Functional assay of *Haemonchus contortus*  
P-glycoprotein-A  
and interaction with macrocyclic lactones

Pablo Godoy

Institute of Parasitology  
McGill University  
Montreal, Canada

Supervisor: Dr. Roger K. Prichard, PhD

A thesis submitted to Faculty of Agricultural and Environmental Sciences, McGill University in partial fulfillment with the requirements of the degree of Master in Science

August 2010

© Pablo Godoy 2010
Abstract

Macro cyclic lactone (ML) resistance has been described in the parasitic nematode, *Haemonchus contortus*. One of the mechanisms involved could be the over expression of P-glycoproteins (Pgps) which are ABC transporters. These proteins may influence the concentration of MLs that reach their target. In *H. contortus* one ABC transporter that is overexpressed in ML resistant parasites is Pgp-A (*HcPgp-A*). The goal of this project was the expression of *HcPgp-A*, in transfected LLC-PK1 cells, and to see the effect of ivermectin and moxidectin on inhibition of rhodamine 123 transport by the transfected cells. Rhodamine123 was actively transported by *HcPgp-A*. Ivermectin was four fold more potent at inhibiting rhodamine 123 transport by *HcPgp-A* than was moxidectin. The work provides the first information showing that MLs can inhibit the transport of Pgp substrates by a parasitic nematode ABC transporter and may indicate an active role for *H. contortus* Pgps in ML resistance.
La résistance aux lactones macrocycliques (LM) est bien connue chez le parasite némate Haemonchus contortus. La surexpression de P-glycoprotéines (Pgp), qui sont des ABC transporteurs, pourrait être impliquée dans un des mécanismes de la résistance aux LM. Ces protéines peuvent influencer la concentration de LM qui atteint leur cible. Pgp-A (HcPgp-A) est un ABC transporteur d’H. contortus qui est surexprimé chez les parasites résistants aux LM. Le but de cette étude était d’exprimer la P-glycoprotéine-A d’H. contortus dans descellules transfectées LLC-PK1 afin d’évaluer les effets de l’ivermectine et de la moxidectine sur l’inhibition du transport de la rhodamine 123. La rhodamine 123 s’est avérée être transportée activement par HcPgp-A. Les effets de l’ivermectine sur l’inhibition du transport de la rhodamine 123 par HcPgp-A étaient quatre fois plus importants que ceux de la moxidectine. L’étude a montré pour la première fois que les LM pouvaient inhiber le transport des substrats de la P-glycoprotéine grâce à un ABC transporteur d’un némate parasite. Cette information pourrait indiquer un rôle actif des Pgps d’H. contortus dans la résistance aux LM.
Acknowledgement

I would like to give my grateful acknowledgement to all the people who I have been working here, at the Institute of Parasitology, McGill University. It has been a great and challenging experience to pursue graduate studies in this academic unit, and I hope to continue contributing and improving my knowledge in science.

I want highlight my enormous gratitude to Dr. Roger K. Prichard, for gave me the opportunity to join to his laboratory. Since my previous arrival to McGill and then during my permanency having his supervision through my graduate studies, Dr. Prichard has been supporting me unconditionally with all kind of topics that research involves. I will be indebted with him, because of the great chance to start a parasitology research career under his mentorship. I also want to acknowledge my advisory committee members, Dr. Robin Beech and Dr. Elias Georges, for their fundamental collaboration and guidance, and time spent in constructive discussions to help me to materialize my project research.

I would like to thank to all my labmates at Dr. Prichard’s lab, certainly starting by Katy Keller for her outstanding support and collaboration for our laboratory. Along I want to thank to my dear labmates: Aissatou, Vijay, Catherine, Virginie, Ludmel, Dr. Yan, Shoaib, Nour and Thangadurai for their camaraderie. Also to my past labmates that I shared many time such as Andrea, Mike and Rodrigo. I want also to really thank to Jing Lian for her extraordinarily help with the experiments and transfered knowledge to complete my project. The whole members of Dr. Prichard’s laboratory, make a multicultural group from what I have learnt and I am still learning from their experience and I have realized that they have become a great supporting team spirit that have encouraged me to carry out with my studies at McGill.
Contribution

With this work I have tried to contribute to the understanding of the mechanisms of resistance of *Haemonchus contortus* to the MLs. In agreement with data in the literature, we have demonstrated the involvement of a ABC transporter, *Hc*Pgp-A. We have contributed to this topic, by being able to express this nematode P-gp and also shown, through a functional assay, its interaction with the MLs, IVM and MOX.

The evidence of *Hc*Pgp-A activity in the efflux of IVM and to a less extent of MOX, could develop into interesting field for further reaearch, in order to study the transport activity of this *H. contortus* ABC transporter and try to modulate its activity for the purpose to enhancing the efficacy of MLs. The expressed *Hc*Pgp-A could be used for screening of different compounds that may inhibit the parasite’s Pgp to enhance anthelmintic activity, and add information and advance parasitology research.
Table of contents

Abstract...........................................................................................................................................2

Abrégé.............................................................................................................................................3

Acknowledgments..........................................................................................................................4

Contribution.....................................................................................................................................5

Table of contents.............................................................................................................................6

List of Abbreviations.......................................................................................................................8

List of Figures and Tables..............................................................................................................9

Chapter I: Introduction..................................................................................................................11

Chapter II: Literature review.........................................................................................................14

1. Haemonchus contortus life cycle.................................................................................................14

2. Anthelmintic chemotherapy........................................................................................................15

3. Mechanisms of anthelmintic resistance.....................................................................................20

3.1 Benimidazoles resistance........................................................................................................21

3.2 Imidiothiazoles resistance........................................................................................................22

3.3 Macro cyclic lactones resistance............................................................................................22

3.3.A. Ligand-gated-ion channels ...................................................................................................23

3.3.B. ABC-binding cassette transporters ....................................................................................23

3.3.C. P-glycoproteins...................................................................................................................25

4. Physiological role of P-glycoproteins.........................................................................................26

5. Mechanisms of P-glycoproteins mediated multidrug resistance............................................29

6. Homologues of P-glycoproteins in nematodes.........................................................................30

Objectives........................................................................................................................................32
Chapter IV: Material and Methods

4.1. Parasites
4.2. RNA extraction
4.3. Reverse transcriptional polymerase reaction (RT-PCR)
4.4. Polymerase chain reaction (PCR)
   4.4.1. Amplification of full length sequence of Haemonchus contortus Pgp-A
4.5. Cloning of Haemonchus contortus Pgp-A
   4.5.1. Purification of Haemonchus contortus Pgp-A
4.6. Mammalian cell culture
   4.6.1. Transfection of Haemonchus contortus Pgp-A in LLC-PK1 cells
   4.6.2. Establishment of LLC-PK1 transformants stably expressing Haemonchus contortus Pgp-A
   4.6.3. CAT/pcDNA 3.1(+) Detection of transfection efficiency
   4.6.4. DNA Integration and Transcription detection
4.7. Drug transport assays
   4.7.1. Fluorescence measurement

Chapter V: Results

5.1. Amplification of full length sequence of Haemonchus contortus Pgp-A
5.2. Cloning of Haemonchus contortus Pgp-A
5.3. Protein alignment and conformational analysis
5.4. Stable transfection of Pgp-A/pcDNA 3.1(+)
5.5. Drug transport assays

Chapter VI: Discussion

Literature references
List of Abbreviations

AAD: amino-acetonitrile derivative
ATP: adenosine-5'-triphosphate
BBB: blood brain barrier
BZ: benzimidazole
CAT: chloramphenicol acetyl transferase
cDNA: complementary deoxyribonucleic acid
CYP450: cytochrome P450
DNA: deoxyribonucleic acid
FBS: fetal bovine serum
GABA: γ-amino-butyric acid
GluCl: glutamine-gated chloride channel
G418®: Neomycin
IC50: half maximal inhibitory concentration
LgIcs: ligand-gated-ion channels
LogP: octanol/water partition coefficient
IMDT: imidothiazole
IVM: ivermectin
L1, L2, L3: larvae stage 1, 2, 3, respectively
LEV: levamisole
Mdr1a: murine multidrug-resistance gene isoform 1a
MDR: multidrug-resistance
ML: macrocyclic lactone
MOX: moxidectin
MRP: multidrug resistance protein
nAchR: nicotinic acetylcholine receptor
NBD: nucleotide binding domain

P-gp: P-glycoprotein
PCR: polymerase chain reaction
Rho123: rhodamine 123
RNA: ribonucleic acid
SNP: single nucleotide polymorphism
STHs: soil-transmitted helminths
RT-PCR: reverse-transcription PCR
TMD: transmembrane domain
THP: tetrahydropyrimidines
VPL: verapamil
VSP: valsapodar
List of Figures and Tables

Chapter III: Literature review

Figure 1. Ivermectin structure..................................................................................................................19
Figure 2. ABC transporter family.............................................................................................................25
Table 1. Substrates of P-glycoprotein transport activity............................................................................27
Table 2. Inhibitors of P-glycoprotein........................................................................................................28

Chapter IV: Material and methods

Figure 1. Subcloning vector Pjet1.2. plasmid structure
multiple cloning sites......................................................................................................................................44
Figure 2. Mammalian expression vector pcDNA3.1(+) plasmid structure with multiple cloning sites 
..........................................................................................................................................................45
Figure 3. Chloramphenicol Acetyl Transferase (CAT), plasmid structure.................................................46
Figure 4. LLC-PK1 cells. Origin: Pig kidney epithelium............................................................................47

Chapter V: Results

Figure 5. Sequence alignment between HcPgp-A/LLC-PK1 and the published sequence of HcPgp-A........................................................................................................................................55-59
Figure 6. Protein alignment of HcPgp-A/LLC-PK1 and the published sequence of HcPgp-A........................................................................................................................................60
Figure 7. A. PCR amplification of full length sequence of HcPgp-A.........................................................61
Figure 7. B. Restriction digestion with Not I and XbaI, on the HcPgp-A construct into Pjet1.2........................61
Figure 8. A. Restriction digestion with Not I and XbaI on construct HcPgp-A/pcDNA3.1(+)........................62
Figure 8. B. Linearisation of HcPgp-A/pcDNA3.1(+) construct with MfeI..............................................62
Figure 9. Transmembrane domains prediction for HcPgp-A.....................................................................63
Figure 10. A. SDS-PAGE CAT/LLC-PK1 cells ................................................................. 64
Figure 10. B. Western blot of CAT/LLC-PK1 cells .................................................... 64
Figure 11. A. Reverse transcription PCR amplification of full length
sequence of HcPgp-A from transfected HcPgp-A/LLC-PK1 cells ........................................ 65
Figure 11 B. PCR amplification of full length sequence from transfected HcPgp-A/LLC-PK1 cells ............................................................ 65
Figure 12. Intracellular accumulation of Rhodamine 123 ............................................. 66
Figure 13. Inhibition of the Rho123 transport by MLs in HcPg-A/LLC-PK1 cells ............ 67
Figure 14. Inhibition of Rho123 transport by MLs in mdr1a/LLC-PK1 cells ..................... 68
Chapter I

Introduction

*Haemonchus contortus* is a parasitic nematode which is spread worldwide and is a major causative agent of decreased animal production along with disease in large animals (O'Connor *et al.*, 2006). There is strong evidence that this nematode has developed drug resistance to most of the anthelmintics available in the market.

