CHARLES UNIVERSITY
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
Department of Biochemical Sciences

Dissertation Thesis:

Metabolism and efficacy of new potential anthelmintics

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Consultant: PharmDr. Ivan Vokřál, Ph.D.

Hradec Králové 2023

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 consultant PharmDr. Ivan Vokřál, 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 konzultanta PharmDr. Ivana Vokřála, 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. Markéta Zajíčková

ACKNOWLEDGEMENTS

The PhD journy is a long process full of oportunities and learning curves and also hard work and occasional obstacles. Therefore, this whole journy would not be possible to finish without support and most importantly without great teachers. To have good teachers I consider as one of the biggest blessings that one can have in life. And I am very greatful, that I have been blessed with such a gift to have many of great teachers throughout my life. And one of those teachers whithout whoom I would not be able to complete this journey is Prof. Lenka Skálová. Her exceptional guidance, unwavering support, remarkable patience, and constant kindness deserve my deepest appreciation and gratitude. Another remarkable teacher who has played a significant role during this journey and to whom I would like to extend my sincere thanks is Prof. Barbora Szotáková.

However, no success would be possible without good friends and colleagues. I am grateful beyond words for the exceptional colleagues and friends I have had the privilege to work with within the Department of Biochemical Sciences. My special thanks belongs to my consultant Dr. Ivan Vokřál, for his profound expertise, sharp critical thinking, willingnes to help and great support in the area of mass spectrometry and others fields. I am also grateful for the opportunity to work with Linh Thuy Nguyen, who has been my inspiration for her extraordinary discipline, hard work and purposefulness. For the extensive knowledge, enthusiasm and encouragement I would like to thank Assoc. Prof. Petra Matoušková. Additionally, most of the experiments would not be doable without extraordinary support of Martina Navrátilová, Michaela Šadibolová, Gabriela Svobodová, and other colleagues. Special thanks belongs to Lukáš Prchal, as without him the data regarding metabolism would not be possible to obtain. I would also like to express my gratitude to Alena Pakostová for support and sense of humor.

My heartfelt appreciation goes out to Dr. Craig Wheelock and Antonio Checa for their remarkable kindness, open-mindedness, and willingnes to genuinly share their extensive knowledge and skills in the area of metabolomics, lipidomics and mass spectrometry. I am truly fortunate to have had the opportunity to learn from them during my internship.

I would like to acknowledge and extend my gratitude to Charles University for their essential financial assistance, without which this journey would not have been feasible. I would like to thank especially projects: GAUK (1568519), Grant Schemes at CU" (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935), Fond mobility FM/a/2021-2-046, and SVV 260 416.

ABSTRACT

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Title of Dissertation Thesis: **Metabolism and efficacy of new potential anthelmintics**

Overpopulation of gastrointestinal nematodes represents global health and economical problem. Therefore, suitable solution in the form of effective anthelmintic treatment is needed. Several anthelmintics with distinct mechanism of action are available on the market, however, the widespread resistance limits their efficacy. As a consequence, finding a new possible alternative becomes important. In present dissertation thesis, the novel anthelmintic candidates were selected and their effect against model parasitic nematode *Haemonchus contortus* was examined. The studies encompassed three approaches within the field of drug discovery: new molecular entity identification and structure modification, drug repurposing, and medicinal plants screening. Newly synthesized compounds designated as BLK127, HBK4 and BLK127 derivatives, already known antipsychotic drug sertraline (SRT) and the extracts from eight European ferns species from genus *Dryopteris*, *Athyrium* and *Blechnum* were the subjects of our interests. Two developmental stages, eggs and adults, of drug-sensitive and drug-resistant strains of *H. contortus* were used for anthelmintic efficacy screening. In order to improve the anthelmintic testing in adult worms, a biochemical method based on the measurement of ATP level was adopted and optimized. BLK127 and several of its derivatives, SRT and three fern extracts: *Athyrium distentifolium*, *Dryopteris aemula*, and *Dryopteris cambrensis* proved anthelmintic activity as they significantly decreased the level of ATP in adults of both, sensitive and resistant strains. Consequently, the hepatotoxicity of all compounds was evaluated, and with the exception of HBK4, which was subsequently eliminated as a potential candidate, none of the compounds exhibited harm to the ovine liver. Lastly, biotransformation of SRT and BLK127 in adult *H. contortus* and sheep liver was assessed. The results revealed extensive biotransformation in sheep liver, whereas *H. contortus* exhibited limited metabolic activity with only traces of metabolized product detected. Some of the obtained results are really promising, and they could be a basis for further studies, including *in vivo* testing.

ABSTRAKT

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Název dizertační práce: **Metabolismus a účinnost nových potenciálních anthelmintik**

Přemnožení gastrointestinálních hlístic představuje globální zdravotní a ekonomický problém. Proto je potřeba nalézt vhodné řešení v podobě účinné anthelmintické léčby. Na trhu je k dispozici několik anthelmintik s odlišným mechanismem účinku, avšak rozšířená léková rezistence omezuje jejich účinnost. Proto je důležité najít novou možnou alternativu. V této disertační práci byla vybrána nová potenciální anthelmintika a byl zkoumán jejich účinek proti modelové parazitické hlístici *Haemonchus contortus*. Studie zahrnovaly tři odlišné přístupy v oblasti vývoje léčiv: identifikace nových molekulárních entit a modifikace struktury, změna indikace již schváleného léčiva a screening léčivých rostlin. Předmětem našeho zájmu byly nově syntetizované sloučeniny označené jako BLK127, HBK4 a deriváty BLK127, dlouhodobě používané antipsychotikum sertralin (SRT) a extrakty z osmi evropských druhů kapradin rodu *Dryopteris, Athyrium* a *Blechnum*. Pro screening anthelmintické účinnosti byla použita dvě vývojová stádia, vajíčka a dospělci, *H. contortus* kmenů citlivých a rezistentních na léčiva. Pro zlepšení anthelmintického testování u dospělých červů byla zavedena a optimalizována biochemická metoda založená na měření hladiny ATP. BLK127 a několik jeho derivátů, SRT a tři extrakty z kapradin: *Athyrium distentifolium, Dryopteris aemula* a *Dryopteris cambrensis* prokázaly anthelmintickou aktivitu protože významně snížily hladinu ATP u dospělých jedinců citlivých i rezistentních kmenů. Následně byla hodnocena hepatotoxicita všech sloučenin a s výjimkou HBK4, která byla následně ze studie eliminována, žádná ze sloučenin nevykazovala toxicitu vůči ovčím játrům. Nakonec byla studována biotransformace SRT a BLK127 u dospělců *H. contortus* a jater ovcí. Výsledky ukázaly rozsáhlou biotransformaci v játrech ovcí, zatímco *H. contortus* vykazoval omezenou metabolickou aktivitu, neboť pouze stopy metabolitů byly detekovány. Některé získané výsledky jsou opravdu slibné a mohly by být základem pro další studie včetně testování *in vivo*.

TABLE OF CONTENT

1 INTRODUCTION

Nematodes are a highly diverse and abundant group of invertebrates that occupy almost the entire world. Among them, gastrointestinal nematode represents health problem to many humans and animals. *Haemonchus contortus* is one of the most economically important parasites of small ruminants around the globe. Therefore, considerable effort has been invested for its management. Even though many preventive strategies are commonly used, treatments using anthelmintics still represent a pivotal role when infection rate rises. However, the efficacy of available anthelmintics becomes lower and lower due to widespread resistance. For this reason, there is a need for new anthelmintics.

Several strategies are available for exploration of new potential anthelmintic: synthesizing new chemical entity, structure modification, drug repurposing or the use of natural products. Each of these strategies carry certain advantages and disadvantages. Synthesizing new chemical entities is long and pricy, however enables discovery of new compounds with new mechanism of action. An alternative approach is drug repurposing, which overcomes those limitations. Medicinal plants are the source of many diverse compounds and played a crucial role in many drugs discovery. Many tests are available to screen for anthelmintic properties of various compounds. Most of those methods are however applicable to free living nematodes or free-living stages of parasitic nematodes and no biochemical method has been developed for screening on adult nematodes, the main targets of anthelmintics.

For this reason, development of new method for viability testing in adult worms was the first aim of presented dissertation thesis. Consequently, an optimized method was applied in investigation of new potential anthelmintics using three different approaches. In addition, potential hepatotoxicity of selected compounds and their metabolism in nematodes as well as in ovine liver was tested with aim to obtain complex information for pre-clinical evaluation of potential candidates.

2 THEORETICAL BACKGROUND

2.1 Gastrointestinal nematodes

Nematodes represent one of the most abundant and morphologically diverse group of invertebrates. Due to the shared characteristic of moulting during development, Nematoda, Arthropoda, and several other smaller phyla are collectively grouped into a larger category known as 'Ecdysozoa', encompassing all animals that undergo moulting [1-4]. So far 25,000 nematode species have been described, but it is estimated that species diversity range from 100,000 to 10 million [2,4,5]. Due to their structure, physiology, diverse reproductive patterns, adaptability, and several survival strategies nematodes are ubiquitous and have been found even in places with harsh environmental conditions. Nematodes spread into more habitats on land and fresh or salt water than any other multicellular group of animals, including insects [4].

Nematodes can be divided into two main groups depending on their lifestyle: free-living and parasitic [5]. The majority of nematodes exist as free-living. Those nematodes occupy terrestrial or aquatic environments, where they feed on bacteria, algae, fungi, dead organisms, or living tissue. By releasing nutrients to the soil, they improve its composition and provide nutrients to the plant. The other group of nematodes lives as parasites of human, animals, plants, or insects. Most of the nematodes infecting vertebrates are localized in gastrointestinal tract, but they can occur also in blood, lymphatic circulation, urogenital tract, respiratory system, cavities and skin [6]. Even though some of the nematodes could be useful as biological pests control, among farmers around the world they are mainly known as threat to stock and crop production and in developing countries cause several health problems [5].

Gastrointestinal nematode (GIN) infection, also known as soil transmitted helminthiasis, with more than billion cases worldwide, represents one of the most common acquired infections in the world. Even though 450 million individuals are seriously ill, those diseases are often neglected by public and research, because most of the infections are not directly lethal or remain asymptomatic [7-9]. People usually get infected from contaminated soil or water by ingesting eggs or infective larvae or by penetration through the skin. The most affected are children and pregnant women in developing countries in sub-Saharan Africa, Asia, Latin America, and Caribbean [8,10,11]. The infections in human are mainly caused by roundworms, hookworms, whipworms and pinworms (threadworms) [5]. This designation is based on the way and place they occupy host body, or their shape [8,12,13]. Generally, all phylum nematodes are

called roundworms because of the round shape of the body, which is common feature to all nematodes [14]. As **roundworms** are denoted the nematodes, which occur freely in small intestine, are curled or round in nature [12,13,15]. **Hookworms** are hooked on to the intestinal wall [12] and parasites in the upper part of human small intestine [15]. **Whipworms** live mainly in cecum of large intestine and received their name based on their whip-like shape [15] as they have thin anterior part containing oesophagus and thick posterior end containing intestine and reproductive organs [16,17]. **Pinworms** refer to roundworms characterized by the presence of a pin-like tail in females [18]. Because pinworms are white and resemble a small piece of thread, the same species is also called **threadworm** [19,20]. The most common species infecting humans are *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), *Ancylostoma duodenale*, *Necator americanus* (hookworms), *Enterobius vermicularis,* and *Strongyloides stercoralis* (pinworms) [9,18].

A. lumbricoides is a giant intestinal worm which can grow up to 30 cm length [13]. The infection begins by ingesting embryonated eggs, which hatch and moult into second-stage larvae (L2) and penetrate the intestinal mucosa to enter pulmonary circulation. Third-stage larvae (L3) then migrate across the alveolar wall up the tracheobronchial tree, where they are swallowed and finally reach the lumen of small intestine where they can mature through fourth-stage larvae (L4) into adults [9]. *T. trichiura* is also transmitted by embryonated eggs, however it completes its life cycle without leaving the intestine. The larvae hatch in small intestine, then migrate to caecum and ascending colon where burrow into the intestinal mucosa and mature into adults [9,16]. Larvae of *A. duodenale* and *N. americanus* enter the host body by penetration of larvae through skin. The larvae then migrate through alveolar wall to the larynx where they are swallowed and finish their development in small intestine. Additionally, *A. duodenale* larvae also cause infection when ingested orally [9,13].

E. vermicularis is occurring mainly in 5 to 10 years old children [21]. Even though the infection is often asymptomatic, it is the most common helminth infection in Europe and the USA. After eggs ingestion the hatching and maturation to adults is taking place within the human body. The adults live freely in the ileum and caecum and the females migrate during the night to lay down eggs in perianal area. By scratching in those area the eggs get attached to the fingers and can be ingested [22].

Compared to other nematodes genera, the biology of *S. stercoralis* and other *Strongyloides* species is more complex and involves alternation of free-living and parasitic generation (Fig. 1) [23-26]. While free-living generation consists of both, males and females, parasitic generation has only females [25]. The **free-living generation** begins by excretion of rhabditiform larvae to the external environment where male larvae develop into adults and rhabditiform females can either develop into free-living adult females or into infective L3 females (filariform larvae). Those free-living adults then sexually reproduce and produce **post-free-living generation** consisting only of females, which develop into infective L3 [23,24,27]. The filariform female larvae from both, post-free-living generation or post-parasitic generation must find a host to complete their life cycle [24,28]. The **parasitic generation** starts when filariform female larvae penetrate the skin of the host and via the lungs or other alternate routes (abdominal viscera or connective tissue) enter the small intestine, where develop into parasitic adults [28]. The parasitic females reproduce by mitotic parthenogenesis and produce eggs that hatch inside of the body and result in rhabditiform larvae. The females from this parasitic generation could start another free-living generation when excreted with faeces or can develop into filariform larvae, which penetrate the intestinal mucosa and reinfect the host again [23,24,27].

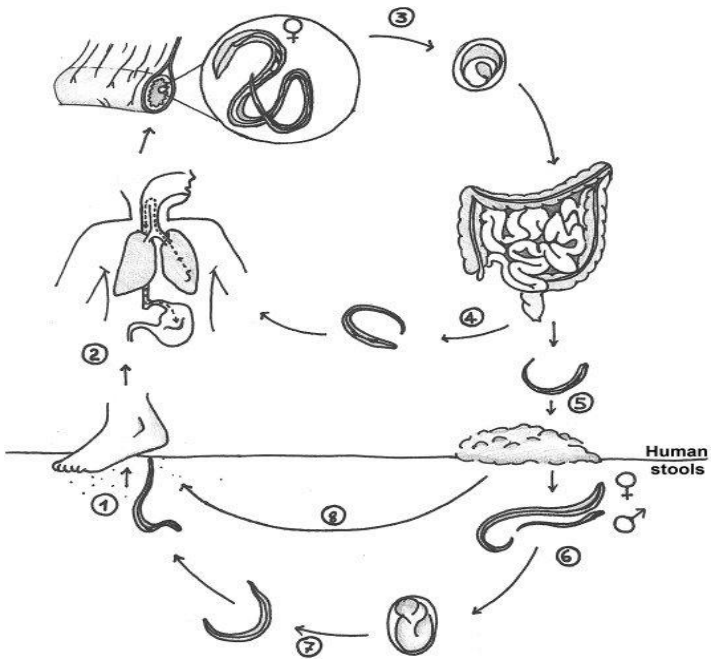

Figure 1: The life cycle of *S. stercoralis*. 1) Filariform larvae; 2) migration to the lungs, trachea, and pharynx; 3) parthenogenetic adult females (2 mm); 4) rhabditiform larvae develop into auto infective filariform larvae; 5) rhabditiform larvae expelled through faeces; 6) free-living male and female adults; 7) sexual reproduction occurs in the soil; 8) filariform larvae mature in the soil, becoming infectious to humans through direct development [26].

