994). Alvinerie *et al.* (1999), have also recently demonstrated that in rats, following topical administration, the systemic exposure to ivermectin measured from the AUC, was 40% higher for ivermectin combined with verapamil than for ivermectin alone. These data indicates that P-glycoprotein can be blocked *in vivo* changing its physiological function.

The data in this research thesis has demonstrated that a pharmacokinetic

interaction between verapamil and ivermectin significantly increase the ivermectin AUC values by 54% and the Cmax by 83% compared with ivermectin alone treatment (Molento *et al.*, 1999b submitted). Thus, the data demonstrates that the host drug-transport physiology can be altered by the use of the ivermectin/verapamil combination strategy. It is possible that the increase of ivermectin bioavailability was caused by the inhibition of P-glycoprotein transport action at the biliary canaliculi, reducing ivermectin bile transport, delaying ivermectin's elimination and possibly enhancing its anthelmintic concentration at the target sites (Fig. 2) with decreased enterohepatic circulation. The mechanism of biliary drug secretion and its blockage by verapamil may be similar to that described by Higgins and Gottesman (1992). They described the multidrug transporter acting as a flipase; carrying its substrate from the inner leaflet of the lipid bilayer to the outer leaflet. The flipase model is supported by the finding that the MDR2 gene product is a phosphatidylcholine translocase essential for extrusion of phosphatidylcholine from the hepatic plasma membrane into the bile (Ruetz and Gros, 1994, and Smit *et al.*, 1993).

The lack of pharmacokinetic effect of verapamil on moxidectin may be explained by moxidectin's faster tissue binding (fat), leading to its longer residence time than ivermectin and that moxidectin is a weaker P-glycoprotein substrate (Pouliot, Prichard, and Georges, unpublished results).

7.4 ALTERNATIVE APPROACH FOR MODULATION MDR *IN VIVO*

Apart from the competition for P-glycoprotein binding sites in various tissues, modulators may also interact in different metabolic pathways, altering the pharmacokinetics of drugs. The involvement of the P450 enzyme CYP3A4 with the P-glycoprotein pump effect is speculative. Interest has focussed on this CYP isoform due to its high abundance in human liver; inducibility by numerous agents, including glucocorticoids and phenobarbital; and prominent role in the metabolism of a large number of clinically relevant drugs including ivermectin (Zeng *et al.*,

1998). Phenotyping of CYP3A4 in preclinical and clinical drug metabolism studies currently represents a major goal towards generating a full understanding of drug pharmacology, toxicology, and pharmacokinetics. These studies are been aided by the development of a mAb that is specific and inhibitory toward CYP3A4 (Mei *et al.*, 1999).

7.5 FOLLOW UP EXPERIMENTS

1. Establish the clinical efficacy of the drug combination strategy in the host:
The objective of this experiment is to test the pharmacokinetic data presented in this thesis. Sheep would be infected with *H. contortus* and treated with the LD50 for ivermectin (alone) and ivermectin plus verapamil (3 treatments).
2. Measure ivermectin at the site of action using the drug combination:
The objective of this experiment is to establish a relationship between plasma concentration and the concentration at the target sites of the active compound in the host animal. This could be demonstrated by measuring the concentration of ivermectin following treatment with ivermectin (alone) or ivermectin plus verapamil treatments (plasma, gastrointestinal fluids, abomasal and intestinal mucosa).
3. Influence of CYP3A4 utilizing the drug combination regime:
The objective of this experiment is to determine the significance (down regulation) of the individual cytochrome CYP 3A4 isoform to the metabolism of the macrocyclic lactones with or without verapamil. This represents an area of considerable importance in the prediction of potential drug/drug interactions because the inhibition of CYP 3A4 may lead to a diminished rate of anthelmintic hydroxylation.

7.6 FINAL DISCUSSION

The purpose of this research was to determine the effects of the use of MDR modulators and of their combination with the macrocyclic lactone anthelmintics employing different assays against unselected and selected *H. contortus* and to relate the results to the overexpression of P-glycoprotein. The same drug

combinations were used to detect any changes in drug pharmacokinetics and correlate this to an alteration of P-glycoprotein physiology in the host organism. A substantial amount of information on P-glycoprotein regarding its structure, modes of activity in normal and tumour tissues, pharmacokinetics, and interaction with modulators was also included. Our group has demonstrated that P-glycoprotein is overexpressed in the early stages of development of resistant *H. contortus*. This data correlates with the findings reported in this thesis using third stage, unselected and selected, larvae and the enhancing results obtained after the employment of the anthelmintics with MDR modulators strategy. Along the same line of investigation, the pharmacokinetic experiment has established an important step to the understanding of the drug interaction and P-glycoprotein. This data demonstrates that the plasma concentration of ivermectin can be increased significantly once in the presence of verapamil. Figure 3 integrates the ivermectin bioavailability curve with a hypothetical parasite-elimination effect under both treatment regimes. The expected result would be that the treatment, ivermectin plus verapamil, would increase the overall antiparasitic efficacy of ivermectin targeting resistant nematodes. It is relevant to mention that not all pharmacokinetic findings in animal studies can be directly translated to the clinical situation, as cross-species differences in drug metabolism and elimination do exist. However, the data reported here may bring a new prospect for the study of drug combination in humans. This would be particularly relevant to the possible emergence of ivermectin resistance in *Onchocerca volvulus*.