Drug resistance in parasites, such as, *H. contortus*, is one of the big problems that is present in modern animal production (Jackson and Coop, 2000). The control of nematodes in livestock depends strongly on anthelmintic drugs and it does not seem that this scenario will change in the foreseeable future. As a result of the reliance on chemotherapy, anthelmintic resistance has arisen in *H. contortus* and other economically important nematodes (Lespine *et al.*, 2008). Thus, it has become critical to try to understand the mechanisms involved in drug resistance related to gastro-intestinal nematodes.

Infection with nematodes has a negative impact on livestock, which although mortality is not high, causes increases in morbidity and decreases in productivity (Perry and Randolph, 1999). Drug resistance is widespread in *H. contortus* and in many parts of the world (Conder and Campbell, 1995). For instance it has been reported in Australia that this parasitic nematode affects ovine production by 12% in terms of the meat and milk (Barton, 1983). Furthermore, *H. contortus* has developed resistance to all of the widely used broad spectrum anthelmintics such the benzimidazoles (BZ), the macrocyclic lactones (MLs) and levamisole (LEV).
Because of the importance of this parasitic pathogen in livestock, it is important to try to find out the mechanisms involved in drug resistance to anthelmintics. It is very expensive for the pharmaceutical industry to find a new drug for helminths, in fact it was estimated that the cost of developing a new anthelmintic drug product is around US$40 million for livestock (Brown et al., 2006). Resistance is a challenge and a new interest in parasitology research is to figure out the complexity of drug resistance in parasitic nematodes such as *H. contortus*.

This parasitic pathogen belongs to the big group of Trichostrongylid worms that affect livestock. It is endemic in many countries in the world, especially those with a subtropical or Mediterranean climate (Vercruysse, 1985). *H. contortus* has been treated for many years with different kinds of anthelmintic such as BZ and LEV, (Green *et al*., 1981). The MLs were developed in the late 1970’s and early 1980’s and introduced commercially in the 1980’s. MLs are important for the control of human and animal helminth infections (Molyneux *et al*., 2003; Kita *et al*., 2007). Following the development and use of modern anthelmintics, last century, drug resistance has been widely described in nematodes (Wolstenholme *et al*., 2004; Kaplan, 2004).

One of the possible mechanisms of drug resistance to MLs in *H. contortus* could involve the presence of homologs of Multi Drug Resistance transporters like P-glycoproteins (P-gps), (Xu *et al*., 1998).

These molecules are present in different nematodes such as the free-living *Ceanorhabditis elegans* and in those who parasitize animals such as *H. contortus* (Prichard and Roulet, 2007). P-gps belong to the super family of ABC transporters that bind to different substrates including drugs like MLs (Schinkel and Jonker, 2003).
The role of the P-gps in drug resistance mechanisms is not very clear. However, these membrane proteins could be facilitating efflux of the MLs to the outside of the cells, producing a decrease in the concentration of the drug in the parasite and thus, affecting the concentration of the anthelmintic available to kill the parasite (Lespine, et al., 2008). Drug resistance to MLs in *H. contortus* may be due to MLs selecting for some alleles of P-glycoprotein in the parasite possibly resulting in over expression of these ABC transporters, thwarting the effect of the drug inside the worm.
1. *Haemonchus contortus* life cycle

*H. contortus* is a member of the phylum *Nematoda*, class *Chromadorea*, order *Rhabditidia*, suborder *Strongylidea*, superfamily *Trichostrongyloidea*, genus *Haemonchus*, and species *contortus* (Hoberg et al., 2004). This nematode as well as other members of the Trichostrongyloidea superfamily, is part of the most pathogenic nematode triad for large animals known as the H-O-T (*Haemonchus, Ostertagia and Trichostongylus spp.*) worms.

Adult worms of *H. contortus* have a length of 2-3 cm. There are sexual dimorphic differences between male and female worms (Taylor, Coop and Wall, 2007). Usually the female is longer than the male worm and the male is characterized by its very well developed copulatory bags. The life cycle of this parasite begins from the adult worms located in the abomasum of the host, usually small ruminants like sheep and goats, where the female and male worm mate and then the female produces eggs which are eliminated in the host stool and disseminate in the environment (Rossanigo and Gruner, 1995). Once the eggs hatch a larva (L1) is released which develops consecutively to L2 and L3, as free-living stages. The L3 or infective stage is developed inside the L2’s cuticle, on the pasture and can be then ingested by the host (O’Connor et al., 2007).
Once the larva reaches the abomasum, the L3 develops to L4 and juvenile adult stage which starts to feed from the blood (Bethony et al., 2006). Finally the adult stage produces eggs around 15 days after the parasite infects its host. Under optimal conditions, the *H. contortus* life cycle as well as other H-O-T worms is completed in three weeks (Rossanigo and Gruner, 1995).

The infection by *H. contortus* produces the disease of hemonchosis. When the load of parasites is high, they produce symptoms in the host such as anaemia, loss of weight and oedema (Jacobson et al., 2009). A self-cure phenomenon, can occur in some sheep (Adams, 1983). Currently it is well known that this cure corresponds to a hypersensitivity reaction which produces a physico-chemical change of the gastro-intestinal mucosa.

2. Anthelmintic chemotherapy

Different and diverse parasitic organisms are found in human and animals, creating a strong, host-parasite relationship, that has allowed parasites to persist through evolution. Helminths including nematodes, cestodes and trematodes, represent a significant group of endoparasites that affect and influence human and domestic animal welfare (James et al., 2009). Although after decades of research and development of alternative control strategies such as grazing management and biological tools like vaccines, helminth infections are still mainly controlled by anthelmintic drugs.

The first pharmacological compound to be used in livestock chemotherapy treatment was Phenothiazine in the 1940’s. Phenothiazine had a broad spectrum activity against microorganisms
it has been shown to possess insecticidal, antifungal, antibacterial and anthelmintic properties (Mitchell, 2006). However, after almost two decades of intense use resistance has developed in human and animal helminths (James et al., 2009).

In the early 1960’s, a new class of drugs that have strong activity against helminths, the Benzimidazoles, came to market. These antiparasitic drugs act by binding to the β-tubulin protein, therefore inhibiting the polymerisation of microtubules affecting the parasite cell structure (Lubega, 1991).

Members of this family include Thiabendazole, which was discovered in 1961 and marked the beginning of the modern anthelmintics, Albendazole, Mebendazole, Oxfendazole and Febendazole. There is also a subgroup of compounds which initially are inactive and then they are metabolized to the benzimidazole-carbamate ring in the host liver. They are known as Probenzimidazoles (Lacey, 1988). Some of these drugs are Febantel, Netobamin and Thiophanate.

After the BZs, a novel group of anthelmintics, the Imidothiazoles (IMDT), were developed. The most important member of these drugs is Levamisole (LEV), which has been extensively used in livestock as well as companion animals (Robertson and Martin, 1993). In addition to LEV having a remarkable effectiveness against trichostrongyloids and other helminths, it has an immunological effect promoting a T-cell immune response. Also there is another group of drugs, the Tetrahydropyrimidines (THP), including Pyrantel, Morantel and Oxantel. Both IMDT and THP, act selectively at synaptic and extra synaptic nicotinic acetylcholine receptors (nAchR) on
nematode muscles cells and produce contraction and spastic paralysis (Martin, 1997). After around a decade of use of the IMDT and THP anthelmintic resistance developed, mainly in gastro-intestinal nematodes of small ruminants such as *H. contortus* (James et al., 2009).

Since their launch to the antiparasitic drug market in the early 1980’s, MLs have shown a remarkable broad-spectrum of action in many parasites as well as good efficiency against ectoparasites of animal and human hosts (Omura, 2008). The success of MLs has been supported in their wide spectrum, safety profile and ease of administration (Prichard and Roulet, 2007). Although the MLs have been used in livestock, their use has been developed in order to treat some human helminth parasitosis such as Onchocerciasis and the Lymphatic filariasis (Osei-Atweneboana et al., 2007).

Macrocyclic lactones are large hydrophobic molecules, highly lipophilic, characterized by a macrocyclic lactone 16-membered ring as a shared structural component (Lespine et al., 2007). These chemotherapy drugs are synthetically derivative products from the fermentation of soil moulds *Streptomyces spp.* (Burg et al., 1979). The MLs are divided in two groups. One group corresponds to the Avermectins in which are included Ivermectin, Abamectin, Doramectin, Epinomectin and Selamectin. In addition to the macrocyclic ring, they share in their structure the presence of a sugar moiety such as oleandrose residues attached in the C13 of the macrocyclic ring (Shoop et al., 1995) (Fig. 1). The other group are the milbemycins, including moxidectin, which do not have the sugar moiety in their structure (Omura, 2008).
From the pharmacokinetic point of view, MLs distribute throughout the body in the blood and the lymph, producing good concentrations in the tissues (Lespine et al., 2006). The ability of MLs to reach an effective concentration for a suitable length of time in the target tissues is correlated with the systemic concentration, and this determines the antiparasitic drug activity (Lifschitz et al., 2000, Craven et al., 2002). The MLs lipophilic properties are important factors in the exchange between tissues and the blood (Bassissi et al., 2004). The mechanism of action of these drugs is to bind the glutamate and GABA receptors producing the opening of the ion channels and the subsequent entry of chloride ions that produce a hyperpolarisation of the nerve cell membrane leading to muscle paralysis (Forrester et al., 2002). Probably the most important effect in worms is the paralysis of the pharynx that causes the starvation of the worms and is followed by death of the helminth (Geary et al., 1993).

The most remarkable member of this kind of antiparasitic drug is Ivermectin (IVM). Since its discovery in the late 1970s, IVM has shown outstanding efficiency in comparison with the older anthelmintics such as the BZs and LEV. Because of its broad spectrum against helminths of humans and animals, IVM has been a leading anthelmintic product. IVM has given good responses against parasites that have become resistance to BZs and LEV. Because of their remarkable efficacy, MLs have been the cornerstone of chemotherapy against human and livestock parasitic diseases over the past 25 years.
Recently, the Amino-acetonitrile derivative (AADs), corresponding to a new class of chemically synthetic compounds with anthelmintic activity have been discovered (Kaminsky et al., 2008). These novel anthelmintic candidates seem to have a model of action, involving a unique clade of acetylcholine receptor sub-units. In C. elegans as well as in H. contortus adult worms, the AADs produced a hyper contraction of the body wall muscles and pharynx, leading to paralysis, spasmodic contractions and death. The AADs show a remarkable efficacy against some H. contortus multidrug resistant strains, and other veterinary nematodes. However, it is still necessary for further studies on AADs to understand the actual mechanism of action and how they can be efficiently used to become a real chemotherapy alternative for gastro-intestinal nematodes.
3. Mechanisms of anthelmintic resistance

Through the years, due to the indiscriminate and poor dosage strategy of anthelmintic drug usage, many parasites, included helminths of human and veterinary importance, have developed resistance to the drugs created to control them (Prichard, 2007). Resistance is described as occurring when there is a greater frequency of individuals in a population able to tolerate doses of a compound than in a normal population of the same species and is heritable, (Prichard et al., 1980). Resistance could also be pointed out as the proportion of worms in a population with increased resistance genotypes.

