CHARLES UNIVERSITY
Faculty of Pharmacy in Hradec Králové
Department of Biochemical Sciences

ADVANCES IN THE DISCOVERY AND TESTING OF ANTHELMINTICS

Dissertation Thesis

Mgr. Thuy Linh Nguyen

Supervisor: Prof. RNDr. Lenka Skálová, Ph.D.
Consultant: Assoc. Prof. Ing. Petra Matoušková, Ph.D.

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STATEMENT OF AUTHORSHIP

I hereby declare that this thesis is my original work which I solely composed by myself under the supervision of Prof. RNDr. Lenka Skálová, Ph.D. and Assoc. Prof. Ing. Petra Matoušková, Ph.D. All used literature and other sources are summarized in the list of references and properly cited. This work has not been submitted for any different or equal degree.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně pod vedením své školitelky prof. RNDr. Lenky Skálové, Ph.D. a konzultantky doc. Ing. Petry Matouškové, Ph.D. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

In Hradec Králové Mgr. Thuy Linh Nguyen

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ABSTRACT

Charles University
Faculty of Pharmacy in Hradec Králové
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Candidate: Mgr. Thuy Linh Nguyen
Supervisor: Prof. RNDr. Lenka Skálová, Ph.D.
Consultant: Assoc. Prof. Ing. Petra Matoušková, Ph.D.
Title of Dissertation Thesis: Advances in the discovery and testing of anthelmintics

The parasitic nematodes cause a considerable problem in human and animal health worldwide. The group of gastrointestinal nematodes is responsible for economic losses in livestock production. The treatment relies on the use of a limited number of anthelmintics, but their efficacy is hampered due to widespread drug resistance. Given the need for new drugs, the present thesis focuses on discovering novel compounds with potential anthelmintic effects and on the development of testing methods.

Based on the literature search, we presented the possible approaches in the current development of new anthelmintics and evaluated their advantages and disadvantages in a review article. For the experiments, we used a model organism *Haemonchus contortus,* which is one of the most important gastrointestinal pathogens of small ruminants. The primary phenotypic screening of a small compound library against larvae identified two 'hit' compounds BLK127 and HBK4. Based on further studies of efficacy against the adult stage and toxicity in sheep, we selected BLK127 for biotransformation studies in *H. contortus* and ovine liver. The promising results may advance this molecule for further drug development. Additionally, we provided a study on the efficacy, biotransformation and toxicity of sertraline in *H. contortus*, which represents the repurposing strategy for the development of novel anthelmintics.

Moreover, the thesis presents an optimised viability assay for testing in adults of *H. contortus* based on bioluminescence determination of adenosine triphosphate concentration. This assay might serve for the detection of drug-resistant isolates. Furthermore, we took advantage of a deep learning algorithm dealing with image recognition tasks, Mask R-CNN. Progress on this front improved the power of automated classification of the nematode including motile/non-motile phenotype.

ABSTRAKT

Univerzita Karlova
Farmaceutická fakulta v Hradci Králové
Katedra biochemických věd

Kandidát: Mgr. Thuy Linh Nguyen
Školitel: Prof. RNDr. Lenka Skálová, Ph.D.
Konzultant: doc. Ing. Petra Matoušková, Ph.D.
Název disertační práce: Pokroky ve výzkumu a testování anthelmintik

Parazitické hlístice způsobují celosvětový problém v humánní i veterinární medicíně. Skupina gastrointestinálních hlístic u hospodářských zvířat je zodpovědná za ekonomické ztráty v živočišné produkci. K léčbě nemocí způsobených hlísticemi se užívají anthelmintika, u nichž se ale v důsledku rozšířené lékové rezistence postupně snižuje účinnost. Vzhledem k potřebě nových léčiv se tato práce zaměřuje na výzkum nových sloučenin s potenciálním anthelmintickým účinkem a na vývoji testovacích metod.

Na základě literární rešerše jsme v přehledovém článku představili možné přístupy v současném vývoji nových anthelmintik a zhodnotili jejich výhody a nevýhody. K následným experimentům jsme využili parazitickou hlístici vlasovku slezovou (*Haemonchus contortus*), která je jedním z nejvýznamnějších gastrointestinálních patogenů malých přežvýkavců. Prvotní fenotypový screening souboru chemických látek prováděný na larvách *H. contortus* identifikoval dvě nadějné sloučeniny BLK127 a HBK4. Na základě následných studií účinnosti na dospělcích a toxicity v játrech hostitele byla sloučenina BLK127 vybrána pro biotransformační studie v *H. contortus* a ovčích játrech. Slibné výsledky ukázaly BLK127 jako vhodnou sloučeninu pro vývoj nového anthelmintika. V další studii jsme testovali účinnost, biotransformaci a toxicitu sertralinu u *H. contortus*, což představuje strategii využití schválených léčiv k novým terapeutickým indikacím.

Práce dále představuje optimalizovaný protokol pro testování životaschopnosti dospělců *H. contortus,* který je založený na bioluminiscenčním stanovení koncentrace adenosin trifosfátu. Tento test může sloužit k detekci izolátů rezistentních vůči léčivům. V další studii jsme využili algoritmus hlubokého učení Mask R-CNN, který se zabývá úlohami rozpoznávání obrazu. Pokrok v této oblasti zlepšil schopnost automatické identifikace hlístice včetně pohyblivého/nepohyblivého fenotypu.

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1 INTRODUCTION

Diseases caused by parasitic helminths substantially impair both human and animal health worldwide. Parasitic nematodes of small ruminants and other livestock are a major constraint on animal welfare and cause severe economic losses to livestock industry production of meat and dairy. To maintain agricultural productivity, the effective control of gastrointestinal nematodes (GINs) in livestock animals is essential (Charlier et al., 2014; Roeber et al., 2013). Key GINs of small ruminants belong to order Strongylida, e.g., *Haemonchus contortus, Teladorsagia* and *Trichostrongylus* spp. The control of these and related nematodes is undertaken mainly by anthelmintic treatment. However, the excessive use of anthelmintics has resulted in widespread problems with anthelmintic resistance (Kaplan, 2004; Wolstenholme et al., 2004). To become practically and ecologically sustainable, the development of integrated parasite management (IPM) strategies aimed to reduce unnecessary anthelmintic treatments and increase reliance on management, environmental and other non-chemical alternatives (Maqbool et al., 2017; O'Connor et al., 2006). Nevertheless, the high prevalence of GIN infection, together with the paucity of vaccines, and the risk of rapidly developing drug resistance to the limited number of widely deployed anthelmintics and their combinations, underlines the essentiality to find new antiparasitic drugs.

Drug discovery and drug development are among the most important translational science activities that aim to convert results in basic research into direct benefit to human and veterinary medicine. Understanding the pathophysiology and epidemiology of a disease is crucial for developing effective drugs, while drug testing methods underpin the speed and success of drug discovery. Despite ongoing manifold efforts put into the search of novel anthelmintics, no new drugs were introduced and the latest commercially available anthelmintics were monepantel (MOP) and derquantel since 2009 and 2010, respectively (Kaminsky et al., 2008b; Little et al., 2011). However, resistance to MOP was reported shortly after in several studies over the globe (Mederos et al., 2014b; Van den Brom et al., 2015b; Viana et al., 2021).

It is recognised that parasite eradication is, in most cases, impracticable and generally not necessary to achieve (Decaestecker & King, 2019; Wood & Johnson, 2015). Moving toward a purposeful goal to attain sustainable control of parasites of small ruminants requires methods for efficient disease control and diagnosis. In the meantime, search for novel drugs using state-of-the-art technologies should be applied. The benefits of using antiparasitic drugs in farm animals are unquestionable, and the present doctoral thesis presents advances in the development and testing of anthelmintics.

2 THEORETICAL BACKGROUND

2.1 Gastrointestinal nematodes of veterinary importance

The phylum Nematoda (roundworms) is morphologically and biologically diverse. Around 30,000 species have been described but their total number is estimated to be a million or more, making them one of the most abundant animals on Earth (Kiontke & Fitch, 2013). While a good number of nematodes are free-living organisms, parasitic nematodes threaten the health of plants, animals, and humans on a global scale (Blaxter et al., 1998). Approximately half of the world's human population, 3.5 billion cases, is infected with gastrointestinal nematodes (GINs), and 8–15% of crop losses are attributed to nematodes worldwide, with a cost of at least $80 billion (Kiontke & Fitch, 2013). The heavy burden to animal health is imputed to the group of GINs. In livestock production systems, GIN infections cause reductions in weight gain, milk production, and carcass quality (Charlier et al., 2014), and even death of animals, all leading to annual economic losses estimated at billions of dollars (Mcleod, 1995; Roeber et al., 2013).

The GINs of greatest importance in small ruminants are members of the nematode order Strongylida and most belong to the superfamily Trichostrongyloidea (Zajac, 2006). Key representatives of GIN that infect small ruminants and are generally associated with production losses and clinical disease include the abomasal nematodes *Haemonchus contortus*, *Teladorsagia circumcincta* (*Ostertagia circumcincta*) and other *Ostertagia* spp, *Trichostrongylus axei*, and the small intestinal species of *Trichostrongylus*, *Nematodirus*, and to a lesser extent *Cooperia* (Vlassoff et al., 2001).

Even though nematodes are ubiquitous, inhabiting aquatic and terrestrial environments, they are remarkably similar in morphology and life stages. Despite their structural complexity, individual nematode species share certain basic principles: nematodes are triploblastic, Pseudocoelomate, bilaterally symmetrical, unsegmented, vermiform, and colourless animals (Decraemer et al., 2013). The aforementioned key representatives have a direct life cycle, i.e., they do not require an intermediate host to develop and/or to reproduce. Their life cycle is similar; mature male and female worms reside in the gastrointestinal tract of the host where they mate and reproduce. Females lay eggs that are passed in the faeces and contaminate the surrounding soil. Here, the eggs embryonate and develop to the first-stage larvae (L1), usually within one day. They hatch and develop via the second larval stage (L2) to become the third-stage larvae (L3), usually in about a week in favourable conditions. The L3s actively migrate from the faeces onto the pasture, from where they are ingested by the host. Afterwards, they

exsheath the outer cuticle becoming the exsheathed L3 (xL3), and develop through a fourth larval stage (L4) to the adult stage often within 3–4 weeks (**Figure 1**).

Interestingly, it is particularly prevalent amongst GIN species with relatively short adult life spans that parasitic larvae can temporarily halt their development at a specific point in the life cycle, usually as xL3s or as early L4s, in the vertebrate host tissues. This arrested development called hypobiosis may affect a small or large proportion of the population, depending on the stimulus (Zajac, 2006). One of the described factors are seasonal influences on infective larvae on pasture during harsh climatic conditions (severe winter, summer heat). The trigger to resume development remains uncertain but appears to have a time component or to be linked to the breeding cycle of the host (Brown et al., 2014). Another factor responsible for the initiation of hypobiosis is the host immune responses that inhibit normal development. And lastly, under natural conditions, it is evident that GINs are part of a 'negative feedback' system which is the physiological response to overcrowding populations. Adult worms or other worm populations inhibit incoming infective larvae which are either rejected immediately or go into hypobiosis until the worm population decreases in number or is eliminated. This is apparent in *H. contortus* infections when established populations of *Trichostrongylus* or *Teladorsagia* spp. reduce the establishment of *H. contortus* L3s in the abomasum (Brown et al., 2014; Hoste et al., 2016).

Teladorsagia circumcincta inhabits the gastric glands of the abomasum. The main pathogenic effects are caused by its larval stages which create nodules in the abomasal mucosa and extensively damage junctions between epithelial cells. By damaging the parietal cells, which normally secrete hydrochloric acid, the pH rises and consequently, plasma concentrations of gastrin and pepsinogen increase while concentrations of albumin decrease. Therefore, the infection causes relative protein deficiency. Females of this species are less fecund than *H. contortus*; on average, they produce 100–200 eggs per female per day. Infection with this nematode can cause a relative protein deficiency and reduce growth rate. Additionally, diarrhoea, poor weight gain, weight loss and reduction in wool production are observed (Stear et al., 2003).

Another important group of parasites of grazing small ruminants are *Trichostrongylus* spp. residing in the small intestine of the host. In Australia and New Zealand, four species are commonly found; *Trichostrongylus colubriformis*, *T. vitrinus, T. rugatus* and *T. axei* (in the stomach) (Brown et al., 2014; Roeber et al., 2013). There are more than 30 species that parasitize herbivores. In general, *Trichostrongylus* affects cattle, sheep, goats, and other ruminants, as well as pigs, horses, poultry, and wildlife. The larvae embed themselves in the mucosa, disrupt the integrity of the intestinal epithelium, and the main pathogenic effects are

caused by villus atrophy leading to reduced absorption and protein leakage into the gut. *T. colubriformis* tends to dominate in summer rainfall zones, whereas *T. vitrinus* occurs in winter rainfall zones and is considered to be the most pathogenic one. *T. colubriformis* and *T. vitrinus* are cosmopolitan parasites of sheep. *T. rugatus* is known only from Australia and South Africa and has not been found in New Zealand (Beveridge et al., 1989; Brown et al., 2014). Although primarily known as parasites of animals, several species of *Trichostrongylus* can infect humans, including *T. orientalis*, *T. colubriformis*, and *T. axei* (Sharifdini et al., 2017).

Nematodirius spp. from the family Molineidae, are identified microscopically by cephalic inflation, very large eggs and long thin spicules in the males. Egg output is low with about 25–30 eggs per day. Interestingly, unlike other roundworms, the first two moults occur in the egg; this allows the larvae to survive from one season to the next. These nematodes can cause severe diarrhoea and even deaths in young lambs and calves; nevertheless, they are not highly pathogenic in adult animals. *N. spathiger* and *N. filicolis* appear in Australia while *N. battus* is common in the United Kingdom (Brown et al., 2014; Hopkins, 2020; Roeber et al., 2013).

Oesophagostomum columbianum and *O. venulosum* from family Chabertiidae reside in the large intestine, causing nodule worm disease in ruminants, non-human primates, and swine. Another representative of Chabertiidae of the large intestine is *Chabertia ovina* (Roeber et al., 2013).

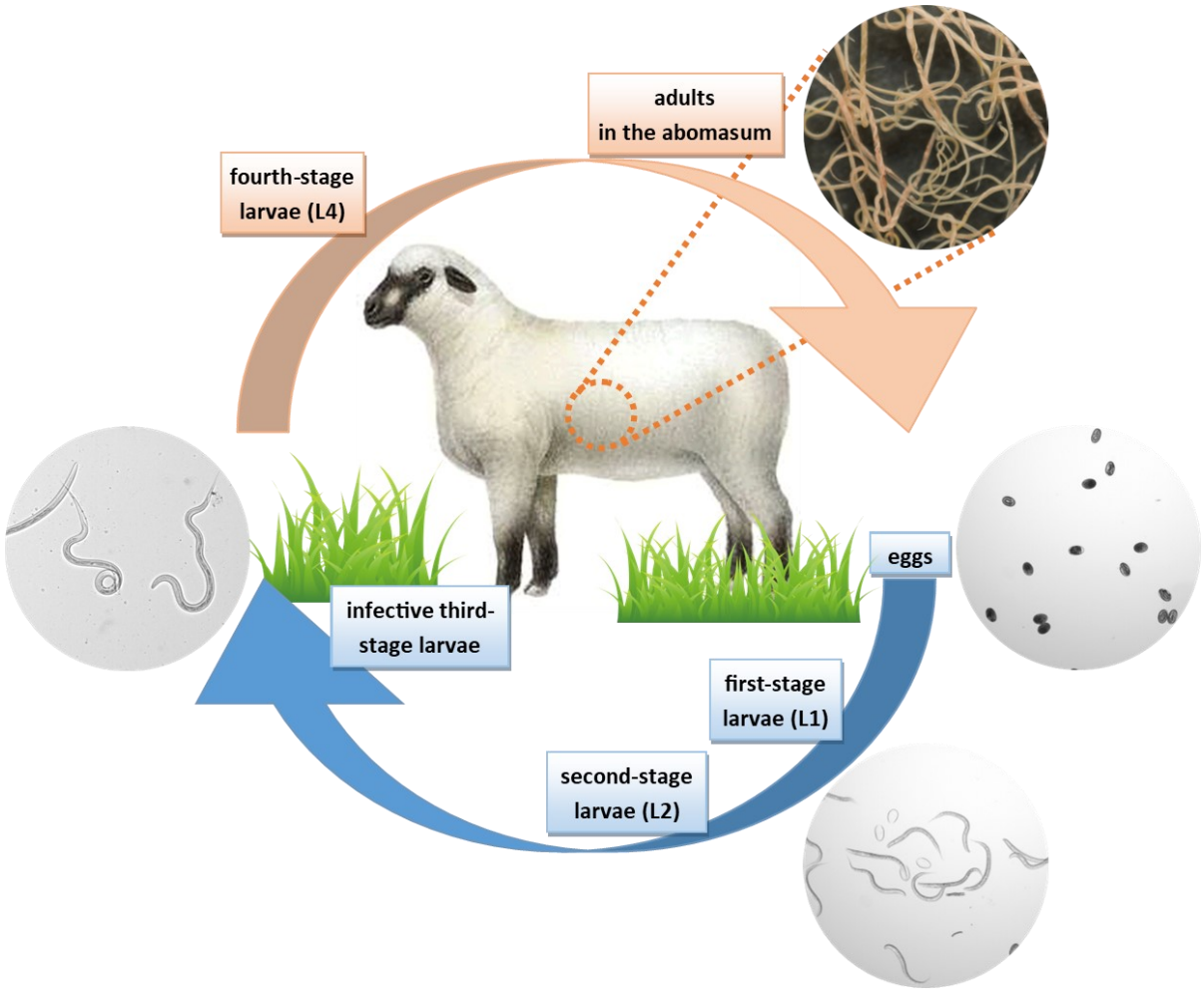


Figure 1 The life cycle of *Haemonchus contortus* with a depiction of free-living stages in blue and parasitic stages in orange.