This thesis research provides information supporting the hypothesis that the transport protein, P-glycoprotein plays an major role in the mechanism of resistance develop by *H. contortus* to the macrocyclic lactone anthelmintics. The use of the MDR modulators with ivermectin and moxidectin using different assays confirm these findings. These facts underscore the relevance of this thesis to the reversal of anthelmintic resistance in nematodes of veterinary importance, and indicates a novel approach to enhance macrocyclic lactones efficacy, maintaining their

usefulness against drug resistant parasites.

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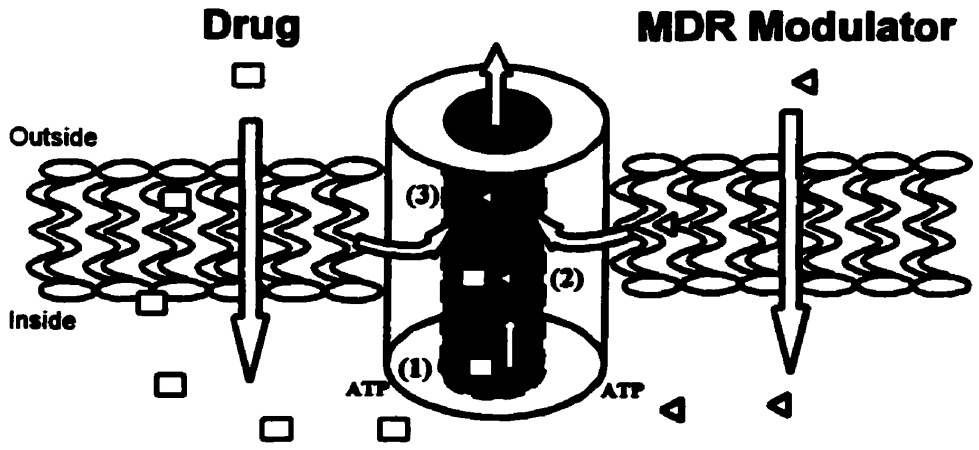

Figure 1. P-glycoprotein transport model. The illustration demonstrates how a drug can be transported from the cell (1), utilizing ATP energy to actively transport drug substrate across the plasma membrane and how the MDR modulator may serve as a competitive inhibitor for the drug-binding site (2), or as a non-competitive inhibitor (3). Adapted from Ford, (1995).