Side resistance is present when parasites resistant to one drug are also resistant to other drugs in the same class. Cross resistance, is when resistance occurs between unrelated chemicals, such as a Benzimidazoles and an organophosphate (Geary, 2003). There are several paths, involving genetic changes that can lead to anthelmintic resistance. Selection of anthelmintic drugs on single genes or alleles may produce a change in the gene encoding region of a target receptor leading to a lack of drug binding for the receptor. Also changes in gene expression may correlate with up-regulation of membrane transporters that extrude chemical compounds such as anthelmintic drugs, affecting a suitable drug concentration to reach the target (Lespine et al., 2008). Some of these genes, directly or indirectly involved in the genetics of anthelmintic resistance have been used as a biomarkers to understand and monitor the development of resistance in human and veterinary nematodes (Prichard and Roulet, 2007).
The research about the molecular mechanisms underlying the drug resistance process in parasitic worms has been focused on the three major and modern anthelmintic classes: MLs, BZs and IMDTs.

3.1. Benzimidazoles resistance

Although BZs have a wide spectrum against many human worms such as the soil-transmitted helminths (STHs), BZs resistance primarily occurs in veterinary nematodes such as *H. contortus* (Prichard, 2001). This resistance involves the β-tubulin isotype 1 gene selected by BZs. This selection is for a single nucleotide polymorphism (SNP) change in the nucleotide sequence in the codon translation of the tubulin protein. In *H. contortus*, the first SNP reported was at the position 200 of the amino acid chain, which corresponds to a switch from phenylalanine (F) to tyrosine (Y); thus changing the binding site of the BZ in the tubulin protein (Kwa et al., 1994). Further, another SNP was discovered at position 167 of the β-tubulin isotype 1 gene, also (F) to (Y), (Silvestre and Cabaret, 2002). Recently a new SNP has been reported from *in vitro* selection of *H. contortus* β-tubulin protein, indicating a point mutation at position 198 of the amino acid sequence switching from glutamate (E) to alanine (A) (de Lourdes Mottier and Prichard, 2008; Ghisi et al., 2007). This single mutation into the β-tubulin protein is reflected in a *H. contortus* BZ resistance phenotype (Rufener et al., 2009).

The mutation at position 200 in isotype 1 β-tubulin, has also been observed in human helminths such as *Trichuris trichura* (Diawara et al., 2009) as well as in veterinary nematodes such as *Teladorsagia circumcincta* (Silvestre and Cabaret, 2002) and *Cooperia oncophora* (Njue and Prichard, 2003). Interestingly, an association of these β-tubulin SNPs, in *H. contortus* ML
selected laboratory and field resistant strains, makes them, good molecular biomarkers to monitor the development of BZ and possibly ML resistance in parasitic nematodes (Mottier and Prichard, 2008).

3.2. Imidothiazoles resistance (IMDT)

The first evidence of resistance to LEV, was described in *C. elegans* mutant strains, after mutagenesis with ethyl methane sulphonate (EMS), when mutant worms could move and feed, even after exposure to a high concentration of LEV (Brenner, 1974). Electrophysiological experiments on the muscles of the pig roundworm *Ascaris suum*, showed that LEV acts as an agonist of nicotinic acetylcholine receptor (nARChs), (Martin et al., 1996). Further work showed that LEV resistance was associated with mutations in the genes that encode for nicotinic acetylcholine receptor (nARChs) subunits (Towels et al, 2005).

In ruminants, field isolates of gastro-intestinal nematodes such as *Trichostongylus colubriformis* and *Ostertagia circumcincta* have been described to have LEV and morantel resistance (Sangster et al., 1979). Usually if a population of parasitic nematodes is resistant to an IMDT like LEV, they also are resistant to a THD such as morantel, due to the fact that they share the same molecular target (Jones et al., 2005).
3.3. Macrocyclic lactones resistance

3.3.A. Ligand-gated-ion channels (LgIcs).

Invertebrates such as worms possess a rudimentary nervous system in comparison with large eukaryotic organisms, like mammals. However, both share a common target for MLs, the γ-amino-butyric acid (GABA) receptors (Campbell, 1985). Also, there is evidence that nematodes have unique kinds of channels, the glutamate-gated chloride channels (GluCls) (Cully et al., 1994). These latter authors characterized, in the free-living nematode *C. elegans* two subunits, GluCl-α and GluCl-β, which had higher sensitivity for MLs than did GABA receptors.

GluCls channel are composed of five subunits. In *H. contortus*, five genes encoding several GluCls subunits have been described (McCavera et al., 2007). ML resistance associated with GluCls in *H. contortus* has been associated with frequency changes of the GluCl-α subunit in laboratory strains alleles (Blackhall et al., 1998b). However, no coding polymorphism in GluCls has been associated so far, with ML resistance in field isolated of *H. contortus* that are resistant to MLs (McCavera et al., 2007).

3.3.B. ABC binding-cassette transporters.

The ABC binding-cassette transporter super family are well conserved cell membrane proteins expressed by both prokaryotes and eukaryotes (Sheps et al., 2004) (Fig. 2). These proteins bind ATP and use its energy to transport different molecules across the membrane. This group is composed of membrane protein transporters that can be classified in some families such as P-glycoproteins (P-gps), the multidrug resistance proteins 1, 2, 3, 4 and 5 (MRPs), and half
ABC transporters related to human neoplasia, such as the breast cancer resistance proteins (Schinkel and Jonker, 2003).

Essentially the conformation of the ABC transporter super family is common to all its members. This corresponds to the presence of transmembrane domains (TMD), which have loops. ABC transporters also have nucleotide binding domains (NBDs), which are present in the intracellular space. In their structure they also present a carbohydrate moiety, which is part of the functionality of the protein efflux (Kage et al., 2002; Cai and Gros 2003).

From the whole family of ABC transporters, the ABC-B subgroup has been associated with multidrug resistance (MDR) in cancer cells (Gottesman et al., 2002). Multidrug resistance can be defined as a simultaneous resistance to structurally unrelated compounds that do not have a common mechanism of action (Ambudkar et al., 1999).
Figure 2. ABC transporters are cell membrane proteins, with a shared basal structure such as: transmembrane domains and ATP-binding sites (Gottesman et al., 2002).

3.3.C. P-glycoproteins (P-gps).

P-glycoproteins are membrane proteins highly conserved in evolution and are associated with multidrug resistance in human cells (Kage et al., 2005). P-gps typically have a molecular weight of 170kD and between 1276 to 1281 amino acid residues (Chen et al., 1986). P-gp was recognized in humans as a multidrug resistant protein (MDR) associated with cancer tumor cells, after chemotherapy treatment failures (Juliano and Ling, 1976). Through lowering the intracellular drug concentration, P-gp causes tumor cells to be resistant to chemotherapy drugs. A wide variety of tumours express P-gp, and in different tumours the expression of P-gp may be different (Cotte et al., 2009). Neoplasias such as colon adenocarcinoma, and hepatocellular cancer cells, express P-gps to a relatively high level (Schinkel and Jonker, 2003), while other neoplasias
such as lung myeloma, lymphoma and acute myeloid leukemia, tumor cells express P-gp only after chemotherapeutic treatment or during relapse (Leith et al., 1999).

Structurally, P-gp is composed of two halves linked by a hydrophilic region. Each half has six transmembrane domains (TMD) and an intra cytoplasmic nucleotide-binding domain (NBD). In each NBD there are two highly conserved sequences: the Walker A and B motifs. These two motifs are thought to contribute to the ATP-binding and hydrolysis. The NBDs provide energy for P-gp to transport substrates out of the cell (Litman et al., 2001).

4. Physiological role of P-glycoprotein

ABC transporters such as P-gp, are expressed in many tissues and organs. In humans, P-gp is mainly expressed in the apical membrane of epithelia of excretory organs, such as the gallbladder, kidney and liver as well as in blood membranes such as the blood brain barrier (BBB) and placenta (Fromm, 2004), modulating the exchange of endogenous substrates and preventing the penetration of cytotoxins across the endothelium (Xie et al., 1999). The multi-resistant protein (MRP); MRP1, is normally expressed in the basolateral surface of Sertoli cells, and protects the sperm within the testicular tubules (Kool et al., 1999).

P-gps influence the pharmacokinetics (absorption, distribution and elimination) and bioavailability of many different drugs (Drescher et al., 2003). In vitro studies have shown that these membrane proteins can actively extrude a wide variety of substrates including: heavy metals, exogenous toxins, endogenous xenobiotic compounds like toxic peptides, steroids and
many chemotherapy drugs such as, anticancer, antibiotics and antiparasitic drugs, as well as fluorophores (Table 1). On the other hand, P-gp function has been studied by following the active transport of fluorophores.

In mammals, some ABC transporters genes associated with MDR, such as the human MDR1 gene that encodes P-gp, have been extensively studied. It has been reported that the disruption of the mdr1a P-glycoprotein gene, in the mouse, leads to a marked increase of sensitivity to IVM into the central nervous system (Schinkel et al., 1995). Alterations in the pharmacokinetics behaviour (a reduced rate of elimination), and altered tissue distribution (increased IVM concentration in the brain) was also observed (Schinkel et al., 1997; Schinkel et al., 1995) in mdr1a knock-out mice. In the absence of drug, knock-out mice did not display phenotypic abnormalities. These results suggested that P-gp genes may provide protection against exogenous compounds

**Table 1.** Substrates of P-glycoprotein transport activity (Gillet and Gottesman, 2010).

<table>
<thead>
<tr>
<th>No.</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Anticancer drugs:</td>
</tr>
<tr>
<td></td>
<td>a) Anthracyclines: Doxorubicin, Epirubicin, Daunorubicin.</td>
</tr>
<tr>
<td></td>
<td>b) Anthracenes: Mitoxantrone, Bisantrene.</td>
</tr>
<tr>
<td></td>
<td>c) Antimicrotubule agents: Colchicine, Paclitaxel, Docetaxel.</td>
</tr>
<tr>
<td></td>
<td>d) Polypeptides: Actinomycin D.</td>
</tr>
<tr>
<td>5.</td>
<td>HIV-protease inhibitors: Ritonaquir, Indinavir, Saquinavir.</td>
</tr>
<tr>
<td>7.</td>
<td>Steroids: Aldosterone, Dexamethasone</td>
</tr>
<tr>
<td>8.</td>
<td>Anthelmintics: Ivermectin</td>
</tr>
<tr>
<td>10.</td>
<td>Other: Emetine, Puromycin, Mithramycin, Ethidium bromide</td>
</tr>
</tbody>
</table>
Because of their influence and preponderant role in unsuccessful cancer chemotherapeutic treatment, P-glycoproteins have been tested with different compound or multi-drug resistance reversing agents that block and decrease P-gp mediated efflux (Fischer et al., 1998). In order to modulate P-glycoprotein drug transport activity, initial clinical trials with P-glycoproteins inhibitors (Table 2), included the calcium-channel blocker verapamil (VPL), the cyclosporin analogue PSC 833 or Valspodar (VSP), have been tried in tumor chemotherapy (Fracasso et al, 2001). VSP is thought to be as an effective inhibitor, because of its high-affinity to MDR1 P-gp and its release from P-gp is very slow (Smith et al., 1998).