2.1.1 Haemonchus contortus and aspects of disease

Haemonchus contortus, also known as barber's pole worm, is a highly pathogenic and one of the most prevalent parasites of small ruminants. Since its apparent evolutionary origins in sub-Saharan Africa, it successfully spread to almost all regions of the world and can infect a variety of domestic and wildlife artiodactyl hosts (Hoberg et al., 2004). The requirement of warm and moist environmental conditions for free-living stages ensures that it is a major problem in the humid tropics and subtropics (Besier et al., 2016b; Peter & Chandrawathani, 2005). Additionally, its high biotic potential, i.e., the maximum reproductive capacity of an organism under optimum environmental conditions, allows the parasite to take advantage of even relatively small changes to external conditions (Besier et al., 2016b). The success of this parasite is also due to its extremely high levels of genetic diversity upon which selection can act; hence, it is attributed the high adaptive capability (Gilleard & Redman, 2016; Laing et al.,

2013; Prichard, 2001). Deciphering its genetic diversity is important for many research areas, including epidemiology, anthelmintic resistance, drug and/or vaccine design, and molecular diagnostics.

H. contortus is transmitted orally from contaminated pasture to the host. The parasite is highly susceptible to cold and desiccation; the favourable conditions for embryonation of eggs are temperatures above 10 °C and high humidity. The infective L3s can survive under warm and moist conditions. Larvae can undergo a period of arrested development called hypobiosis if environmental conditions are unfavourable, usually at the end of the grazing season. The L4s and sexually dioecious adults feed on blood in the abomasum. The females are 2.5–3 cm in length with a barber pole appearance of the white uterus filled with eggs wrapped around the blood-filled intestine. The males are 1–2 cm in length and have a copulatory bursa at the posterior end, a feature characteristic of strongylids (Veglia, 1915). *H. contortus* has the shortest life cycle of any GIN (Emery et al., 2016). In sheep, the prepatent period of *H. contortus* is about 18–21 days, i.e., the length of time elapsing between infection of the host with a parasite and parasite maturity to first reproduction (Roeber et al., 2013). Additionally, it is one of the most prolific nematodes; a single female may produce thousands (5,000–15,000) eggs per day. The life span of adult worms is short; they survive in the host for only a few months (Zajac, 2006). Each worm can remove up to 30–50 µL of blood per day, and the burden of hundreds or thousands of worms can rapidly cause anaemia (Arsenopoulos et al., 2021; Emery et al., 2016).

The clinical signs of *H. contortus* infection depend on the number of present parasites and, to an extent, on variation in susceptibility among individual animals and their nutritional status. Unlike many other gastrointestinal parasites, *H. contortus* is not a primary cause of diarrhoea. The pathophysiology of haemonchosis and associated clinical signs are mainly connected with anaemia that develops as a consequence of the blood-feeding activity of L4s and adults of *H. contortus*. Based on the worm burden and host response, haemonchosis has been categorized into three general forms: hyperacute, acute and chronic. The hyperacute form is relatively rare; the blood loss from infection with as many as 30,000 *H. contortus* causes haemorrhagic gastritis, in addition to terminal anaemia. If antiparasitic treatment is not commenced, signs of hypoproteinaemia appear. Submandibular oedema (‘bottle jaw’) is a common clinical finding, although not unique to this infection (Arsenopoulos et al., 2021). In acute haemonchosis, significant anaemia develops over a relatively longer period; however, deaths may occur within 4–6 weeks of infection. Clinical signs are associated with haemorrhagic anaemia, dark-coloured faeces, oedema, hypoproteinaemia, weakness, reduced production of wool and muscle mass, or

sometimes sudden death. The chronic haemonchosis is characterized by infections of small but persistent *H. contortus* burdens and is most common in environments that are marginal for the development of the free-living stages, during less favourable periods in seasonally endemic zones, but also where partially effective control measures prevent the emergence of acute stage. In these cases, clinical symptoms resemble the malnutrition syndromes (i.e., reduction of meat and milk production), and decreased food intake, weight loss and anaemia are commonly observed (Besier et al., 2016b; Roeber et al., 2013). Nevertheless, grazing animals are commonly infected with one or more GINs; therefore, the intensity of infection and clinical signs can vary considerably. The losses of individual host animals differ greatly depending on the regions, years and seasons, environmental conditions and the effectiveness of control measures, including the impact of anthelmintic resistance (Besier et al., 2016b). In traditional livestock systems, animal losses are often exacerbated by poor nutrition and the limited availability and affordability of anthelmintics (Vatta & Lindberg, 2006).

2.2 Treatment and control of GIN infections

The control of GIN infections is critical for the sustainable livestock production system worldwide (Busin et al., 2013). The farmers should implement IPM as a practical approach to reducing chemical use whilst achieving parasite control. Sustainable control is challenging due to growing anthelmintic resistance, hence keeping the efficient anthelmintic therapy in the right animals, right dose, and the right time is undisputedly crucial. Moreover, the selection of the best approaches or their combinations also depends on parasite and disease epidemiology, herd/flock size, season/climate, the conditions on a farm, and associated costs (Jack et al., 2017; Learmount et al., 2015).

2.2.1 Anthelmintic drugs and their mode of action

Anthelmintics offer an affordable and simple means to manage parasitic nematodes and remains the mainstay in the control of GINs infections. Nevertheless, several factors should be considered when selecting a drug or drug combinations, starting with the necessity of treatment or prevention, then the level of severity and type of infection, as well as the prevalence and severity of anthelmintic resistance (Besier et al., 2016a). The anthelmintic efficacy must be preserved since there are no equally effective methods of nematode control available.

For the treatment of GINs of small ruminants, there are a number of classes used, namely benzimidazoles (BZs), imidazothiazoles/tetrahydropyrimidines, macrocyclic lactones (MLs), salicylanilides, amino-acetonitrile derivatives, and spiroindoles (Besier et al., 2016a). Each chemical group has unique targets in the parasites.

BZs are a chemical class with a broad anthelmintic activity; some are effective not only against nematodes but also against cestodes (tapeworms) or trematodes (flukes). They were first introduced in 1960s (e.g., thiabendazole, parbendazole, oxibendazole) and later in 1970s, newer BZs such as albendazole, fenbendazole, mebendazole, and oxfendazole were released for commercial use. Chemically, they are thiazoles or carbamates. There is also a group of prodrugs derived from BZs such as febantel. BZs act on nematodes at the cellular level by binding to β-tubulin subunits, hence inhibiting the polymerization of microtubules, eventually causing cell death. BZs also have ovicidal activity (Kotze & Prichard, 2016).

Most of the commercially available anthelmintics exert their effect on the nervous system of the parasite. Imidazothiazoles together with tetrahydropyrimidines belong to one family of anthelmintics with the same mechanism of action. They act as an agonist for nicotinic acetylcholine receptors (nAChR) on the body wall musculature of nematodes, causing a prolonged activation of the receptor. Consequently, the effect alters the neuromuscular coordination of the nematode and causes paralysis, leading to worm-expulsion from the host (Lanusse et al., 2016). They were also introduced in the late 1960s. Levamisole (LEV) is the L-isomer of tetramisole, and it is the most widely used representative of imidazothiazoles; this relatively broad-spectrum drug acts against adult stages of many gastrointestinal nematodes. Pyrantel and morantel, the tetrahydropyrimidines, are available in some countries for use in sheep.

Another class represents two closely related 16-membered ML groups, the avermectins and the milbemycins, which exhibit broad-spectrum activity in most nematodes and some ectoparasites. The avermectins were introduced in the 1980s and include a series of natural (fermentation product of bacterium *Streptomyces avermilitis*) and semisynthetic molecules such as ivermectin, its precursor abamectin, doramectin or selamectin. Later, another natural fermentation product of *Streptomyces cyanogriseus* moxidectin became the commonly used anthelmintic. Together with milbemycin, these drugs belong to the milbemycin group (Besier et al., 2016a; Lanusse et al., 2016). In general, macrocyclic lactones bind to glutamate-gated chloride ion channels (GluCls) in nerve or muscle cells of parasites. The increased permeability of cell membrane to chloride results in hyperpolarisation of cells, which leads to paralysis and subsequent death of the parasite (Prichard, 2001). As GluCls are expressed on motor neurons,

interneurons and pharyngeal muscle cells, MLs inhibit motility, egg laying and pharyngeal pumping.

Another group called salicylanilides comprises compounds with activity against nematodes, trematodes and/or cestodes as well as some ectoparasites; nevertheless, the main drugs with activity against parasitic nematodes include closantel and rafoxanide (Besier et al., 2016a; Lanusse et al., 2016). These compounds act as uncouplers of oxidative phosphorylation, thus inhibiting energy metabolism.

In the late 2000s, a new anthelmintic class called amino-acetonitrile derivatives with the only representative MOP (trade name Zolvix®) was introduced (Kaminsky et al., 2008a). It was launched firstly in New Zealand in 2009 and then in Australia and the United Kingdom in 2010. MOP has a unique mode of action relating to one or more nematode-specific nAChR called MPTL-1 and another receptor representing DEG-3 and DES-2 molecules in *H. contortus* (Kaminsky et al., 2008b).

A semi-synthetic derquantel represents the last class of anthelmintic used in the treatment of GINs, spiroindoles. This compound is derived from paraherquamide, a fermentation product of *Penicillium simplicissimum*. Derquantel was first described in the patent WO1997/03988 and by Lee et al. in 2001 (Epe & Kaminsky, 2013; Lee et al., 2001). It is produced only in combination with abamectin (trade name Startect®), due to its limited efficacy on some nematodes (Little et al., 2011). Derquantel acts as an antagonist on the B-subtype nAChR, which block cation channels in muscle cell membranes and exert flaccid paralysis in nematodes (Lanusse et al., 2016).

This anthelmintic arsenal can be highly beneficial in controlling infections of GINs and if given at the recommended doses, they have a wide safety margin to the host animal (Sargison, 2012). Nevertheless, the major threat is anthelmintic resistance. Even though the combinations of drugs, i.e., various mixtures of BZs, LEV, MLs and closantel, can ensure higher efficacy against helminths resistant to one or more of the components as well as reduce the rate of selection for anthelmintic resistance, they are not accepted in all countries (Besier et al., 2016a; Lanusse et al., 2015; Leathwick, 2012) and still, the risk of cross-resistance between drug classes cannot be excluded (Falzon et al., 2014). Plainly, the control of multi-resistant worms will be difficult, and procedures to prevent this from occurring are an essential part of sustainable management programmes.

In addition to the resistance problem, chemicals present a source of environmental pollution and have other potential drawbacks, including toxicity to off-target organisms in both terrestrial and aquatic ecosystems (Horvat et al., 2012). In the recent study by Navrátilová et al. mimicking

a real farm situation, the circulation of albendazole and its metabolites was monitored. The detection of active albendazole metabolite (albendazole-sulfoxide) in ovine plasma and rumen content but also in dung, fodder plants and faeces proved chronic environmental contamination, posing threat to ecosystems and food-chain (Navrátilová et al., 2021). Moreover, our study in *H. contortus* showed that contact with sub-lethal albendazole traces improves the ability of the worm to protect itself via induction of drug-metabolizing enzymes and this parasite may gradually become less sensitive to BZ drugs (Dimunová et al., 2022). Still, there is scarce information on the concentrations of other anthelmintics in the environment, and on toxicity in off-target organisms, including plants (Bártíková et al., 2016).

2.2.2 Non-chemical control

The presence of anthelmintic resistance has reactivated thinking about other non-chemical control options. While there is abundant literature on the topic of anthelmintic resistance in sheep GINs, only a few studies have explicitly investigated the putative risk or protective factors in relation to the emergence of anthelmintic resistance. Consequently, some recommendations for non-chemical controls were not evidence-based (Falzon et al., 2014). A wide variety of control measures are available, particularly involving IPM. Non-chemical control encompasses several methods which refer to three main principles of action (i) limit the contact between the hosts and the infective larvae in the field (grazing management), (ii) stimulate the host response (vaccination, nutritional management, and genetic selection of resistant host species), and (iii) use non-conventional anthelmintic materials to eliminate the worms in the host (biological control) (Hoste & Torres-Acosta, 2011).

Grazing management is based on controlling the free-living stages on the pasture to reduce the contamination of pasture and uptake of infective larvae by host animals. Under rotational grazing, only a subdivided paddock is grazed at a time while the remainder of the pasture is spelling (i.e., resting). Infective L3s are reduced or eliminated from unstocked pasture by environmental means (e.g., desiccation); hence spelling during a hot, dry period is more effective in comparison to the winter or spring season when larval survival is good (Barger et al., 1994). In case of mixed-species grazing, simultaneous grazing of goats/sheep most commonly with cattle or horses is practised. While this approach has long been hypothesized as a protective factor for anthelmintic resistance for the reason that intestinal parasites are relatively host specific, it is not completely free of risk as some parasites such as *Trichostrongylus axei* can infect both sheep and horses. Nevertheless, this strategy requires

further studies to prove the association between mixed-species grazing and anthelmintic resistance (Falzon et al., 2014). Another approach is grazing or rotation of young animals with adults since adult animals have acquired immunity to parasites and therefore do not accumulate large burdens of worms.

Among the non-chemical approaches, vaccinations, improving the nutritional status of host animals, and breeding of resistant animals are focusing on stimulating the host response against GINs. Until 2014, the only vaccine available for ruminant nematodes has been for bovine lungworm *Dictyocaulus viviparus* (Jarrett et al., 1960), and to date, only one vaccine (Barbervax®) has been produced to protect host animals against *H. contortus.* It was developed at the Moredun Research Institute in Edinburgh and is only registered in sheep in Australia, but it may be used as 'off-label' in goats (Besier et al., 2016a). The vaccine contains 'hidden antigen' extracted from worm intestinal membranes, in particular native gut internal proteins enriched for H-gal-GP and H-11 of *H. contortus* (Smith et al., 2001). Due to this composition, repeated vaccinations are required to stimulate relatively high levels of circulating antibodies; protection is achieved after the third dose and lasts for approximately six weeks. During the summer barber's pole worm risk period, five or six doses are generally required (Kebeta et al., 2021).

Adequate nutrition is essential for the development and expression of an optimal immune response, but it can also increase resilience which is the ability to maintain productivity (growth, production, reproduction) during parasitic burden (Coop & Kyriazakis, 2001). In several studies, it was shown that sheep and goats respond to supplementary feeding with improvement in resilience and resistance, i.e., the ability of the host to contain and eventually to overcome parasitism (Abbott et al., 1985; Etter et al., 2000; Kahn, 2003). Among the commercialised feed supplement, copper oxide wire particles have confirmed efficacy against *H. contortus* in sheep or goats. In contrast, the anthelmintic effect against other main nematode genera such as *Teladorsagia* and *Trichostrongylus* was less obvious in both small ruminant species (Soli et al., 2010). The question of potential toxicity due to the accumulation of copper in the liver has been addressed, showing the lower risk in goats compared to sheep (Martínez Ortiz de Montellano et al., 2007).