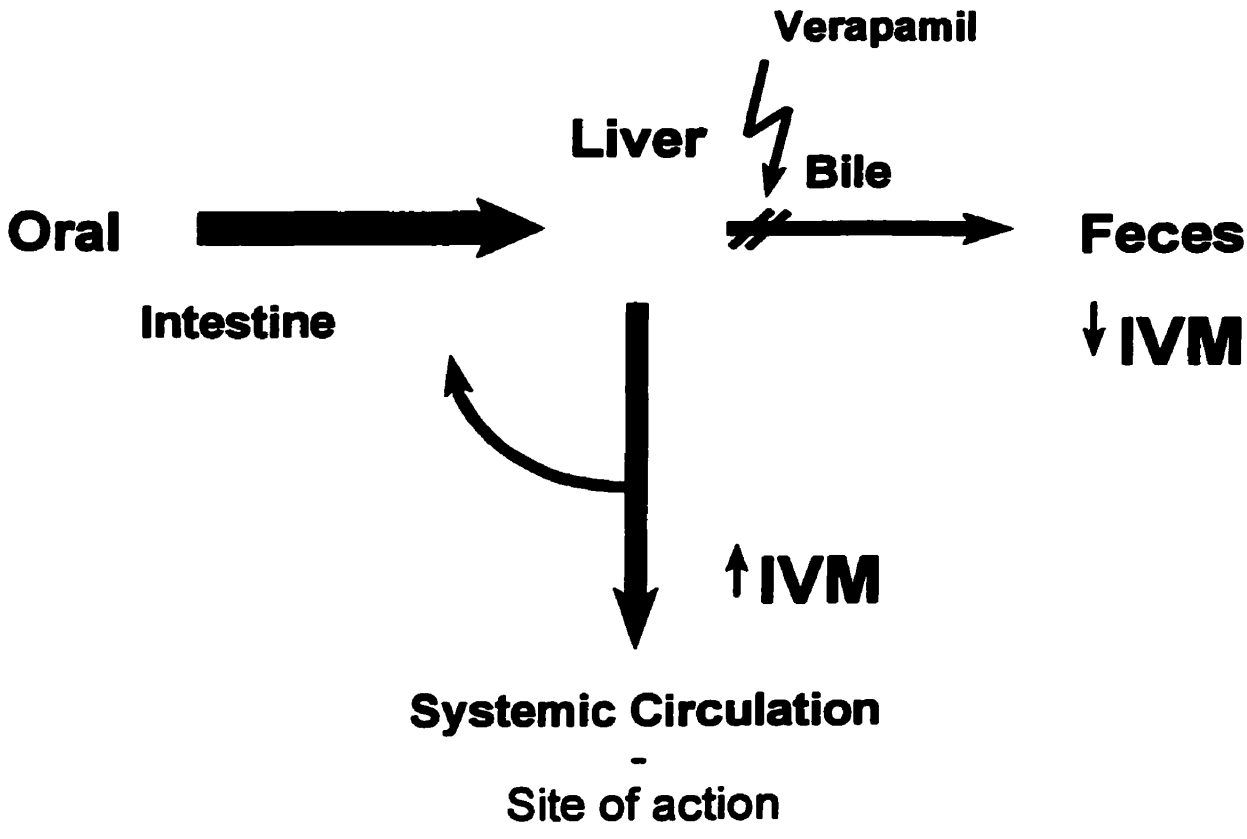

Figure 2. Possible alteration of pharmacokinetics of ivermectin by verapamil, blocking P-glycoprotein transport mechanism at the biliary caniculi. Verapamil is probably reducing the drug transit; liver → bile → feces and thus, indirectly increasing ivermectin systemic concentration and concentration at its site of action.

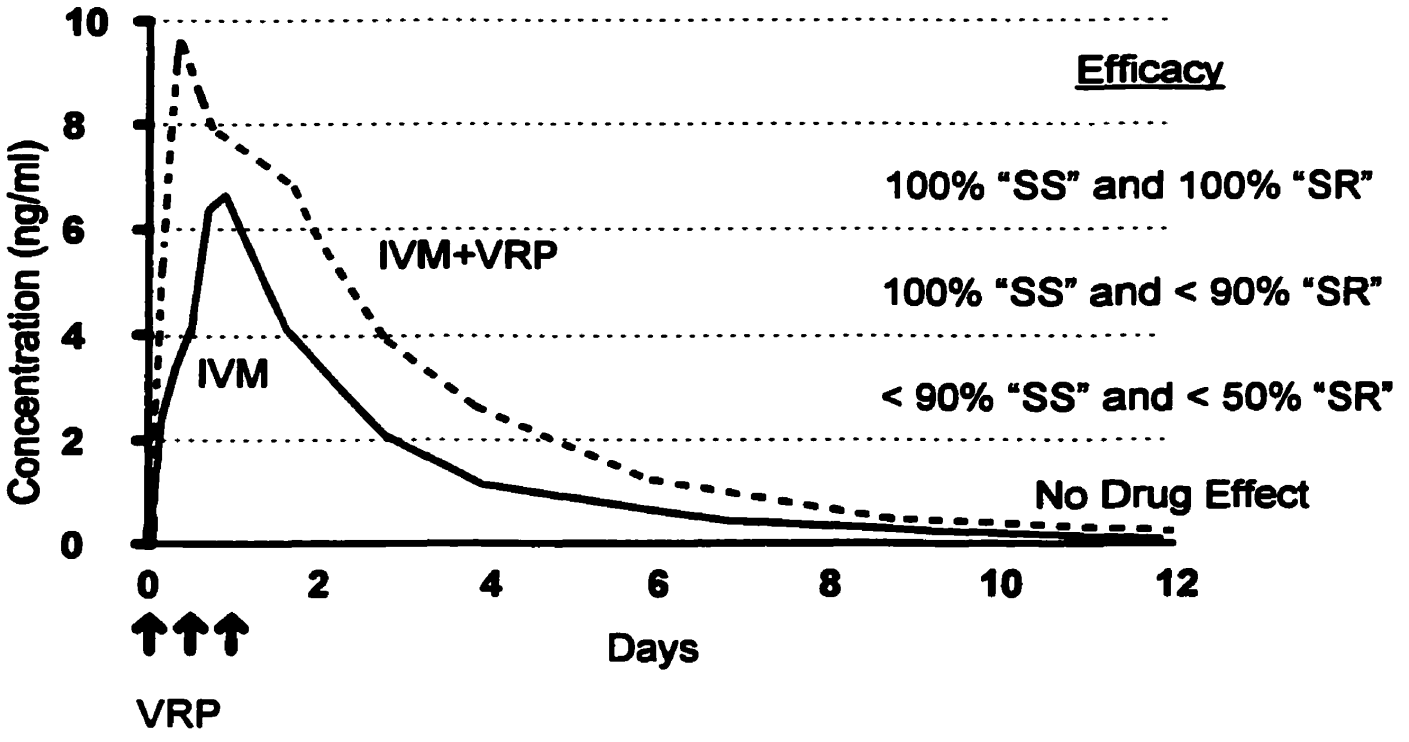

Figure 3. Concentration of ivermectin in plasma with or without verapamil (3 treatments) in sheep. Note the increase of ivermectin concentration using the drug combination and its longer bioavailability. This strategy may enable ivermectin to eliminate both susceptible (SS), and partially resistant (SR) parasites, for a longer period and to a greater extent in the host animal.