Table 2. Inhibitors of P-glycoprotein (Choi, C.H., 2005).

<table>
<thead>
<tr>
<th>Inhibitors of P-glycoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Calcium channel blockers: Verapamil, Nifedipine, Azidopine, Diltiazem</td>
</tr>
<tr>
<td>2. Cyclosporin A analogues: PSC 833</td>
</tr>
<tr>
<td>3. Antifungals: Ketoconazole, Itraconazole</td>
</tr>
<tr>
<td>4. Antiarrhythmics: Digoxine, Quinidine, Propafenone, Amidarone</td>
</tr>
<tr>
<td>5. Antimalarial drugs: Mefloquine, Quinine</td>
</tr>
<tr>
<td>6. Hormones: Progesterone, Spirinolactone</td>
</tr>
<tr>
<td>7. Calmodulin antagonists: Trifluoperazine, Chlorpromazine, Fluphenazine</td>
</tr>
<tr>
<td>8. Other Drugs: GF120918, N-acetyl daunorubicin, Quercetin, Pluronic 85</td>
</tr>
</tbody>
</table>
5. Mechanisms of P-glycoprotein mediated multidrug resistance

Constitutive evidence from MDR tumor cells over expressing MDR1 P-gp indicates a major role for this transporter in the outcome of cancer chemotherapy. Increased gene amplification leads to increased gene copies, or changes in the promoter region of the gene cause high levels of RNA transcription (Kantharidis et al., 2000). An alternative pathway which may lead to over expression of the MDR1 gene in humans, such as the modification or alteration near the MDR1 locus, as can occur with abnormalities, has been proposed. For instance an up regulation of P-gp expression has been observed in patients who have been under exposure to irradiation or chemotherapy. P-gp is more often expressed in association with chromosome 7 abnormalities (de Silva et al., 1996).

On the other hand, P-gp may be involved in multidrug resistance as a result of changes in its amino acid sequence. These changes may modify the substrate specificity or cause an increase in the drug pumping efficiency of P-gp (Sangster, 1994). Structural analyses of the P-gp composition and folding conformation, predict that substrates may bind to some particular regions in the P-gp structure.

In humans, the transmembrane (TM) domains 5, 6, 11 and 12 are involved in drug binding (Loo and Clarke, 1999). Mutational analyses in human P-gp also indicated that point mutations in the TMs such as those addressed above, could alter substrate and binding specificity of P-gp (Zhang et al., 1995).
6. Homologues of P-gps in Nematodes

In helminths, the free-living nematode *Caenorhabditis elegans*, has been used as a model to understand the physiological role of ABC transporters such as P-gp and its involvement in drug resistance (Broeks et al., 1995). In *C. elegans* at least 14 P-gps gene have been described, whereas in humans and rodent, 2 and 3 Pgp-s genes, respectively, related to MDR in tumor cells have been described (Bosch and Croop, 1998).

The presence of a high abundance of P-gps in *C. elegans*, may imply that these membrane proteins represent a complex mechanism to protect this free-living nematode against the multitude of toxins in the environment. The presence of ABC transporters have also been described in parasitic nematodes of livestock such as *Ostertagia ostertagi*, *Trichostrongylus spp*, and *Cyathostomus spp* (Drogemuller et al., 2004). Additionally, there are ABC transporters in human parasitic nematodes such as the filarial worm *Onchocerca volvulus*, which is responsible for River blindness; a disease affecting human population in many tropical countries in the world (Ardelli et al., 2005). After sequence analysis of resistance markers to IVM in the *O. volvulus* genome of filarial worms of patients treated in Ghana, Ardelli and Prichard, (2004) found a decrease in genetic polymorphisms of two ABC transporter genes, which were designated OvMDR-1 and Ov-ABC-1. The presence of two other ABC transporters, Ov-PGP-1 and Ov-PLP appear to be involved in ML resistance in *O. volvulus* (Ardelli and Prichard, 2004).

*H. contortus* was the first veterinary nematode that was described to have a P-glycoprotein homolog that may be involved in ML resistance (Blackhall et al., 1998b; Le Jambre et al., 1999; Xu et al., 1998). Moreover, these latter authors found evidence of a higher level of
expression in IVM-resistant *H. contortus* isolates in comparison with an unselected parental strain. Furthermore, *in vivo* pharmacokinetics experiments in jirds infected with *H. contortus* ML resistant strains, using the MDR reversing agent Verapamil, enhanced the efficacy of IVM and MOX against these resistant strains (Molento and Prichard, 1999).

From the *H. contortus* genome, six putative P-gps, have been identified in the literature, including the full-length cDNA sequence of *HePgp*-A (Prichard and Roulet, 2007). A linker region that joins the two P-gp halves has been described in *H. contortus*. This region is the most variable region in terms of amino acid sequence (Roulet et al, manuscript in preparation). Therefore, this section in the P-gp structure allows the differentiation between the six P-glycoproteins previously described in *H. contortus* (Prichard and Roulet, 2007). Moreover, these authors pointed out that after an induction with MLs (IVM or MOX) in different *H. contortus* experimentally selected ML resistance strains (IVM23 and MOX23) and also in a field resistance strain (Wallangra 2003) there was an up-regulation of three P-glycoproteins, namely P-gp A, E and C taking as a reference the PF or ML susceptible *H. contortus* (Roulet et al., manuscript in preparation).

Because of the numerous associations of Pgps with ML resistance in parasitic nematodes, it was of interest to express *H. contortus* Pgps in a cell expression system and study the effect of MLs on transport properties of the expressed nematode Pgp.
Objectives

1. The expression of *Haemochus contortus* P-glycoprotein-A in LLC-PK1 cells.

2. To assess inhibition by IVM or MOX of Rho123 transport by *H. contortus* P-glycoprotein-A.

3. To determine whether there are any differences between MOX and the avermectins in *H. contortus* P-glycoprotein-A transport.

4. Confirmation of the relevant role of *H. contortus* P-glycoproteins in the interaction with MLs (IVM + MOX) in the drug resistance process.
Chapter IV

Material and Methods

4.1. Parasites.

The parasites were obtained from adult sheep infected with *H. contortus* PF strain. This strain is the parental line which has been maintained in sheep without anthelmintic treatment and is susceptible to IVM and other MLs (Wang, 2002). Worms were collected from the abomasum of sheep and incubated in PBS (phosphate saline buffer) for 2 hours at 37ºC. Worms were originally supplied by Fort Dodge Animal Health, Princeton, NJ, USA and are maintained by our laboratory.

4.2. RNA extraction

Worms were homogenized and the homogenate lysed according to the manufacturer's instruction using the Trizol® Reagent (Invitrogen Life Technologies Burlington, ON). For the RNA tissue extraction, 400µl per worm were used. Then, the suspension was incubated at room temperature for 5 minutes and then 100µl chloroform added per 400µl of Trizol used and the tube shaken vigorously for 15 seconds. Then, the extract was incubated at room temperature for 2-3 minutes. The sample was centrifuged at 12,000x g for 15 minutes at 4ºC. 400µl of the colorless upper phase was taken and transferred to a new tube. An equal volume of isopropyl alcohol was added to the sample and then incubated at room temperature for 10 minutes. After, the sample was centrifuged at 12,000x g for 10 minutes at 4 ºC temperature. The supernatant and the RNA pellet were washed with 1ml of 75% ethanol. Then, the sample was mixed, vortexed and centrifuged at 7,500 x g for 5 minutes at 4 ºC. After that, the RNA pellet was dried for 5-10 minutes. The RNA pellet was eluted in 50µl of RNase free water. The RNA concentration was measured spectrophotometrically at 280nm wave length.
4.3. Reverse Transcriptional Polymerase Reaction (RT-PCR).

The reverse transcription reaction was executed following the Omniscript® Reverse Transcription protocol, using DNA wipeout buffer (Qiagen, Hilden, Germany). Prior to the RT-PCR reaction, 5ng of the RNA extract were taken, and 1µl of EDTA was added to the reaction mix and maintained at 70° C for 10 minutes. The solution was then used for RT-PCR in a thermocycler. In order to get a single strand of complementary DNA (cDNA), 2µl of reverse transcriptional first strand reaction buffer were added to the mixture, 2µl of dNTP mix (5mM), 1 µl RNase, 1 µl Omniscript RT and RNase free water to complete 20µl for each tube. The reaction was incubated at 37ºC for 90 minutes and then at 70 ºC for 10 minutes.

4.4 Polymerase Chain Reaction (PCR)

4.4.1. Amplification of full length *H. contortus* P-glycoprotein A cDNA.

From the freshly extracted *H. contortus* RNA and cDNA, specific primers to amplify the full length of P-glycoprotein A (Accession number: AF003908, GenBank) were designed using the software Generunner version 3.05 as follow: Forward primer 5’GTGCACAAGCTTGCCAACCATGTTTGCAGAAAAGGCAAGA’3, in order to improve the translation efficiency, a Kozak sequence was included (Kozak, 1987), shown underlined before the starting codon. The reverse primer was: 5’TCATTGTGATTCAACGAGTGT’3.

The reaction mixture was composed of 2µl of the cDNA product, 2µl of PCR buffer 10X, 0.4µl dNTP mix 10mM, 0.6µl MgCl 50mM, 0.5µl of each primer described above, 0.2µl of proof-reading Taq polymerase and pure water to a volume of 50µl of reaction. The procedure for the reaction was a denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 57
°C for 45 sec, and 72 °C for 6 min with a final extension of 72 °C for 10 min. PCR products were run in a 1% agarose gel electrophoresis.

4.5 Cloning of *H. contortus* P-glycoprotein-A gene.

The full length cDNA sequence of PF *H. contortus* P-glycoprotein A, was cloned into a sub-cloning vector (pJet 1.2, Fermentas, International Inc., Burlington, ON) (Fig. 1). Prior to cloning, *HcPgp*-A PCR product was run in 0.7% agarose-gel and purified using the GenJET gel extraction kit (Fermentas) according to the manufacturer’s instructions. The purified PCR product was resuspended in RNA-free water. The cloning reaction was made from the following components: 10µl of 2X reaction buffer; 1µl of PCR purified product, 1µl of pJet1.2/blunt cloning vector (50ng/µl), 1µl of T4 Ligase and 7µl of RNA-free water to a final volume of 20µl. The mixture was incubated at room temperature for 5 minutes. Then a chemical transformation reaction using *Escherichia coli* bacteria, strain TOP10F’competent cells (Invitrogen Life Technologies, Burlington, ON) was undertaken. The bacteria were cultured in LB agar with Ampicillin (100µg/ml) in an overnight incubation at 37 °C. Positive transformants were identified as white colonies carrying plasmids with the correct insert.
4.5.1. Purification of the *H. contortus* P-glycoprotein A.