Infections by GIN are also known for its detrimental effect on health of many domestic animals such as cattle, equines, pigs, fowls, sheep, and goats. The result of poor health is lower production of various animal products like milk, eggs, meat etc. [29,30]. GIN similarly as other parasites can be distinguish to **specialists**, with narrow host specificity limited to species or family and **generalists**, capable to infect heterogenous host [31]. For example, species infecting cattle are similar to those of small ruminants (sheep and goat) however the particular species will differ and the one infecting cattle does not readily infect sheep and goats and vice versa [32]. This is the reason why grazing cattle and sheep or goat together can be integrated in parasite control programs [33].

The infection is rarely caused by a single species, but most of the time it is a mixture of many [32,34,35]. The species responsible for disease varying with the region. While some species are predominant in warm and moist environment the other prefer temperate or colder climates [36]. Clinical symptoms and their severity are the result of species, burdens of worm occupying the gastrointestinal tract and general condition and age of the animal. The most susceptible to helminth infection are young stock till 18-20 month [32,37,38], however adults can carry small burdens of worm and represent a reservoir for pasture contamination [39].

The most important species of ruminant (sheep, goats and cattle) are *Haemonchus* spp. (hookworm), *Teladorsagia* spp. (*Ostertagia*, roundworm), *Cooperia* spp. (roundworm), *Oesophagostomum* spp. (nodule worm) and *Trichostrongylus* spp. (pseudohookworm) [29,32,40]. *Haemonchus*, *Teladorsagia*, *Cooperia* and *Trichostrongylus* belongs to the same family *Trichostrongylidae* and *Oesophagostomum* to family *Chabertiidae* [38].

In **small ruminants** (sheep and goats) the major species **in cool and temperate regions** with uniform rainfall are *Teladorsagia circumcincta*, *Trichostrongylus colubriformis* and *Trichostrongylus vitrinus* [41-44]. The name *Teladorsagia* was introduced to the genus *Ostertagia* in goats and sheep in 1990s while *Ostertagia* is still commonly used for the individuals infecting cattle [37,45].

T. circumcincta is uniformly brown (for this reason also call “brown stomach worm”) small (10 mm) worm infecting the abomasum [34,45]. Unlike some other GIN, *Teladorsagia* does not feed on blood and the harmful effect to the host are caused by its larval stages [38]. After being ingested, the L3 larvae enter the abomasal glandular lumen, where they undergo transformation into the fifth-stage larvae (L5). From there, the L5 larvae emerge and return to the surface of the abomasal mucosa, where they undergo further maturation into adult worms [35,41,46]. The

adults line up on the abomasal surface as umbilicated nodules resembling morocco leather appearance [41,43]. During the migration L5 release waste-secretory products which are responsible for pathophysiological changes in the abomasum such as edema, sloughing of the abomasal folds and reduced HCl production [43,46].

T. circumcincta often occurs as mixed infection with *Trichostrongylus* spp. Various *Trichostrongylus* spp. infect different segments of gastrointestinal tract. While *T. colubriformis* and *T. vitrinus* infect small intestine of sheep and goats, one less common species, *T. axei*, which is also shared with cattle occurs in abomasum [42,43]. Both species, *Teladorsagia* and *Trichostrongylus* will contribute to the clinical signs, however *Teladorsagia* will play the major role [34,43]. Some of the *Trichostrongylus* spp., including *T. colubriformis*, and *T. axei* can occasionally also infect humans [47-49].

The most common species in **warm and humid areas** with the greatest economic importance infecting **small ruminants** is *Haemonchus contortus*. *H. contortus* is the largest worm in the abomasum and due to the livestock translocation, its ability to adapt wide range of host species and climatic zones it is now distributed globally [43,50-52].

In **cattle** the most important species in temperate regions are *Ostertagia ostertagi* and *Cooperia oncophora* [40,53]. The biology and live cycle of *O. ostertagi* is essentially comparable to *T. circumcincta* [35]. *C. oncophora* inhabits small intestine where do not burrow into the intestine as for other trichostrongyles, but instead reside between villi and cause pressure necrosis [34]. *Haemonchus* spp. usually do not affect cattle, but young calves may be susceptible to infection. However, they develop immunity much more easily than sheep and goats. While *H. placei* is known to be a bovine parasite, other species such as *H. similis* and *H. contortus* can be also observed [33,43].

Oesophagostomum spp. are also distributed globally among mammals and similarly as *H. contortus* prefer warm and wet weather for its development [54,55]. Major species infecting ruminants are *O. columbianum* and *O. venulosum* [34,54]. *Oesophagostomum* primarily occupy large intestine and similarly as *Teladorsagia*, they do not suck blood, but they are feeding on gut content [34,56,57]. In some cases L4 persist within the ileum wall or undergo a secondary tissue phase referred to as 'histotrophic' within the large intestine wall, where they become encapsulated in thick-walled, caseous (cheesy) nodules [57]. Similarly as *Trichostrongylus*, some of the *Oesophagostomum* species are cause of zoonotic disease [58].

2.2 *Haemonchus contortus*

H. contortus originates from sub-Saharan Africa but now is spread almost worldwide. High level of genetic variation in parasite population is responsible for its ability to adapt wide range of host species and climatic zones [51]. Genetic diversity can exist either among individuals within a population or between populations, which have been geographically separated or undergone drug-induced selection. *H. contortus* exhibits remarkable level of genetic diversity both within and among population isolates. Mutations occur at the level of nuclear and mitochondrial DNA, and the mutation rate in mitochondrial DNA in *H. contortus* is up to ten times higher than in vertebrates [51,59,60]. Moreover nematodes can increase their physiological diversity by employing posttranslational modification, or alternative splicing of mRNA, which enables to produce different proteins from the same genes [59]. The genetic diversity is influenced by a range of factors such as population size, geographical or environmental barriers, gene flow, selection pressures, population crashes or bottlenecks [51,59].

The distribution and diversity of genes within and among groups of population are more complex for parasites than for free-living organisms as the interaction with host can impact their genetic structure [51]. *H. contortus* during its life cycle undergoes both free-living and parasitic stages. While free-living stages are exposed to significant temperature and humidity fluctuations, the host environment represents in that sense much more stable environment and allows portion of *H. contortus* population to survive unfavourable external environmental conditions and it is one of the reasons behind its worldwide distribution. On the other hand, inside of the host, the parasite has to face host's immune system or drug treatment which may be administered for the parasite control [51].

The population of *H. contortus* within a host is large and one ruminant can carry from hundreds to thousands of adult worms and the population size on pasture is even greater than within a host. The females of *H. contortus* are one of the most fecund strongyle nematodes and one female can lay up to 10,000 eggs per day [59]. In contrast, females of *T. circumcincta* produce around 100-200 eggs per day [38].

2.2.1 Morphology and life cycle

The beginning of *H. contortus* life cycle (Fig. 2) is copulation of adult males and females in the abomasum of the ruminant which results in the production of many fertilized eggs [33]. The

eggs are laid at the 4-cell stage and they develop into 11- to 26-cell stage referred to as morula by the time of excretion together with faeces into the environment [61]. The hatching and development of the larvae are occurring within the faecal mass, which provides certain level of protection from unfavourable environmental conditions [33]. The egg hatching is influenced by many factors, which are unique to each nematode species. Those factors can be divided into two main categories: intrinsic and extrinsic factors. Intrinsic factors are the factors related to individuals such us expression of particular genes which are responsible for the progressing in the egg hatching cascade. However, those factors in many nematode species are not yet well understood. The extrinsic factors are related to environmental conditions, such as temperature and the level of moisture. Those factors prevent the larvae to hatch under unfavorable conditions [62]. Nevertheless, *H. contortus* eggs exhibit a limited ability to resist the effect of drying or freezing. The optimal temperature range for eggs development is 25-30°C [63]. Under the optimal conditions the eggs hatch within 14 – 17 hours after releasing onto the pasture [61]. Lower temperature slow down the process and if they are kept at 5°C continuously, the eggs die within 4 to 6 days. If the eggs reach maturity to prehatch stage before exposure to low temperature, the minority of them can survive up to two months, although they won't hatch until temperature rises to 9°C [63].

The **first-stage larvae (L1)** of *H. contortus* emerge from the eggshell by using its sharp tail and by production of lipases from pharyngeal glands which break down the eggshell durability [62]. Once the larvae hatch they start to feed on bacteria and continue growing through two molting cycles until they reach the infective **L3** [33]. Before each molt the larvae experience a period of inactivity known as **lethargus stage** during which significant structural changes are taking place [61,64]. The L3 leave the fecal mass and move onto forage from where they are available for consumption by the host. The larval migration onto forage is influenced by air temperature, soil moisture and relative humidity. In dry and hot weather, the larvae are unable to travel and remain in the faecal mass. Most of the larvae stays within 10 cm from the faecal pellet, but they can travel up to 90 cm.

In every molt the cuticle is shed, except L3, which maintain their L2 cuticle as a protective shield. However, this protective layer prevents the larvae from feeding, so they must rely on their metabolic reserves. The cool and dry weather reduces movement of the larvae which consequently prolongs the period of surviving up to several months. On the other hand, hot and moist conditions lead to exhaustion in much shorter time [33]. The temperature also

influences the period required for development into L3. At a temperature of 35°C it takes about 3-4 days, at 21°C 6-14 days and around 10°C it can take 3-4 weeks [63].

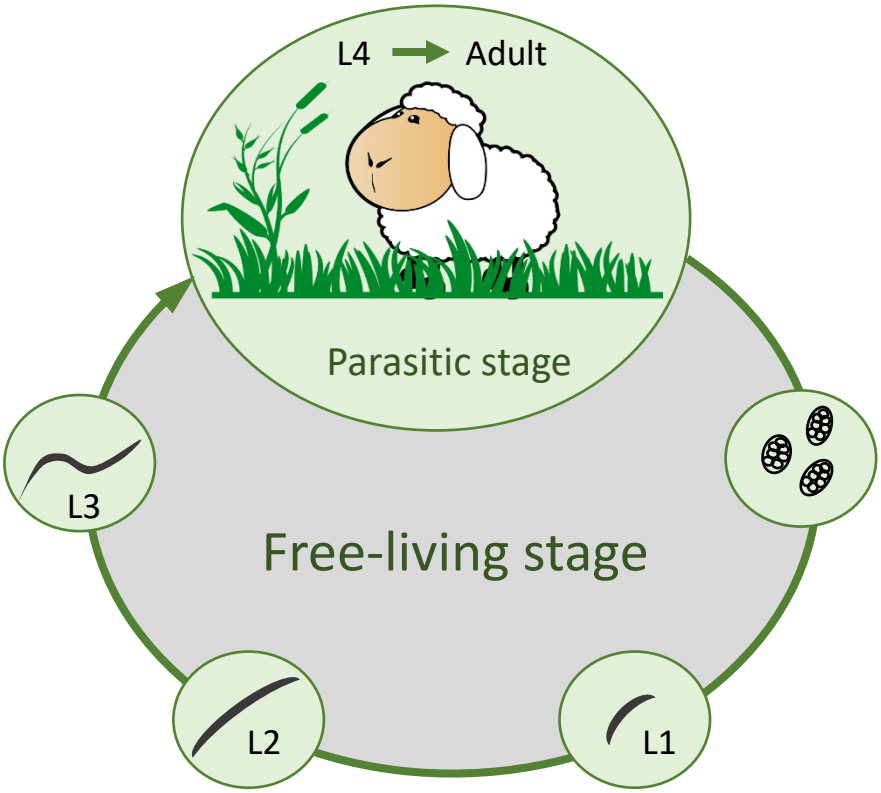

Figure 2: The schematic representation of life cycle of *H. contortus*.

Directly after ingestion, L3 lose their protective coating and can start to feed again [64]. For exsheathment the L3 digest particular region of anterior end of the L2 cuticle by 44-kDa proteases [65]. When the L3 enter the abomasum, they shelter themselves into the villi of abomasal mucosa with assistance of a thin layer of mucus. Around 30 to 36 hours post-ingestion the larvae enter **third lethargus stage** lasting approximately 12 hours. During that stage the larvae are immobile and undergo transformation to the **L4**. The main purpose of this metamorphosis is not to grow in size, but to form structures which are important for beginning their parasitic life as blood suckers. The main change is development of lancet in buccal cavity enabling perforation of stomach mucosa. The L4 is also the first stage where the beginning of sexual differentiation is observable by the length of the larvae and position of primordium. The shorter larvae with longer primordium located farther from the anus indicates future males and longer larvae with shorter primordium located closer to the anus indicates future females. In the beginning of blood feeding, the L4 cause a hemorrhage from abomasal mucosa however

this stops over time. The leaking blood forms a coagulum which together with food particles and mucus surround the L4 anchored to the mucosa by its mouth. The L4 rapidly grow in size and 6 days post infection males and females are clearly distinguishable. Between 9- and 11- days post infection the larvae undergo **final lethargus** stage ongoing for about 24 hours resulting in molting of adult worm. Over the time the **adults** grow, and mouth apparatus become better developed, therefore the blood leaking does not occur during mucosa perforation. The copulation of males and females starts around 18 days post-infection [64]. Adult worms then live within a host for a few months and die [33,38].

Adult females are much bigger than males and measure between 17-30 mm, depending on the age, species and conditions influencing the host [64]. The main visible feature of the adult females is the twisting of the white ovarian tubes filled with eggs around blood-filled intestine giving it red-brown colour (Fig. 3B). Due to this spiralling the *H. contortus* received the name „barbers pole“ worm [64,66]. In the posterior third of the body the females are equipped with vulva [67]. The vulva is generally hidden behind a predominant anatomical feature called the linguiform process or vulva flap.

The **males** are smaller (10-20 mm), and the reddish-brown colour is consistent throughout all their body. They have a well-developed copulatory bursa consisting of two symmetrical lateral lobes and one asymmetrical lobe placed dorsally (Fig. 3A) [66,68]. The surface of the worm is covered by cuticle which protects the parasite against digestive system of the host. The cuticle consists of three different layers made mainly with collagen-like proteins. On the surface of the cuticle glycoprotein macromolecules with antigenic functions are located [64,68,69].

Under certain conditions *H. contortus* can enter a dormant phase known as **hypobiosis**. Hypobiosis is the state when the larvae become metabolically inactive for several months before continuing their development. Although the host's immunity can play a role in hypobiosis rates, larvae usually enter into arrested development when environmental conditions are not optimal for eggs and larvae survival, for example during a cold winter season [33]. If the larvae diapause their development, they stay in the third lethargus stage. As hypobiotic larvae get older the rod-like crystalline inclusions are accumulating in their intestine. The purpose of those crystals remains unknown, but it has been suggested that they are generated during larval development as waist product of metabolism [70,71].