APPENDICES

I. PROTOCOLS USED FOR THE *IN VIVO* EXPERIMENTS

1.1 Food re-pelletizing process

1. Grind the commercial food in a Thomas-Wiley (model 4- U.S.A.) grinder.
2. Mix the food plus 0.02% of hydrocortisone for 60 minutes (The Hobart U.S.A.).
3. Repeletize using a I801, 22 shopper plate (The Hobart, U.S.A.) gradually adding 0.3 % of molasses (warm it up before mix), and 3 ltr. of water.
4. Let dry at a 60° C. for 24 hrs.
5. Maintain the mixed food at - 20° C. freezer.

1.2 Aliquoting larvae for infection

1. Take the flask containing the L_3 from the refrigerator, and mix it to get a homogeneous solution.
2. Take a sample of 5 or 10 microliter, put it on a slide, and count the larva under the microscope.
3. From the result, calculate the final amount for the infection of 1,000 L_3 per animal, plus 20% more due to deaths during the process. Dead larvae are immotile have a "C" shape.

* If necessary, take some solution out of the flask to increase the larval concentration.

5. Place the final volume to infect the animals in a Erlenmeyer and start the exsheathment.

1.3 Larval exsheathment G. Conder, (personal communication, 1995)

1. Aliquots of 10^5-10^6 L_3 of the parasite in 10 ml of Earle's balanced salt solution; incubate at 37°C in a shaker bath for 1 hour.
2. Immediately prior to incubation, CO_2 should be bubbled into the tube containing the larvae and the salt solution for 10 minutes.
3. The tube can be covered with parafilm while bubbling the medium.
4. Use screw-top centrifuge tubes to which the cap can be added during the incubation.
5. Count the L_3 concentration per 5 μl before the infection and calculate the number of exsheathed L_3 to give to each animal (average of 80 μl/animal).
6#. Using a micropipette (200 μl), introduce it laterally into the mouth of the gerbils, until reaching the esophagus, and deposit the larvae.
7#. During the gerbil`s infection, maintain the tube that contains the larvae in a water bath (37°C) to prevent the larvae from clumping together thereby ensuring a homogeneous sample.

1.4 Larval recovery from stomach

1. Remove the stomachs, open longitudinally and place in scintillation vials containing 14 ml of distilled water.
2. Vortex and place in a water bath at 37° C for 5 hours.
3. Following incubation, vortex again, remove the stomachs, and add 1 ml of formaldehyde solution to each vial and store the contents for subsequent examination.
#. After taken the stomachs from the animals, cut them transversely in half, leaving just the posterior part to be processed (glandular portion).

1.5 Total worm count (TWC) after necropsy

1. Mix the contents of each vial.
2. The material should be examined in 2-5 aliquots (depending on clarity) using a dissecting microscope (15-45 x).
3#. During the counting, wash the vials with tap water `briefly` to get the 1-2% of larvae that remain attached to the vials.
4#. After opening the stomachs longitudinally, some worms will be cut in half; count just one head end, as 1 worm.
5. The percentage clearance for each drug will be determined based on the method of Coles *et al.* (1992).

#. Improvements to the gerbil model were made by the author.

II. PERSISTENT EFFICACY OF DORAMECTIN POUR-ON AGAINST ARTIFICIALLY INDUCED INFECTIONS OF NEMATODES IN CATTLE

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Abstract

Two studies were conducted to determine the persistent efficacy of doramectin pour-on against an artificial, trickle challenge of mixed nematodes in calves. In each study, forty-two, four- to eight-month-old calves, were randomly assigned into 4 groups of 10 animals each (T1 - T4), plus two larval-viability monitor animals. All animals were treated with fenbendazole (10 mg kg^{-1}) 14 days prior to the start of the study to clear any existing infection. Doramectin pour-on at 500 µg kg^{-1} was used on each animal in Groups T2, T3, and T4 with intervals of one week (Day 0, 7, and 14, respectively). Calves in Group T1 were treated with saline solution on Day 0 and at the same volumetric rate (1 mL 10 kg^{-1}) as the doramectin treated animals. All treatments were applied in a single passage along the midline of the back, from the withers to the tailhead. Subsequently, trickle inoculations with infective larvae were administered to all calves for 22 consecutive days (Days 14-35). Doramectin pour-on provided ≥91.9% efficacy against challenge with *Dictyocaulus viviparus, Haemonchus* spp., and *Ostertagia ostertagi* for up to 35 days post-treatment and against challenge with *Cooperia oncophora, Cooperia punctata,* and *Oesophagostomum radiatum* for up to 28 days post-treatment.

Keywords: Doramectin, Pour-On, Cattle-Nematode, Endectocide, Persistent Efficacy.