From those selected clones, the DNA of *HcP-gpA* was purified using a plasmid purification kit Miniprep, (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. A 1% agarose gel electrophoresis was run to identify the full length sequence of *HcP-gpA* (DNA band of approx. 4.0Kb), and the plasmid vector pJet1.2 (≈3.0Kb). In order to cut and isolate the sequence of *HcP-gpA*, the restriction sites *Not I*, located upstream the *H. contortus* P-glycoprotein A and *Xba I*, found downstream of the multiple cloning site in pJet 1.2 cloning vector were used.

After restriction analysis, positive clones with the correct size and orientation were selected and sent for sequencing to Genome Quebec, McGill University. Once the correct sequence was confirmed, 10μg of DNA construct *HcPgp-A/pJet1.2* were digested using 10U of *NotI* and 10U *XbaI*, in 10X buffer 4, supplemented with BSA (100μg/ml). A 0.7% agarose gel was run with the digested plasmids and purified using the Zymo gel DNA recovery kit (Zymo research, Switzerland), following the manufacturer’s instructions. *HcPgp-A* purified product was resuspended in RNA-free water. In parallel, 5μg of DNA plasmid pcDNA 3.1(+) were digested with 5U of *NotI-HF* and 5U of *XbaI* in 10X buffer 4, supplemented with BSA (100μg/ml) (Fig. 2). All components from the DNA restriction digestions were from NEB (New England Biolabs, Ipswich, MA, USA). RNA-free water was added to reach a final reaction volume of 500μl reaction. Restriction digestion was incubated for 2 hours at 37°C. After that, 10X Antarctic phosphatase reaction buffer (NEB), was added to the mixture plus 5U of Antarctic phosphatase enzyme and incubated for 15 minutes at 37°C. Subsequently, both restriction endonucleases and Antarctic phosphatase were heat inactivated at 65°C for 20 minutes. After, a 0.7% agarose gel was run with the digested pcDNA 3.1(+) plasmid and then purified and recovered with Zymo gel DNA recovery kit (Zymo research, Switzerland). Both purified *HcPgp-A* and pcDNA 3.1 (+),
were mixed in a ligation reaction using a 3:1 molar ratio of insert/vector (50ng/μl) as follow: 1.2μl of pcDNA 3.1 (+); 3.1μl of HcPgp-A; 0.5μl of concentrated T4 Ligase, (20U/ml, Promega, USA); 1μl 10X T4 ligase buffer and 4.2 μl of RNA-free water to complete a 10μl final volume. The ligation mixture was incubated for 18 hours at 16°C.

From the ligation mix, 2μl were taken and added to 50μl of Top10’F competent cells (Invitrogen Life Technologies, Burlington, ON) and proceeded with transformation reaction. Positive colonies were selected by PCR reaction using the primers to amplified the full length cDNA sequence of HcPgp-A, and also by restriction digestion of the construct with NotI and XbaI. Positive transformants were grown in LB media and the plasmid isolated with the purification Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After, DNA samples of HcPgp-A/pcDNA 3.1(+) construct were sent for sequencing to Genome Quebec, McGill University to confirm the right sequence and orientation of the construct.

Prior to transfection, the HcPgp-A/pcDNA 3.1(+) construct was replicated in 500ml of LB media with Ampicillin (100mg/ml) and around 800μg of DNA isolated with Midiprep kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. After, 80μg of plasmid were linearized with the restriction enzyme MfeI (NEB, MA, USA).
4.6 Mammalian cells culture.

As host cells the pig kidney epithelial cell line LLC-PK1 was selected. This cell line has shown low drug transport activity due to low endogenous level of P-glycoproteins and MRP’s making them suitable for over expression of *H. contortus* P-glycoprotein A for drug transport assays with macrocyclic lactones (Goh et al., 2002; Lespine et al., 2007).

4.6.1. Transfection of LLC-PK1 cells

LLC-PK1 cells were seeded in 24 well plates, at a density of $2 \times 10^5$ cells per well. Lipofectamine 2000® (Invitrogen Lifescience Technologies, Burlington, ON), was selected as method of transfection according to the literature (Takeuchi et al., 2006). In order to test the cytotoxicity of Lipofectamine 2000® against the host cells, a range of increasing concentrations (0.5; 0.8; 1; 2; 3 μg/μl) of this reagent was tested in one 24 well plate with LLC-PK1 cells at a confluency of 90%. After 24 hours of incubation, cells were microscopically examined for vitality and morphology and their survival estimated.

Those cells cultivated with 0.8 μg/μl of Lipofectamine 2000®, showed a good survival rate and normal morphology, thus, this concentration was selected to use for the stable transfection.

LLC-PK1 cells were grown in 24 well plates until almost 100% confluency. Twenty four hours before transfection, cells were incubated in 199 media without serum. DNA and Lipofectamine 2000® were diluted in Opti-MEM I Medium (Invitrogen Life Technologies,
Burlington, ON), according to instructions. Then the transfection complex was prepared in the ratio DNA/Lipofectamine 2000® 1:1, mixed and incubated for 15 minutes. Medium was removed from the cells and substituted by complex solution, using 500μl per well and cells were incubated for twenty four hours.

After checking the survival of the cells, the transfection solution was change for 199 media supplemented with 10% of fetal bovine serum (FBS) and 5% Ampicillin plus Streptomycin. Forty eight hours after transfection, the media was changed and Neomycin, (G418, Gibco, Invitrogen cell culture, Burlington, ON ), added as selective antibiotic for positive transformants in a concentration of 400μg/L. Complementarily, the chloramphenicol acetyl transferase (CAT) gene in pcDNA3.1(+), was used as positive control for transfection efficiency, (Nieto et al., 2005), following the same transfection protocol as the HcPgp-A/pDNA3.1(+) construct (Fig. 3). The original LLC-PK1 cells (Fig. 4) selected with G418 (400mg/L) were used as negative control.

4.6.2. Establishment of LLC-PK1 transformants stably expressing *H. contortus* P-glycoprotein A

Cells, after transfection, were cultured further, to increase their number and selected with G418 (400mg/L) for 4 weeks, until positive resistant cell patches were identified. Cells were then trypsinized and collected, counted and seeded individually as single cell cultures in 48 well plates with continued selection with G418 (400mg/l) for 4 weeks until wells with almost 100 % confluency of cells were present.
4.6.3. CAT/pcDNA 3.1(+) Detection of transfection efficiency.

After 2 and 4 weeks of selection respectively, cells transfected with CAT/pcDNA 3.1(+) construct, were collected and lysed with lysis buffer (20mM Tris pH8;100mM NaCl; 1mM EDTA; 0.5% Triton-X), plus proteases inhibitor cocktail (Roche, Mannheim, Germany).

Aliquots from these two extractions and one aliquot from untransfected cells, were measured for protein concentration by the Bradford method (Bradford, 1976). 30μg of protein from each sample were analyzed in two 12% SDS-PAGE, according to the Laemmli method, modified by Thomas (Thomas and Kornberg, 1975). One gel was stained with Coomassie blue to see the protein pattern from each sample, and the other gel was transferred to Hybond ECL membrane (Amersham, Piscataway, NJ, USA), in order to detect the CAT protein by Western-blots, as a positive control for transfection.

The Hybond ECL membrane was blocked overnight at 4°C, in 3% blocking solution (Amersham, Piscataway, NJ, USA). Twenty four hours later, the membrane was washed 3 times for 10 minutes each with 0.1% T-TBS. Then the membrane was incubated overnight at 4°C, with the first antibody α-CAT protein, raised in goat (ABCAM, Cambridge, MA, USA) in a 1/4000 dilution (1μl of α-CAT stock solution into 4ml of blocking solution). Then the membrane was washed 3 times for 10 times for each with 0.1% T-TBS. After, the membrane was incubated with a secondary antibody, Ig-G α-goat (ABCAM, Cambridge, MA, USA) in a dilution 1/12000 (1μl of α-goat stock solution into 12ml of blocking solution). Further, the membrane was developed using the Hybond ECL developing kit (Amersham, Piscataway, NJ, USA), detecting a protein band of around 27 KDa, which is the expected size for the CAT protein.
4.7. DNA Integration and Transcription detection

After continued selection, DNA from the *HcPgp-A*/pcDNA 3.1(+) construct was detected. Cells cultured in a 24 well plate were trypsiniزed and collected for DNA and RNA extraction. From 2x10^5 cells, DNA was extracted using the DNA Easy extraction kit (Qiagen, Hilden, Germany), according to manufacturer’s instructions. Isolated DNA was used as template for PCR reactions using the specific primers to amplify the full length sequence of *HcPgp-A*. From an aliquot of 1x10^7 cells RNA was extracted with RNeasy ® Plus (Qiagen, Hilden, Germany), following the manufacturer’s recommendations. This isolated RNA was frozen at -80°C until further analysis. Twenty four hours later 2μg of RNA were used as a template for RT-PCR, with the Omniscript® Reverse Transcription protocol, (Qiagen, Hilden, Germany). From the cDNA synthesized, an aliquot for PCR was taken, using the specific primers to amplify the full length sequence of *HcPgp-A*. Both RT-PCR and PCR products were analyzed in a 0.9% agarose gel, visualizing a DNA band of 4Kb, according to the expected molecular size of *HcPgp-A*. Both products were send for sequencing at Genome Quebec, McGill University, obtaining the correct sequence for *HcPgp-A*.


*HcPgp-A*/LLC-PK1 transgenic cells, after continued culture under G418 selection, were seeded in 24 well plates for 48 hours in G418-free growing media, until confluency was almost reached. Once the cells were ready, the growing media was removed and they were incubated with the fluorophore Rhodamine 123 (Rho123), (Hamada et al., 1987; Kerboeuf et al., 2003b), in a single concentration of 10μM, to test P-glycoprotein function.
Rho123 was prepared in HBSS media (Invitrogen Life Technologies, Burlington, ON), supplemented with 1% of BSA (Sigma-Aldrich, WGK, Germany) with increasing concentrations of IVM (0.0625-10µM) and MOX (0.5-16µM). All the drugs were dissolved in DMSO (Sigma-Aldrich, WGK, Germany) and diluted in medium 199 with a final DMSO concentration of <0.2%. Each ML concentration used, was incubated in 3 wells. In addition, 3 blank wells, with HBSS media, and 3 wells as baseline just with 10µM Rh0123, were included in the incubation.