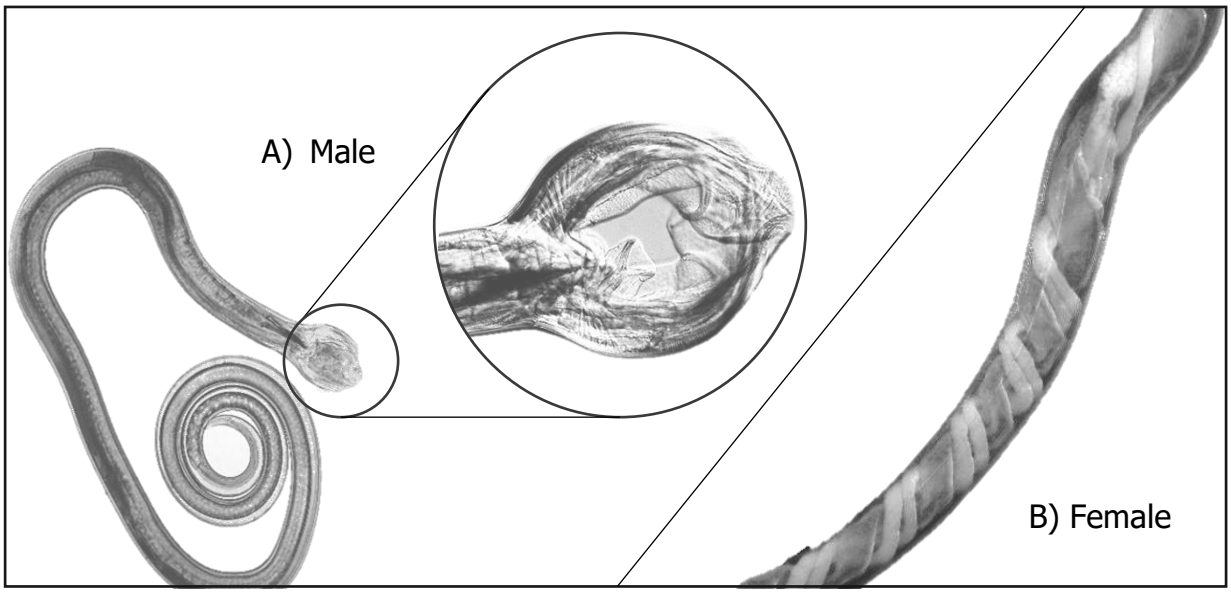

Figure 3: Morphological features of adults of *H. contortus.* The photographs were taken by Linh Thuy Nguyen on a confocal microscope at the Faculty of Pharmacy in Hradec Králové, Charles University.

2.2.2 Metabolism

The parasitic lifestyle of *H. contortus* and other endoparasites results in development of unique alteration of metabolic pathways. While some of the synthetic pathways were simplified as the host supplies most of the nutritional requirements for parasites, the other related to substrate absorption and interconversion have been extended [72]. For example, nematodes, including *H. contortus* have a limited ability to synthesize polyamines from ornithine, therefore rely on acquiring polyamines from the host. Nevertheless, they can transform absorbed polyamines into other polyamines by oxidizing acetylated intermediates, such as conversion of spermine into spermidine or spermidine into putrescine. The precursors for purine synthesis are also dependent on host supply, as *H. contortus* and other nematodes are not able to synthesize purines *de novo*. Purine nucleotides are then formed from purine bases by phosphoribosyltransferases or by action of nucleoside phosphorylases and nucleoside kinases [73].

H. contortus must possess certain level of adaptation as free-living and parasitic stage face two completely different environments during their complex life cycle [72]. This adaptation is associated with changes in gene transcription and expression affecting metabolism of nucleic acids, nitrogen, lipids, amino acids and energy metabolism within each stage. For example,

in the eggs the gene expression related to oxidoreductase activity, apoptosis, body morphogenesis and development of embryo and larvae, DNA replication and/or chromosome organisation are upregulated. During the transition of nematode eggs into the L1 stage, enhanced motility becomes necessary to facilitate active feeding. This prompts the upregulation of genes associated with muscle development and motor activity. On the other hand, the transition from L2 to L3 stage is accompanied by downregulation of genes linked to myosin complex and motor activity. However, those genes are again upregulated during transition from L3 to L4. Additionally, several changes that are sex-specific occur during the transition from L4 to adulthood. Those changes are associated with embryogenesis in females and sperm production in males. Parasites growth is also associated with changes related to development of cuticle. The cuticle is composed of cross-linked collagen fibres, glycolipids, lipids and several enzymes [73]. Collagen, which is the main protein in the nematode's cuticle, is encoded by more than 20 genes. Although there are changes occurring throughout the developmental stages, the most prominent variations in the cuticular protein composition occur during the shift from free-living to parasitic stages. For example, cuticular proteins in adults have more basic and fewer glycine residues compared to larval stages. Moreover, lipophilicity of lipoproteins on the L3 surface change in response to different pH within the host [61,74].

H. contortus, similarly as other living organisms, use molecules with 'highly-energy bonds' such as ATP, GTP or NAD(P)H to run its metabolic processes. However, the generation of those molecules differ between free-living and parasitic stages [73,75,76]. In general, those molecules are generated during the process of degradation of carbohydrates, lipids, or proteins via **aerobic** or **anaerobic** strategies [72,76,77]. The primary energy source and storage for free-living larval stages of *H. contortus* are lipids, which are degraded via aerobic pathway [73,75,78]. In contrast, lipid metabolism of adults is limited thus carbohydrates serve as their main energy source and due to the low oxygen abundance in the gut, those substrates are degraded under anaerobic conditions [73]. In carbohydrate metabolism both aerobic and anaerobic pathways begin with the breakdown of glucose into phosphoenolpyruvate (PEP) and consequently into pyruvate through many consecutive enzymatic reactions. In **aerobic** condition, the pyruvate is transported to mitochondria where it is completely oxidized to carbon dioxide and water through coordinated action of tricarboxylic acid cycle (TCA) and mitochondrial respiratory chain. Under **anaerobic** conditions, pyruvate is converted into various end-products dependent on the organism. It can be converted into lactate or ethanol through process of lactic or alcoholic fermentation [72,76,77,79]. However, anaerobic substrate degradation is far less efficient with

yield of only 2 mol ATP molecules per 1 mol of glucose compared to aerobic substrate degradation gaining 30 mol of ATP per 1 mol of glucose. However, some parasites including *H. contortus,* evolved another approach called **malate dismutation** (Fig. 4). In this process PEP is not converted to pyruvate but by phosphoenolpyruvate carboxykinase (PEPCK) is transformed to oxaloacetate, which is subsequently reduced to malate. Unlike lactate, malate is not excreted but enters mitochondria where part of the malate is oxidized to acetate and another portion is reduced to succinate which is then further metabolized to propionate. The ratio of production between acetate and propionate is 1:2. This pathway is much more efficient compared to lactic or alcoholic fermentation and yields 5 mol of ATP per 1 mol of glucose [72].

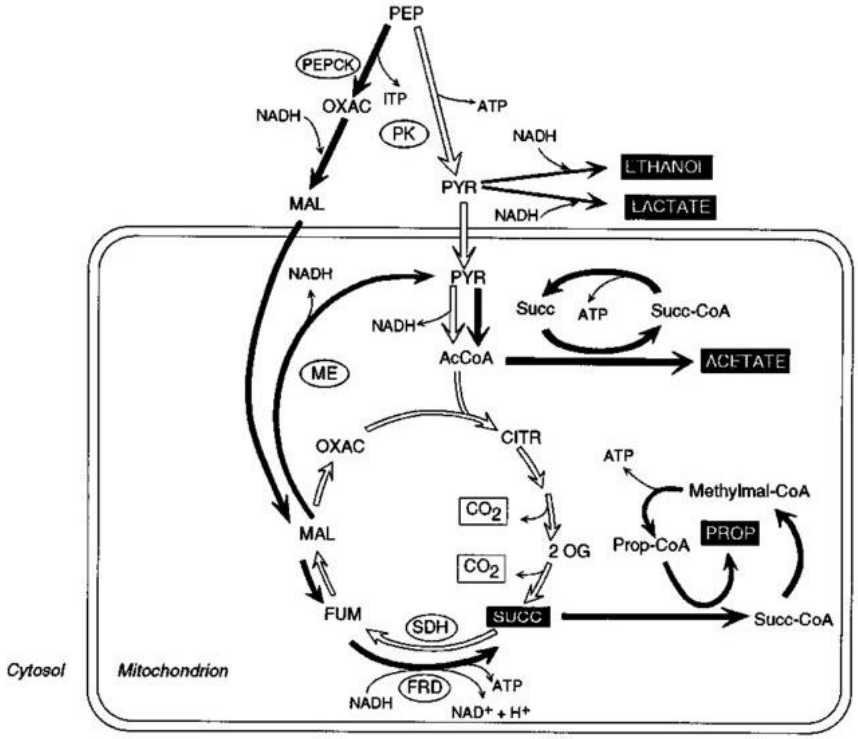

Figure 4: General aerobic and anaerobic carbohydrate metabolism in eucaryotes. Aerobic degradation is denoted by open arrows, while malate dismutation and simple fermentation are denoted by closed arrows. End products are represented in boxes. Abbreviations: AcCoA (acetyl-CoA), CITR (citrate), FRD (fumarate reductase), FUM (fumarate), MAL (malate), Methylmal-CoA (methylmalonyl-CoA), ME (malic enzyme), 2 OG (2-oxoglutarate), OXAC (oxaloacetate), PEP (phosphoenolpyruvate), PEPCK (phosphoenolpyruvate carboxykinase), PK (pyruvate kinase), PROP (propionate), Prop-CoA (propionyl-CoA), PYR (pyruvate), SDH (succinate dehydrogenase), SUCC (succinate), Succ-CoA (succinyl-CoA) [72].

2.2.3 Diagnosis, symptoms, and clinical manifestation

The typical symptoms indicating infection by *H. contortus* are related to blood loss due to blood-feeding activity of the adult and advanced larval stages [80]. The anaemia typically appears 10-12 days post-infection and single adult worm is estimated to consume 30-50 μL blood daily [36]. The clinical manifestation of the disease is directly linked to the number of *H. contortus* larvae that successfully colonize host's abomasum and those numbers are the result of quantity of ingested larvae and resilience of the host. The most severe symptoms occur when animal is infected with large number of *H. contortus*. Infection with up to 30,000 individuals is classified as **hyperacute** form and leads to sudden death caused by massive blood loss. However, this form is relatively rare. Typically, the haemonchosis is present as an **acute** form with 2,000-20,000 individuals present in abomasum of one sheep [36,80]. In this case anaemia develops more slowly and death may occur within 4-6 weeks post-infection [36]. The anaemia is visible as a paleness of mucous membranes noticeable especially in conjunctivae. Animals become gradually weaker and less active and spend more time resting. If left untreated, hypoproteinaemia develops due to the blood loss in some animals. The low protein levels lead to oedema, which is recognizable mainly in submandibular region known also as "bottle jaw" and in the cervical area [80].

In less favourable condition for larval development, the animals can carry small burdens of *H. contortus* individuals (around 100-1,000) for a longer period of time (2-6 months), referred to as **chronic** haemonchosis [81,82]. Chronic haemonchosis is very often asymptomatic, however over time symptoms similar to malnutrition, such as reduced weight gains, reduced milk and wool production may appear in poorly nourished animals. The symptoms can also manifest when number of worms escalates or the host loses the ability to tolerate the infection due to inadequate physical condition [36,81].

Detecting the presence of developing *H. contortus* burdens in small ruminants is a relatively simple task that can be accomplished through clinical or laboratory assessment or can be verified through necropsy in case of mortality. Based on visible symptoms of anaemia, the simple and fast diagnostic method called FAMACHA (FAffa MAlan CHArt) was developed in south Africa to identify animals that require treatment. The system is based on evaluation of the colour of conjunctival membrane against set of five colours ranging from red-pink, indicating no anaemia, to white in terminal anaemia. Assessing level of anaemia and identification of individuals that require treatment, rather than treating entire animal groups

helps to decrease the use of anthelmintic drugs and thus reduce the risk of anthelmintic resistance development. However, frequent monitoring required to identify animals in early stage of haemonchosis limits the use of this approach to smaller animal population or adequate labour resources are needed to perform the task. Although the presence of anaemia can suggest *H. contortus* infection, it is not specific, as several other circumstances can lead to similar symptoms. Therefore, the confirmation of *H. contortus* infection needs to be done through other tests.

One of the useful and relatively easy tests is examination of eggs in faecal mass. In *H. contortus*, compared to other trichostrongyles, the egg output strongly correlates with total worm burdens present in abomasum of sheep and goats. While a count of 3,000 eggs per gram (epg) in an adult sheep suggests a "light" infection, a range of 2,000-20,000 epg indicates a "moderate" infection, and 30,000 epg indicates a "severe" infection. Nevertheless, the faecal worm egg counts (FWECs) have a certain limitation. In early stages of infection or when species differentiation is not possible, even lower values of epg can indicate severe infection.

The two most commonly used techniques to estimate FWECs are McMaster and FLOTAC [80]. Both methods are based on the same principle: microscopical examination of eggs in defined volume of faecal suspension prepared from known weight of faeces and known volume of flotation solution. The FLOTAC technique is newer and requires more complex procedures, more expensive equipment, and longer examination time compared to other methods; however, its higher level of accuracy compensates for these drawbacks. Since first reports of the McMaster method in 1939 it underwent many modifications such as different types of flotation solution and faecal weight, flotation times, sample dilution, adding extra centrifugation steps to clarify the suspension, different centrifugation times and speeds, and modification of numbers of McMaster slide sections for counting. Both methods use some kind of counting chamber which differ in the capacity for final egg suspension. While in the McMaster method the counting is taking place in 0.15 mL chambers, the FLOTAC apparatus can examine much larger volume (2 x 5 mL) and enables centrifugation due to its more complex design which increase the sensitivity of the method [83,84].

The identification of eggs of *H. contortus* from other trichostrongyles species is not that easy task and cannot be reliably done by determination of size or morphology. Instead, the cultivation of eggs to L3 is required to identify specific morphological features of the larvae. However, the identification becomes complicated when more species are presented in the faecal

sample. Moreover, morphology of the worms may be influenced by cultivation conditions, it is time consuming and experienced labour is also required to perform the task. The lectin-binding assay provides solution to those limitations. The method is based on specific binding of fluorescent dye-labelled lectin to *H. contortu*s eggs [80]. Nevertheless, the main enhancement of distinguishing parasitic worms in different stages came with emergence of molecular technique, particularly polymerase chain reaction (PCR) [85].

2.2.4 Treatment, prevention, and host defense mechanisms

Even though parasites are generally viewed as entities causing disease and mortality in humans and animals, they are important parts of the ecosystem and play significant role in biological evolution [86-88]. The parasite-parasite and parasite-host relationships are very complex and understanding of how the presence of one species can impact the health of environment and the host is indispensable for effective management [86,89]. Multiple strategies can be employed to control the burden of *H. contortus*, but it is essential to evaluate the optimal timing and implementation of each strategy [90]. Naturally, animals harbour colonies of diverse parasite species that compete with each other and the host about food resources. The presence of certain parasites can stimulate the host's immune system and decrease susceptibility to other parasitic species (i.e., cross-immunity). Conversely, certain parasitic species can act as agonists for one another [86,89]. For example, European wild rabbits display a lower susceptibility to the helminth *Graphidium strigosum* after being infected with *Trichostrongylus retortaeformis* [86,91]. Another example of cross-protection is development of high-level immunity against *H. contortus* in young lambs after previous infection with *O. circumcincta* or *T. axei* [92-95]. An intriguing relationship is between GIN *Heligmosomoides polygyrus* (roundworm) and coccidia *Eimeria hungaryensis*. When wood mouse (*Apodemus sylvaticus*) is coinfected with both of these parasitic species, the burdens of *H. polygyrus* is 2.5-time higher compared to single infection. On the other hand, the presence of *H. polygyrus* supress the population of *E. hungaryensis* and by removing *H. polygyrus* from the gut the population of *E. hungaryensis* grows 15-times [96,97]. The fact that modification in the abundance of target species can have an impact on other non-target species should be consider when applying anthelmintic treatment.