Introduction

Doramectin [25-cyclohexyl-5-*O*-demethyl-25-de (1-methylpropyl) avermectin A1a], a macrocyclic lactone of the avermectin sub-class, is produced by *Streptomyces avermitilis* using a mutational biosynthetic process (Dutton *et al.*, 1991; Goudie *et al.*, 1993). The anthelmintic activity of doramectin has been demonstrated against natural and induced infections of a broad spectrum of

nematodes in cattle under different environmental conditions in both an injectable (Eddi *et al.*, 1993; Jones *et al.*, 1993; Kennedy and Phillips, 1993; Vercruysse *et al.*, 1993; Phillips *et al.*, 1996) and a pour-on (Conder *et al.*, 1998; Marley *et al.*, 1998) formulation. Doramectin in the injectable formulation also has been shown to have persistent efficacy against re-infection by nematodes for an extended period of time post-treatment (Weatherley *et al.*, 1993; Barton *et al.*, 1995; Rew, 1995; Burden and Ellis, 1997; Conder *et al.*, 1997; Rolfe *et al.*, 1997). The objective of this study was to evaluate the persistent efficacy of doramectin pour-on at its recommended dosage of 500 μg kg^{-1} against artificially induced nematode infections of calves.

Materials and Methods

Study sites and animals

Two studies run to a common protocol were conducted at Macdonald Campus of McGill University, Quebec, Canada and at Johnson Research, Parma, Idaho, USA. Animals were housed indoors in concrete-floored pens. Hay and water were provided *ad libitum* along with restricted supplementation with grain (Quebec) or feeder ration (Idaho). Forty-two bull and steer Holstein calves, 4 - 8 months old, weighing approximately 90-250 kg were used in each study. Cattle were penned separately by treatment to prevent any physical contact. All animals were treated orally with 10 mg kg^{-1} of fenbendazole 14 days prior to the start of the study (Day 0) to clear existing infections.

Treatment

Doramectin-treated animals received a pour-on formulation at 500 mg kg^{-1} (1 mL 10 kg^{-1}) in a single passage extending from the withers to the tailhead. A control group of cattle was treated in an identical manner with saline solution in a volume equivalent to the doramectin treatments (1 mL 10 kg^{-1}). All calves were

observed at approximately 1, 4, and 24 hours after treatment for the assessment of any adverse reactions.

Experimental procedures

On Day -1, animals were determined, by fecal egg counts, to be clear of any existing trichostrongylid infections. In addition, each of the 42 animals was weighed, ear-tagged and randomly allocated to one of four treatment groups (T1 - T4; 10 animals/group) or as a larval-viability monitor (2 animals). The experimental design for both studies is outlined in Table 1. Calves in groups T2, T3, and T4 were treated with doramectin pour-on on Days 0, 7, and 14, respectively. The control group (T1) received saline treatment on Day 0. General health observations were recorded on Days -1, 21, 28, 35, 42, and 49.

Each of the animals was inoculated orally by gavage with approximately 50 L_3/day of *Dictyocaulus viviparus* and 1,000 L_3/day each of *Ostertagia ostertagi* and *Cooperia* spp., concomitantly for 22 consecutive days (Days 14-35) to simulate a natural daily ingestion of larvae from a contaminated pasture. The two larval-viability monitor animals were given approximately 2,000 *D. viviparus* and 30,000 each of *O. ostertagi* and *Cooperia* spp. on Day 35 to confirm the viability of the inoculum at the end of the infection phase of the study. All isolates were obtained from the field during 1994 or 1995 (Tables 2-7). The *O. ostertagi* and *Cooperia* spp. isolates were mixed cultures containing other nematodes.

On each of Days 49 and 50 (Idaho) or 49-51 (Quebec), an equal proportion of cattle from each group were humanely killed for necropsy in accordance with the "Guide for the Care and Use of Laboratory Animals", DHEW Publication No. 86-23. The two larval-viability monitor animals were killed one each on days 49 and 50 (Idaho) or both on day 51 (Quebec). The lungs of each animal were cut open down all air passages as far as possible, squeezed under warm, running tap water over a collection container, and incubated overnight in the container at 37°C. After

incubation, the lungs were squeezed in the container and discarded. The incubate was then divided into two 50% aliquots, and each aliquot was sieved using a 200-mesh seive. Both aliquot residues retained by the sieve were preserved in formalin and later examined in their entirety for *D. viviparus*. Separately, the abomasum, small intestine, and large intestine were cut open longitudinally, their surfaces rubbed/stripped into a container, and the container filled to a known volume with tap water. Following stirring, two 2% aliquots were taken for each gastrointestinal organ. Each aliquot was washed over a 400-mesh sieve and the retained material was collected and preserved in formalin. A single 2% aliquot from each organ was subsequently examined for nematodes. All data are reported as total by species without breakdown by stage. Where males of only one species of a genus were found in a study, all females and larvae of the genus were considered to be the same species as the males. Females and larvae of the genus *Cooperia*, where males of more than one species were found in each study, were proportionally assigned to species based on the proportion as determined by the speciated males.