The selective breeding of animals that are resistant to GINs presents another approach. It is employed in experimental and commercial flocks in developed countries rather than in developing countries due to the associated costs with complex collection of information about phenotypes and pedigree. The genetic control relies on the existence of host genetic variation and the predominating environmental condition. In the classical approach, animals can be

selected based on the low egg counts, whereas in the genomic approach, the selection is based on the identification of quantitative trait loci or single nucleotide polymorphisms associated with nematode resistance. Nevertheless, it seems that the continuous development of new classes of anthelmintics in several last decades has compensated for parallel development of this approach for key genera of GIN in sheep and goats (Zvinorova et al., 2016).

The biological control is relatively recent innovation which aims to break the parasite's life cycle by targeting or removing the free-living stages using nematophagous fungi or bioactive pasture plants (Besier et al., 2016a). In the first case, spores of the nematophagous fungi are ingested by host animals. Owing to the thick cell wall, fungi are passed through the gut into the faeces where they colonize and form trapping structures in the presence of nematodes. In both *in vitro* and field studies, nematode-trapping fungi *Duddingtonia flagrans* reduced nematode larvae, including *H. contortus*, in sheep faeces (e.g., Chandrawathani et al., 2002; Chandrawathani et al., 2003; Larsen et al., 1998; Pena et al., 2002) and in goats (Maingi et al., 2006). However promising and environmentally benign, *D. flagrans* has not been widely commercialized and due to the need for daily supplementation of fungal material, biological control is more time-consuming and can be inconsistent compared to the chemical control (Waller, 2003). In the second case, some plants, mainly those rich in tannin, can serve as bioactive forages. For instance, the L3 exsheathment was impaired and motility was inhibited in *H. contortus* when incubated with extracts obtained from tropical plants *Havardia albicans* and *Acacia gaumeri* (Alonso-Diaz et al., 2011). Under scanning electron microscopy, the dramatic changes to the cuticle of the adult of *H. contortus* exposed to tannin-rich material was observed (Martinez-Ortiz-de-Montellano et al., 2013). Anti-parasitic action of chicory (*Cichorium intybus*), sulla (*Hedysarum coronarium*), sainfoin (*Onobrychis viciifolia*) and sericea lespedeza have been also demonstrated (*Lespedeza cuneata*) (Houdijk et al., 2012). Despite the promising results, these biological control strategies are not commonly used (reviewed by Kearney et al., 2016).

For disease control, the aim is to identify animals or flocks that are sufficiently heavily infected or show reduced production. Disease caused by GINs manifests itself in a range of clinical signs described above in **Chapter 2.1**. Several approaches were developed to interpret signs linked to body condition and anaemia scoring. Even though they are subjective and lack specificity, they can still serve as a quick conventional diagnostic technique. For infections caused by *H. contortus,* the degree of anaemia can be assessed by the FAMACHA© test, which is based on the evaluation of the mucous membranes of the conjunctivae using a five-colour chart score: red, red-pink, pink, pink-white or white (categories 1–5, normal–severe anaemia,

respectively). It is an acronym derived from the name of the originator of the idea, Dr. Faffa Malan (FAffa MAlan CHArt) (van Wyk & Bath, 2002). The worsening infection level of an individual animal can also be monitored by the reduction in packed cell volume and increase in faecal egg count (Kearney et al., 2016).

Collectively, the integration of multifaceted IPM measures with strategic drenching coupled with monitoring approaches including faecal egg count reduction (FECR) assist, to some extent, also in managing resistance (Coles et al., 1992). Here, it is important to mention the term *refugium*, which defines a proportion of parasites not exposed to a particular control measure, thus constituting a reservoir of susceptible genes. The lack of these susceptible parasites has been suggested as an essential factor in resistance development; therefore, *refugia* should be incorporated into rational anthelmintic use as a means of slowing the spread of resistance (Hodgkinson et al., 2019; Kearney et al., 2016; Van Wyk, 2001). Overall, given that there is no effective alternative to chemical control of parasitic helminths (Wolstenholme et al., 2004), there is a need to discover and develop novel anthelmintic classes with a new mode of action unless effective vaccines are developed in the future.

2.3 Anthelmintic resistance in GINs with emphasis on *H. contortus*

The occurrence of resistance in parasitic nematodes has been defined in the early 1980s as 'when a greater frequency of individuals within a population is able to tolerate doses of a compound than in a normal population of the same species and is heritable' (Prichard et al., 1980). Several features are considered to influence the rate at which anthelmintic resistance arises and spreads. These include inappropriate dosing (dosing too often or not administering the correct dose quantity), the proportion of nematodes in *refugia*, the gene frequency for resistance in untreated populations, and the biological fitness of resistant worms compared with susceptible isolates (Coles, 2005; Falzon et al., 2014). With regard to the mechanism of anthelmintic resistance, resistance to one member of the anthelmintic class usually confers resistance to the other members and it is increasingly common to have multiple resistances (Wolstenholme et al., 2004). Anthelmintic resistance has been documented in most sheep-rearing countries and notably, resistance to individual anthelmintics has been reported within several years of the introduction to the market in small ruminants (Kaplan, 2004; Sutherland & Leathwick, 2011).

The reduction in drug efficacy is associated with change(s) in: (i) the drug target, (ii) alterations in parasite metabolism, and/or (iii) drug distribution (Wolstenholme et al., 2004).

The pharmacodynamic-mediated drug resistance of the BZ group relates to the point mutations in the β-tubulin that prevent the drug binding. Specifically, polymorphisms in the β-tubulin isotype 1 gene leading the substitution of phenylalanine with tyrosine at codons 200 (F200Y) and 167 (F167Y), and a glutamate-to-alanine polymorphism at codon 198 (E198A) have been associated with resistance in trichostrongyles (von Samson-Himmelstjerna et al., 2007). The highly resistant populations of *H. contortus* were also known to possess a deletion in β-tubulin isotype 2, which can confer BZ resistance (Kwa et al., 1993), although this phenomenon has not been observed in all GIN species (von Samson-Himmelstjerna et al., 2007). A recent study revealed five novel polymorphisms at codon 198 in BZ-resistant *H. contortus* from goats (Mohammedsalih et al., 2020).

In the imidazothiazole class, the mechanism of resistance to LEV is polygenic and associated with molecular changes in its binding to the parasite's pentameric nAChR. In *H. contortus* studies, resistant isolates had increased levels of truncated transcript of two nAChR subunit genes (*Hco-unc-63b* and *Hco-acr-8b*), indicating that these changes may lead to differences in drug sensitivity (Williamson et al., 2011). Moreover, transcription levels of many nAChR subunit genes *Hco-unc-63* and *Hco-unc-29* were downregulated in resistant compared to susceptible isolates (Sarai et al., 2014). The same study described reduced transcription of three ancillary protein genes involved in receptor assembly. Nematodes resistant to LEV are also resistant to other nicotinic agonists such as morantel and pyrantel. However, the exact nature of the resistance to this class remains unclear (Wolstenholme et al., 2004).

The mechanism of development of resistance to the macrocyclic lactones is still being explored. Rather than being associated with qualitative and/or quantitative changes in ligand-gated chloride ion channels, the resistance mechanism is attributed to the efflux pumps P-glycoproteins, which lead to more rapid removal of the drug from the worm (Kotze & Prichard, 2016; Prichard, 2001).

MOP is considered an important alternative treatment in cases of multiple resistance to other classes. However, in 2013, the first resistance report in *T. circumcincta* and *T. colubriformis* was reported from New Zealand (Scott et al., 2013) and since then in *H. contortus* in many other countries (Martins et al., 2017; Mederos et al., 2014a; Sales & Love, 2016; Van den Brom et al., 2015a). The mechanism of MOP resistance has not yet been described. In the experiments of artificial selection under anthelmintic pressure in *H. contortus* larvae, the resistant lines showed mutations in two nAChR subunit genes, namely *Hco-mptl-1* (previously known as *Hco-acr-23H*) and *Hco-des-2H* (de Albuquerque et al., 2017; Kaminsky et al., 2008a).

As mentioned, apart from the mutations in the sites where anthelmintics work, there is pharmacokinetic-mediated drug resistance, i.e., differences in the metabolism of a host or a parasite may affect the biotransformation or transport of the anthelmintics (James et al., 2009). Increased enzyme activity in the host causes the decrease of the anthelmintic concentration in plasma which may result in pharmacotherapy failure. Therefore, the induction of the host's enzymes may indirectly contribute to the development of resistance. The major biotransformation pathways are well studied in vertebrates, but xenobiotic-metabolizing enzymes also deserve detailed research in helminths. In this context, the annotated parasites' genomes played a substantial role in advancing the progress in this field. The literature indicates that the presence, expression and activities of the drug-metabolizing enzymes in helminths are distinct from most of those in mammals. Several studies, including those published by our research group, focused on the expression and activities of drug-metabolizing enzymes of phase I and phase II of the biotransformation process, as well as the efflux transporter proteins in parasite strains with different levels of drug susceptibility (reviewed by Ardelli, 2013; Cvilink et al., 2009; Matoušková et al., 2016). From the metabolic studies, it is clear that GINs can protect themselves against the toxic action of anthelmintics via the increased level of biotransformation.

2.4 Anthelmintic drug discovery

In the face of widespread anthelmintic resistance, there is an urgency to discover new anthelmintic drugs with a novel mode of action to preserve animal health and economy in the small ruminant industry. Drug discovery and development is a multifaceted process that consists of roughly four major stages, called phases. In essence, preclinical phase 0 comprises basic research, drug discovery, and preclinical tests which aim at assessing the efficacy of the drug candidate. Clinical tests aiming at establishing safety and dosage, efficacy and side effects then follow (phase I–III). If there is evidence that the drug is safe and effective, it undergoes regulatory approval. In case a drug is introduced to the market, the post-market surveillance is monitored in phase IV (Réda et al., 2020). Drug discovery and development is a lengthy, complex, and costly process, with a high degree of uncertainty that a drug will succeed and be amenable to pharmacological action (**Figure 2**). The extent of failure rate in drug development is over 96 %, including a 90% failure rate during clinical development (Hingorani et al., 2019). The recent cited estimate suggests that it costs on average $1.3 billion and usually takes 10 years and longer to advance a new drug from initial concept to market for human drugs (Wouters et

al., 2020), whereas the research and development cost for a new animal health drug is about $50–100 million (Nixon et al., 2020). While these numbers are daunting, ultimately, the success of drug discovery brings hope and relief to patients and animals in human and veterinary medicine, respectively. Interestingly, most of the anthelmintics currently used in public health have been developed in veterinary parasitology (Keiser & Utzinger, 2010).

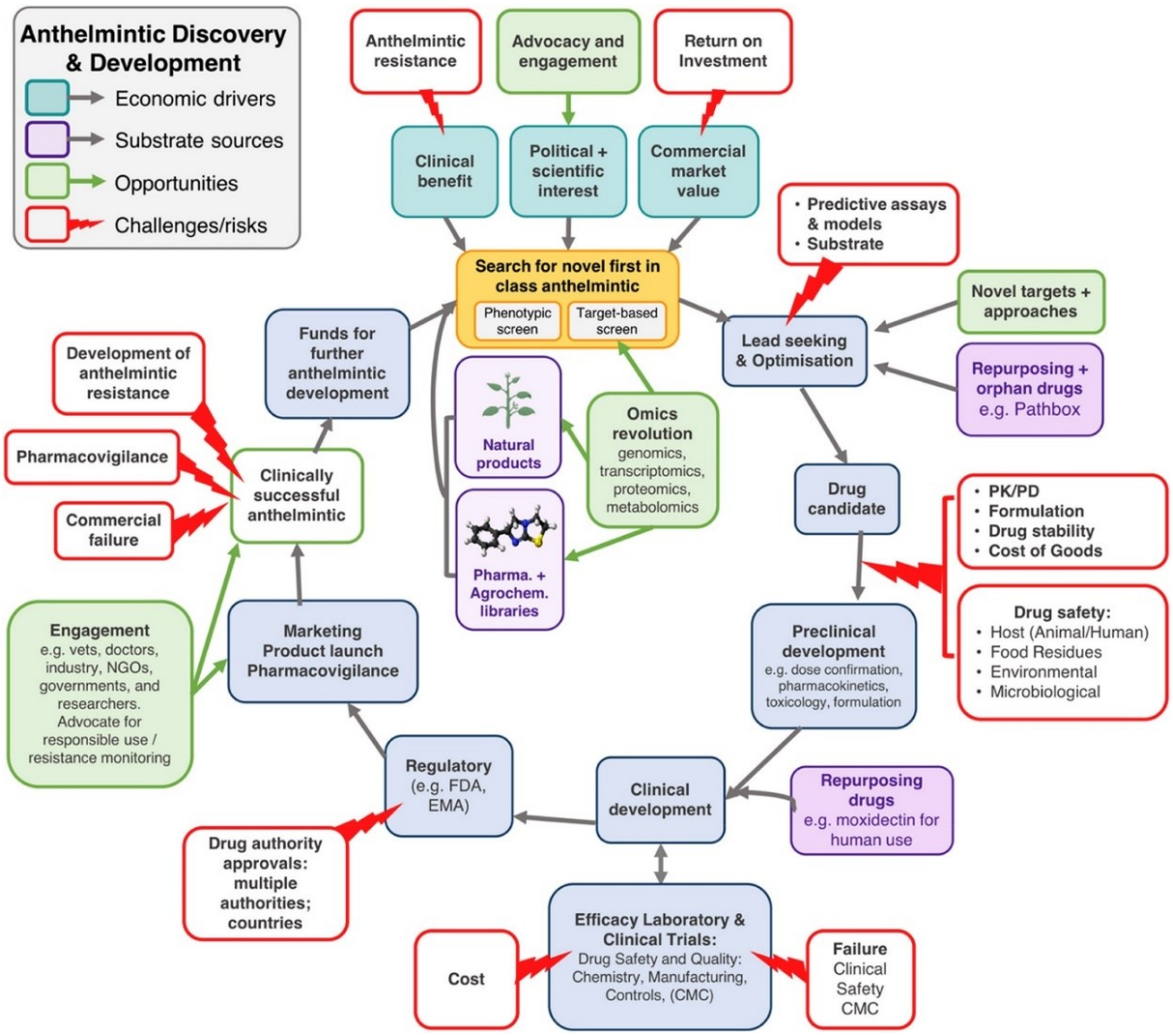


Figure 2 An overview of the anthelmintic discovery and development process. The economic drivers, substrate sources, opportunities, and challenges are illustrated (Nixon et al., 2020).

Like all drug discovery efforts, veterinary parasite drug discovery is an iterative process, beginning with a screening of large compound libraries which contain diverse or scaffold-specific collections of synthetic chemicals or natural products. For discovery purposes, the attempts are to develop cost-efficient and high-throughput screening (HTS), which enables

screening in parallel. For medium-throughput screening, the sample size generally lies between 500 and 10,000, whereas in the case of high- and ultrahigh-throughput, the sample size goes up to 10,000–100,000 and > 100,000, respectively (Wildey et al., 2017). The concept of HTS first appeared in the mid- 1980s and has evolved over the past years. Nowadays, various HTS platforms and technologies differ, for example, in the detection system and analysis, some of which are described below (see **Chapter 2.5.1**). Nevertheless, it is necessary to realise that HTS rarely identifies a new drug but rather a chemical starting point around which a 'hit-to-lead' chemistry process can be initiated (Preston et al., 2016). Another accepted drawback of random screening assays is that a single arbitrarily selected concentration is used for the particular screening which increases the risk of false negatives.

In anthelmintic drug discovery, different strategies have been employed. In the past, the discovery has been conducted using infected animal models, which were treated with experimental compounds, and consequent changes in parasite burdens were measured. This low-throughput model led to the discovery of LEV, pyrantel, and ivermectin (Geary, 2016). Nevertheless, this paradigm has been largely abandoned in favour of more economically appealing procedures. Nowadays, two strategies prevail, both using parasites *in vitro*.