The central role in the prevention and treatment of parasitic diseases still retain **anthelmintic drugs** due to their affordability, ease of administration, and lack of satisfactory alternative solutions [98]. Multiple classes of anthelmintic drugs with diverse modes of action are

commercially available for treatment of haemonchosis and other helminth infections. These classes encompass benzimidazoles (albendazole, fenbendazole, oxfendazole, mebendazole, thiabendazole), imidazothiazoles (levamisole, morantel), macrocyclic lactones (ivermectin, moxidectin, doramectin), salicylanilides (closantel, rafoxanide), amino-acetonitrile derivatives (monepantel) and spiroindoles (draquantel). For the treatment, the anthelmintics can be used alone or in combination [80]. Before the first benzimidazole drug, thiabendazole, was introduced to the market in 1961, many chemical compounds such as organophosphates and plant metabolites were used. However, those compounds are toxic to mammalian host, too [80,99]. **Benzimidazoles** are broad spectrum anthelmintics effective also against some trematodes and arthropods [80]. Their mode of action is based on the creation of complex with β-tubulin subunits in cytoplasm. Formation of this complex prevents microtubules polymerization and numerous cellular functions including egg laying, egg hatching, larval development, substrate transport, enzyme activity and secretion are then affected [73]. **Imidazothiazoles**, introduced in early 1970s, cause prolonged muscle contraction and spastic paralysis of the nematode by acting as cholinergic agonist at the nicotinic neuromuscular junction. Nevertheless, imidazothiazoles exhibit nicotinic agonistic activity also in mammals, therefore they have narrower therapeutic index compared to other broad-spectrum anthelmintics. In the early 1980s, ivermectin (IVM), the first **macrocyclic lactone**, was commercially released as a representative of the avermectins. It originates from the natural fermentation product of *Streptomyces avermitilis* [99]. IVM is effective against majority of nematode species in all developmental stages and some ectoparasites. However, its effectiveness does not include cestodes or trematodes. While the macrocyclic lactone (ML) group includes various active substances, they all act primarily by potentiating glutamate-gated chloride channels, leading to the disruption of nervous transmission. Significant role in *H. contortus* management is played by members of **salicylanilide** group, as they target specifically blood-feeding helminths. Moreover, certain compounds like closantel and disophenol exhibit an extended duration of activity lasting several weeks following administration. **Amino-acetonitrile derivatives** include just one representative – monepantel (MOP). MOP was introduced in late 2000s and exhibits wide spectrum of activity comparable to MLs [80]. MOP is binding to subunits of nicotinic acetylcholine receptors which are specific to nematodes but do not occur in mammalian host. Semi-synthetic **spiroindole**, draquantel, released in 2011 act also as acetylcholine receptor agonist inducing fast muscle paralysis and mortality of nematodes [99]. Draquantel is commercially available only in combination with abamectin due to the potential

limitations of derquantel alone in effectively targeting all nematodes, particularly the larval stages of *T. circumcincta* [80].

Various strategies can be applied in administration of anthelmintic drugs. Some of those strategies are less specific with periodic administration of anthelmintics to all flocks, while others are more targeted, focusing only on animals or groups of animals that require treatment. Routine deworming of all flocks is used mainly in tropical regions, where anthelmintics are administered with regular interval during rainy season. However, this strategy is expensive, restricts the opportunity for immune response development and intensifies the risk of drug resistance emergence in contrast to targeted regime [100]. For this reason, the effective and sustainable control of *H. contortus* burden cannot rely just on the use of anthelmintic drugs but other strategies such us reasonable pasture management, immunomodulation, nutrition management and the use of bioactive forages should be involved [90,99].

Pasture management has been used for decades and it is very often combined with anthelmintic treatment. Several approaches are used in pasture management. One of the approaches is **rotative grazing** when animals are not returned to the same contaminated pasture after anthelmintic treatment. Instead, they are transferred to a fresh pasture that has been left ungrazed for a duration based on the environmental condition or it has been grazed with other type of animal (for example cattle) which are not susceptible to *H. contortus*. This practice prevents immediate reinfection of animals, thereby reducing the reliance on veterinary chemicals, and lowers the contamination rate of the new pasture. On the other hand, if resistant population of *H. contortus* is present, the implementation of this strategy would favour the propagation of resistant worms, leading to the next generation being entirely composed of resistant individuals. When used in conjunction with anthelmintics, it is crucial to administer the treatment in a targeted manner to preserve sensitive strains within the population (refugia strategy) to mitigate the development of drug resistance [80,90]. **Co-and multi-species grazing** when cattle and sheep or/and goats are sharing the pasture is another approach which results in many benefits. While sheep and goats share common parasites, cattle harbour the set of parasites which is distinct from sheep's and goat's. Thus, grazing them together reduces available infective larvae for preferred host by consuming parasites of one another. Moreover, different feeding habits increase the productivity and quality of the pasture [101].

For centuries, **medicinal plants** have been employed for the treatment of diverse health conditions and many of them have been recognized for their anthelmintic properties. However,

insufficient knowledge regarding actual effectiveness of plant compounds against particular parasites, suitable dosing protocols, various methods of preparation and administration for different livestock species and potential toxicity risk limit their use for anthelmintic treatment in broader scale. Nevertheless, some of the active compounds which have been recognized for its anthelmintic activity were also used as prototypes for synthetic drugs. Many plant secondary metabolites participate in defence against parasitic nematodes; however, phenolic compounds, especially condensed tannins have received the most attention among them [90]. Similarly, as anthelmintic drugs, several mechanisms of action are responsible for anthelmintic effect of the plant. Moreover, plant can act not only by direct action towards parasite structure, but also indirectly by boosting immunity response and/or nutrition of the host. For example, protein binding activity of condensed tannins to essential amino acids enable their better absorption from lower gut, while binding to proteins in the nematode cuticle or some enzymes can affect function and development of parasites [102].

To find another alternative solution which could substitute the use of anthelmintic drugs, considerable effort has been invested into **vaccine** development. The availability of efficient vaccines would provide long-term protection against accumulation of detrimental parasite loads, which would consequently reduce the likelihood of animal mortalities and help alleviate the severity of anthelmintic resistance [80]. Recently, the only vaccine available, Barbervax®, is registered for the use in lambs only and it is produced by Albany laboratories in Western Australia [80,90]. The vaccine is made of intestinal membranes of *H. contortus* and consist of native integral gut antigens H-gal-GP and H11 [103]. There were many attempts to produce those antigens by recombinant technology, but they were unsuccessful [80]. However, several studies have been published to provide significant protection in goats after vaccination with recombinant DNA vaccines. The antigen present in the Barbervax® is hidden, therefore it requires repeated vaccination to stimulate the immune response. To maintain protective immunity in lambs during the high-risk season, it is recommended to administer the first three initial injections before the season, followed by two further injections spaced 6 weeks apart. On the other hand, the use of hidden antigens can result in protective effect in scenarios where natural immunity is insufficient. The mechanism of action is based on increasing levels of antigen-specific circulating IgG levels, which differ from natural immunity developed through exposure to infective larvae; however, this immunity could be additionally obtained during the season [103].

Many **immune-mediated mechanisms** are involved in the response to various stages of *H. contortus*. One of the non-specific mechanisms is **rapid rejection** of infective L3s. This immune response involves the presence of antibodies (IgE), mucosal mast cells and intra-epithelial mast cells (GLs) and it occurs in the sheep that have undergone hypersensitization due to repeated larval infections over a prolonged period and results in the elimination of a sequential L3 infection within 48 hours. If L3s are not eliminated by hypersensitization and penetrate host's tissue, another mechanism called **delayed rejection** takes place. In this case the immune response is mediated by recruiting eosinophils, which directly kill the larvae through antibody-dependent cell cytotoxicity (ADCC). Additionally, γδ T cells, $CD4^+CD25^+$ T cells and B cells are also involved in the process. The immune-based defence against L4 and adults is however not that deeply understood. Recently, the mammalian galectins, especially galectin-11 have been investigated for the role in GIN defence. *In vitro*, galectin-11 inhibited feeding, exsheathment and growth of *H. contortus* L4s. IgAs are also shown to play a role in protection against another GIN *T. circumcincta*; however, their role in *H. contortus* remain unclear. The studies focused on the immune response modulated by adults showed minimal alteration in leucocyte population, which supports the theory that adult parasites might possess the ability to supress the immune response. It is also thought, that for clearing of adults are responsible the specific and nonspecific mechanism which are modulated during earlier stages [103].

2.2.5 Anthelmintic resistance

Due to widespread administration of anthelmintics, *H. contortus* developed resistance to all of them [104]. On the level of individual, the resistance can be understood as a change in the organism's reaction to drug treatment, resulting in decreased efficacy compared to when the drug was used for the first time. On the level of population, the resistance is observed when there is a higher occurrence of individuals within the community that can endure anthelmintic treatment [104-106].

Several distinct assessments have been employed to identify anthelmintic resistance in nematodes. Even though many molecular markers to detect anthelmintic resistance have been already described, **molecular-based tests** are not in routine use as they are expensive, and the knowledge is mainly restricted to benzimidazoles. For other drug classes the molecular mechanism contributing to anthelmintic resistance is not yet adequately understood, thus

appropriate molecular markers have not been identified [104,107]. Therefore, the two most simplest methods - **Faecal egg count reduction test** (FECRT) and **egg hatch test** (EHT), are the most frequently used ones [105]. The **FECRT** evaluates the effectiveness of anthelmintics by comparing FWECs before and after treatment or between treated and untreated control group [107,108]. The faecal samples are examined from the group consisting of at least 10 animals displaying positive egg counts and the period between the treatment and sample collection should be at least 10-14 days to prevent false negative results [107]. FECRT can be used in examination of all nematode species which expelled their eggs in faecal matter in ruminants, horses and pigs [105]. Anthelmintics resistance is considered to be present if percentage reduction in egg count following drug treatment is less than 95 %, and the lower 95 % confidence level is below 90 % [104,108]. The **EHT** is used for detection of resistance of benzimidazole drugs and can only be used in the nematode species whose eggs hatch quickly. [105]. The test is based on counting the percentage of eggs that successfully hatched (or do not hatch) at increasing concentration of anthelmintic (usually thiabendazole due to its better solubility in water) in comparison to untreated control. The relationship between drug concentration and response is than plotted to calculate ED_{50} values (the threshold concentration of anthelmintic that leads to the death of 50 % of the eggs). The susceptible strains rarely hatch above concentration 0.1 μg/mL which is often referred to as discriminative dose [107]. However, neither FECRT nor EHT is sensitive enough to detect resistance below 25 % [105].

Nematodes employ various specific and non-specific mechanisms to decrease susceptibility to anthelmintic drugs. **Target site mechanisms** are associated with changes in drug target and affect the response only to specific drug class. On the other hand, **non-target site mechanism**, which includes accelerated drug metabolism and drug efflux, results in resistance against broad spectrum of different drugs [73,109]. A nucleotide alteration at a single position can cause the substitution of an amino acid in the drug target protein, which consequently modifies the drug's binding affinity to the target. For example, **benzimidazole resistant strains** change β-tubulin coding sequence at one position, codon 200, from TTC_{200} to TAC_{200}. This modification then leads to replacement of phenylalanine (presented in susceptible strains) with tyrosine in amino acid sequence of β-tubulin. β-tubulin variants with tyrosine at position 200 exhibit notably lower benzimidazole binding affinity compared to those expressing phenylalanine [73]. Another example of changing drug target structure is connected to levamisole (LEV) resistance. **LEV resistant strain** exhibits lower binding affinity to nicotinic acetylcholine receptor (nAChR), which comes as an outcome of many molecular changes.

Those changes involve the presence of truncated forms of nAChR subunit genes *unc*-63 and *Hco-acr*-8 and downregulation of many nAChR subunit genes (*co-unc*-63*a,* -63*b,* -29.2, -29.4, -26 and -*acr*-8*a)* and ancillary protein genes (*Hco-unc*-74, *unc*-50, -*ric*-3.1 and -*ric*-3.2) [104]. Similarly, the **resistance to macrocyclic lactones** is linked to mutation in extracellular domain of invertebrate specific glutamate-gated chloride channels (GluCl) and γ-aminobutyric acid (GABA) receptor [104,109].

2.2.6 Metabolism of xenobiotics

H. contortus, similarly as other organisms, possesses a wide range of complex detoxification systems to defend itself against the action of foreign compounds referred to as xenobiotics. Metabolism of xenobiotics involves three main detoxification reactions: phase I biotransformation, phase II biotransformation, and transport (Fig. 5) [110]. Phase I biotransformation reactions usually leading to slight increase in hydrophilicity by incorporating or exposure of hydrophilic functional groups (-OH, -NH2, -SH, or -COOH) [111]. In phase II, the xenobiotics, or their product from phase I are conjugated with endogenous compounds or functional groups such as saccharide, glucuronide, sulfone, acetyl, methyl, glutathione, or amino acids (e.g., glycine, taurine, glutamic acid).

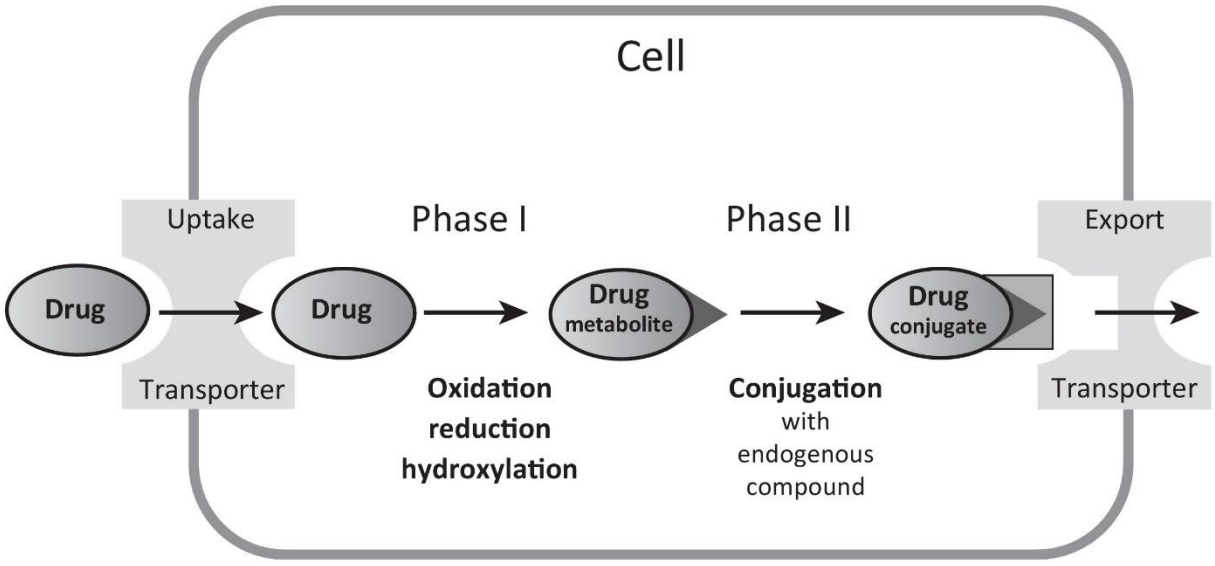

Figure 5: A schematic representation of xenobiotic metabolism. Initially, upon uptake, the drug undergoes biotransformation in the phase I, where it is oxidized, reduced, or hydroxylated. This leads to the addition or exposure of a reactive hydrophilic component. In the phase II, this reactive component is conjugated with endogenous compounds [110].