Statistical analysis

For each study as well as across the both studies, geometric mean worm burdens by species were calculated from the natural log (worm burden + 1), where worm burden equals actual count (*D. viviparus*) or aliquot count x 50 (gastrointestinal nematodes). The percentage reduction for each nematode was calculated for the doramectin-treated groups (T2 - T4) using the following formula:

$$\text{Percentage Reduction} = \left[\frac{\begin{array}{l}\text{(Geometric mean worm burden in saline-treated group) -} \\ \text{(Geometric mean worm burden in doramectin-treated group)}\end{array}}{\text{(Geometric mean worm burden in saline-treated group)}} \right] \times 100$$

Analysis of variance was used to determine if the mean natural log (worm burden +1) for each parasite species differed significantly ($P<0.05$ two tailed)

between the saline- and doramectin-treated groups. For each study, saline-treated animals with a parasite burden of zero for a particular species and an equal proportion of zero burden animals from the doramectin-treated groups were excluded from the analysis for that species. No cross-study analysis was done for the two *Haemonchus* species, since each was present in only one study. With the exception of *Haemonchus* spp., only cross-study data are presented, since individual study and cross-study data were very similar.

Results and Discussion

No adverse reaction to treatment was observed in any of the calves. At the end of the Quebec study, however, some animals in the saline-treated group (T1) were showing signs of heavy parasite infection.

In addition to the three species targeted for efficacy evaluation (*D. viviparus*, *O. ostertagi*, and *C. punctata*), due to the use of field isolates, *Haemonchus placei* (Idaho), *Haemonchus contortus* (Quebec), *Cooperia oncophora*, and *Oesophagostomum radiatum*, were present in each study in sufficient numbers to provide useful information. Data on other contaminating species are not reported herein, since they were present in numbers too low to provide for meaningful assessment. Results for worm counts and percentage reductions at the various persistence intervals (time from treatment to last day of parasite challenge) in both studies are given in Table 2. In the Quebec study, the target parasites, *D. viviparus*, *O. ostertagi*, and *C. punctata* were recovered from 6, 10, and 10 saline-treated animals, respectively. In the Idaho study, *D. viviparus*, *O. ostertagi*, and *C. punctata* were recovered from 9, 9, and 10 saline-treated animals, respectively. Based on geometric means, through Day 35 post-treatment (PT), doramectin was ³91.9% (³98.1% overall) efficacious against *D. viviparus* and ³98.7% (³99.8% overall) effective against *O. ostertagi*, and through Day 28 PT, doramectin was ³95.1% (³99.0% overall) efficacious against *C. punctata* in the two studies. In

addition to the three target species, *Haemonchus* spp., *Cooperia oncophora*, and *Oesophagostomum radiatum*, were present in both studies in sufficient numbers to provide meaningful data (Table 2). In each case, 9-10 saline-treated animals harbored these contaminating species. Based on geometric means, doramectin provided ≥97.4% efficacy against the two *Haemonchus* species up to 35 days PT and ≥93.2% efficacy (≥98.7% overall) against *C. oncophora* and >99.9% efficacy (>99.9% overall) against *O. radiatum* for 28 days PT in each of the two studies. One or both larval viability monitors in each study harbored each of the nematode species discussed above, with the exception of *Ostertagia ostertagi* in the Idaho study. It is worth noting that even after 22 days of inoculations some control animals did not harbor certain species (Table 2), so it is not surprising that the single inoculation given to larval viability monitors did not establish in some animals.

Persistent efficacy of >87% has been reported for the injectable formulation of doramectin from necropsy studies conducted against *D. viviparus* (Weatherley *et al.*, 1993; Barton *et al.*, 1995; Burden and Ellis, 1997), *O. ostertagi* (Weatherley *et al.*, 1993; Conder *et al.*, 1997), and *C. oncophora* (Weatherley *et al.*) in cattle for up to 28 days for each species except *C. oncophora* where activity persisted for up to 21 days. In contrast, Rolfe and colleagues (1997) found similar levels of persistence (≥86%) only to days 21, 21, and 14 for *H. placei*, *O. ostertagi*, and *Cooperia* spp., respectively. Our results for the pour-on formulation of doramectin suggest that the topical formulation has at least an additional week of persistence compared to the injectable formulation against those species where data are available (*D. viviparus*, *H. placei*, *O. ostertagi*, and *C. oncophora*), except for *O. radiatum* where persistence intervals appear to be similar between the two formulations.

Reducing the number of anthelmintic treatments per year is one strategy for reducing drug pressure and combating the development of drug resistance. The persistent efficacy of doramectin allows for an extension of periods between

treatment, hence reducing drug pressure. In addition, persistent efficacy serves to reduce pasture contamination with infective larvae, directly improving productivity and reducing expenses.