The first approach is mechanism-based screening or also called target-based screening. Only together with the in-depth knowledge of the basic biology of the parasite can the target-based screening strategy be successful. Owing to advances in molecular biology, the principle of this screening is to measure the interaction of the drug with a specific target protein. Nevertheless, this approach can miss the lead compounds where specific targets have not yet been characterised to the readily assayable extent (Kotze, 2012). The current anthelmintics target the neuromuscular system of nematodes, except BZs. However, it is apparent that there are more potential anthelmintic targets within nematodes, the promising one being, for example, the worm gut and cuticle, the signalling pathways such as ecdysone, kininase inhibitors, and metabolic pathway chokepoints (Kotze, 2012; Taylor et al., 2013). The approach of 'one disease–one target–one drug' enables greater screening capacity, but it oversimplifies disease mechanisms. Moreover, drug discovery evolves toward network pharmacology (Geary et al., 2015; Hopkins, 2008). Even though this strategy has merits and HTS could be applied to identify the best-in-class drugs (Swinney, 2013), no compound discovered in a mechanism-based screen has yet been commercialised as an anthelmintic (Geary et al., 2015).

The second approach is whole-worm screening, where drugs are deemed successful by virtue of their effects on viability or behaviour, regardless of the previous knowledge about the drug target. Whole-worm screening has been revealed to be a more successful strategy, and

conventional whole-worm screening assays have been utilised even for detecting anthelmintic resistance. Most assays use free-living stages, including egg hatch assay, larval development assay, larval feeding assay, larval migration inhibition assay and larval motility assay. For instance, the first BZ anthelmintic, thiabendazole, was discovered in a trichostrongyloid larval assay (Brown et al., 1961). However, most of these assays are time- and labour-consuming. The manual recording, e.g., in the larval development and motility assays, is inherently subjective and can influence the measurement of the activity of compounds. Moreover, these methods also rely on the preparation of the biological material (egg, larvae, adults) and keeping the parasitic culture under experimental conditions (Nixon et al., 2020). Nonetheless, there have been recent improvements in the screening assays for the parasitic nematodes such as automated recording of the nematode phenotype (see **Chapter 5.4.2**).

Typically, researchers discover new drugs through novel insights into a disease process. The era of system biology puts relevance to the use of advanced molecular and informatics technologies to assist the anthelmintic discovery. The information gained from genomic, transcriptomic, proteomic, and metabolomic investigations provides a solid foundation for bioinformatic-guided discovery and understanding molecular mechanisms involved in metabolism, parasite-host interaction, immune system evasion and molecular evolution, among other topics.

The main strategies and recent advances in the drug discovery of anthelmintics have been reviewed (see **Chapter 5.1**). Thenceforth, there were novel studies and reviews published on the topic of anthelmintic drug discovery and development, which underline the importance of this issue. A remarkable study was done by Taki et al.; the authors screened 80,500 small molecules against xL3 of *H. contortus*. This study achieved not only an identification of three small molecules that reproducibly inhibited larval motility and/or development (IC_{50} values of ~4 to 41 μM) but also provided with a standardised screening assay with ≥ 10-times higher throughput (i.e., 10,000 compounds per week) compared to the most previous assay (Taki et al., 2021). Furthermore, in the most recent book series of Advances in Parasitology, several chapters were dedicated to *H. contortus* and its role in drug discovery (Herath et al., 2021; Jiao et al., 2020; Ma et al., 2020). Additionally, natural products are also highly considered for anthelmintic drug discovery. A systematic review on comparative analysis, toxicology and pharmacology of medicinal plants against *H. contortus* assessed 458 research studies from 1,480 articles published in 40 years since 1980 (Ali et al., 2021).

Moreover, in recent years, attention has been paid towards the helminth secretome with an aim to explore interactions between hosts and pathogens, but it has also been viewed as

a potential source for novel anthelmintic and vaccination discovery (Ditgen et al., 2014; Moreno et al., 2021). Nevertheless, there is only scarce information about GINs of small ruminants relative to other helminths which parasitize in humans (Ditgen et al., 2014; Drurey & Maizels, 2021). The excretory-secretory products from larvae of *H. contortus* were studied (Gamble & Mansfield, 1996) and more comprehensive proteomic analysis of secretome from L3s, L4s, females and males of *H. contortus* after 12h *in vitro* culture was characterised (Wang et al., 2019a). Understanding the host-parasite relationship at such level might support the discovery of novel drugs and vaccine targets.

2.4.1 H. contortus as a model for anthelmintic drug discovery

A central assumption of the model-organism approach is that genetic model organisms produce information that is relevant to target species (Gilleard et al., 2005). Among pathogens for anthelmintic deployment, trichostrongyloid nematodes parasitizing small ruminants are arguably the most important. Although several species in this family have been used for drug discovery and pharmacological investigations, *H. contortus* has been the most prominent (Geary, 2016). For many reasons described below, it attracts the attention of researchers endeavouring to develop new anthelmintic drugs.

H. contortus shares the same clade V as another established model in the anthelmintic screening *Caenorhabditis elegans* (Blaxter, 2003). *C. elegans* is well described on a molecular level, and since its introduction to the study of development and neurobiology in 1965, it facilitated and accelerated research of a broad array of biological problems (Brenner, 1974; Nigon & Felix, 2017). However, it is a free-living soil nematode, hence suitable for the study of nematode biology and not for parasitism (Geary & Thompson, 2001).

The short, direct life cycle and easy infection maintenance in experimental animals make *H. contortus* a good model. Since females are highly fecund, it is simple to produce L3s by incubating collected faeces from the infected animals at 27–28 °C in humid conditions for 7 days. L3s are free-living and can be stored in water or physiological saline at 7–10 °C for several months for experiments and/or artificial reinfection with a single dose. The L4s can be produced *in vitro* after exsheathment of L3s with 0.15% (v/v) of sodium hypochlorite for 20 min at 38 °C and incubation of these xL3s for a week in a humidified environment (38 °C, 10% CO_2) (Preston et al., 2015). These larval stages allow compounds to be screened for their effects in a high-throughput fashion.

At present, there are many genetically divergent strains derived from distant geographical locations used for experimental needs that vary with the susceptibility to the common anthelmintics (Sallé et al., 2019). For our studies, we maintain the life cycle of the Inbred Susceptible Edinburgh (ISE; MHco3) strain and two resistant strains. Inbred Resistant Edinburgh (IRE; MHco5) is derivative of ISE strain, and it is BZ and ivermectin resistant (Redman et al., 2008; Roos et al., 2004). The White River (WR; MHco4) multi-resistant strain is a field strain derived from South Africa, and besides the BZs, it also demonstrated resistance to ivermectin, LEV, closantel, and rafoxanide (van Wyk & Malan, 1988). Notably, two genomes and transcriptomes of *H. contortus* are available; one is the MHco3 strain (Laing et al., 2013) and the other is McMaster strain from Australia (Schwarz et al., 2013). At that time, the genome was sequenced using the second-generation Illumina technology, and both assemblies were missing approximately 7% of the haploid genome. Nevertheless, the employment of novel third-generation genome sequencing, which generates long reads even from a single DNA molecule, can complement the available assembly of the short-read dataset (van Dijk et al., 2018). Clearly, genomes now present a solid foundation for fundamental research in areas such as pathogenesis and should facilitate applied research toward the development of new interventions and diagnostic methods as well as investigation of anthelmintic resistance (Doyle & Cotton, 2019). Besides, the transcriptomes also helped to identify the xenobiotic detoxification enzymes in *H. contortus* (Matoušková et al., 2016). In the integrative 'multi-omics' approach, *H. contortus* can readily provide miRNome (Winter et al., 2012), secretome (Wang et al., 2019a), proteome (Dicker et al., 2014; Hart et al., 2012; Wang et al., 2016; Wang et al., 2019b), phosphoproteome (Wang et al., 2020) and lipidome (Wang et al., 2018). These ample entry points will be of major benefit as resources for the scientific community working on this and related parasitic nematodes.

2.5 Drug testing in *H. contortus*

Whether it is a drug for human or veterinary usage, it must be tested for its efficacy and toxicity. This is connected with pharmacokinetic data, which report the fate of the drug from absorption to metabolism, distribution, and excretion, and with pharmacodynamic data, which describe the drug effect on the body in a dose-dependent manner, including anticipated and adverse responses (Visser, 2018). The following chapters provide an account of the significant techniques used in drug efficacy testing ordered by the nature of the assays and further by the life cycle stage of *H. contortus*. These assays are used in drug discovery but also in the diagnosis

of anthelmintic resistance, which is critical for delaying and controlling resistance development. In some areas, only the representatives and principles of the anthelmintic assays are described rather than exhaustive examples of alternatives in the methodology but still with the intention to be comprehensive.

2.5.1 Developmental and behavioural bioassays

Historically, drug discovery was based on testing compounds in infected hosts *in vivo*. Widely used across GIN parasites of livestock, **faecal egg count reduction test (FECRT)** provides an estimation of the anthelmintic efficacy by comparing egg per gram (epg) from treated or untreated hosts, before and after treatment. Although the standardised FECRT method was proposed (Coles et al., 1992), several authors used different evaluation protocols (Cabaret & Berrag, 2004). Moreover, the method uses the McMaster method for counting the eggs microscopically in a special counting chamber with a detection limit of 10–50 epg; nevertheless, many modifications to this method were introduced for diagnostics purposes (Vadlejch et al., 2011). The GIN species may also affect the results. As mentioned above, drug discovery nowadays rather uses screening assays.

On the contrary, the following assays monitor the worm development and/or behaviour *in vitro* and the drug efficacy is based on a dose-response curve. **Egg hatch test (EHT)** was first described by Le Jambre and is used for testing the ovicidal activity, which was proven in the BZ group, hence EHT is also used to detect BZ resistance (Le Jambre, 1976). Eggs are incubated with the various concentrations of a drug for 48–72 h at 25–27 °C. Thiabendazole is used as a positive control due to its high solubility in water. In the case of incubation for 24 h, the evaluation of embryonation can be assessed. After the incubation time, the reaction is stopped by adding a drop of Lugol's iodine solution, and a number of larvae and unhatched eggs are microscopically evaluated. The procedure also obtained minor modifications, but an important step is the preceding isolation of eggs from freshly collected faeces which can be done by various techniques, e.g., saturated sodium chloride, magnesium or zinc sulfate flotation, sugar or glycerol gradient centrifugation (von Samson-Himmelstjerna et al., 2009a).

In the case of infective L3s, there is an abundance of assays for drug testing, including larval development, migration and motility tests. **Larval development test (LDT)** describes culturing of eggs in the presence of heat-treated lyophilised *Escherichia coli* and/or yeast extract, as a food source, and the anthelmintic for 7 days at 25 °C to the development to L3s (Hubert & Kerboeuf, 1992). The second version of LDT is the **micro-agar larval development test**

(MALDT) where an agar matrix containing the anthelmintic is used. While the results of LDT and MALDT are comparable, LDT has the advantage of being less time-consuming (Várady et al., 2009). LDT also brought a fruitful result from a screen of compounds active in agrochemical screens and found the precursor of MOP (Ducray et al., 2008). Nowadays, LDT and MALDT are routinely applied to various anthelmintic groups as an inexpensive test for the investigation of anthelmintic resistance with limits to MLs. For this inability, a **larval migration test** was prompted. It measures the inhibition effects of anthelmintics on the ability of the L3 stage to migrate through an agar layer and a 20 μm filter mesh. The advantage is the direct counting of physically separated migrated worms rather than relying on the assessment of individual worms as either motile or non-motile. In the study of Kotze et al., contrary to LDT, larval migration assay failed to show activity against thiabendazole (Kotze et al., 2006). This indicates a need for applying different assay types to drug discovery to prevent erroneous elimination of candidates at the early screening stages. Larval migration test was also used in LEV and ivermectin drug efficacy testing (Raza et al., 2015).

The ease of using larval stages of *H. contortus* for the identification of novel compounds has led to the continuing employment of this model. **Larval feeding inhibition assay** is employed in L1s and is adopted from the same assay on adults. The assay is based on the reduction of food ingestion containing *E. coli* labelled with fluorescein-5-isothiocyanate, which can be quantified using fluorescent microscopy (Alvarez-Sánchez et al., 2005). This method can be used for detection of resistance to MLs and imidazothiazoles. In avermectin, the disruption of larval feeding and growth were both affected by relatively low drug concentrations which were between 10- and 100-fold lower than concentrations required to inhibit larval motility (Geary et al., 1993; Gill et al., 1995). Nevertheless, a more recent study opposed this relative sensitivity reporting that worm motility was affected at drug concentrations below those required to inhibit feeding. This was explained by a detailed analysis of subtle versus more profound changes in worm motility (Kotze et al., 2012). This study highlights the complexity of worm movement and the nuances that can be easily overlooked by machine scoring. In imidazothiazoles, the feeding inhibition is a result of muscular spastic paralysis. Larval feeding inhibition assay was used for testing of a small number of novel compounds such as plant lectins and extracts from tropical plants (Ríos-de Álvarez et al., 2012).

Worm motility is considered a health marker and is often used as a readout of the effect of anthelmintics. Motility can be measured in several time points with the need to agitate the larvae by shaking prior to the measurements. **Larval motility assay** was firstly evaluated directly by observation. Further improvements such as multi-well plate format and employment of the

image analysis platforms made the manual microscopy-based motility assays more or less automated and feasible from a modest- to high-throughput scale. The first such system was the micromotility meter in the 1980s. The movement of L3s after incubation with anthelmintics (usually for 24–72 h) in the culture tube was measured via the altered light refraction from the meniscus of the solution. The signal entered the photodiode, where the information passed to a computer to give a motility index (Bennett & Pax, 1987). A dose-related-anthelmintic-induced paralysis can again differentiate between the susceptible and resistant isolates; however, this does not apply to all drugs (George et al., 2018). It is worth noting that motility assay is not able to discriminate between drugs causing worm paralysis versus those that are directly lethal. They only provide a rapid readout after the exposure to the drug regardless of giving the information whether the paralysis can be reversible or not. Another example is a low-cost motility which was already applied in many screening initiatives of various compounds libraries (Preston et al., 2015). Many motility platforms were developed for *C. elegans* (Buckingham & Sattelle, 2009; Partridge et al., 2018; Ramot et al., 2008). Nevertheless, taking into account that all nematode larvae move in a sinusoid fashion, the algorithms might be applied to other nematodes, including *H. contortus*, after adjusting the open-source and the parameters (see **Chapter 5.4.2**). Another study by Smout et al. described an apparatus that did not need microscopy but instead used special microtiter plates (xCELLigence and E-plate, Roche Inc.) to measure movement in real time via the electrical impedance (Smout et al., 2010).

Unlike the larval stage, the inability to maintain viable adults of *H. contortus* in *ex vivo* conditions precludes their use in a primary HTS, even though thousands of adults can be obtained from a single infected sheep. Nonetheless, there are studies of **motility assays in adults** of *H. contortus*. In the study by O'Grady, motility was scored manually by observing the form and degree of movement after 24 h, 48 h and 72 h (O'Grady & Kotze, 2004). The same study also monitored **feeding** by measuring effects on the ingestion of radioactively labelled [^{3}H]inulin after 24 h. The calculation of the amount of ingested [^{3}H]inulin required subtraction of the radioactivity recovered from worms whose pharynx was prevented from functioning by pre-treatment with ivermectin. Similar to larvae, the feeding assay can be based on measuring fluorescence; in that case, the indicators are dextran or *E. coli* labelled with fluorescein-5-isothiocyanate. Overall, these assays have not proved to be suitable for routine use.

2.5.2 Biochemical assays

Generally, biochemical assays are valuable and scalable tools even for drug discovery and development. Biochemical assays in conjunction with the colorimetric, fluorimetric or luminesce readout can offer a fast quantitative assessment of drug efficacy in an objective manner (Hughes et al., 2011). Unlike a variety of biochemical assays well validated for viability testing in mammalian cell cultures or other organisms, for instance in *C. elegans*, there have been relatively sporadic studies in *H. contortus*. The major caveat of this approach is that compounds must bio-accumulate in parasites to demonstrate efficacy. The **MTT assay** is a colorimetric method that uses metabolic activity as a marker of viability. Mitochondrial dehydrogenases convert the pale-yellow water-soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple insoluble product formazan. This assay was optimised in L1s of *C. elegans* in a high concentration of MTT (5 mg/mL) with incubation for 3 h; however, the uptake of MTT was not observed in L3s of *H. contortus* (James & Davey, 2007). The same study provided a correlation of formazan production and the number of L1s of *H. contortus* but did not show the viability of L1s after the ivermectin treatment. The modified MTT assay was used for testing plant products in xL3s. After the 42 °C incubation for 14 h with a coloration solution of five reagents, blue larvae were counted using the light microscope. Based on the available literature, MTT reduction assay failed in other life cycle stages. The successful coloration of larvae in this study was probably due to the exsheathment, as the cuticle protects the larvae from environmental contact (Hordegen et al., 2006).