Oxidation, reduction, and hydrolysis are three main reactions involved in **phase I xenobiotic biotransformation** and products of all three reactions have been detected in *H. contortus* and other helminths [110,111]. The main enzyme catalysing monooxygenation of wide range of endogenous and exogenous substrates represents family of cytochrome P450 enzymes (CYPs). Another group of xenobiotic metabolizing enzymes (XMEs) represent family of flavine-containing monooxygenases (FMOs) which catalyse **oxidation** of substrates containing nitrogen or sulphur atom in their molecule [110,112]. For many years it has been thought that helminths are the only organisms lacking CYPs, however with advancement of molecular technique CYPs genes were identified in several helminths; nevertheless, the role of most of them remains unknown [110,112,113]. The genome of *H. contortus* encodes significant number of CYP genes with variable expression in different life stages. Higher activity of CYPs was observed in L1-L3 compared to adults, which could be due to transition to anaerobic conditions inside of the host. Despite this, the adult worm shows relatively elevated expression of a few selected CYP genes, along with NADPH-cytochrome P450 reductase, which indicate potential significance of CYP-mediated metabolism in the adult worm, too [113]. Oxidation of anthelmintics has been described in multiple helminth species, including *H. contortus*. Furthermore, higher level of oxidized metabolites in resistant strains were observed in some cases reflecting the involvement of drug metabolism as the mechanism of resistance. For example, more metabolites of MOP were found in *H. contortus* multidrug-resistant strain (White River, WR) compared to the sensitive one (inbred-susceptible-Edinburg isolate, ISE). While ISE strain of *H. contortus* formed only MOP-sulfoxide (MOPSO), the WR strain metabolized MOPSO further to MOP-sulfone ($MOPSO_2$) [110,114].

Xenobiotics containing carbonyl-, azo- or nitro- group, quinone, N- and S-oxides are the main substrate for **reductive metabolism**. The reduction of substrate can happen by two ways: enzymatically or by interaction with reducing agent, such as the reduced form of glutathione, FAD, FMN and NAD(P) [111]. Reductases/dehydrogenases, which play a significant role in biotransformation of alcohols, aldehydes, and ketones are categorized into three protein classes: medium-chain dehydrogenases (MDRs), short-chain dehydrogenases (SDRs), and aldo-keto reductases (AKRs) [112]. However, CYPs can also act as reductases, especially for azo- and nitro- compounds in the condition of low oxygen tension [111]. In helminths, SDR and AKR genes are present in relatively high quantity, compared to oxidases, which emphasizes their importance in xenobiotic metabolism. In *H. contortus* the reductase activity was observed in metabolism of mebendazole, flubendazole, naloxone, metyrapone

and 4-pyridinecarboxaldehyde (4-PCA), and similarly as in the oxidative metabolism, the level of some of the metabolites were higher in resistant strains [110,112].

The biotransformation of esters, amides, and epoxides frequently occurs through **hydrolysis** as a major metabolic pathway. This process is facilitated by the enzymatic activity of various hydrolases, which have also been detected in helminths [112]. In *H. contortus*, hydrolysis was observed as one of the biotransformation pathways of MOP, albendazole (ABZ) and flubendazole (FLU) [115,116].

Phase II biotransformation reactions, except for methylation and acetylation, lead to a significant enhancement in the hydrophilicity and their rate is much faster compared to phase I reactions. Therefore, the rate of xenobiotic elimination that undergoes biotransformation via phase I followed by phase II conjugation is primarily determined by the rate of the initial phase I reaction. The main conjugation reaction in mammals is glucuronidation [111]. The reaction is catalysed by **UDP-glycosyltransferases (UGTs)**, which catalyse the covalent attachment of hexose (glucose, glucuronide, galactose, xylose, acetylglucosamine) from activated sugar donors in the form of uridine diphosphate (UDP) sugars into substrate. Typical substrates for glycosylation reactions are alcohols, phenols, carboxylic acids, primary and secondary aromatic and aliphatic amines and free sulfhydryl groups [110,117]. Sugar conjugation is also used as deactivation strategy in *H. contortus*; however, they form glycoside instead of glucuronide as a product. Glycosyl metabolites were identified in metabolism of FLU and ABZ with their increased levels in resistant strains [116]. Another superfamily of multifunctional xenobiotic-metabolizing enzymes (XMEs) is **glutathione S-transferases (GSTs)**. Although the primary function of GSTs is the conjugation of compounds containing electrophilic centers with the tripeptide glutathione (GSH), GSTs have been associated with numerous other functions. In helminths, GSTs are involved in the detoxification of lipid hydroperoxides, and carbonyl compounds generated during oxidative stress. Therefore, they are attractive as a potential drug target or vaccine candidates. Even though **GSTs** are able to bind GSH also to xenobiotic compounds, no conjugates of anthelmintic drugs have been identified in helminths [110].

Transport is also included as important part of the metabolic process. Molecules can be transported through membranes actively or passively by four types of membrane transport proteins: ion channels, transporters, aquaporins and ATP-powered pumps [118]. Metabolism of xenobiotics is usually related to efflux by families of efflux transporters. One of the most studied and largest superfamily of efflux transporters represents **ATP-binding cassette (ABC)**

transporters. ABC transporters are generally composed of two nucleotide-binding domains (NBDs or ABCs), that are situated in cytoplasm and serve to bind and hydrolyse ATP, and two transmembrane domains (TMDs) which participate in substrate recognition and translocation across the membrane [118,119]. In terms of drug resistance, the most frequently studied ABC transporters were ABCB1 (P-glycoprotein, Pgp, or multidrug resistance protein 1, MDR1), ABCC1 (multidrug resistance associated protein, MRP1) and ABCG2 (known as breast cancer resistance protein, BCRP). In nematodes, including *H. contortus*, ABCB1 transporter is the most important due to its role in drug resistance, mainly to macrocyclic lactones [110,120].

2.3 Drug development

Drug development is long and costly process. To bring new molecular entity (NME) to the market for human treatment usually takes about 12 to 15 years and the cost varies between $161 million to $4,54 billion (depending on therapeutic areas) [121-123]. There are many steps in drug development process. In the beginning of this process is **drug discovery** which involves screening thousands of compounds to identify new potential medicines and requires collaboration of multiple scientific disciplines including biology, chemistry and pharmacology [123]. The result of the drug discovery is the emergence of one or more drug candidates with substantial biological activity and adequate safety profile [124]. Many strategies are used to screen for effective compound. One of the approaches is **phenotypic screening**. Phenotypic screening uses cultured cell lines (*in vitro*), isolated tissues/organs (*ex vivo*), or entire organisms (*in vivo*) to determine the effect of the compound. This approach is more aligned with physiological relevance as it takes place within biological systems, where multiple factors can influence pharmacological effect [125]. One of the commonly used models are cell culture-based methods, which allow high throughput screening (HTS) and provide significant amount of information throughout the screening. Due to the technological progress, some of the platforms can carry out over 100,000 assays per day [126]. Nevertheless, in the recent decades, the predominant strategy for screening represents **target-based screening**. In target-based approach compounds are screened directly on purified target proteins *in vitro*. The approach incorporates state-of-the-art molecular technologies and biological methods that enables the effective utilization of HTS platforms. The benefits of target-based approach are its simplicity, faster development timelines and the ability to uncover mechanism of action. On the other hand, simplifying the biological system can introduce a discrepancy between the observed effect on the target and the actual effect in complex biological system [125].

Considerable acceleration of the drug discovery speed represents **computer aided drug design** (CADD). CADD can be implemented at various stages of the drug development process. In the early stage of drug discovery CADD assists in the virtual screening of extensive compound libraries, effectively narrowing down the selection of potentially active compounds for synthesis and subsequent testing. Moreover, CADD can identify drug safety concerns, efficacy issues, and absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties. In the other words, CADD utilizes construction algorithms that help to design compounds with anticipated high activity, that are easy to synthesize, demonstrate favourable properties and avoid issues including poor ADMET [126].

Indispensable part of the drug development is assessment of potential toxicity risks. Toxicological screening takes part in both, early and late phase of drug development. The screening method used for identification of drug candidates with most favourable safety profiles during discovery phase is much easier and relatively fast and usually employs *in vitro* systems. *In vitro* systems display varying degrees of complexity ranging from the simplest forms like subcellular preparations or cell cultures, to more sophisticated ones such as tissue slices or isolated perfused organs. On the other hand follow up toxicological studies are more complex, expensive and time consuming and require testing on whole-animal models [127].

After the drug discovery process, once suitable candidates with desirable properties are identified, they progress to **preclinical studies**. During this phase, *in vivo* evaluations are conducted on animal models to assess toxicity, pharmacokinetics, and pharmacodynamics parameters. If a compound exhibits a promising profile and receives approval from regulatory authorities like the FDA (Food and Drug Administration) or EMA (European Medicines Agency), it can proceed to **clinical trials**. The clinical trials are structured into four sequential phases. In the initial Phase I, small number of healthy volunteers are recruited to assess pharmacodynamic and pharmacokinetic parameters. Phase II is then carried out with a limited number of patients who have the specific disease or conditions of interest. Phase III, on the other hand, is performed on a larger group of patients to confirm the safety and efficacy of the treatment and optimize the dosing range. The fourth phase is conducted after regulatory approval and involves post-marketing surveillance to collect supplementary safety information [122,123,125].

An alternative to the traditional drug development approach is **drug repurposing**, where researchers identify new therapeutic applications for drugs that have already been approved.

The main advantages of this approach are cost and time reduction [126]. The pool of candidates for repurposing includes not only existing drugs but also potential medicines that have faced challenges, discontinuation, or have reached only the clinical trial stage without achieving the desired outcomes [102]. However, from a commercial standpoint, drug repurposing may not be as attractive as other methods due to lack of legal framework specifically designed to protect the intellectual property associated with repurposed drugs [126].

Nonetheless, there are notable differences between the development processes of human drugs and **veterinary drugs**. One significant advantage in favour of veterinary drugs development is the possibility of testing directly on intended target species from the early stage of the development. This fact makes the process much faster and cheaper (the estimated cost for development of veterinary drugs is around $50-100 million) and increases the rate of success of new potential candidates [128,129]. However, human health care can play a significant role in generating novel anthelmintic drugs. During development of human drugs, a significant number of pharmacokinetic/pharmacodynamic, safety, and efficacy data are generated and revisiting these studies can be used for the approval of animal drugs [128]. In contrast to human medicine, veterinary medicine employs multiple routes for administration (such as oral, topical, and injectable methods) and this complexity is further magnified by diverse range of animal species treated. Due to the smaller market size, the main focus in animal drugs development is on identifying agents that effectively target parasites of economic significance to favour the expectation of investment return [130]. Additionally, drugs developed for farm animals need to satisfy safety requirements to protect consumers of animal products, and it is crucial to establish a defined withdrawal period [129].

2.3.1 Discovery of drugs against nematodes

One of the most significant target pathogens for anthelmintic treatment are undoubtedly trichostrongyloid nematodes of small ruminants. While various species in this family have contributed to drug discovery and pharmacological studies, *H. contortus* received the most attention and became an essential model and target organism within the field of anthelmintic discovery [131]. The suitability of *H. contortus* as a model for anthelmintic drug and drug-target discovery is underscored by its ability to maintain infection in a natural host under laboratory conditions, the *in vitro* cultivation, and maintenance of various larval stages for reliable screening assays, the abundance of genome and transcriptome data, and its close association

with other GIN as well as free-living nematode *Caenorhabditis elegans* [132]. Moreover, enormous egg production by females enables the collection of sufficient amount of material for testing [131].

In development of new anthelmintics, whole organism screening assays maintain their pivotal role over the target-based strategies as many targets have not been yet fully characterized. Over time, different whole nematode screening method have been employed, each carrying its own strengths and weaknesses. The most commonly used *in vitro* **phenotypic screening methods** involve **motility test**, **larval development test** (LDT) and **EHT** [133]. Larval motility testing can be conducted on both L3 and L4 stages of worms. Implementation of automated or semi-automated techniques enables their application on a large scale, facilitating HTS in multi-well formats [131,134,135]. LDT test is more time and labour demanding compared to motility test; however, in some cases it reveals better sensitivity. There are two variations of the LDT. While one of the variations examines the impact of the drug on the development of eggs into the L3 stage, the other focuses on the transition from exsheathed L3 (xL3) to the L4 stage. Since xL3 and L4 are parasitic stages, the outcomes obtained from xL3 to L4 test may provide a closer representation of the pharmacological sensitivity of adult worms [136,137].

Additional assays, such as the **larval migration inhibition test** (LMIT) and the **larval feeding inhibition test** (LFIT), can be utilized to evaluate the efficacy of anthelmintic treatments. The LFIT assesses the efficacy of the drug by examination of the feeding patterns of L1 or L2, utilizing fluorescently labelled *Escherichia coli* as a feeding stimulus. The differentiation between actively feeding or non-feeding larvae is then determined by analysing the presence or absence of fluorescent signals, using confocal microscopy. On the other hand, LMIT assesses the migration of L3 larvae outside of the agar gel [131,138].

Unlike larval stages, adult worm screening is associated with various constraints. While it is feasible to collect a significant quantity of worms from a single sheep, their short-lived nature in culture, typically lasting no more than 3 days, limit their use [131]. However, efficacy of antiparasitic drugs on the adult stage is essential, considering that it is the primary target for treatment and drug responses can differ between adults and larvae. An attempt was made to develop a feeding assay using radiolabelled [^{3}H]inulin, but this approach was found to be unsuitable for screening purposes. Therefore, until recent work, the only available method for adult worm assessment was the adaptation of motility tests [131,133].

In vitro evaluation of cellular viability in mammalian cell culture systems commonly involves a range of **biochemical assays**. Introducing a quantitative biochemical approach to measure worm viability and reproduction would be a valuable analytical tool [139]. One of the commonly used methods for viability screening represents **MTT assay**. This assay is based on the conversion of water soluble yellow dye MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into insoluble purple formazan by the action of mitochondrial reductase [140]. The generation of dark-blue water-insoluble formazan crystals by viable mammalian cells exhibits a direct relationship with cell quantity across a wide span [139]. One of the main strengths of this assay compared to other techniques is its reliance on a straightforward and fast procedure, which generates objective quantitative data [141]. Hence, this method has been adjusted to measure viability at different stages of parasitic or free-living nematodes [142]. Successful optimization of these assays has been achieved for the free-living nematode *C. elegans* and the L1 and xL3 stages of *H. contortus*. However, they could not be adapted for use in the L3 stage of *H. contortus* due to the presence of a protective sheath of L3 larvae [139,142,143]. Moreover, these assays can be applied to protozoans *Tetrahymena pyriformis*, and filarial nematodes such as *Onchocerca* spp., *Brugia pahangi*, *Dipetalonema viteae*, *Acanthocheilonema viteae*, and *Litomosoides carinii* [139].

3 AIMS OF THE DISSERTATION THESIS

The aim of this work was to investigate the efficacy, biotransformation, and hepatotoxicity of new potential anthelmintics. Parasitic nematode *H. contortus* and its host, sheep, were used as model organisms.

The specific goals of this dissertation thesis were as follows:

- Summary of recent findings and approaches in the field of discovery and development of new drugs against gastrointestinal nematodes
- Development of biochemical method which would enable screening of new potential anthelmintics in parasitic stages of *H. contortus*
- Testing of anthelmintic activity, potential hepatotoxicity, and metabolism of newly synthesized compounds HBK4 and BLK127
- Testing of anthelmintic activity and potential hepatotoxicity of BLK127 derivatives
- Testing of anthelmintic activity, potential hepatotoxicity, and metabolism of antipsychotic drug SRT
- Testing of anthelmintic activity and potential hepatotoxicity of methanolic extracts from 8 European ferns

4 RESULTS AND DISCUSSION

4.1 Current trends in the discovery and development of new drugs against gastrointestinal nematodes

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 Discovery Today*, 25(2), 430-437

* These authors contributed equally to this work.