Conclusion

A single topical application of doramectin pour-on, administered at a dosage of 500 µg kg^{-1}, provided persistent efficacy against challenge infections of *D. viviparus*, *Haemonchus* spp., and *O. ostertagi* for up to 35 days PT and against challenge with *C. oncophora, C. punctata,* and *O. radiatum* for up to 28 days PT. No adverse reaction to treatment was observed during these studies.

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Table 1 Experimental design

Group[a,b]	*n*	Treatment	Dose	Regimen	Route	Day of Treatment	Days of challenge with nematodes	Days of Slaughter[c]
T1	10	Saline	1 mL 10 kg^{-1}	SID x 1	Topical	0	14-35	49-50/49-51
T2	10	Doramectin	500 µg kg^{-1} (1 mL 10 kg^{-1})	SID x 1	Topical	0	14-35	49-50/49-51
T3	10	Doramectin	500 µg kg^{-1} (1 mL 10 kg^{-1})	SID x 1	Topical	7	14-35	49-50/49-51
T4	10	Doramectin	500 µg kg^{-1} (1 mL 10 kg^{-1})	SID x 1	Topical	14	14-35	49-50/49-51

[a] Two additional animals (non-treated) were included in the study to evaluate viability of larval inoculum on the last day of challenge.
[b] All animals were treated on Day -14 with fenbendazole orally at 10 mg kg^{-1}, and none exhibited trichostrongyle-type eggs in fecal samples taken on Day -1.
[c] Animals in the Idaho study were slaughtered on Days 49-50; those in the Quebec study were slaughtered on Days 49-51.

Table 2 Persistent Efficacy of Doramectin Pour-on at 500 µg kg^{-1} Against Daily Challenge with Various Nematodes on Days 14-35 - Worm Burdens and Percentage Efficacies (Based on Geometric Means) at Persistence Intervals of 21, 28, and 35 Days Post-treatment

Parasite	Treatment	Group Size[a]	Persistence Interval (Days)	Worm Burdens			Percentage Efficacy
				Geometric Mean	Range	*P* value[b]	
Dictyocaulus viviparus	T1-Saline	15	–	21	1 - 75	–	–
	T2-Doramectin	15	35	<1	0 - 4	0.0109	98.7
	T3-Doramectin	15	28	<1	0 - 14	0.0121	98.1
	T4-Doramectin	15	21	0	0 - 0	0.0087	100.0
Haemonchus placei	T1-Saline	9	–	106	40 - 240	–	–
	T2-Doramectin	9	35	3	0 - 60	0.0001	97.4
	T3-Doramectin	9	28	0	0 - 0	0.0001	100.0
	T4-Doramectin	9	21	0	0 - 0	0.0001	100.0
Haemonchus contortus	T1-Saline	10	–	960	300 - 2,450	–	–
	T2-Doramectin	10	35	4	0 - 300	0.0001	99.6
	T3-Doramectin	10	28	4	0 - 350	0.0001	99.6
	T4-Doramectin	10	21	2	0 - 100	0.0001	99.8
Ostertagia ostertagi	T1-Saline	19	–	419	20 - 2,800	–	–
	T2-Doramectin	19	35	1	0 - 100	0.0090	99.8
	T3-Doramectin	19	28	<1	0 - 100	0.0071	99.9
	T4-Doramectin	19	21	0	0 - 0	0.0063	100.0

[a] All saline-treated animals with zero burdens for a parasite species and an equal number of zero-burden animals from each doramectin-treated group were excluded from the analysis for the respective parasite species.

[b] Significance level of testing the null hypothesis (H_o) on mean natural log (worm count + 1) (H_o:saline-treated group = doramectin-treated group).

Table 2 (Cont'd) Persistent Efficacy of Doramectin Pour-on at 500 µg kg^{-1} Against Daily Challenge with Various Nematodes on Days 14-35 - Worm Burdens and Percentage Efficacies (Based on Geometric Means) at Persistence Intervals of 21, 28, and 35 Days Posttreatment

Parasite	Treatment	Group Size[a]	Persistence Interval (Days)	Worm Burdens			Percentage Efficacy
				Geometric Mean	Range	*P* value[b]	
Cooperia oncophora	T1-Saline	20	--	1,426	34 - 11,133	--	--
	T2-Doramectin	20	35	87	0 - 5,429	0.1724	93.9
	T3-Doramectin	20	28	19	0 - 6,870	0.0710	98.7
	T4-Doramectin	20	21	2	0 - 603	0.0280	99.
Cooperia punctata	T1-Saline	20	--	1,037	66 - 4,565	--	--
	T2-Doramectin	20	35	50	0 - 1,819	0.1209	95.2
	T3-Doramectin	20	28	10	0 - 2,226	0.0490	99.0
	T4-Doramectin	20	21	1	0 - 253	0.0226	99.9
Oesophagostomum radiatum	T1-Saline	20	--	655	64 - 3,316	--	--
	T2-Doramectin	20	35	11	0 - 780	0.0763	98.3
	T3-Doramectin	20	28	<1	0 - 12	0.0250	>99.9
	T4-Doramectin	20	21	<1	0 - 12	0.0241	>99.9

[a] All saline-treated animals with zero burdens for a parasite species and an equal number of zero-burden animals from each doramectin-treated group were excluded from the analysis for the respective parasite species.