A recent article applied the common cytotoxicity **lactate dehydrogenase (LDH) assay** to measure the anthelmintic effect of three tropical plant extracts. Nevertheless, the authors did not measure the activity of LDH released from damaged cells into the medium as in the standard procedure, but rather the inhibition of LDH activity as it is discussed that anthelmintics such as LEV and BZs might alter the parasite LDH activity (Davuluri et al., 2020). The authors used this enzyme assay as a complement to other routine methods, but the methodology and results alone did not yet seem convincing that LDH assay might be used for screening of novel anthelmintics.

In vitro **enzymatic activities** can be measured from the **subcellular fractions** of the parasite. For instance, a study by Kotze studied oxidase activities in L3s and adults after ivermectin treatment of ML-susceptible and resistant strains. He conducted two assays commonly used to examine cytochrome P450 activities, namely aldrin epoxidation and *O*-deethylation of 7-ethoxycoumarin. Higher levels in drug-resistant strains were assumed, but

no significant differences were measured in the isolates for any of the activities in either life stage. The author also stated the absence of activity toward a number of general cytochrome P450 substrates (methoxyresorufin, ethoxyresorufin, benzo[*a*]pyrene, aniline) in *H. contortus* (Kotze, 2000).

Another biochemical assay is used in association with P-glycoproteins which have been implicated in resistance, particularly to ivermectin and LEV (Ardelli, 2013). The functional consequence of these drugs exposure was examined by measuring the efflux of the dye rhodamine-123 from larvae. The rhodamine from the media is detected using a fluorescence spectrophotometer (Raza et al., 2016).

In our laboratory, we optimised the adenosine triphosphate as the viability marker in *H. contortus* with **ATP bioluminescence assay** (see **Chapter 5.4.1**). Previously, ATP level was used for measurement of metabolic activity in *H. contortus* adults incubated with ivermectin. Results of that study showed that ATP levels were unaffected by ivermectin even at concentrations ≤ 10 μM, which exceeded concentrations required to inhibit ingestion by 10,000-fold (Geary et al., 1993).

2.5.3 Molecular diagnostic tests

Concerning testing of drug efficacy, molecular tests are not used for the screening of novel compounds but for diagnostic purposes of parasitic infection and as an adjunct to the FECRT. The molecular diagnostic tests are able to detect and/or analyse nucleic acid molecules (DNA or RNA), hence directly measure genetic differences between susceptible and resistant populations. Nevertheless, the degree to which the molecular mechanisms of resistance are understood varies considerably among the different anthelmintic classes and parasite species. The genetic determinants of BZ resistance are perhaps the best characterised of all anthelmintics. The BZ resistance can be diagnosed with the **tubulin binding assay**. The test uses the radioactive measurement of tritiated mebendazole that binds to a measured amount of nematode protein (Lacey & Snowdon, 1988). The extent of resistance was expressed as the ratio of resistant isolate binding to the control susceptible isolate binding. Molecular analysis of the F167Y, E198A and F200Y markers associated with BZ resistance can be analysed from DNA extracted from eggs and larvae, followed by **pyrosequencing** and **amplicon sequencing** of PCR products. These two methods show concordance, albeit higher at codon 200 compared to codon 167 (Morrison et al., 2022; von Samson-Himmelstjerna et al., 2009b). Pyrosequencing is based on the sequencing-by-synthesis principle with the luminescence detection of

pyrophosphate released on nucleotide incorporation. Nowadays, **genome-wide approaches** provide broader applications than candidate gene-based or gene expression changes. They are now accessible, and with the decrease in the cost, they are increasingly applied to characterise traits including drug resistance (Doyle & Cotton, 2019). The results of the molecular tests show good agreement with the biological tests (EHT, LDT) when high levels of resistance are present as molecular assays are generally more sensitive (Barrère et al., 2013; Doyle & Cotton, 2019).

The first report of a putative DNA marker for detection of LEV resistance in *H. contortus* was provided by the DNA sequencing of *Hco-acr-8* gene from many LEV-susceptible and resistant field strains (Barrère et al., 2014). The absence of a 63 bp index in the exon 3b sequence has been found to be correlated with the LEV resistance phenotype. Other DNA-based techniques such as allele-specific PCR, real-time-PCR, pyrosequencing, which can distinguish between genomic DNA with and without this insertion/deletion, could also be used to monitor for LEV resistance.

2.5.4 Challenges and research perspectives

To address the challenges of anthelmintic discovery, we must search for and apply new opportunities and technologies. The challenge of the spreading resistance in prevalence and severity was already referred to in the previous chapters. In summary, pharmacology-parasitological knowledge is critical to maintain the efficacy of both traditional and more recent anthelmintic compounds. For that, further understanding of the host-parasite-environment interaction is important to complement control measures (Emery et al., 2016; Falzon et al., 2014). Additionally, pharmacokinetic-based optimisation of drug activity and pharmacodynamic interactions between drug combinations need deeper research to identify the best treatment strategies (Lanusse et al., 2018). Moreover, only evidence-based management recommendations should be implemented to reduce the risk of further development and impact of the anthelmintic resistance (Falzon et al., 2014).

Other challenges are associated with inherent difficulties in maintaining the life cycle of *H. contortus* and the current screening strategies. Even though the '3R' principles (Replacement, Reduction and Refinement) are increasingly prioritized as a framework for conducting science in the academic and industrial sectors with a high focus on developing alternative approaches that avoid the use of experimental animals (Tannenbaum & Bennett, 2015), so far the *in vitro* egg-to-egg culture system for this parasite has not yet been fully developed. The necessity of maintaining sheep infected with *H. contortus* as a source of eggs

and larvae for drug testing is an impediment, and the viability of *ex vivo* adults is also unsustainable. Still, there would be a risk of higher stress due to the *in vitro* conditions; therefore, parasites in culture might be more susceptible to the drug than parasites in a host, leading to the routine discovery of false positives. It would be necessary to look into the gene expression profile of worms freshly removed from the host and compare it with the parasites maintained in culture (Geary, 2016). In case the expression profiles of *in vitro* developed L4s would be similar to the profile of those isolated from the sheep abomasum, it would be possible to substitute the more readily available L4s for adults in the secondary screens, hence eliminating the need for adults from sacrificed sheep hosts. Nevertheless, each life cycle stage may vary in the response to the drug; therefore, the studies should provide separate testing on different stages.

In the second half of the last century, there were attempts to culture the parasitic stages of *H. contortus* (Silverman, 1959). A laborious technique for the *in vitro* culture of mature males and females of *H. contortus* from xL3s in 28 and 36 days, respectively, has been reported (Stringfellow, 1986). The study described and compared 10 systems of complex culture medium containing bovine and ovine gastric content and many other supplements. Mating was not observed, but the eggs that passed from the female were smaller, unsegmented and underwent only partial development to the 8-cell stages. Generally, the morphogenesis of larval and adults *in vitro* was identical to that observed *in vivo*. The factor that enhanced development in adults was not identified, but the crucial role of hemin was stated.

Other studies characterised the immunosuppressed rodent models with a successful evaluation of several anthelmintics from BZ and ML groups. The development of xL3s to L4s, albeit slower, was established in gerbils (*Meriones unguiculatus*) and mice (Conder et al., 1990; Sommerville, 1977). These models may provide an effective tool for studying host-parasite interactions or for the preliminary assessment of the activity of the experimental drugs; for instance, *H. contortus*-infected gerbil model was also used to test the anthelmintic effects of different plant extracts (Squires et al., 2011; Squires et al., 2010). Even though the small laboratory animals can offer convenience in handling and feeding, as well as lowering the research cost, the full life cycle of *H. contortus* cannot be successfully established in them.

The empirical drug discovery in the parasite realm has evolved over the past century. Nowadays, there has been a revival of interest in whole-worm screening. Interpretation of anthelmintic activity in these assays commonly relies on parameters such as nematode development and motility. To summarise the aforementioned drug testing assays, there is no single correct method to assess drug efficacy given the variety of mechanisms of action of the

drugs. Nevertheless, the motility assays have proved to be highly efficient in terms of robustness, time and speed in primary screening. Moreover, the system supports high-throughput toxicity screening in an alive, metabolizing organism. Even though the phenotypic drug discovery has considerable challenges, such as validation of the 'hits' and target deconvolution, this approach may address the incompletely understood complexity of diseases (Moffat et al., 2017). Furthermore, greater implementation of the state-of-the-art technologies in material handling platforms and automated parasite phenotyping with image recognition systems can conceivably improve the predictive power of *in vitro* whole-organism screening. The main resources of compound screening libraries originate from chemical synthesis or natural product-inspirited sets. In the former case, libraries contain small molecules which were scientifically curated with significant structural diversity, lead-likeness and overall solubility profile. In the latter case, the natural products as drug candidates are experiencing re-emergence, which is also facilitated by advances in analytical methods such as chromatography, spectrometry and spectroscopy (Herath et al., 2021). Towards the mechanism-based screening, bioinformatic-driven prioritising of compounds *in silico* based on transcriptomics and proteomics analyses has certainly made the drug discovery faster and more efficient. It remains to be determined whether this prioritisation of initial hits can improve the success rate in the mechanism-based screens using *H. contortus* (Taylor et al., 2013; Wang et al., 2015).

Clearly, the need for the development of drug efficacy methods was intensified in relation to anthelmintic resistance. In terms of the diagnostic tools to detect anthelmintic resistance, FECRT is the only assay used in the field; however, it suffers from a lack of sensitivity and labour-intensive sampling procedures. *In vitro* assays remain as laboratory tools only and currently lack utility across different drug classes and parasite species. On the other hand, molecular tests are used as research tools. They offer considerable advantages in terms of sensitivity, cost, sampling procedures, and speed; thereby, there is an opportunity for commercial use of molecular tests in the near future (Kotze et al., 2020).

Economic drivers are another impetus of the drug discovery process. Not exclusively, but the phenotypic screening approach has been undertaken by industrial laboratories while the mechanism-based strategy has traditionally been the focus of the academic institutions (Campbell, 2016). Nonetheless, the line of research is too important to cede solely to the industry or academia, hence the partnerships are crucial. The vital role of basic research was advocated (Nixon et al., 2020). However, it seems that the visibility is very low for GIN diseases in contrast to the neglected tropical diseases or parasitic infections of humans in general. The

public-private partnerships comprise research institutes, universities, not-for-profit organizations, and pharmaceutical companies. They can facilitate anthelmintic discovery and development and should be further supported and built (Preston & Gasser, 2018). For instance, the researchers now work on screening collections of > 200,000 synthetic and natural compounds in collaboration with philanthropic partners, including the Medicines for Malaria Venture and Griffith Institute of Drug Discovery (Taki et al., 2021).

In the light of the widespread drug resistance, paucity of vaccines and a low number of novel anthelmintics, it is vital to maintain the efficacy of the available drugs. The opportunities on the path to new anthelmintics lie in the effort put into the drug discovery and development which is connected with reliable assays for drug testing. With this, we aspired to address some of the stated challenges in the following chapters.

3 AIMS OF THE DISSERTATION THESIS

The objective of the present thesis was to examine various directions in the discovery and testing of new potential anthelmintics using parasitic nematode *H. contortus* as a model organism.

Specific aims of this dissertation thesis included:

i. characterization of current trends in the discovery and development of new anthelmintics;
ii. primary screening of chemical compound library for potential drugs with anthelmintic activity in larval stages of *H. contortus*, and in case of positive 'hit' compounds, conducting further studies on the effect in other life cycle stages as well as the study of biotransformation and toxicity of 'hit' compounds;
iii. determination of the effect and biotransformation of sertraline (SRT) as a repurposed drug in *H. contortus* and study of hepatotoxicity and biotransformation in sheep;
iv. establishing and optimisation of methods for anthelmintic testing in *H. contortus*.

4 PUBLICATIONS RELATED TO THE TOPIC WITH CANDIDATE'S CONTRIBUTION

This dissertation thesis is presented in the form of an annotated set of peer-reviewed articles published in or submitted to the impacted international scientific journals. Information about the 2020 journal impact factor (IF) and quartile (Q) based on journal IF is indicated for each category. Apart from the description and commentary of the original research findings, the thesis also includes a general discussion.

4.1 Publication I

Zajíčková, M.*, **Nguyen, L. T.***, Skálová, L., Raisová Stuchlíková, L., & Matoušková, P. (2020). Anthelmintics in the future: current trends in the discovery and development of new drugs against gastrointestinal nematodes. *Drug Discov Today*, *25*(2), 430-437 **(IF = 7.851, Q1)**

* These authors contributed equally to this work.

Candidate's contribution

- literature research and analysis
- responsible for the 'Novel compounds' and 'Modification of chemical structure and combination of known anthelmintics' parts
- revision of the manuscript

4.2 Publication II

Nguyen, L. T., Preston, S., Brueckmann, H., Lungerich, B., Dilrukshi Herath, H. M. P., Koehler, A. V., Wang, T., Skálová, L., Jabbar, A., & Gasser, R. B. (2019). Phenotypic screening of the 'Kurz-box' of chemicals identifies two compounds (BLK127 and HBK4) with anthelmintic activity *in vitro* against parasitic larval stages of *Haemonchus contortus*. *Parasit Vectors*, *12*(1), 191 **(IF = 3.876, Q1/Q1 first decile)**

Candidate's contribution

- design and performance of the experiments
- data analysis, interpretation of results, data visualization

- writing of the manuscript and preparation for submission

4.3 Publication III

Zajíčková, M., Prchal, L., Vokřál, I., **Nguyen, L. T.**, Kurz, T., Gasser, R. B., Bednářová, K., Mičundová, M., Lungerich, B., Michel, O., & Skálová, L. (2022). Assessing the anthelmintic candidates BLK127 and HBK4 for their efficacy on *Haemonchus contortus* adults and eggs, and their hepatotoxicity and biotransformation. *Pharmaceutics*, 14, 754 **(IF = 6.321, Q1**)

Candidate's contribution

- collection of biological material, preparation of samples for the ATP assays
- investigation, data curation
- review and editing of the manuscript

4.4 Publication IV

Zajíčková, M., Prchal, L., Navrátilová, M., Vodvárková, N., Matoušková, P., Vokřál, I., **Nguyen, L. T.**, & Skálová, L. (2021). Sertraline as a new potential anthelmintic against *Haemonchus contortus*: toxicity, efficacy, and biotransformation. *Vet Res*, *52*(1), 143 **(IF = 3.699, Q1 first decile)**

Candidate's contribution

- collection of biological material
- participation at experiments and data analysis of Egg hatch test and Viability test of *H. contortus* adults
- revision of the manuscript

4.5 Publication V

Nguyen, L. T., Zajíčková, M., Mašátová, E., Matoušková, P., & Skálová, L. (2021). The ATP bioluminescence assay: a new application and optimization for viability testing in the parasitic nematode *Haemonchus contortus*. *Vet Res*, *52*(1), 124 **(IF = 3.699, Q1 first decile)**

Candidate's contribution

- design and performance of the experiments
- data analysis, interpretation of results, data visualization
- writing of the manuscript and preparation for submission

4.6 Publication VI

Žofka, M., **Nguyen, L. T.**, Mašátová, E., & Matoušková, P. Image recognition based on deep learning in *Haemonchus contortus* motility assays. *bioRxiv*, 2021.12.01.470699 (Under review in *Comp Struct Biotechnol J*, **IF = 7.271, Q1**)

Candidate's contribution

- participation in the design of the study
- obtaining and annotation of the images used for training of deep learning model
- performance of the experiments and results analysis
- writing of the manuscript and preparation for submission
- creation of a graphical abstract

5 RESULTS

5.1 Current trends in the discovery and development of new anthelmintics

Publication I: Zajíčková, M.*, **Nguyen, L. T.***, Skálová, L., Raisová Stuchlíková, L., & Matoušková, P. (2020). Anthelmintics in the future: current trends in the discovery and development of new drugs against gastrointestinal nematodes. *Drug Discov Today*, *25*(2), 430-437 * These authors contributed equally to this work.