The review provides an overview of the latest advancements in the development of new anthelmintics, highlighting the advantages and disadvantages of each approach. Four main approaches are discussed in the review: novel compounds, chemical structure modification, drug repurposing, and exploration of natural products.

The first chapter is focused on the identification of **NME** and application of various strategies used for screening. The discussed strategies include target-based approaches, HTS of compound libraries using different life-stages of nematodes, and genomic methodologies. Additionally, the chapter includes a table providing a summary of representative novel compounds, the species screened, and the library size. The long and costly process of this approach is mentioned as the main limitation. On the other hand, the possibility to find a compound with new mechanism of action, which would be effective against resistant isolates, is considered to be vital in drug development process.

The next chapter highlights the importance of **chemical structure modification** in discovery of new anthelmintic drugs. As an example, tenvermectin (TVM), the fermentation product of *Streptomyces avermitilis,* and its efficacy against swine nematodes is discussed. In addition, the review explores the modification of benzimidazoles, imidazoles, and the cyclooctadepsipeptide analogue of emodepside as other examples. It is also noted that while this strategy can result in development of drugs with improved properties, there is also higher risk of drug resistance emerging. **Combination of anthelmintics** is also described in this chapter as possible strategy to slow down the resistance development and minimize possible side effects.

Drug repurposing is mentioned as alternative to the drug development approach. Similarly, as in other chapters, pros and cons of this strategy, together with several examples have been discussed. The main benefits include: speed and cost of the approval process and the possibility to find new mechanism of action. In contrast, the main limitation is its effectiveness at doses that go beyond the toxicity thresholds examined during the registration process, thus repeated testing is needed. The examples included the results of the screening of various libraries, such as FDA compound library, 'Pathogen box' and 'Stasis Box' and small-molecule library of compounds used in human clinical trials. The review also mentions the case study of the organophosphorus insecticide trichlorfon, which regardless of its effectivity exhibited toxicity towards environment and human health.

Since **medicinal plants** are important for majority of human population as well as for drug discovery, their use against GIN was also included in the review. In the beginning of this section the reasons behind their importance and the role of primary and secondary plant metabolites in anthelmintic efficacy are discussed. The chapter continues by description mechanisms of action of some secondary metabolites involved in anthelmintic effect, such us tannins, terpenes, and alkaloids. Many plants with significant effect against GIN as well as other sources of natural anthelmintics, like marine sponges and higher mushrooms are given as an example. Lastly, the review acknowledges the limitations associated with the use of medicinal plant while also exploring potential solutions to overcome those challenges. Those limitations include inconsistent composition of active compounds, collection of rare species, and environmental contamination. The possible solution could be controlled plant cultivation or use of agro-industrial by-products.

4.2 Method development

Publication II: 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*. *Veterinary Research*, 52(1), 124

The current method for evaluation of anthelmintic drug efficacy predominantly relies on observation of motility or development of larvae using microscopy or other monitoring system. Even though those methods enable HTS, most of them are not possible to use on adult worms, the main target of anthelmintic drugs. Consequently, there has been an effort to explore alternative methodologies in addition to the traditional technique. Many colorimetric and fluorometric methods are widely used for viability testing in various cell lines and adaptation of some of those methods shown their potential for application in toxicity testing in some larvae of helminths [139,142,143]. However, those methods were not suitable for use in adult worms neither. Therefore, our study was aimed to establish a biochemical method that would be applicable to parasitic stages - xL3 and adult nematodes.

In our study, the level of ATP was chosen as biochemical marker to distinguish between dead and alive individuals. The most common method to measure ATP level is based on catalytic activity of luciferase, which facilitates oxidation of d-luciferin into oxyluciferin, resulting in light emission. Since ATP is the limiting component in the luciferase reaction, the intensity of the emitted light is directly proportional to the concentration of ATP. This method has been widely used in cell lines and *in vitro* models such as hepatic and intestinal slices [144]; however, there is a limited number of studies investigating its use in the field of parasitology. ATP-based viability assays have demonstrated their efficacy in HTS for evaluating viability in unicellular parasitic protozoa, including *Trypanosoma brucei*, *Entamoeba histolytica*, *Plasmodium berghei*, and in *Leishmania donovani*. In terms of multicellular organisms the assay has been successfully used in trematodes of the genus Schistosomes [145] and the free-living nematode *C. elegans* [146]. Nevertheless, to the best of our knowledge, it has not been conducted in any GIN.

Optimization of the ATP assay in nematodes consisted of series of four main steps: determine the minimum number of larvae/adults, necessary for conducting ATP assay and subsequent normalization of protein concentration measured by bicinchonic acid (BCA) assay (i); number of homogenization cycles (ii); centrifugation time and speed (iii); influence of various ratios of

water and buffer tris(hydroxymethyl)aminomethane hydrochloride/ethylenediaminetetraacetic acid (Tris/EDTA) for xL3 measurement (iv). Once the optimal conditions were evaluated, the assay was applied for assessment of the efficacy of known anthelmintic drugs LEV and MOP in xL3s and adults. In larvae, 50 xL3s were enough for detection of luminescence signal; however, subsequent experiments revealed optimal conditions with 400 xL3s in 200 µL of water. In adults, only one female or two males were needed for detection. The release of ATP from the cells was achieved with a single homogenization cycle (20 s, 8.0 m/s speed). Subsequent homogenization cycles did not lead to an increased ATP concentration. Also, no significant differences were observed when comparing centrifugation speed and time. Thus, in the further experiments the samples were centrifuged at 13,200 rpm for a duration of 10 minutes. Lastly, the comparison between water and Tris/EDTA ratios showed the strongest luminescence signal in pure water only.

The results obtained from incubation of xL3 of ISE and WR strains with LEV indicate that the method is well-suited for detecting anthelmintic activity, as well as drug resistance. However, non-proportional ATP decrease and interindividual differences make this method unsuitable for evaluation of half inhibitory concentration values (IC_{50}).

In adults, two anthelmintics with distinct mechanism of action – LEV and MOP were used for testing. While in males, MOP and LEV caused significant dose-dependent decrease of ATP levels, the effect in females was much milder and not significant due to inter-individual variability. Nevertheless, in males, this method is considered suitable for evaluation of anthelmintic efficacy of new potential drugs.

In conclusion, when compared to other HTS methods, the ATP is not ideal for screening large compound libraries, but it could be used for secondary screening of hit compounds. The main advantages are its sensitivity and possibility to use in adult worms. Additionally, ATP-based viability assays can overcome the limitation posed by other colorimetric methods, that rely on reagent absorption into the parasite's body, which is often prevented by the presence of a cuticle or external protective layer [142].

4.3 Screening of new potential anthelmintic candidates

4.3.1 Drug repurposing

Publication III: **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. *Veterinary Research*, 52(1), 143

A screening of small-molecule library of NIH Clinical Collection on *C. elegans* by Weeks et al. [147] revealed three neuromodulatory drugs with anthelmintic potential: chlorpromazine, paroxetine, and sertraline. Further investigations within the same study demonstrated their efficacy in reducing motility in adult *Trichuris muris* (whipworms), inhibiting hatching and development of *Ancylostoma caninum* (hookworms), and induced mortality of *Schistosoma mansoni* (flatworms). Moreover, these drugs also displayed efficacy against anthelmintic resistant mutants of *C. elegans*. Among the tested compounds SRT emerged as the most promising candidate, therefore, it was selected in this study.

The study was designed to test: the efficacy against eggs and adults of *H. contortus* (i), potential hepatotoxicity to the host (ii), and biotransformation in adults of *H. contortus* and in the host (iii). The effect of SRT on adults was performed on drug-sensitive (ISE) and drug-resistant (IRE, Inbred-Resistant-Edinburgh; MHco5) strains of *H. contortus* and the effect was compared to commonly used anthelmintic drugs LEV and MOP. Recently developed ATP bioluminescent assay was used for viability measurements. For biotransformation and hepatotoxicity experiments in the host (sheep) two liver models were used: isolated hepatocytes and precision-cut liver slices (PCLS).

(i) Although in the previous findings of Weeks et all (2018) SRT inhibited hatching of *A. caninum* eggs, no impact on the eggs of *H. contortus* was observed in this study. However, with the exception of benzimidazoles, most anthelmintics do not possess ovicidal properties as they mainly target parasitic stages. On the other hand, SRT significantly decreased viability of males and females of ISE strain and males of IRE strain and the effect of SRT was comparable to MOP and LEV. The average IC_{50} values for both males and females in this study are consistent with the findings reported by Weeks et al. in other nematodes and *Schistosoma mansoni*.

(ii) In terms of hepatotoxicity, SRT was concluded as non-toxic to ovine liver in predicted therapeutic concentrations.

(iii) STR was extensively metabolized in both of the ovine liver models by desmethylation and hydroxylation followed by glucuronidation (Fig. 7). On the other hand, *H. contortus* adults metabolized SRT in very limited amount. The predominant metabolites were two positional isomers of hydroxy-SRT (SRT-OH). Only traces of other metabolites were detected (Fig. 6). When compared to the production of SRT-OH between sensitive and resistant strains, no differences have been observed. These finding indicate, that *H. contortus* is not able to effectively protect itself against the effect of SRT by biotransformation. Those findings are in contrast to previous studies evaluating biotransformation of commonly used anthelmintic drugs [116,148].

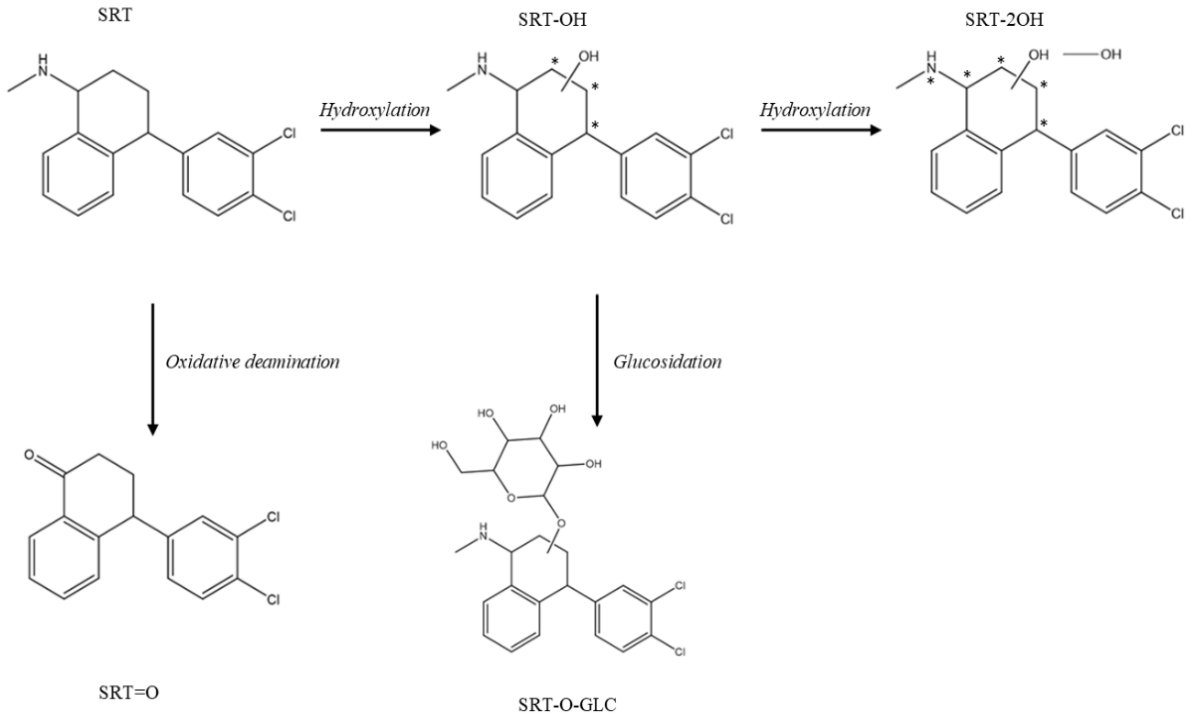

Figure 6: The proposed metabolic pathway of SRT in *H. contortus* (ISE and IRE strain) adults. The * marks possible location of the functional group. Abbreviations: SRT (sertraline), SRT=O (SRT-ketone), SRT-*O*-GLC (SRT-*O*-glucoside), SRT-OH (Hydroxy-SRT), SRT-2OH (Dihydroxy-SRT).

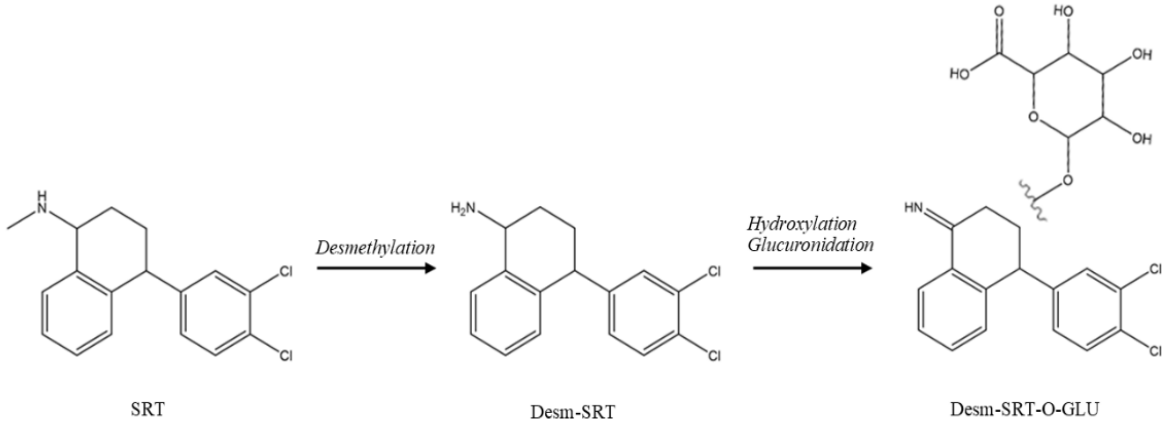

Figure 7: The proposed metabolic pathway of SRT in ovine liver (liver slices and isolated hepatocytes). Abbreviations: SRT (sertraline), Desm-SRT (desmethyl-SRT), Desm-SRT-*O*-GLU (Desmethyl-SRT-O-glucuronide.

4.3.2 New chemical entity

Publication IV: **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 is a continuation of the research carried out by Nguyen et al. [149], in which two promising compounds, **BLK127** (N-(benzyloxy)-4-(pentyloxy)benzamide) and **HBK4** (N-(4-(5-(phenylsulfonamido)-1H-benzo[d]imidazole-2-yl)phenyl)benzenesulfonamide) (Fig. 8), were identified as "hit" compounds through a phenotypic screening of the 'Kurz-box' compound library against *H. contortus* xL3 and L4 stages. The aim of this study was to investigate efficacy of those compounds against other stages of H. *contortus*, particularly adults and eggs as well as to evaluate their biotransformation and hepatotoxicity.

Even though none of the compounds demonstrated the ability to inhibit **eggs** hatching, **BLK127** significantly reduced ATP levels at a concentration of 40 µM in **females** and 20 µM in **males** of drug-sensitive ISE strain. In two resistant strains, WR and IRE, BLK127 (at a concentration of 1 µM) significantly reduced the viability of females. In males of the WR strain, a notable

decrease in viability was observed at BLK127 concentration of 10 µM. In contrast, **HBK4**, did not exhibit any activity against adults.