In parallel, as positive control, LLC-PK1/mdr1a overexpressing cells (kindly provided by Dr. A.H. Schinkel, The Netherlands Cancer Institute, Netherlands), were used and incubated with Rho123 and increasing concentrations of either IVM or MOX. As negative control LLC-PK1 untransfected cells incubated with Rho123 10µM, were used. All cells were incubated for 2 hours in a cell culture incubator (Forma Scientific, Asheville, NC, USA) at 37°C and 5%CO₂. Subsequently, cells were washed with PBS three times and then the cells, in each well lysed in PBS-0.5%SDS (Lespine et al., 2007). Aliquots of lysed cells from HcPgp-A/LLC-PK1 and controls were frozen at -20°C until further analysis.

4.8.1. Fluorescence measurement.

The intracellular accumulation of Rho123 was measured by using a flurometer (BMG FLUOstarII Galaxy, USA). The reading parameters were \( \lambda_{\text{max}} \) excitation= 507nm, \( \lambda_{\text{max}} \) emission=529nm, mechanical slit = 3.5nm. The values were normalized by measuring the protein concentration using bovine serum albumin (BSA) by the Bradford method (Bradford, 1976).

The inhibition of transport of Rho123 through the inhibition of the P-glycoprotein function by the MLs (IVM and MOX) was compared against the maximum effect produced by
valspodar (VSP) (kindly provided by Dr. E. Georges) and expressed as percent of total VSP inhibition as follow:

\[
%\text{VSP} = \frac{(f_x/F_0) - 1}{(F_{\text{VSP}}/F_0) - 1} \times 100
\]

Where \( f_x \) was the normalized fluorescence in cells treated with the ML of interest (\( x \)); \( F_0 \) was the mean normalized fluorescence in the control (Rho123 alone); \( F_{\text{VSP}} \) was the mean normalized fluorescence in cells treated with VSP. The data generated was fitted with Graphpad Prism software according to the Michaelis-Menten equation (Lespine et al., 2007)
**Figure 1.** Subcloning vector PJet1.2. (Fermentas), plasmid structure. multiple cloning sites.
Figure 2. Mammalian expression vector pcDNA3.1(+) (Invitrogen) plasmid structure with multiple cloning sites.
Figure 3. Chloramphenicol Acetyl Transferase (CAT), (Invitrogen), plasmid structure. Positive control for mammalian transfection.
Figure 4. LLC-PK1 cells. Origin: Pig kidney epithelium. (Wohlwend et al., 1986).
Chapter V

Results

5.1. Amplification of the full length sequence of *H. contortus* P-glycoprotein A.

In order to investigate the possible involvement of *H. contortus* Pgp-A in ML resistance in this parasite, its full length sequence was cloned. After PCR, an aliquot of this product was analysed in 1% Agarose gel, stained with Ethidium bromide (0.1mg/ml). A product of 3.9Kb was obtained in the gel (Figure 7A).

After sequence analysis, the sequence amplified from the *H. contortus* PF unselected strain, was found to have a highly similar profile with the published sequence (AF003908 GenBank); sharing 97% identity at the level of nucleotide composition. Also, the sequencing confirmed the presence of a Kozak sequence around the start codon, which had been added to improve the translation in the mammalian host and which helps to stabilize the secondary structure of the HcPgp-A protein expressed. The sequence downstream of the Kozak insert corresponds to the whole structure of this ABC transporter, ending in a stop codon.
2. Cloning of full length sequence of *HcPgp-A*.

The full length sequence of *HcPgp-A* was amplified and inserted into the subcloning vector pJET1.2. After selection, positive transformants were checked by restriction digestion with *NotI* and *XbaI*, which are located up and downstream of the *HcPgp-A* insert, respectively. Following restriction digestion and separation of the digest on a 0.9% agarose gel, a band of 4Kb, corresponding to the *HcPgp-A* and another band close to 3Kb, which corresponds to the pJET1.2 plasmid DNA were detected (Figure 7B).

In order to express *HcPgp-A* in LLC-PK1 mammalian cells, the mammalian expression vector pcDNA 3.1(+) was selected. To make this construct, the restriction sites *NotI* and *XbaI*, present in the multiple cloning site of pcDNA 3.1(+) were also used. After ligation and transformation, positive colonies were screened. Restriction digestion, with the enzymes described above, was used to verify the presence of the insert *HcPgp-A* in pcDNA 3.1(+). In Figure 8A, a 0.9% agarose gel shows the pattern of this restriction digestion, featuring an upper band between 5 and 6Kb, corresponding to the plasmid pcDNA 3.1(+). Also a lower band of 4Kb, representing *HcPgp-A* was present. The plasmid was sequenced to confirm the presence of the insert. Subsequently, the plasmid was linearized which *MfeI* enzyme (Fig. 8B) to prepare the construct for stable transfection into LLC-PK1 cells.
3. Protein sequence alignment and conformational analysis.

The full length DNA sequenced of the construct $Hc$pgp-A/pcDNA 3.1(+), was translated into amino acid sequence, as well as the translation of published sequence of $Hc$Pgp-A (Accession number : AF003908). In Figure 6 is shown a global alignment of the protein sequences, sharing a 99.4% identity. There were a few amino acid differences particularly in the first half of the $Hc$Pgp-A region compared with the published sequence in GenBank. This could be explained due to the mixed RNA material extracted from multiple of $H. contortus$ worms. Also, a characteristic of the genomes of nematodes, and well established in $H. contortus$, is their highly polymorphic genetic profile (Prichard, 2001).

The translated sequence of $Hc$Pgp-A/pcDNA3.1(+), was subjected to transmembrane prediction analysis through TMHMM prediction tool, version 3.2, Biology workbench (Figure 6). A protein pattern composed of 12 transmembrane domains, with two intracellular portions, corresponding to the nucleotide binding domain (NBD) was predicted. Both the C and N-terminals of the $Hc$Pgp-A, were predicted to be found intracytoplasmatic. This is in agreement with the predicted protein patterns of different mammalian P-glycoproteins (Kast et al., 1995). These analyses indicate that the $Hc$Pgp-A belongs to the ABC transporter family.

The stable transfection of *HcPgp-A/*pcDNA 3.1(+), was developed in parallel with a positive control; such as the chloramphenicol acetyl transferase (CAT) protein. After transfection of both constructs separately, in LLC-PK1 cells, G418 (Neomycin) was used for selection. After several weeks of selection of both transfected gene constructs in the cells, an aliquot of the cells with the construct CAT/pcDNA 3.1(+), was lysed. In Figure 10A, the 12% SDS-PAGE protein migration pattern from the protein aliquots extracted from CAT/LLC-PK1 cells after 4 weeks of selection (Lane1) is shown; CAT/LLC-PK1 cells after 2 weeks of selection (Lane 2) and LLC-PK1 non-transfected cells (Lane3). In Lane 1 and 2, a protein band with a molecular size close to 25KDa, which may indicate the presence of the CAT transfected protein, is shown. This was validated by Western-blot. In Figure 10B, in Lanes 1 and 2, is shown the presence of a band of 25 KDa molecular size, corresponding to the CAT protein. After the transfection and further selection of the stable transformants, the detection of the positive transfection control (CAT protein), indicates the probable transfection for *HcPgp-A* into LLC-PK1.

After selection of stable transformants and further propagation of individual cells in 24 well plates, a RNA extraction was made. From this material a Reverse-transcription PCR was prepared, using the specific primers that were used to amplify the full length sequence of *HcPgp-A*. Figures 11A and 11B, present a 0.9% agarose gel, showing a RT-PCR and PCR product of each 4Kb size respectively, in agreement with the expected size of the full length sequence of *HcPgp-A*. This confirmed the insertion of the transgene, *HcPgp-A*, into the nucleus of the host mammalian cells and its transcriptional activation.
5. Drug transport assays.

The propagation of stable transfected cells, after continued selection pressure with G418, allowed the assessment of the \( HcPgp \)-A expression. The role of P-glycoprotein in relation to the mechanism of resistance in parasitic nematodes is not well understood. The role of ABC transporters in mammalian cells is well documented, including the active transport of different substrates such as xenobiotic compounds. In order to test the expression and ability of the \( HcPgp \)-A in the LLC-PK1 to transport Pgp substrate, a drug transport assay was developed, using the fluorophore Rhodamine 123. This compound has a planar, hydrophobic and poorly charged structure that, make it an ideal substrate to measure P-glycoprotein function (Kerboeuf et al., 2003a).

\( HcPgp \)-A/LLC-PK1 stably transfected cells along with mdr1a/LLC-PK1 cells and non-transfected LLC-PK1 cells were incubated in triplicate for 2 hours with Rho123, then washed and lysed until further analysis. In Figure 12, is shown the histogram representing the intracellular accumulation of Rho123. To measure the maximum possible accumulation (100%), the fluorescence of the non transfected LLC-PK1 cells (Lane 1) was taken. When compared with non-transfected cells, the fluorescence accumulated into \( HcPgp \)-A/LLC-PK1 cells reached 60% (Lane 2), indicating transport function activity. Mdr1a/LLC-PK1 overexpressing cells, showed around 30% (Lane 3) of fluorescence accumulation in comparison with non-transfected cells. This indicated an actively transport function by the \( HcPgp \)-A and the mdr1a gene products. Both ABC transporter transfected cells showed a significantly lower fluorescence than the untransfected cells, validating the use of Rho123 as testing compound for P-glycoprotein function.
Once the expression and transport activity by \( HcPgp-A \), was established, the inhibition of the Rho123 \( HcPgp-A \) transport activity by the MLs, in the LLC-PK1 cells was assessed. In order to assess, whether IVM or MOX had some effect on the transport of Rho123, \( HcPgp-A/LLC-PK1 \), cells were incubated with these compounds and then the fluorescence in the cells was determined. In Figure 13, the drug-response curve for IVM (red) and MOX (blue) is shown. For IVM there was an increased effect on the inhibition of Rho123 transport, in direct relationship with increasing concentration of this ML. IVM produced a maximum effect at 2μM concentration. This maximum effect of IVM represented 80% of the VSP effect on the inhibition of the transport of Rho123.

Also shown is the drug response curve obtained with MOX. Although there was an effect by MOX, it was variable and less marked than IVM. Nevertheless, the fluorescence in the \( HcPgp-A/LLC-PK1 \) cells decreases as the MOX concentration increased. Although the response to MOX was rather flat, a maximum inhibitory effect was reached at approximately at 4μM concentration, representing around 70% of the VSP effect. However, after this apparent peak in the inhibitory effect, the MOX response appeared to fall to some extent with higher concentrations. MOX showed an effect on the inhibition of Rho123 transport with a minimum concentration of 0.25μM, whereas IVM produces a measurable inhibitory effect at 0.0625μM; a fourfold difference between the two drugs in the inhibition of the Rho123 transport into \( HcPgp-A/LLC-PK1 \) cells.