Anthelmintic discovery is an endeavour of rare success. With the presence of several reviews on anthelmintics used in treatment of helminthiasis, including their mechanism of action, the challenges of development, treatment and control strategies (e.g., Besier et al., 2016a; Geary et al., 2015; Hoste & Torres-Acosta, 2011; Taman & Azab, 2014; Waller, 2004), this review did not attempt to revisit areas that have already been described, rather to explore and comprehensively present the approaches for identifying novel targets for anthelmintic therapy against GINs. Even though lacking the characters of systematic review, four different approaches to identify drug candidates were summarized. Among other things, we considered the recently identified novel compounds that derived from industry and academic research. Further, we described derivatives of known anthelmintics and treatment based on the drug combination. Development of drug combinations can be less costly in comparison to other mentioned approaches; however, the problem arises with the need to match the pharmacokinetics of the individual components, drug-drug interactions or chemical compatibility in the pharmaceutical preparation. Specifically, we discussed the target-based approach through genomics, transcriptomics, and metabolomics can enable the identification of essential enzymes as targets for new chemical entities; still, this approach is empirical. We discussed several compounds in which derivative synthesis explores the scientific knowledge and anthelmintic effects of known compounds potentiated by hit-to-lead optimisation.

Another chapter was dedicated to drug repurposing. Even though it might seem to be the fast and cheap route of approval of a new anthelmintic drug for veterinary use, the process is challenging due to unknown pharmacokinetics in the target species, and toxicity studies may need to be repeated at higher doses. The review also gave insight into the strategy of returning to natural plants and usage of complex bioactive mixtures with various modes of action, which might emphasize the anthelmintic effect and limits resistance development.

Even though distinct examples of new formulations and promising compounds were described, the main priority is the *in vivo* activity and cost-benefit ratio. From the up-to-date results summarized in this review and lack of solid publications on chemicals that would make it into final clinical studies, we can conclude that the development of anthelmintics has been slow and laborious and, to date, not very successful in introducing novel anthelmintics to the market. Hence this review highlighted the need for ongoing research and development of novel anthelmintics and might serve as a collective knowledge and guide further investigation.

5.2 Primary screening of a small chemical library and studies of the 'hit' compounds

The following two chapters describe the main findings from the primary screening of 236 chemically diverse compounds and the subsequent studies on efficacy, hepatotoxicity and biotransformation of the 'hit' compounds. The library was kindly provided by Prof. Kurz from Heinrich-Heine University Düsseldorf, Germany. This work has initiated collaboration with the Australian and German research groups.

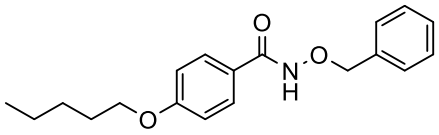
5.2.1 Primary screening

Publication II: **Nguyen, L. T.**, Preston, S., Brueckmann, H., Lungerich, B., Dilrukshi Herath, H. M. P., Koehler, A. V., Wang, T., Skálová, L., Jabbar, A., & Gasser, R. B. (2019). Phenotypic screening of the 'Kurz-box' of chemicals identifies two compounds (BLK127 and HBK4) with anthelmintic activity *in vitro* against parasitic larval stages of *Haemonchus contortus*. *Parasit Vectors*, *12*(1), 191

The screening of the 'Kurz-box' library against xL3s of *H. contortus* identified two 'hit' compounds denoted as BLK127 and HBK4 ('hit' rate of 0.8 %) (**Figure 3**). We used optimised motility screening and included the medium with DMSO as a negative control and two anthelmintics, MOP and moxidectin, as positive controls (Preston et al., 2015). None of 236 compounds reduced the xL3 motility by more than 70 % even after 72 h; however, incubation of the plate for 4 more days revealed that two compounds induced phenotypic changes. Compound BLK127 induced an 'eviscerated' phenotype in the xL3 stage (Jiao et al., 2019) and also inhibited L4 development. The affected larvae exerted severe morphological changes, concretely an anterior protrusion in xL3s that did not allow the larva to moult to the next stage.

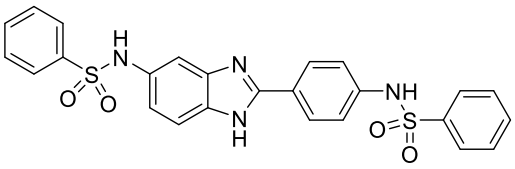
The second compound HBK4 exerted a ‘curved’ phenotype in both xL3s and L4s. By further examination, we noticed cuticular wrinkling and the presence of numerous vacuoles mainly in the intestinal cells which were also present in the MOP-treated larvae; however, MOP exerted a ‘coiled’ phenotype (**Figure 4**).

Both compounds inhibited xL3 motility at 7 days with BLK127 being more potent than HBK4, with IC_{50} of 7.45 ± 1.76 μM to 12.17 ± 2.28 μM, respectively. The IC_{50} values were not possible to calculate from the dose-response curves from the L4 motility experiments; nevertheless, BLK127 and HBK4 reproducibly inhibited L4 motility at concentrations from 12.5 μM to 100 μM and from 25 μM to 100 μM, respectively.



BLK127

N-(benzyloxy)-4-(pentyloxy)benzamide



HBK4

N-(4-(5-(phenylsulfonamido)-1*H*-benzo[*d*]imidazol-2-yl)phenyl)benzenesulfonamide

Figure 3 Structural formula and IUPAC names of two ‘hit’ compounds.

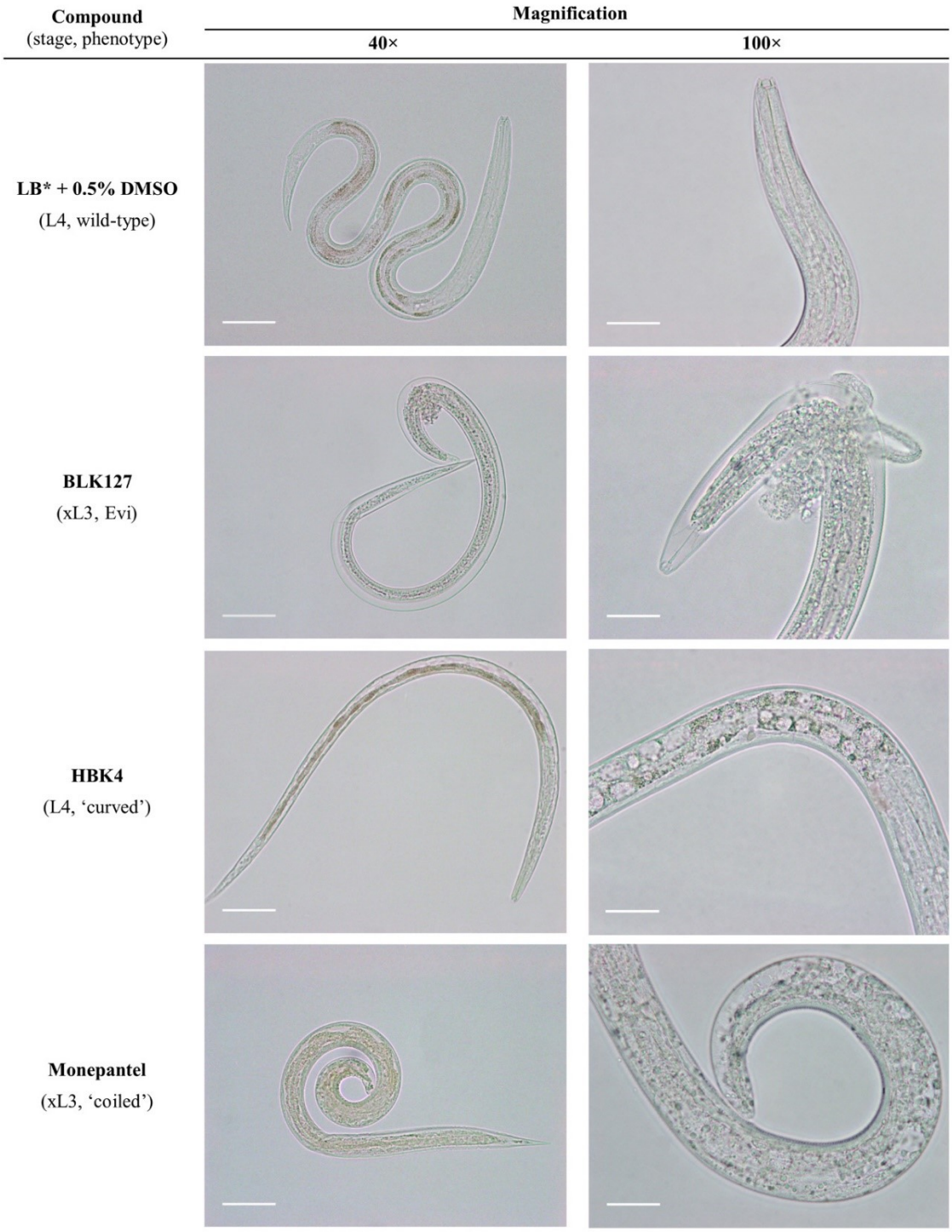


Figure 4 Phenotypic changes after 7 days following exposure to two 'hit' compounds, BLK127 or HBK4. The details of the developed pharynx in the negative control, the anterior protrusion in the 'eviscerated' (Evi) phenotype and the presence of vacuoles in the 'curved' and 'coiled' phenotype are shown. LB* + 0.5% DMSO served as a negative control, 20 µM monepantel served as a positive control. Scale-bars are 50 µm and 20 µm for 40× and 100× magnification, respectively. *Abbreviations: xL3 – exsheathed third-stage larva; L4 – fourth-stage larva; LB – Luria Bertani medium.*

5.2.2 Evaluation of 'hit' compounds

Publication III: Zajíčková, M., Prchal, L., Vokřál, I., **Nguyen, L. T.**, Kurz, T., Gasser, R. B., Bednářová, K., Mičundová, M., Lungerich, B., Michel, O., & Skálová, L. (2022). Assessing the anthelmintic candidates BLK127 and HBK4 for their efficacy on *Haemonchus contortus* adults and eggs, and their hepatotoxicity and biotransformation. *Pharmaceutics*, 14, 754

This study provided an extension of the phenotypic screening of the 'Kurz-box' library with two 'hit' compounds. Further studies on assessing the activity of BLK127 and HBK4 in eggs and adult stages of *H. contortus*, and cytotoxicity test in ovine hepatocytes and precision-cut liver slices (PCLS) were conducted. The study also described biotransformation in sheep and *ex vivo H. contortus* adults.

None of the compounds affected egg hatching. For measuring the viability in adults, we used the bioluminescence ATP assay (see **Chapter 5.4.1**). BLK127 significantly decreased ATP level at 40 μM ($P < 0.05$) and 20 μM ($P < 0.01$) in females and males from the susceptible ISE strain, respectively. We further tested the effect of the drugs in resistant IRE and WR strains. In females, BLK 127 significantly reduced the ATP level at 1 μM concentration in both strains ($P < 0.0001$ and $P < 0.05$ for IRE and WR, respectively). In WR males, BLK127 decreased significantly ($P < 0.01$) at 10 μM. The compound HBK4 did not show any effect compared to the control with DMSO only.

Further experiments concerned testing hepatotoxicity. For that purpose, two liver models were used, PCLS and isolated hepatocytes, using two animal species sheep and rats. From our findings, BLK127 did not show any negative impact on any of the liver models, while HBK4 exerted a hepatotoxic effect.

Therefore, the subsequent experiments of studying biotransformation in ovine PCLS and isolated hepatocytes and in males and females of *H. contortus* was conducted only for BLK127. The metabolic pathways in ovine liver models varied from *H. contortus*, with hydroxylation, hydrolysis and *N*-glycosidation being present in *H. contortus* while hydrolysis and subsequent glycine conjugation of BLK127 being present in the ovine liver. The higher biotransformation rate in sheep might suggest that *H. contortus* is not able to protect itself against BLK127 via biotransformation and supports its efficacy and non-toxicity results. Within the framework of drug development and chemical optimisation, following experiments on efficacy and safety of 13 synthesised analogues of BLK127 are a subject of research of my colleague, PhD candidate Markéta Zajíčková.

5.3 Drug repurposing of sertraline

Publication IV: Zajíčková, M., Prchal, L., Navrátilová, M., Vodvárková, N., Matoušková, P., Vokřál, I., **Nguyen, L. T.**, & Skálová, L. (2021). Sertraline as a new potential anthelmintic against *Haemonchus contortus*: toxicity, efficacy, and biotransformation. *Vet Res*, *52*(1), 143

The need for the development of novel anthelmintics is urgent due to the widespread drug resistance in parasitic nematodes. In this context, it is critical to find a new or repurposed drug. This article presented drug repurposing of the neuromodulatory drug sertraline (SRT), which has shown efficacy against other parasites of socio-economic importance, namely whipworm *Trichuris muris*, hookworm *Ancylostoma caninum*, and flatworm *Schistosoma mansoni* (Weeks et al., 2018). In the original paper by Weeks et al., the authors surveyed 281 compounds from the NIH Clinical Collection and tested SRT with two other FDA-approved drugs, paroxetine and chlorpromazine, on eggs, larvae and fertile adults of the model organism *C. elegans*.

In our experiments, the effect of SRT was tested on eggs and adults of *H. contortus*. SRT at concentrations 0–200 μM did not prevent egg hatching. We then used the optimised protocol of ATP bioluminescence assay (see **Chapter 5.4.1**) to measure the viability of males and females after exposure to SRT at concentrations of 0–50 μM. Two strains were included in the study, ISE and IRE. In ISE females, ISE males, and IRE males, we observed significantly decreased viability in a concentration-dependent manner. In IRE females, the ATP level was increased up to 20 μM and significantly decreased at concentrations 40 and 50 μM ($P < 0.05$). The elevation in lower concentrations could be explained as a reaction to stress after exposure to SRT (Calabrese & Mattson, 2017; Picard et al., 2018). Additionally, we compared the effect of SRT to other two commonly used anthelmintics LEV and MOP; nevertheless, the effect of all three drugs on *H. contortus* ISE adult worms was similar.

We used two *in vitro* models to test hepatotoxicity of SRT, PCLS and primary culture of isolated hepatocytes. SRT did not significantly decrease viability in PCLS even at the highest test concentration of 100 μM. In the hepatocytes, 25 μM SRT increased the viability, while 75 μM and 100 μM SRT decreased the viability ($P < 0.05$).

Another aim of the study was to examine the biotransformation of SRT in *H. contortus* and the ovine liver. For that purpose, we incubated females and males of ISE and IRE strains with 10 μM SRT for 24 h. The same concentration and incubation time was used in ovine liver slices and hepatocytes. The results revealed weak biotransformation of the parent compound in both *H. contortus* strains. The main metabolite was hydroxy-SRT and minor metabolites were

SRT-*O*-glucoside, dihydroxy-SRT, and SRT-ketone. In contrast, the ovine liver metabolized SRT more extensively, mainly via desmethylation and glucuronidation.

In conclusion, our results have shown *ex vivo* efficacy against *H. contortus* adults of ISE and IRE strains. SRT in anthelmintically active concentrations showed no hepatotoxic effect in the ovine liver. Moreover, *H. contortus* is not able to protect itself against SRT via extensive biotransformation. This all collectively encourages examining SRT as a potential anthelmintic drug.

5.4 Methods for the anthelmintic testing

H. contortus serves as an excellent model for parasitic nematodes of the order Strongylida which is one of the largest groups of pathogenic worms of animals. Most of the drug screening and drug testing is done on the free-living stages, such as eggs and L3s. Not many methods are conducted on the actual parasitic stages which will eventually become the targets of anthelmintics. Moreover, most of the available assays require microscopy, which might be time-consuming. The following two chapters address these gaps. The first article provides the optimisation of the ATP bioluminescence assay in *H. contortus*. Still, the gold standard for testing the anthelmintic effect is the motility assay which is suitable for HTS. Thereby, in the second article, we therefore provided a novel strategy in analysing the motility video by applying a deep learning algorithm.

5.4.1 The ATP viability assay in H. contortus

Publication V: **Nguyen, L. T.**, Zajíčková, M., Mašátová, E., Matoušková, P., & Skálová, L. (2021). The ATP bioluminescence assay: a new application and optimization for viability testing in the parasitic nematode *Haemonchus contortus*. *Vet Res*, *52*(1), 124

In this article, we presented the optimised protocol for viability testing in xL3 and adults of *H. contortus*. The method is based on measuring the intracellular concentration of ATP which catalyses the oxidation of D-luciferin to oxyluciferin. We assessed the minimum number of worms needed for the experiment, which was only one female or two males (males are almost twice smaller than female worms), and for larvae, we opted for 400 xL3s. After determining the viability of *ex vivo* adults, we incubated the female and male worms with anthelmintics LEV and MOP for 48 hours. Although huge interindividual differences in ATP level among the

biological replicates were detected, the content of ATP related to protein decreased significantly in males incubated with 1 µM, 5 µM and 10 µM LEV ($P < 0.05$). A significant decrease was also observed in males incubated with all concentrations of MOP ($P < 0.05$). However, only an insignificant decrease was found in the females. One reason might be that females metabolize, hence deactivate drugs more extensively than males, as described in other studies.