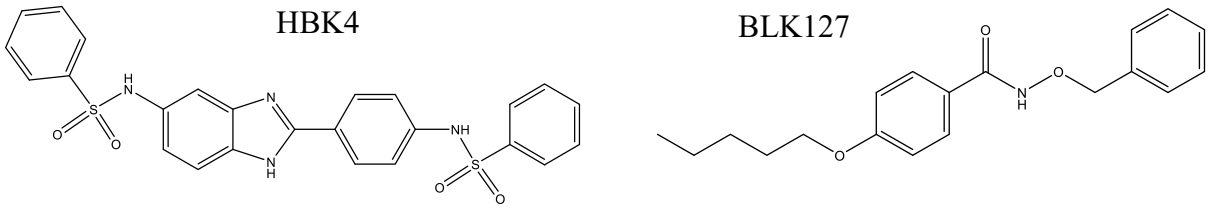

Figure 8: Structural formulas of two 'hit' compounds.

The assessment of **hepatotoxicity** for BLK127 and HBK4 was performed on PCLS and isolated hepatocytes of two animal species - sheep and rats. While BLK127 did not display any toxicity to any of those models, HBK4 exhibited significant toxicity in all of them, with concentrations ranging from 1 to 10 µM, depending on the specific models. Therefore, the subsequent experiments focused solely on BLK127, and HBK4 was excluded from further investigation.

In *H. contortus,* BLK127 is **metabolized** via hydroxylation, hydrolysis, and *N*-glycosidation, and *O*-glycosidation (Fig. 10). However, similar to SRT, most of the parent compound remains unmetabolized. Those metabolic pathways are not exceptional for *H. contortus* and they have been observed in the biotransformation of other anthelmintics, too [116].

On the other hand, in the sheep liver models, the only identified metabolite of BLK127 was glycine conjugate of the hydrolysed BLK127 intermediate (Fig. 9). Conjugation with glycine is commonly observed as metabolic pathway for carboxylic acids in sheep, cats, and gerbils.

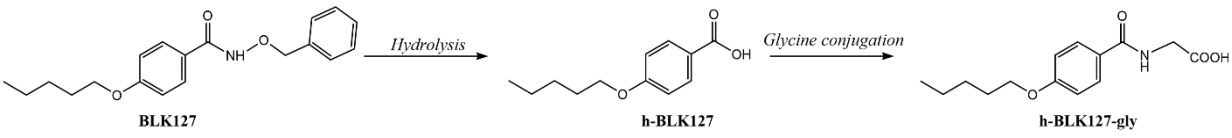

Figure 9: The proposed metabolic pathways of compound BLK127 in the ovine liver (liver slices and isolated hepatocytes). Abbreviations: h-BLK127 (hydrolysed BLK127), h-BLK127-gly (hydrolysed BLK127-glycine).

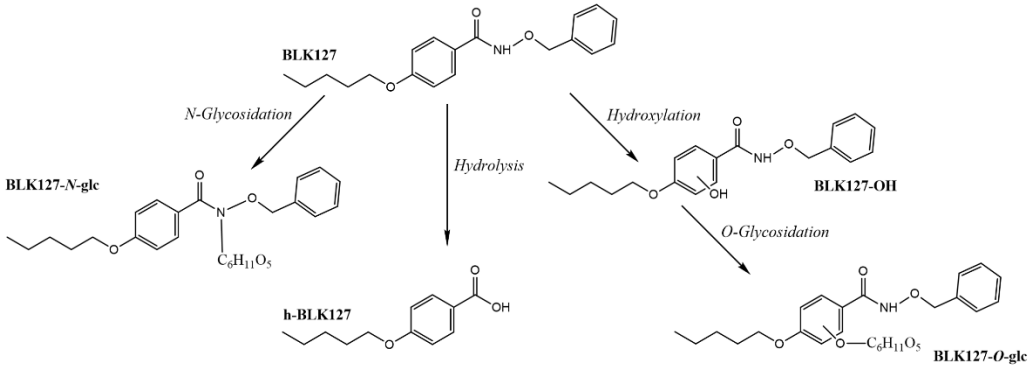

Figure 10: The proposed metabolic pathways of compound BLK127 in *H. contortus* adults. Abbreviations: BLK127-*N*-glc (BLK127-*N*-glycoside), BLK127-*O*-glc (BLK127 *O*-glycoside), BLK127-OH (Hydroxy-BLK127), h-BLK127 (hydrolysed BLK127).

In conclusion, our findings suggest, that BLK127 could be promising candidate for anthelmintic treatment. Moreover, we assessed the properties based on Lipinski's rule of five, a widely used guideline in rational drug design, and BLK127 satisfies all five rules, which suggests its potential for oral administration.

4.3.3 Chemical structure modification

Based on the findings from our previous study [150], 13 derivatives of our potential candidate BLK127 were synthesized by our collaborators at Heinrich-Heine-University, Düsseldorf and investigated for their anthelmintic effect against adults and eggs of *H. contortus*. The candidates revealed in this study are currently under investigation, therefore these results have not been published yet. However, they were included in diploma thesis of Mgr. Magdalena Mičundová.

From all 13 compounds performing EHT, one compound designated as BLK8 significantly decreased egg hatching from 30 µM concentration in sensitive (ISE) and two resistant strains (WR and ISE) (Fig. 11). Experiments performed on ISE strain of *H. contortus* adults support the findings from EHT for the compound BLK8 as potential candidate. Moreover, those tests led to the discovery of other compounds: BLK7, and BLK12. These candidates were subjected to further investigation to evaluate their potential hepatotoxicity (Fig. 12) and were also tested against adults of resistant strains (Fig. 13). The effects of these compounds were also compared with those of LEV.

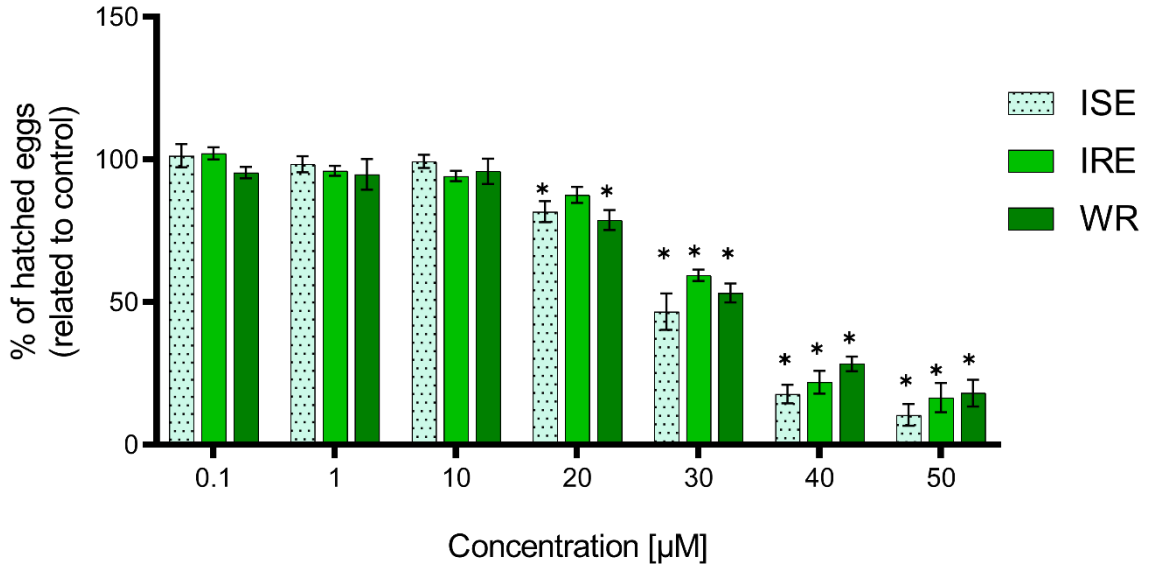

Figure 11: Effect of compound BLK8 on eggs hatching of ISE, IRE and WR strains of *H. contortus*. Data are presented as means ± SEM. The data were obtained in 4 independent experiments with three technical replicates in each experiment (n = 4). For statistical analysis, two-way ANOVA with Dunnett's multiple comparison test was used. * $p < 0.05$ in comparison to control.

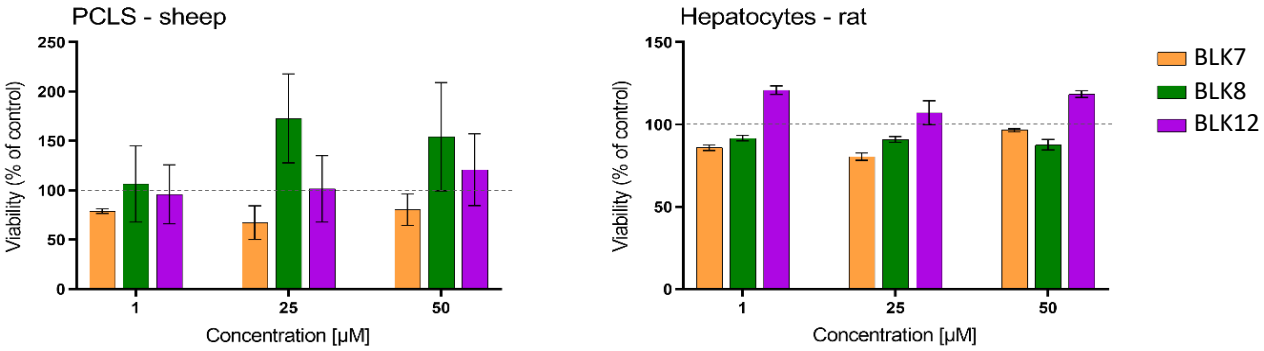

Figure 12: Hepatotoxicity test on sheep PCLS and rat hepatocytes. Data are presented as means ± SEM. The results for sheep PCLS were performed in three independent experiments with 4 technical replicates in each of the experiment (n = 4). The data for rat hepatocytes were obtained from one experiment with 4 technical replicates (n = 1).

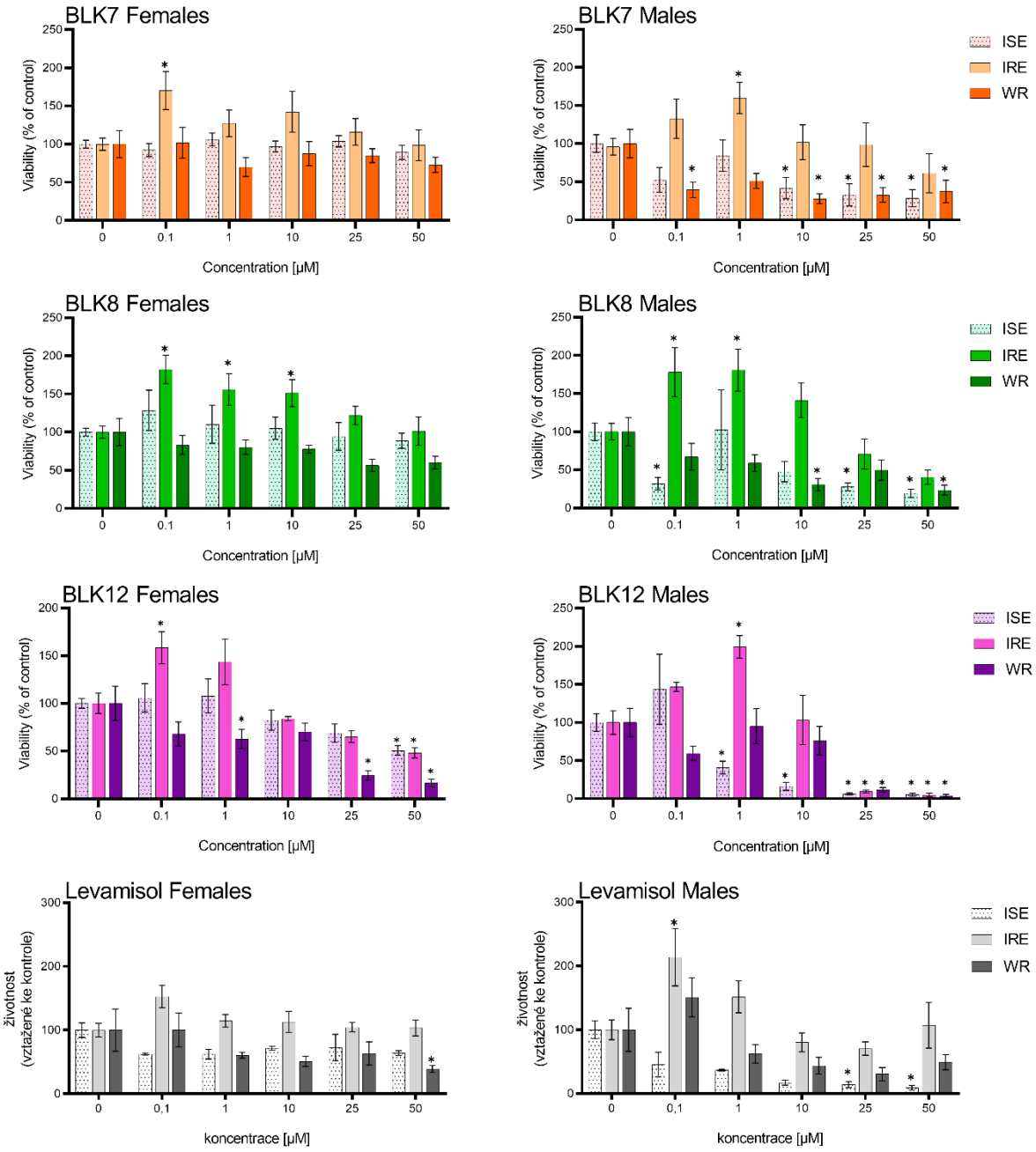

Figure 13: Comparison of three selected compounds and levamisole on viability of *H. contortus* adults of ISE, IRE and WR strains. Data are presented as means ± SEM. The results were performed in two independent experiments with four replicates in each of the experiment (n = 8). For statistical analysis, two-way ANOVA with Dunnett's multiple comparison test was used. * $p < 0.05$ in comparison to control

4.3.4 Medicinal plants

Publication V: Pavičič, A., **Zajíčková, M**., Šadibolová, M., Svobodová, G., Matoušková, P., Szotáková, B., Langhansová, L., Maršík, P., & Skálová, L., (2023). Anthelmintic activity of European fern extracts against *Haemonchus contortus*. *Veterinary research*

In general, ferns have been showed as a rich source of nutrients and plant secondary metabolites. Ferns have also been traditionally used for medicinal purposes, particularly in the treatment of ailments such as ascarid infection. Furthermore, ferns have been recognized for their antioxidant properties, and as antimicrobial, antiviral, anti-inflammatory agents as well as for their potential against cancer and HIV-infection [151].

Moreover, the anthelmintic effects of specific fern species against certain nematodes and trematodes have been reported. For instance, the study of Kalpana et al. [152] demonstrated significant activity of extracts *Dicranopteris linearis* and *Microlepia speluncae*, and moderate activity of *Blechnum orientale* against trematode *Gastrothylax crumenifer* during 60 min incubation at concentrations ranging from 1 to 5 mg/mL. The effect of *B. orientale* was also investigated in another study performed by Devi et al. [151], where the extract fraction at concentration 5 mg/mL conducted 100 % of mortality of *G. crumenifer* after 15 min of incubation. The moderate activity against L3 of *T. colubriformis* was also observed with *D. filix-mas* at concentration of 2 mg/mL by Urban et al. [153]. Moreover, Socolsky et al. [154] reported that the acylphloroglucinol isolated from *Dryopteris wallichiana* displayed moderate activity in experiments conducted on L4 of rat nematode *Nippostrongylus brasiliensis.* However, the diversity of ferns in Europe is relatively poor (only 1,5 % of fern diversity worldwide) and therefore European ferns are often overlooked [155].