[b] Significance level of testing the null hypothesis (H_o) on mean natural log (worm count + 1) (H_o:saline-treated group = doramectin-treated group).

III. PUBLICATIONS, CONFERENCE PRESENTATIONS AND AWARDS ARISING FROM THIS THESIS RESEARCH

Publications:

Molento, M. B., Lifschitz, A., Sallovitz, J., Lanusse, C., and Prichard, R. Influence of verapamil on the pharmacokinetics of the antiparasitic drugs ivermectin and moxidectin in sheep. Journal of Pharmacology and Experimental Therapeutics, submitted

Molento, M. B., and Prichard, R. K., 1999. Effect of multidrug resistance modulators on the activity of ivermectin and moxidectin against larvae of ivermectin- or moxidectin-selected *Haemonchus contortus*. Parasitology Research, submitted

Molento, M. B., and Prichard, R. K. Nematode control and the possible development of anthelmintic resistance. Journal of the Brazilian Society of Veterinary Parasitology, in press

Molento, M. B., and Prichard, R. K., 1999. Effects of the multidrug-resistance-reversing agent, verapamil and CL 347,099, on the efficacy of ivermectin or moxidectin against unselected and drug-selected strains of *Haemonchus contortus* in jirds (*Meriones unguiculatus*). Parasitology Research, 85: 1007-1011

Molento, M. B., Wang, G. T., and Prichard, R. K., 1999. Decrease ivermectin and moxidectin sensitivity in *Haemonchus contortus* selected with moxidectin over fourteen generations. Veterinary Parasitology, 86: 77-81

Molento, M. B., Trudeau, C., Prichard, R. K., Zimmerman, G., Johnson, E., Marley, S., and Conder, G., 1999. Persistent efficacy of doramectin pour-on against

artificially induced infections of nematodes in cattle. Veterinary Parasitology, 82: 297-303

Xu, M., **Molento, M.**, Blackhall, W., Ribeiro, P., Beech, R. and Prichard, R. 1998. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Molecular and Biochemical Parasitology, 91: 327-335

Conference presentations:

Molento, M. B., Lifschitz, A., Sallovitz, J., Lanusse, C., and Prichard, R. Verapamil modifies the pharmacokinetics of ivermectin in sheep. 17th Conference of the World Association for the Advancement of Veterinary Parasitology, Aug/1999. Copenhagen, Denmark.

Molento, M. B., and Prichard, R. K. Comparative activity of MDR modulators against resistant *Haemonchus contortus*. 73rd Annual Meeting of the American Society of Parasitologists, Aug/1998. Kona, Hawaii, USA.

Molento, M. B., and Prichard, R. K. Reversal of resistance in ivermectin and moxidectin-selected strains of *Haemonchus contortus* by co-administration of MDR modulators. 10th Meeting of the Brazilian Society of Veterinary Parasitology, Oct/1997. Itapema, Santa Catarina, Brazil.

Molento, M., Wang, G., and Prichard, R. Activity of moxidectin and ivermectin against moxidectin selected strains of *Haemonchus contortus* in jirds. 16th Conference of the World Association for the Advancement of Veterinary Parasitology, Aug/1997. Sun City, South Africa.

Molento, M., and Prichard, R. Enhanced activity of moxidectin and ivermectin against selected strains of *Haemonchus contortus* in jirds by co-administration of the MDR-reversing agent verapamil. 16th Conference of the World Association for the Advancement of Veterinary Parasitology, Aug/1997. Sun City, South Africa.

Zimmerman, G., Johnson, E., Prichard, R., **Molento, M.**, Trudeau, C., Marley, S., and Conder, G. Persistent efficacy of doramectin pour-on against artificially-induced infections of nematodes in cattle. 42nd Annual Meeting of the American Association of Veterinary Parasitologists, Jul/1997. Reno, Nevada, USA.

Awards and Scholarships:

1999 McGill Alma Mater Student Travel Award - McGill University, Canada

1999 World Association for the Advancement of Veterinary Parasitology - Local Organizing Committee Scholarship, Copenhagen, Denmark

1998 Blair Postgraduate Fellowship - Blair Farm Estate, Chateauguay Valley, Canada

1997 Blair Postgraduate Fellowship - Blair Farm Estate, Chateauguay Valley, Canada

1997 World Association for the Advancement of Veterinary Parasitology - Local Organizing Committee Scholarship, Sandown, Gauteng, South Africa

1997 McGill Alma Mater Student Travel Award - McGill University, Canada

1996 Blair Postgraduate Fellowship - Blair Farm Estate, Chateauguay Valley, Canada

1996-1999 Differential Fee Waiver - McGill University, Montreal, Canada

1994-1996 International Fee Exemption - Brazil and Quebec Government, Quebec City, Canada