From the drug response curves observed by both, IVM and MOX, the relative half maximum inhibitory concentration effect (\( IC_{50} \)) was calculated. From the IVM drug response curve, the \( IC_{50} \) was calculated to be 0.45μM. This result is similar to what has been described with mammalian P-glycoprotein (Lespine et al., 2007). In comparison, the MOX drug response
curve, gave an IC$_{50}$ of approximately 1.96μM. Although the MOX response was somewhat variable, there was a 4 fold difference in inhibitory effect on HcPgp-A/LLC-PK1 cells, in terms of Rho123 transport, between IVM and MOX. Again this evidence, suggests an active role by HcPgp-A in the transport of at least IVM.

Complementarily, these same assays were applied to the (mammalian) mdr1a/LLC-PK1 cells. In Figure14 the inhibition of the Rho123 transport by IVM (red) and MOX (blue) is shown, on the cells overexpressing the mdr1a gene product. IVM showed a clear drug response curve, reaching a maximum inhibitory effect, at 4μM, that represented approximately 90% of the VSP maximum inhibitory effect. In the case of MOX, the drug response curve had maximal inhibitory effect at 8μM, producing approximately 80% of the VSP inhibitory effect. This difference was clearly marked when the IC$_{50}$ values were compared for these two drugs. While IVM had an IC$_{50}$ of 0.32μM, MOX had an IC$_{50}$ of 2.60μM, in mdr1a overexpressing cells. There was an approximately 8 fold of difference between the inhibitory transport concentration of IVM and MOX in the mdr1a/LLC-PK1 overexpressing cells.

These results indicate that there are differences between IVM and MOX in the inhibition of Rho123 transport into HcPgp-A/LLC-PK1 cells and in mdr1a/LLC-PK1 cells. These results are similar to those that have been described in the literature for active transport of MLs by mammalian P-glycoproteins (Lespine et al., 2007). Certainly this work suggests a more active role for HcPgp-A in the transport of IVM than MOX. However further work needs to be done to confirm these differences.
<table>
<thead>
<tr>
<th>Hspg-ALLC-PK1</th>
<th>AF003508</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,590</td>
</tr>
<tr>
<td>2,590</td>
<td>2,570</td>
</tr>
<tr>
<td>2,580</td>
<td>2,590</td>
</tr>
<tr>
<td>2,600</td>
<td>2,600</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,610</td>
</tr>
<tr>
<td>2,620</td>
<td>2,630</td>
</tr>
<tr>
<td>2,640</td>
<td>2,650</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,660</td>
</tr>
<tr>
<td>2,670</td>
<td>2,680</td>
</tr>
<tr>
<td>2,690</td>
<td>2,700</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,710</td>
</tr>
<tr>
<td>2,720</td>
<td>2,730</td>
</tr>
<tr>
<td>2,740</td>
<td>2,750</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,760</td>
</tr>
<tr>
<td>2,770</td>
<td>2,780</td>
</tr>
<tr>
<td>2,790</td>
<td>2,800</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,810</td>
</tr>
<tr>
<td>2,820</td>
<td>2,830</td>
</tr>
<tr>
<td>2,840</td>
<td>2,850</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,860</td>
</tr>
<tr>
<td>2,870</td>
<td>2,880</td>
</tr>
<tr>
<td>2,890</td>
<td>2,900</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,910</td>
</tr>
<tr>
<td>2,920</td>
<td>2,930</td>
</tr>
<tr>
<td>2,940</td>
<td>2,950</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>2,960</td>
</tr>
<tr>
<td>2,970</td>
<td>2,980</td>
</tr>
<tr>
<td>2,990</td>
<td>3,000</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,010</td>
</tr>
<tr>
<td>3,020</td>
<td>3,030</td>
</tr>
<tr>
<td>3,040</td>
<td>3,050</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,060</td>
</tr>
<tr>
<td>3,070</td>
<td>3,080</td>
</tr>
<tr>
<td>3,090</td>
<td>3,100</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,110</td>
</tr>
<tr>
<td>3,120</td>
<td>3,130</td>
</tr>
<tr>
<td>3,140</td>
<td>3,150</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,160</td>
</tr>
<tr>
<td>3,170</td>
<td>3,180</td>
</tr>
<tr>
<td>3,190</td>
<td>3,200</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,210</td>
</tr>
<tr>
<td>3,220</td>
<td>3,230</td>
</tr>
<tr>
<td>3,240</td>
<td>3,250</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,260</td>
</tr>
<tr>
<td>3,270</td>
<td>3,280</td>
</tr>
<tr>
<td>3,290</td>
<td>3,300</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,310</td>
</tr>
<tr>
<td>3,320</td>
<td>3,330</td>
</tr>
<tr>
<td>3,340</td>
<td>3,350</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,360</td>
</tr>
<tr>
<td>3,370</td>
<td>3,380</td>
</tr>
<tr>
<td>3,390</td>
<td>3,400</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,410</td>
</tr>
<tr>
<td>3,420</td>
<td>3,430</td>
</tr>
<tr>
<td>3,440</td>
<td>3,450</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,460</td>
</tr>
<tr>
<td>3,470</td>
<td>3,480</td>
</tr>
<tr>
<td>3,490</td>
<td>3,500</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,510</td>
</tr>
<tr>
<td>3,520</td>
<td>3,530</td>
</tr>
<tr>
<td>3,540</td>
<td>3,550</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,560</td>
</tr>
<tr>
<td>3,570</td>
<td>3,580</td>
</tr>
<tr>
<td>3,590</td>
<td>3,600</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,610</td>
</tr>
<tr>
<td>3,620</td>
<td>3,630</td>
</tr>
<tr>
<td>3,640</td>
<td>3,650</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,660</td>
</tr>
<tr>
<td>3,670</td>
<td>3,680</td>
</tr>
<tr>
<td>3,690</td>
<td>3,700</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,710</td>
</tr>
<tr>
<td>3,720</td>
<td>3,730</td>
</tr>
<tr>
<td>3,740</td>
<td>3,750</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,760</td>
</tr>
<tr>
<td>3,770</td>
<td>3,780</td>
</tr>
<tr>
<td>3,790</td>
<td>3,800</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,810</td>
</tr>
<tr>
<td>3,820</td>
<td>3,830</td>
</tr>
<tr>
<td>3,840</td>
<td>3,850</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,860</td>
</tr>
<tr>
<td>3,870</td>
<td>3,880</td>
</tr>
<tr>
<td>3,890</td>
<td>3,900</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,910</td>
</tr>
<tr>
<td>3,920</td>
<td>3,930</td>
</tr>
<tr>
<td>3,940</td>
<td>3,950</td>
</tr>
<tr>
<td>RGACCTGGATCAATTGACATTCTCATTGCTACCTG</td>
<td>3,960</td>
</tr>
<tr>
<td>3,970</td>
<td>3,980</td>
</tr>
<tr>
<td>3,990</td>
<td>4,000</td>
</tr>
</tbody>
</table>
Figure 5. Sequence alignment between *HcPgp*-A/LLC-PK1 and the published sequence of *HcPgp*-A (Accession number: AF003908). Alignment made with Geneious software version 5.4.
Figure 6. Protein alignment of *HcPgp*-A/LLC-PK1 and the published sequence of *HcPgp*-A (Accession number: AF003908). Alignment made with Multi sequence alignment (Corpet, 1998).
Figure 7. **A.** PCR amplification of full length sequence of \(\text{HcPgp-A}\).

**B.** Restriction digestion with \(\text{Not I}\) and \(\text{XbaI}\), on the \(\text{HcPgp-A}\) construct into Pjet1.2 (cut lane); plasmid of the \(\text{HcPgp-A}\) construct into Pjet1.2 (uncut lane).
Figure 8. A. Restriction digestion with *Not I* and *XbaI* of construct *HcPgp-A/pcDNA3.1(+) (cut lane)*. Plasmid of construct *HcPgp-A/pcDNA3.1(+) (uncut lane)*

B. Linearisation of *HcPgp-A/pcDNA3.1(+) construct with *MfeI (cut lane)*. Plasmid of construct *HcPgp-A/pcDNA3.1(+) (uncut lane).*
Figure 9. Transmembrane domains prediction for HePgp-A. TMHMM protein tool from Biology Workbench. Version 3.2 (http://workbench.sdsc.edu.)
Figure 10.  

A. SDS-PAGE CAT/LLC-PK1 cells  
B. Western blot CAT/LLC-PK1 cells  

MW: Protein molecular marker  
Lane 1: CAT/LLC-PK1 cells after 4 weeks of selection with G418  
Lane 2: CAT/LLC-PK1 cells after 2 weeks of selection with G418  
Lane 3: LLC-PK1 cells after 4 weeks of selection with G418
Figure 11. A. Reverse transcription PCR amplification of full length sequence of *HcPgp*-A from transfected *HcPgp*-A/LLC-PK1 cells.

B. PCR amplification of full length sequence from transfected *HcPgp*-A/LLC-PK1 cells.
Figure 12. Intracellular accumulation of Rhodamine 123
1: Fluorescence in non-transfected cells LLC-PK1 cells.
2: Fluorescence in HcPgp-A/LLC-PK1 cells.
3: Fluorescence in mdr1a/LLC-PK1 cells.
Mean of three replicates ± S.E.M.
Figure 13. Inhibition of the Rho123 transport by MLs into HcPg-A/LLC-PK1 cells.
Red: IVM drug-response curve.
Blue: MOX drug-response curve.
IC$_{50}$: Half maximal inhibitory concentration effect.
Mean of three replicates ± S.E.M.
Figure 14. Inhibition of Rho123 transport by MLs into mdr1a/LLC-PK1 cells. Red: IVM drug-response curve. Blue: MOX drug-response curve. IC₅₀: Half maximal inhibitory concentration effect. Mean of three replicates ± S.E.M.
Chapter VI
Discussion

Drug resistance in parasitic nematodes has become an unsolved problem and much remains to elucidate; what are the genetic bases that allow helminths to survive against control strategies such as chemotherapy. *H. contortus*, a veterinary nematode of small ruminants, representing one of the most dangerous infective pathogens, responsible for animal production failure in many farms worldwide (van Wyk et al., 2006).

*H. contortus* has been well studied in order to try to understand the mechanisms of resistance to different classes of anthelmintics. Macrocyclic lactones (ML) continue to have a great endectocidal effect against both internal and external parasites (Prichard, 2007). Nonetheless, the appearance of resistance occurred rapidly after the anthelmintics were employed. ML resistance in nematodes, seems to have a multigenic origin (Prichard et al., 2007), which may include mainly changes at the target receptors (Dent et al., 2000; Gillard, 2006), and possibly drug metabolism (James et al., 2009) among other mechanisms. Perhaps the most important mechanisms include the major role of ABC transporters, which have been described to influence the final concentration of the ML in the host and the parasite, thus affecting the efficacy of these drugs (Lespine et al., 2008). P-glycoproteins in *H. contortus*, may provide a first line mechanism of resistance to MLs in this worm. Supporting this concept has been evidence in *H. contortus*, of ML resistance selection on *HcPgp-A*, (Blackhall et al., 1998a; Xu et al., 1998; Le Jambre et al., 1999; Sangster et al., 1999). Furthermore, in *C. elegans* selected with IVM, there is evidence of the involvement of ABC transporters in the resultant IVM resistance (James and Davey, 2009). The constitutive over expression of ABC transporters may be generated by ML selection for specific alleles. In eukaryotes gene expression involves regulatory elements located upstream of
the coding sequence (Kantharidis et al., 2000). It has been well established that these regulatory structures correspond to the nuclear receptors which bind to promoter elements and induce gene expression (Nagy et al., 2006). For instance, in mammals the nuclear pregnane X receptor (PXR) upregulates transcription of ABC transporters in concomitance with cytochrome P450 (CYP450) detoxification enzymes in response to direct binding to structurally diverse xenobiotic compounds (Kliewer et al., 2002).