We also used our optimised protocol on larvae obtained from ISE strain and WR strain. When we incubated the ISE larvae with LEV, the level of ATP was not different from the control with DMSO only after 24 h. However, after 48 h, the ATP concentration increased in the lowest tested concentration, whereas it significantly decreased at 10 µM LEV. In the WR strain, the ATP level decreased only at 10 µM LEV after 48 h. With longer incubation time, in ISE, we observed a concentration-dependent decrease in the ATP level. On the contrary, in WR, ATP decreased only at the highest concentration of 10 µM. Also, we observed an increase at 1 µM LEV after 96 h. We can speculate that the significant elevations ($P < 0.05$) can also reflect a stress response, hence stimulate ATP synthesis at low concentrations, which are not yet lethal. Overall, a significant difference ($P < 0.05$) between the susceptible and resistant strain was observed at 72 h and 96 h treatments, where ISE exhibited reduced viability in all concentrations. These results show the potential of this method for the detection of drug-resistant isolates.

The advantage of this method is its sensitivity, hence the low amount of biological material, although a higher number of biological replicates are necessary due to high inter-individual differences. In terms of high-throughput methods, our protocol is not ideal for the primary screening of large compound libraries. Nevertheless, this assay can be useful for the secondary screening of 'hit' compounds.

5.4.2 Implementation of a deep learning algorithm in the motility assay

Publication VI: Žofka, M., **Nguyen, L. T.**, Mašátová, E., & Matoušková, P. Image recognition based on deep learning in *Haemonchus contortus* motility assays. *bioRxiv*, 2021.12.01.470699 (Under review in *Comp Struct Biotechnol J*)

In this article, we facilitated the evaluation part of the motility assays by implementing deep learning algorithm. Deep learning, a subset of machine learning, has made significant improvements over the past years, reaching a level where models can successfully detect

heterogeneous object instances in an image. We used a state-of-the-art region-based convolutional neural network called Mask R-CNN (**Figure 5**).

Firstly, we have annotated ~10,400 objects representing different developmental stages of *H. contortus*, namely eggs, L1s and L3s, and retrained our model over time. Secondly, we have trained the deep learning model. These two steps are time-intensive; nevertheless, they are required only once. Moreover, this annotated dataset can be utilised by researcher for future automated evaluation of common *in vitro* assays which require microscopy, such as egg hatch test or larval development test.

Furthermore, we compared our model to the two available motility algorithms, Wiggle Index and Wide Field-of-View Nematode Tracking Platform (WF-NTP). In our experiment, we prepared five motility groups with predefined ratios of motile and non-motile worms in 9 replicates, each containing on average 60 worms in total. We manually counted the worms and assessed the motility phenotype of each worm from the 10-second video recordings, which served as a referential method for comparison of accuracy among the three models. For applying the Mask R-CNN algorithm on motility assays, we used the 'intersection over union' parameter. This metric was shown to be applicable in place of body bends per minute, which is commonly used in *C. elegans*. In assessing the motile/non-motile phenotype, Mask R-CNN reached an overall accuracy of 89 %.

The Mask R-CNN had the lowest statistical error metrics, a mean absolute percentage error of 7.6% and a mean absolute error of 5.6% for the detection and motility forecasts, respectively. Unlike Wiggle Index, Mask R-CNN and WF-NTP algorithms are able to detect each worm in the image. Even though both algorithms had a tendency to underestimate the number of worms on average, Mask R-CNN resulted in a smaller worm count error than WF-NTP.

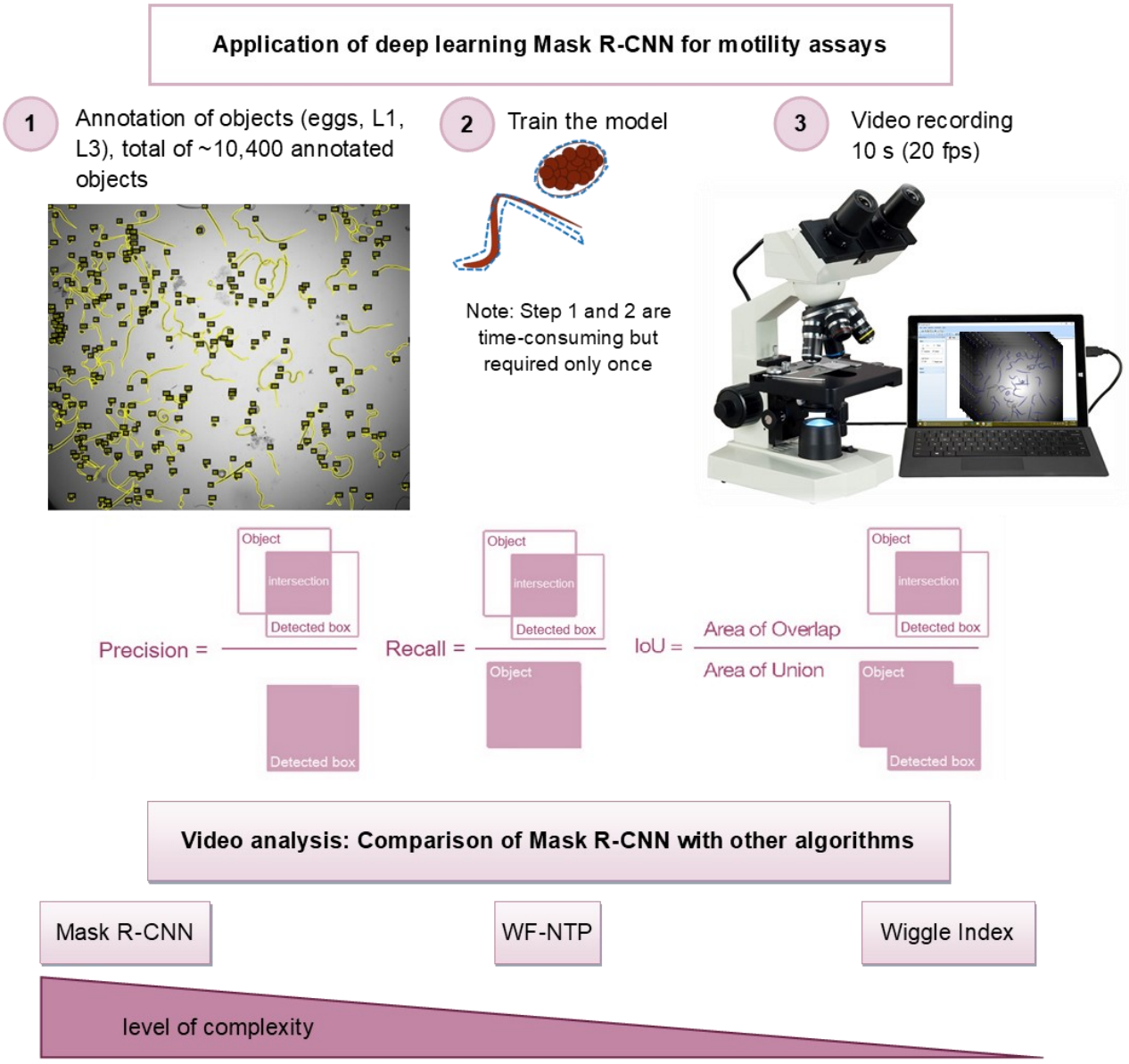


Figure 5 Graphical abstract depicting the strategy of application Mask R-CNN for motility assays with the presentation of deep learning metrics. Mask R-CNN was compared with two other algorithms with different levels of complexity. *Abbreviations: fps – frames per second IoU – intersection over union; Mask R-CNN – region-based convolutional neural network; WF-NTP – Wide Field-of-View Nematode Tracking Platform.*

6 DISCUSSION

There are concerns about the widespread drug resistance which comprises the treatment and control of parasitic GINs. It is important to embrace the fact that zero levels of infections are mostly neither achievable nor desirable and that the repertoire of new chemical treatments is limited. Since chemical treatment remains the mainstay of anthelmintic control, the principle is to reduce the selection pressure for resistance and prolong the life of chemical agents. In the same vein, the discovery and development of novel anthelmintics are of great importance for the sustainable control of such nematodes. The purpose of the present chapter is to discuss the achieved results in a broader context.

One aim of this thesis was to explore anthelmintic candidates from the 'Kurz-box' compound library. We used established phenotypic screening with the advantage of being able to assess the nematostatic effects of the chemical on whole worms in real time. This assay was designed to quantify the reduction in motility of larvae (Preston et al., 2015). However, our two identified compounds BLK127 and HBK4 did not reduce the motility of xL3s below the arbitrarily selected threshold but rather caused phenotypic changes. Thereby, motility reduction is not always a suitable parameter and can lead to false-negative results. In our study, a close inspection of treated larvae by light microscopy and comparison to wildtype phenotype led to the identification of 'hit' compounds. Therefore, in the future, the phenotype of treated worms should be recorded as a complementary endpoint to improve the identification of 'hit' compounds, even though this might be challenging for screening of large compounds libraries and might restrict screening throughput as well as data storage requirements. Since we selected a target-blind approach, the mechanisms of action of these active compounds are unknown. The study was conducted on *in vitro* produced xL3s and L4s. Nevertheless, considering that the adult stages are the target stages for the anthelmintic action, the inclusion of adults in our next study allowed a comparative assessment of potency among developmental stages.

We also provided results on toxicity and biotransformation of BLK127 and HBK4 in *H. contortus* as well as in sheep liver as an important link to drug safety. Based on our results, BLK127 was further considered as a 'lead' compound. This compound also fulfils Lipinski's 'rule of 5', which has become influential in reducing attrition due to poor pharmacokinetics. In the HTS, these *in silico* guidelines are applied to prioritise compounds with an increased likelihood of high oral absorption and enhance rational drug design as well as reduce the number of chemicals if they fail two or more 'rules'. Nevertheless, less stringent criteria would allow more lead compounds to be advanced in a field such as anthelmintic discovery, where

there are few leads, as admitted by the authors (McKerrow & Lipinski, 2017). In early phases of drug discovery and development process, drug properties related to the fate of the candidate molecule in an organism are assessed, i.e., ADMET (absorption, distribution, metabolism, excretion and toxicity). Structure-activity relationships can also be used to predict biological activity from molecular structure. These *in vitro* assays provide important information for prioritising compounds with low-risk profiles for further development prior to expensive preclinical and clinical testing, thereby reducing the risks and improving the probability of success in the drug development pipeline. However, translating *in vitro* to clinical trials is challenging due to differences often referred to as the 'valley of death' (Cha et al., 2018). In the next phase of medicinal chemistry, we involved a synthesis of a small library of 13 analogues of BLK127 and conducted structure-activity relationship studies, which interrogated their efficiency and safety in *in vitro* studies. This part was not a subject of the present thesis.

The drug discovery is undergoing a resurgence of phenotypic screening that follows a diversity-led paradigm in high-throughput assays. This approach is also suitable for complex diseases (Al-Ali, 2016). Compared to the development of *de novo* compounds, drug repurposing provides already defined mechanisms of action, *in vivo* pharmacokinetic profile, toxicity and dosing information which could help in faster processing into clinical trials. Given the cost and time involved in developing a new drug, the approach of repurposing can be a promising alternative. Nevertheless, drug repurposing via screening is confined to a relatively small number of compounds, such as there are only ~13,000 human and ~3,000 animal FDA-registered drugs (FDA, 2021). Drug repurposing is of higher relevance for human medicine, including human helminthiasis, compared to veterinary medicine. Interestingly, the available anthelmintics have been considered for drug repurposing in human cancer, many being in phase II/III of clinical trials (Alavi & Shahmabadi, 2021). The second example is ivermectin against viral infections including SARS-CoV-2; however, the clinical effect is poorly understood at the moment (Kinobe & Owens, 2021). Our study tested SRT, an antidepressant known as a selective serotonin reuptake inhibitor, on *H. contortus* viability and examined toxicity in the ovine liver. A caveat of drug repurposing for antiparasitic use is a requirement for testing concentrations that exceed the ones achieved during the toxicity studies for registration. For SRT, the therapeutic concentrations are considered to be < 1.5 μM (Kirchherr & Kuhn-Velten, 2006). In the original paper, the authors used 100-fold higher concentrations for testing and determined IC_{50} were 18.2 μM, 8.8 μM and 52 μM for lethality (48 h), motility (24 h) and pharyngeal pumping inhibition (60 min), respectively (Weeks et al., 2018). In our study, we assessed the viability with ATP bioluminescence assay and the IC_{50} were 15.9 μM and 4.15 μM

for females and males of ISE strain, respectively. In potential next steps, it would be important to assess the ability of SRT to reduce worm burdens in mammalian hosts as well as determination of dosages and administration schedules. The treatment protocol for administration of anthelmintics is usually single dose or very few doses which are in contrast with SRT chronic administration in humans. The initial maximum recommended starting doses for drug development, which are determined in toxicity studies as no adverse effect level, can be converted from human to animal and vice versa. The doses between species should not be extrapolated solely based on body weight but should be normalised using body surface area. Likewise, a safety factor should be taken into consideration when deciding on high doses in animal toxicology studies (Nair & Jacob, 2016). Another notable finding of the original study was the potential mode(s) of action of SRT in helminths. The mutation in the genes responsible for the SRT anti-depressant effect in humans did not eliminate their anthelmintic activity in *C. elegans*. Also, *C. elegans* mutants resistant to existing anthelmintics retained sensitivity to all three drugs, suggesting that they act on different targets. In our study, we also observed concentration-dependent decreased viability of IRE males after treatment with SRT. Nonetheless, it is worth mentioning that pharmaceuticals are concerning environmental contaminants. Among anti-depressants, SRT is one of the most widely prescribed anti-depressants of its group. With broadening the medical indication due to drug repurposing, there is a higher chance of exposure to this chemical at low concentrations which can induce adverse effects in off-target aquatic and terrestrial organisms (Horvat et al., 2012; Chen et al., 2021; Styrishave et al., 2011). For this reason and also in the context of emerging drug resistance, the control strategies should be based on effective preventive programmes that should prevent disease outbreaks while maintaining anthelmintic efficacy (Besier et al., 2016a).

Generally, the choice of screening strategy and predictive efficiency assays is the major determinant of success. To our knowledge, the ATP assay was not applied in adults of *H. contortus* or other key GINs of small ruminants with one exception (Geary et al., 1993). Even though this assay provides an alternative to other drug efficacy tests, the inability to maintain *ex vivo* adults has not been overcome. The viability of adults decreases significantly after 48 hours. Taking this together, it would be difficult to read the IC_{50} from a dose-response curve using ATP assay. We also discussed the sensitivity of the assay. It is possible to detect the luminescence signal even from one female or two males; however, for the statistical analysis, it is more suitable to include more biological replicates, which on the other hand comes with a negative trade in time and speed with sample handling and required amount of testing compound. Since the readout of the assay is luminescence-based, scalability is possible.

However, in our assay setting with intermediate steps of homogenization and centrifugation of samples in the micro-test tubes, our protocol is unsuitable for parallel testing of tens or more compounds. Still, we demonstrated that our methodology enables the objective measurement of xL3s and adult viability and represents an alternative assay for assessing drug efficacy as well as the potential to detect drug resistance. As the majority of diagnostic tools for drug resistance are based on labour-intensive bioassays, molecular or biochemical assays would be useful. Since definitive molecular tests remain elusive for most anthelmintic groups and helminth species, this gap that impedes progress may be overcome partially with biochemical assays.

Additionally, effective and practical whole-organism screening joined with automated image-based analysis tools has allowed for the establishment of medium to high-throughput assays. For parasites, the toxicity effect of a compound can be expressed with complex defects in motility, development and/or feeding (Preston et al., 2015). We took advantage of a deep learning algorithm dealing with image recognition tasks, Mask R-CNN. Progress on this front improved the power of detection of worm and motile/non-motile phenotype. Since we annotated eggs, L1s and L3s, this method may help the researchers automate the evaluation of EHT or LDT. Nonetheless, machine-learning algorithms are only as good as the training datasets on which they are based. The most important aspect of these applications is the possibility to automate these *in vitro* assays and scale them, therefore allowing researchers to avoid tedious, repetitive tasks and focus on activities where they provide a higher added value.