Based on those findings, the main objective of this study was to investigate the anthelminthic properties of eight European ferns selected from the *Dryopteris*, *Athyrium*, and *Blechnum* genera. The methanolic extract of those ferns was assessed on eggs and adults of ISE and WR strains of *H. contortus*.

No effect on eggs hatching for any of the fern extracts was observed in concentrations up to 100 µg/mL. Only one species, *Dryopteris aemula* exhibited 25 % decrease at concentration of 200 µg/mL in ISE strain. However, similar inhibition was not observed in the eggs of WR strain.

The experiments on adults of ISE strain revealed three promising candidates: *Athyrium distentifolium*, *Dryopteris aemula*, and *Dryopteris cambrensis*, which significantly lowered ATP level content. Moreover, *A. distentifolium* and *D. cambrensis* also exhibited activity against WR strain. In those three promising candidates, similarly as in our previous studies, the toxicity to the ovine liver was tested, but none of them display any negative impact.

As secondary metabolites represent the main active components, the polyphenols content of the three species that exhibit efficacy was determined. The main components found in all three extracts were quercetin, 3-hydroxybenzoic acid, kaempferol, and protocatechuic acid. Two extracts also contained luteolin and taxifolin. Caffeic acid and p-coumaric acid were specific to the *D. aemula* extract, and vitexin was exclusively detected in the *A. distentifolium* extract. When multiple polyphenols were tested on *H. contortus* larvae, taxifolin showed no efficacy, whereas quercetin and luteolin were highly effective [156].

While anthelmintic effect is a result of synergic action of many polyphenols, the application of whole plant would be therefore more beneficial. Based on our findings and consideration of other factors such as volume of the ovine plasma and polyphenols bioavailability, it is estimated that one sheep should consume at least 100 g of fresh leaves to achieve anthelmintic effect.

5 CONCLUSIONS

The promising candidates for anthelmintic treatment discovered in this work are essential to meet the global requirement for effective solutions to combat gastrointestinal nematodes. Furthermore, the development of a biochemical method tailored to the parasitic stages of *H. contortus* represents a significant breakthrough, enabling more comprehensive exploration in future studies.

- The review summarizes the recent findings in three main areas of drug development: new chemical entity, drug repurposing, and natural product. In each chapter the strengths and weaknesses are discussed, providing relevant examples of recent discoveries.

- To assess toxicity towards advanced stages of *H. contortus*, we adapted a method commonly used in cell cultures and tissue models. The method is based on measurement of ATP level as viability marker by bioluminescence assay. Even though the method is not suitable for HTS, the revealed outcome could better reflect *in vivo* conditions compared to other HTS methods. Therefore, the main benefit would be in combination with other methods to narrow the range of potential candidates for consequent *in vivo* tests.

- Among the assessed candidates, BLK127 exhibited remarkable anthelmintic activity against both susceptible and resistant strains of adult *H. contortus.* Subsequent metabolism studies revealed that *H. contortus* has a limited ability to metabolize BLK127, compared to its host. On the other hand, HBK4 did not demonstrate significant anthelmintic activity and showed notable toxicity, therefore was not suggested as potential anthelmintic candidate. Furthermore, neither compound demonstrated an impact on eggs.

- From 13 BLK127 derivatives, three of them: BLK7, BLK8 and BLK12 showed to be promising candidates of new anthelmintic drugs as they effectively targeted sensitive and resistant strains of adults of *H. contortus.* Moreover, compound BLK8 was also effective against eggs. Those results are promising and suggest further testing.

- In both susceptible and resistant isolates, SRT demonstrated remarkable activity against adult *H. contortus*, with no notable differences in metabolism between the isolates. Nevertheless, no effects were observed on eggs. Moreover, *H. contortus* exhibited limited ability to metabolize SRT compared to its host. Importantly, SRT exhibited no hepatotoxicity at anthelmintically active concentrations. Considering these results, SRT emerges as a promising candidate for potential anthelmintic treatment.

- The study of European ferns revealed two species: *A. distentifolium* and *D. cambrensis,* which decreased ATP level in adults of susceptible and resistant strain. However, no effect was observed on eggs. European ferns did not display toxic effect to any of the liver models. Anthelmintic potential of *A. distentifolium* and *D. cambrensis* deserves further study.

6 LIST OF RELATED PUBLICATIONS

6.1 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 Discovery Today*, 25(2), 430-437 **(IF = 7.4*, Q1)**

* These authors contributed equally to this work.

Candidate's contribution

- responsible for the chapter "Natural products and compounds with anthelmintic efficacy "
- revising the manuscript

6.2 Publication II

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. Veterinary Research*, 52(1), 124 **(IF = 4.4*, Q1 first decile)**

Candidate's contribution

- experimental design and performance
- data collection and analysis
- revising the manuscript

6.3 Publication III

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. *Veterinary Research*, 52, 143 **(IF = 4.4*, Q1 first decile)**

Candidate's contribution

- experimental design and performance
- data collection and analysis
- data visualization
- writing of the manuscript

6.4 Publication IV

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(4), 754 **(IF = 5.4*, Q1**)

Candidate's contribution

- experimental design and performance
- data collection and analysis
- data visualization
- writing of the manuscript

6.5 Publication V

Pavičić, A., **Zajíčková, M**., Šadibolová, M., Svobodová, G., Matoušková, P., Szotáková, B., Langhansová, L., Maršík, P., & Skálová, L., (2023). Anthelmintic activity of European fern extracts against *Haemonchus contortus*. *Veterinary research,* 54(1), 59*,* **(IF = 4.4*, Q1 first decile)**

Candidate's contribution

- design and performance of the experiments related to adult viability
- data collection and analysis
- data visualization
- responsible for the section "materials and methods"
- revising the manuscript

***** Journal Impact Factor 2022

7 OTHER OUTCOMES

7.1 Participation in scientific conferences

25th Helminthological Days, Rejčkov, Czechia (2019); Current trends in the discovery and development of new drugs against gastrointestinal nematode; Zajíčková, M., Nguyen, L.T., Skálová, L., Raisová Stuchlíková, L., Matoušková, P. Oral presentation

Anthelmintics: Discovery to Resistance IV meeting, Santa Monica Bay, California, USA (2020); Metabolism of new potential anthelmintics in *Haemonchus contortus*; Zajíčková, M., Nguyen, L.T., Raisová Stuchlíková, L., Navrátilová, M., Kellerová, P., Skálová, L. Poster

22nd School of Mass Spectrometry, Srní, Czechia (2021); Use of UHPLC-MS in identification of sertraline metabolites in *Haemonchus contortus* and in ovine liver; Zajíčková, M., Prchal, L., Vokřál, I., Vodvárková, N., Navrátilová, M, Skálová, L. Poster

26th Helminthological Day, Deštné v Orlických horách, Czechia (2021); Toxicity, efficacy, and biotransformation of sertraline as a new potential anthelmintic against *Haemonchus contortus*; Zajíčková, M., Prchal, L., Vokřál, I., Vodvárková, N., Navrátilová, M., Nguyen, L.T., Skálová, L. Oral presentation

10th Postgraduate and 8th Postdoc Conference, Hradec Králové, Czechia (2020); Metabolic pathways of new potential anthelmintics in *Haemonchus contortus* and its host; Zajíčková M., Navrátilová M., Nguyen L.T., Prchal L., Stuchlíková R.L., Kellerová P., Skálová L. Oral presentation

11th Postgraduate and 9th Postdoc Conference, (2021); Sertraline as new potential anthelmintics against *Haemonchus contortus*?; Zajíčková M., Navrátilová M., Prchal L., Nguyen L.T., Stuchlíková R.L., Skálová L. Oral presentation

12th Postgraduate and 10th Postdoc Conference, Hradec Králové, Czechia (2022); New potential anthelmintics against *Haemonchus contortus* - BLK127 and HBK4: toxicity, efficacy, and biotransformation; Zajíčková, M., Prchal, L., Vokřál, I., Nguyen, L.T., Kurz, T., Gasser, R.B., Bednářová, K., Mičundová, M., Skálová, L. Oral presentation

13th Postgraduate and 11th Postdoc Conference, Hradec Králové, Czechia (2023); the potential of ferns extracts against *Haemonchus contortus*; Zajíčková, M., Pavičić, A., Navrátilová, M., Šadibolová, M., Svobodová, G., Skálová, L., Matoušková, P. Oral presentation

7.2 Publications not related to the topic

Kellerová P., Matoušková P., Lamka J., Vokřál I., Szotáková B., **Zajíčková M.**, Pasák M., Skálová L. (2019) Ivermectin-induced changes in the expression of cytochromes P450 and efflux transporters in *Haemonchus contortus* female and male adults. *Veterinary Parasitology*, 273, 24-31. (IF = 2.6*, Q2/Q1)

Langhansová, L., Pumprová, K., Haisel, D., Ekrt, L., Pavičić, A., **Zajíčková, M**., Vaněk, T, Dvořáková, M., (2021). European ferns as rich sources of antioxidants in the human diet. *Food Chemistry*, 356. (IF = 8.8*, Q1)

7.3 Grant projects

2018 – 2021 GAUK 1568519 - Metabolism of new potential anthelmintics in *Haemonchus contortus* and in its host, principal researcher

2021 – 2023 START/SCI/052 - Anthelmintics in the environment – detection, consequences, a possible solution, team member

7.4 Internships and courses

4 – 18 October 2019 Course "BIOANALYSIS-Forefront Technologies and Applications", University of Oslo, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Oslo, Norway (funded by SVV 260 416)

23 – 27 August 2021 Course "Samer school of statistical method", Faculty of Mechanical Engineering, Brno, Czechia (funded by Grant Schemes at CU" (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935))

February 2022 – March 2022 One-month internship at research group of Dr. Craig Wheelock, Karolinska Institutet, Department of Medical Biochemistry and Biophysics, Sweden (funded by Grant Schemes at CU" (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935))

March 2022 – September 2022 Six-month internship at research group of Dr. Craig Wheelock, Karolinska Institutet, Department of Medical Biochemistry and Biophysics, Sweden. (funded by Fond mobility (FM/a/2021-2-046))

7.5 Teaching experience

Classes

2018-2022 - Czech practical courses of General Biochemistry

2022-2023 - English practical courses of General Biochemistry

Consultantion of undergraduate students´ diploma thesis:

Nikola Vodvárková (2021), The study of possible use of sertraline against gastrointestinal nematodes, Diploma thesis.

Magdalena Mičundová (2022), The use of new *ex vivo* tests to find synthetic compounds with anthelmintic effect, Diploma thesis.

Klára Bednářová (2022), The use of new *ex vivo* tests to find ferns with anthelmintic effect, Diploma thesis.

8 ABBREVIATIONS

ABC	ATP-binding cassette
ABZ	Albendazol
AcCoA	Acetyl-coenzyme A
ADCC	Antibody dependent cell cytotoxicity
ADMET	Absorption, distribution, metabolism, excretion, toxicity
AKR	Aldo-keto reductase
ATP	Adenosine triphosphate
B cells	B lymphocyte
BCA	Bicinchoninic acid
BLK127-*N*-glc	BLK127 *N*-glycoside
BLK127-*O*-glc	BLK127 *O*-glycoside
BLK127-OH	Hydroxy-BLK127
CADD	Computer aided drug design
$CD25^+$	Cluster of differentiation 25
CD4	Cluster of differentiation 4
CITR	Citrate
CYP	Cytochrome P450 enzyme
Desm-SRT	Desmethyl-sertraline
Desm-SRT-*O*-GLU	Desmethyl-sertraline -*O*-glucuronide
DNA	Deoxyribonucleic acid
ED_{50}	Median effective dose
EDTA	Ethylenediaminetetraacetic acid
EHT	Egg hatch test
EMA	European Medicines Agency
epg	Eggs per gram
FAD	Flavin adenine dinucleotide
FAMACHA	FAffa MAlan CHArt
FDA	Food and Drug Administration
FECRT	Faecal egg count reduction test
FLU	Flubendazole
FMN	Flavinmononucleotide
FMO	Flavine containing monooxygenase
FRD	Fumarate reductase
FUM	Fumarate
FWEC	Faecal worm egg count
GABA	Gamma-aminobutyric acid

GAUK	Charles University Grant Agency
GIN	Gastrointestinal nematode
GL	Globule leucocyte
GluCl	Glutamate gated chloride channel
GSH	Glutathione
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
h-BLK127	hydrolysed BLK127
h-BLK127-gly	Hydrolysed BLK127-glycine
HTS	Hight throughput screening
IC_{50}	Half inhibitory concentration value
IF	Impact factor
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IRE	Inbred Resistant Edinburgh strain of *H. Contortus,* MHco3
ISE	Inbred Susceptible Edinburgh strain of *H. Contortus,* MHco5
IVM	Ivermectine
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
L4	Four-stage larvae
L5	Fifth-stage larvae
LDT	Larval development test
LEV	Levamisol
LFIT	Larval feeding inhibition test
LMIT	Larval migration inhibition test
MAL	Malate
MDR	Multidrug resistance protein
ME	Malic enzyme
Methylmal-CoA	Methylmalonyl-coenzyme A
ML	Macrocyclic lactone
MOP	Monepantel
MOPSO	Monepantel sulfoxide
$MOPSO_2$	Monepantel sulfone
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NBD	Nucleotide-binding domain

nAChR	Nicotinic acetylcholine receptor
NME	New molecular entity
2 OG	2-oxoglutarate
OXAC	Oxaloacetate
4-PCA	4-pyridinecarboxaldehyde
PCR	Polymerase chain reaction
PCLS	Precision-cut liver slices
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
Pgp	P-glycoprotein
PK	Pyruvate kinase
PROP	Propionate
Prop-CoA	Propionyl-CoA
PYR	Pyruvate
Q	Quartile
SDH	Succinate dehydrogenase
SEM	Standard error of the mean
SDR	Short-chain dehydrogenase
SRT	Sertraline
SRT=O	Sertraline ketone
SRT-OH	Hydroxy sertraline
SRT-*O*-GLC	Sertraline-*O*-glucoside
SRT-2OH	Dihydroxy sertraline
SUCC	Succinate
Succ-CoA	Succinyl-CoA
TAC	Thymine-Adenine- Cytosine
T cells	T lymphocyte
TCA	Tricarboxylic acid cycle
TMD	Transmembrane domain
Tris	Tris(hydroxymethyl)aminomethane
TTC	Thymine-Thymin- Cytosine
TVM	Tenvermectin
UDP	Uridine diphosphate
UGT	Uridine diphosphate glycosyltransferase
WR	White River strain of *H. Contortus*, MHco4
XME	Xenobiotic metabolizing enzyme
xL3	Exsheated third-stage larvae
γδ T cells	Gamma delta T cells

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10 SUPPLEMENTS

10.1 Supplement I

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 Discovery Today*, 25(2), 430-437.

10.2 Supplement II

Publication II: 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. Veterinary Research*, 52(1), 124.

10.3 Supplement III

Publication III: **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. *Veterinary Research*, 52, 143.

10.4 Supplement IV

Publication IV**: 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(4), 754.

10.5 Supplement V

Publication V: Pavičić, A., **Zajíčková, M**., Šadibolová, M., Svobodová, G., Matoušková, P., Szotáková, B., Langhansová, L., Maršík, P., & Skálová, L., (2023). Anthelmintic activity of European fern extracts against *Haemonchus contortus*. *Veterinary research,* 54(1), 59.