In nematodes, ABC transporters such as P-glycoproteins are believed to play a role in the detoxification of xenobiotic compounds, originally made by the host or occurring in the environment. Also P-glycoproteins have a physiological role transporting natural substrates such as lipophilic peptides across the membrane, and excluding toxins from the parasite’s metabolism (Sangster, 1994). At present, fourteen genes and one pseudogene of P-glycoprotein, have been reported in C. elegans (Lincke et al., 1992), whereas in H. contortus, at least 6 P-glycoprotein genes (Prichard and Roulet, 2007) have been described. However, when the incomplete sequence available for another putative H. contortus P-glycoprotein were compared against the data base available for C. elegans P-glycoproteins sequences, it appeared that some H. contortus P-glycoproteins, could have more than one copy that could encode for the same P-glycoprotein (Roulet et al., manuscript in preparation), suggesting that there is a whole battery of these ABC transporters present in this helminth. The reason for this abundance could be explained as due to the lack of a central nervous system in nematodes that can be protected by a complex structure like the blood brain barrier found in mammals (Schinkel, 1997). In addition, nematode neurons are likely to be more exposed to the environment, thus a protective mechanism, could be the presence of ABC transporters such as P-glycoproteins, effluxing different kinds of substrates. Another interesting hypothesis about this extensive repertoire of ABC transporters in nematodes, involves the divergence of P-glycoproteins in regard to evolution-conservation functional role.
Accordingly, there is an adaptative group of diverse P-glycoproteins which have strong divergence and have been duplicated in order to adapt and evolved to the environment conditions. These P-glycoproteins either in the free-living nematode *C. elegans*, as well as in the parasitic nematode *H. contortus*, would have a possible role to transport xenobiotics compounds and minerals (Broeks et al., 1995). This group would be particularly important in the larvae stages of *H. contortus*, which develop outside the host and are more exposed to environmental conditions.

On the other hand, there is an intrinsic group of P-glycoproteins, which have low variation in their sequences and may play a physiological function (Roulet et al., manuscript in preparation). This later group may include *HcPgp-A*. Its ortholog in *C. elegans*, Celpgp-2, has a role of transporting lipids and lipophilic substrates. MLs including IVM, are very lipophilic and should be present at considerable concentrations in the lipid bilayer of the cell membrane, where *HcPgp-A* should be interacting with them and transport these substrates to the extracellular compartment.

In line with this observation, our functional assay demonstrated the expression of *HcPgp-A* in LLC-PK1 and showed a clear transport activity of Rho123, an excellent substrate for measuring P-glycoprotein function, reflected in the reduced accumulation of the fluorophore in the *HcPgp-A*/LLC-PK1 cells, in comparison with non-transfected cells. On the other hand, when the fluorescence from the *HcPgp-A*/LLC-PK1 cells was compared with the fluorescence accumulated into mdr1a/LLC-PK1 cells, the latter was a half of that of the *HcPgp-A*/LLCPK1 cells. An explanation for that could be the level of expression of each ABC transporter. The *HcPgp-A* may not have reached a comparable level of expression in the mammalian cells as the mammalian P-glycoprotein (mdr1a), due to differences in the nucleotide level affecting codon usage or at the amino acid level. It has been calculated that *HcPgp-A* has 64% identity, at the amino acid level, with the mdr1a gene product (Xu et al., 1998). In the mammalian heterologous expression system, the *HcPgp-A* codon composition may not be efficiently read by the translation machinery in the mammalian host. Also, it is known in studies on mammalian P-glycoproteins, that just one
amino acid change may change the transport specificity of these cell membrane proteins for the substrate (Kwan and Gros, 1998; Loo and Clarke, 2002). Furthermore, HcPgp-A at the level of nucleotide or amino acid sequences, may be polymorphic, affecting expression and transport kinetics. Nonetheless, our result show a marked reduction of Rho123 transport in the HcPgp-A/LLC-PK1 cells in comparison with non transfected cells, demonstrating that there is HcPgp-A expression and that it is transporting this substrate out of the cell.

In addition, once the expression and functional activity of HcPgp-A in LLC-PK1 cells was established, it was of interest to see its interaction with the MLs, IVM or MOX. Valspodar (VSP), was used as maximum reference of P-glycoprotein inhibitor activity, and this substance was found to inhibit the transport of Rho123 by HcPgp-A, confirming the interaction and strong inhibition with this compound. Then, when either IVM or MOX was used to inhibit the Rho123 transport, a difference between these two drugs was seen. IVM induced a stronger inhibition of Rho123 transport than MOX in the HcPgp-A expressing cells. This was in agreement with what has been described between mammalian P-glycoprotein and MLs (Griffin et al., 2005; Lespine et al., 2007).

The basis of this difference between IVM and MOX may lie in their structure. All MLs have a 16-ring macrocyclic backbone (Shoop et al., 1995). However, IVM unlike MOX, has a disaccharide structure at the level of C-13 in the macrocyclic ring, (Burg et al., 1979). The presence of this carbohydrate influences the hydrophobicity of these molecules, affecting the octanol/water partition coefficient (logP). This parameter can be defined as the ratio of solute concentrations in two phases (organic and aqueous) and the solute (drug) (Ruelle and Kesserling, 1998). MLs such as IVM and MOX are highly lipophilic (Craven et al., 2002). However, IVM (an avermectin) has a disaccharide group which decreases its hydrophobicity, relative to the
aglycone. According to Vellarkad et al., (1989), using atomic parameters, a logP for IVM and MOX of 4.8 and 6, respectively were calculated (Lespine et al., 2007), whereas for VSP the logP was estimated to be around 3.65 (Wang et al., 2003), meaning that the higher the logP, the higher the lipophilicity and tendency to remain in the cell membrane. Thus, IVM may be interacting from the intracellular space with the HcPgp-A rather than from the membrane. Also it has been presumed that the disaccharide structure in IVM enhances its binding to P-glycoproteins, and transport activity to efflux the drug outside of the cell. The varied response of MOX in the inhibition of the Rho123 transport, suggests that it may be a relatively poor substrate for HcPgp-A, compared with IVM and VSP. However further work is needed to confirm this observation.

The expression and transport activity observed confirms the relevance of HcPgp-A to the IVM resistance process, as shown in this work by the inhibition of Rho123 transport by IVM and to a lesser extent by MOX. Interestingly, evidence of the involvement of ABC transporters in IVM resistance mechanism in C. elegans has been described (James and Davey, 2009). Experiments from these latter authors show an increased expression of P-glycoproteins and MRPs after continuous selection with IVM of C. elegans resistant strains. Also they noted that CelPgp-2, the ortholog of HcPgp-A in H. contortus, had increased expression after several weeks of exposure to IVM in comparison with the susceptible C. elegans strain. These authors tested the involvement of ABC transport proteins in these IVM resistant C elegans worms, using MDR-reversing agents such as Verapamil (VPL), PSC-833 or VSP and MK571. After incubation with these drugs, a reversal to a susceptible phenotype to IVM, but not to MOX, suggested clear evidence of the involvement of ABC transporters such as P-glycoproteins, in IVM resistance but his situation is less clear with MOX. This work was also consistent with reports that described in vivo that the use of MDR-reversing agent restored efficacy against H. contortus ML strains
(Molento and Prichard, 1999) and confirms that IVM is an excellent substrate of both P-glycoprotein (Pouliot et al., 1997) and MRPs (Lespine et al., 2006).

Our findings support the proposition that ABC transporters play a major role in ML resistance in parasitic nematodes. In order to improve ML chemotherapy against *H. contortus* and possibly other gastro-intestinal helminths, it will be important to understand the kinetics of these ABC transporters in these parasites. In regard to this, the literature describes many compounds that can modulate the transport activity of P-glycoproteins (Teodori et al., 2002; Toffoli et al., 1995). However, most of this research has been carried out in MDR human cancer cells. Nonetheless, the scenario involving nematode P-glycoproteins in ML resistance could be dramatically altered with selective MDR reversing agents.

It is important to try to test and screen compounds available in the veterinary market, for their capacity to modulate *HcPgp*-A transport activity and to search for the compounds that maximize the bioavailability in the host and increase the efficacy of MLs against resistant parasites. Among the compounds present in animal health, that have been tested *in vivo* as P-glycoproteins substrate and enhanced ML bioavailability, are the antimicotics, ketoconazole and itraconzole (Hugnet et al., 2007; Ballent et al., 2007), the natural flavonoid, quercetine (Dupuy et al., 2003) and the antihistaminic, loperamide (Lofshitz et al., 2002). Additionally some of these drugs have been tested in *H. contortus* IVM resistant and susceptible larvae, modulating and restoring partially IVM efficacy (Bartley et al., 2009). Despite these last results, there is still a need to further assess the modulation and transport activity of *HcPgp*-A and other *H. contortus* ABC transporters in order to characterize their structure-affinity for the purpose of enhancing chemotherapy with MLs against resistant parasites.
Literature references


Hamada, H., Hagiwara, K., Nakajima, T., Tsuruo, T., 1987, Phosphorylation of the Mr 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumor cells: effects of verapamil, trifluoperazine, and phorbol esters. Cancer Res 47, 2860-2865.


O'Connor, L.J., Kahn, L.P., Walkden-Brown, S.W., 2007, Moisture requirements for the free-
living development of Haemonchus contortus: Quantitative and temporal effects under
conditions of low evaporation. Vet Parasitol 150, 128-138.


Prevalence and intensity of Onchocerca volvulus infection and efficacy of ivermectin in
endemic communities in Ghana: a two-phase epidemiological study. Lancet 369, 2021-
2029.

Perry, B.D., Randolph, T.F., 1999, Improving the assessment of the economic impact of parasitic
diseases and of their control in production animals. Vet Parasitol 84, 145-168.

Pouliot, J.-F., L'Heureux, F., Liu, Z., Prichard, R.K., Georges, E., 1997, Reversal of P-
glycoprotein-associated multidrug resistance by ivermectin. Biochem Pharmacol 53, 17-
25.

Prichard, R., 2001, Genetic variability following selection of Haemonchus contortus with

Prichard, R.K., 2007, Markers for benzimidazole resistance in human parasitic nematodes?
Parasitology 134, 1087-1092.


towards markers for anthelmintic resistance in helminths of importance in animal and

Robertson, S.J., Martin, R.J., 1993, Levamisole-activated single-channel currents from muscle of