Nevertheless, the application of machine learning is not limited only to the area of high-content imaging. The 'post-genomics' era can underpin applied areas, including the new drug design, vaccines, and diagnostics. Certainly, the 'omics' approach is powerful; however, with big data, the analysis might become challenging to a human researcher. As an astonishingly high number of compounds in which drug-like characteristics can be made, to navigate the direction, the role of artificial intelligence can be praised. The latest frontier in computational drug discovery is machine learning, in which algorithms use data and experience to teach themselves which compounds bind to which targets, finding patterns invisible to the human eye. Still, advances in machine learning application in drug discovery may be expected (Tkatchenko, 2020; Zamanian & Chan, 2021).

The present thesis contributes to the field of anthelmintic discovery and testing and offers prospects for anthelmintic development. The drug discovery and testing of anthelmintics in *H. contortus* have evolved over the past decades and the processes in this area will certainly develop at a faster pace. Whether the new strategies will deliver the quality and quantity of

potential anthelmintics provided in this and related drug discovery and drug testing studies remains to be seen. It is encouraging to note that new screening initiatives facilitated by public-private partnerships are underway that hold promise to yield new anthelmintics.

7 CONCLUSIONS

The present dissertation thesis focused on the prominent parasitic nematode of small ruminants *H. contortus* which is, therefore, an important target for anthelmintic chemotherapy. The original results of the present thesis study can be summarized in the following points:

i. The thesis synthesised and critically analysed the information in the available literature giving a comprehensive review of current trends and potential opportunities in drug development and testing of anthelmintics with the main emphasis on gastrointestinal nematodes. The role of *H. contortus* as a model organism for drug discovery and development was discussed.

ii. Using whole-organism phenotypic assay, the primary screening of 236 chemicals of diverse structures revealed two compounds BLK127 and HBK4, with promising anthelmintic effects against larvae of *H. contortus*. Further evaluation of BLK127 and HBK4 was undertaken. None of the compounds was ovicidal. BLK127 reduced the viability of *H. contortus* adults from susceptible and resistant strains, and it did not show a negative impact on the viability of liver models in sheep and rats. In contrast, HBK4 was not effective in adults and was hepatotoxic. The main metabolic pathways and metabolites were characterised for BLK127 in *H. contortus* and in sheep. Positively, BLK127 was metabolized more extensively in sheep compared to adults of *H. contortus*. In conclusion, BLK127 may be a promising candidate for further anthelmintic development.

iii. Anti-depressant SRT was not ovicidal but decreased viability in both males and females of *H. contortus* from ISE and IRE strains. SRT was not hepatotoxic in the anthelmintically active concentrations in sheep. The main metabolic pathways and metabolites were identified, showing that *H. contortus* did not metabolize SRT extensively, unlike the ovine liver. Altogether, SRT should certainly be further tested as a potential anthelmintic drug.

iv. We optimised the viability assay based on bioluminescence measurement of ATP in xL3 and adults of *H. contortus*. This assay might also be used for the detection of drug resistance isolates. In another study, we enhanced a method for analysing motility videos by applying the deep learning algorithm Mask R-CNN. Our model was able to outperform the other two algorithms, WF-NTP and Wiggle Index algorithms, in motility and worm counts prediction.

Collectively, our findings have contributed by providing entirely new insights and direction into further research, development, and testing of novel anthelmintics.

8 DISSEMINATION OF RESEARCH FINDINGS

8.1 Presentations related to the topic of the dissertation thesis

Oral presentations:

- **Nguyen, L. T.**, Herath, H. M. P. D., Preston, S., Kurz, T., Skálová, L., & Gasser, B. R. (Jan 2019). Phenotypic screening of a chemically diverse compound library identified two compounds with anthelmintic activity against *Haemonchus contortus*. *9th Postgraduate and 7th Postdoc Conference*, Hradec Králové, Czechia
- **Nguyen, L. T.**, Žofka, M., Mašátová, E., & Skálová, L. (Jan 2021). Using machine learning in evaluation of *in vitro* viability tests in *Haemonchus contortus*. *11th Postgraduate and 9th Postdoc Conference*, Hradec Králové, Czechia
- **Nguyen, L. T.**, Zajíčková, M., Matoušková, P., & Skálová, L. (Sept 2021). Optimization of ATP assay for viability testing in the parasitic nematode *Haemonchus contortus*. *26th Helminthological Days*, Deštné in Orlické hory, Czechia
    - awarded the 1st prize for the best oral postgraduate presentation
- **Nguyen, L. T.**, Zajíčková, M., Matoušková, P., & Skálová, L. (Feb 2022) Biochemical assay for viability testing in susceptible and resistant strains of *Haemonchus contortus*. *12th Postgraduate and 10th Postdoc Conference*, Hradec Králové, Czechia
- **Nguyen, L. T.**, Zajíčková, M., Kurz, T., Gasser, R. B., & Skálová, L. (May 2022). Story from hit identification to lead optimization of potential anthelmintics against *Haemonchus contortus. XIV. Czech and Slovak Parasitological days*, Vysočina, Czechia

8.2 Publications unrelated to the topic of the dissertation thesis

Most of the publications are related to the topic of *H. contortus*, e.g., biotransformation of anthelmintics, but are not related to the topic of the dissertation thesis.

- **Nguyen, L. T.**, Myslivečková, Z., Szotáková, B., Špičáková, A., Lněničková, K., Ambrož, M., Kubíček, V., K., K., Anzenbacher, P., & Skálová, L. (2017). The inhibitory effects of beta-caryophyllene, beta-caryophyllene oxide and alpha-humulene on the activities of the main drug-metabolizing enzymes in rat and human liver *in vitro*. *Chemico-Biological Interactions*, 278, 123-128 **(IF = 5.194, Q1/Q2/Q1)**
- Matoušková, P., Lecová, L., Laing, R., Dimunová, D., Vogel, H., Raisová Stuchlíková, L., **Nguyen, L. T.**, Kellerová, P., Vokřál, I., Lamka, J., Szotáková, B., Várady, M., & Skálová,

L. (2018). UDP-glycosyltransferase family in *Haemonchus contortus*: Phylogenetic analysis, constitutive expression, sex-differences and resistance-related differences. *Int J Parasitol Drugs Drug Resist*, *8*(3), 420-429 **(IF = 4.077, Q1/Q2)**

- Raisová Stuchlíková, L., Matoušková, P., Vokřál, I., Lamka, J., Szotáková, B., Sečkařová, A., Dimunová, D., **Nguyen, L. T.**, Várady, M., & Skálová, L. (2018). Metabolism of albendazole, ricobendazole and flubendazole in *Haemonchus contortus* adults: Sex differences, resistance-related differences and the identification of new metabolites. *Int J Parasitol Drugs Drug Resist*, *8*(1), 50-58 **(IF = 4.077, Q1/Q2)**
- Kellerová, P., Navrátilová, M., **Nguyen, L. T.**, Dimunová, D., Raisová Stuchlíková, L., Štěrbová, K., Skálová, L., & Matoušková, P. (2020). UDP-Glycosyltransferases and Albendazole Metabolism in the Juvenile Stages of *Haemonchus contortus*. *Front Physiol*, *11*, 594116 **(IF = 4.566 Q1)**
- Dimunová, D., Matoušková, P., Navrátilová, M., **Nguyen, L. T.**, Ambrož, M., Vokřál, I., Szotáková, B., & Skálová, L. (2022). Environmental circulation of the anthelmintic drug albendazole affects expression and activity of resistance-related genes in the parasitic nematode *Haemonchus contortus. Sci Total Environ, 822*, 153527 **(IF = 7.963 Q1 first decile)**
- White, R.*, Sotillo, J.*, Ancarola, M. E., Borup, A., Boysen, A. T., Brindley, P. J., Buzás, E. I., Cavallero, S., Chaiyadet, S., Chalmers, I. W., Cucher, M. A., Dagenais, M., Davis, Ch. N., Devaney, E., Duque-Correa, M. A., Eichenberger, R. M., Fontenla, S., Gasan, T. A., Hokke, C. H., Kuipers, M. E., Laha, T., Loukas, A., Maizels, R. M., Marcilla, A., Mazanec, H., Morphew, R. M., Neophytou, K., **Nguyen, L. T.**, Nolte-'t Hoen, E., Povelones, M., Robinson, M. W., Rojas, A., Schabussova, I., Smits, H. H., Sungpradit, S., Tritten, L., Whitehead, B., Zakeri, A., Nejsum, P., Amy H. Buck, A. H., & Hoffmann, K. F. Minimal information for studies of extracellular vesicles from parasitic helminths: a community-led roadmap to increase rigor and reproducibility in non-mammalian species. (Under review in *J Extracell Vesicles* **IF = 25.841, Q1 first decile**)
  * These authors contributed equally to this work.

8.3 Presentations unrelated to the topic of the dissertation thesis

Oral presentations:

- **Nguyen, L. T.**, Matoušková, P., & Skálová, L. (May 2019). MicroRNA expression in susceptible and resistant *Haemonchus contortus* isolates. *25th Helminthological Days*, Rejčkov, Czechia
    - awarded the 1st prize for the best oral postgraduate presentation
- **Nguyen, L. T.** (Oct 2019). Sekvenační metody – zdroje bioinformatických dat (Eng. Sequencing methods – sources of bioinformatics data). *Code Week*, Hradec Králové, Czechia
- **Nguyen, L. T.**, Matoušková, P., & Skálová, L. (Jan 2020). Profiling microRNA expression in susceptible and resistant strains of *Haemonchus contortus* using small RNA sequencing. *10th Postgraduate and 8th Postdoc Conference*, Hradec Králové, Czechia

Posters:

- **Nguyen, L. T.**, Matoušková, P., & Skálová L. (Sept 2019). Comparative analysis of microRNA levels in *Haemonchus contortus* strains with different drug susceptibility. *Parasitic Helminths: New Perspectives in Biology and Infection*, Hydra, Greece
    - contributed to the position paper as the output of this conference's workshop *Helminth Extracellular Vesicles Workshop* (White et al., 2022) (see **Chapter 8.2**)
- **Nguyen, L. T.**, Matoušková, P., & Skálová, L. (Feb 2020). Small RNA-Seq differential expression analysis of microRNAs in susceptible and resistant strains of *Haemonchus contortus*. *Anthelmintics IV: From Discovery to Resistance*, Santa Monica Bay, California, USA
    - awarded the 2nd prize for the best poster

8.4 Grant projects

Team member:

- 2020–2021 Grant Agency of Charles University No. 1171620 Efflux transporters of *Haemonchus contortus* and their role in resistance development
- 2021–2022 START/MED/065 Novel therapeutic approaches to the treatment of hepatic diseases; involved mainly in bioinformatic part

8.5 International scientific experience

- 6-month internship at the Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Australia, the research group of Prof. Robin B. Gasser, Feb–July 2018
    - funded by Charles University (Fond mobility FM/c/2017-2-076)
- 3-week internship at the Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Australia, the research group of Prof. Robin B. Gasser, Sept 2018
    - scholarship attained by Charles University, 'Project of Support for strategic partnerships'
- 2-week Workshop on genomics, Český Krumlov, Czechia, Jan 2020
    - organised by the world's leading universities (from USA and EU)
    - funded by the Bioinformatics Centre of Hradec Králové (BioInfoHK 2019-1-CZ01-KA203-061433 funded by project KA2 Erasmus+)
- 1-week League of European Research Universities (LERU) Summer School, Trinity College Dublin, Ireland – online due to covid-19, Aug 2021
    - one of two representants for Charles University
    - collaboration on writing a policy paper on the topic of 'The role of the expert and implications for early-career researchers' (In preparation)

8.6 Teaching experience

Consultant of undergraduate students:

- Hegrová, L. (2020). Differences in expression of selected microRNA in sensitive and resistant nematodes. Diploma thesis
- Mašátová, E. (2021). Optimization of the method for viability testing in *Haemonchus contortus*. Diploma thesis
- Kačerová, J. (2022). Ferns as potential anthelmintics. Students' Professional Activities
    - So far in the 44th year of the Students' Professional Activities competition, school and district rounds were held. Her project took the 1st place in the Biology section (Apr 2022).

Lectures:

- 2017–2021 Czech and English practical courses of General Biochemistry
- 2020–2022 Czech lecture on topic Biochemistry of blood clotting, muscle contraction and vision, subject Basic Biochemistry

9 LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BZ	benzimidazole
DNA	deoxyribonucleic acid
EHT	egg hatch test
epg	eggs per gram
FDA	the United States Food and Drug Administration
FECR(T)	faecal egg count reduction (test)
GIN	gastrointestinal nematode
GluCls	glutamate-gated chloride ion channels
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory concentrations
IF	impact factor
IPM	integrated parasite management
ISE	Inbred Susceptible Edinburgh strain of *H. contortus*; MHco3
IRE	Inbred Resistant Edinburgh strain of *H. contortus*; MHco5
IUPAC	International Union of Pure and Applied Chemistry
L1–L4	first–fourth-stage larva
LDH	lactate dehydrogenase
LEV	levamisole
MALDT	micro-agar larval development test
Mask R-CNN	region-based convolutional neural network
ML	macrocyclic lactone
MOP	monepantel
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nAChR	nicotinic acetylcholine receptor
NADH	nicotinamide adenine dinucleotide
NIH	National Institutes of Health
PCLS	precision-cut liver slices
PCR	polymerase chain reaction
Q	quartile
RNA	ribonucleic acid
SRT	sertraline

WR	White River strain of *H. contortus*; MHco4
xL3	exsheathed third larval stage
WF-NTP	Wide Field-of-View Nematode Tracking Platform

10 REFERENCES

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11 SUPPLEMENTS

Copies of articles related to the topic of this dissertation thesis

I. Zajíčková, M.*, **Nguyen, L. T.***, Skálová, L., Raisová Stuchlíková, L., & Matoušková, P. (2020). Anthelmintics in the future: current trends in the discovery and development of new drugs against gastrointestinal nematodes. *Drug Discov Today*, *25*(2), 430-437 **(IF = 7.851, Q1)** * These authors contributed equally to this work.

II. **Nguyen, L. T.**, Kurz, T., Preston, S., Brueckmann, H., Lungerich, B., Dilrukshi Herath, H. M. P., Koehler, A. V., Wang, T., Skálová, L., Jabbar, A., & Gasser, R. B. (2019). Phenotypic screening of the 'Kurz-box' of chemicals identifies two compounds (BLK127 and HBK4) with anthelmintic activity *in vitro* against parasitic larval stages of *Haemonchus contortus*. *Parasit Vectors*, *12*(1), 191 **(IF = 3.876, Q1/Q1 first decile)**

III. Zajíčková, M., Prchal, L., Vokřál, I., **Nguyen, L. T.**, Kurz, T., Gasser, R. B., Bednářová, K., Mičundová, M., Lungerich, B., Michel, O., & Skálová, L. (2022). Assessing the anthelmintic candidates BLK127 and HBK4 for their efficacy on *Haemonchus contortus* adults and eggs, and their hepatotoxicity and biotransformation. *Pharmaceutics*, 14, 754 **(IF = 6.321, Q1**)

IV. Zajíčková, M., Prchal, L., Navrátilová, M., Vodvárková, N., Matoušková, P., Vokřál, I., **Nguyen, L. T.**, & Skálová, L. (2021). Sertraline as a new potential anthelmintic against *Haemonchus contortus*: toxicity, efficacy, and biotransformation. *Vet Res*, *52*(1), 143 **(IF = 3.699, Q1 first decile)**

V. **Nguyen, L. T.**, Zajíčková, M., Mašátová, E., Matoušková, P., & Skálová, L. (2021). The ATP bioluminescence assay: a new application and optimization for viability testing in the parasitic nematode *Haemonchus contortus*. *Vet Res*, *52*(1), 124 **(IF = 3.699, Q1 first decile)**

VI. Žofka, M., **Nguyen, L. T.**, Mašátová, E., & Matoušková, P. Image recognition based on deep learning in *Haemonchus contortus* motility assays. *bioRxiv*, 2021.12.01.470699 (Under review in *Comp Struct Biotechnol J*, **IF = 7.271, Q1**)