Literature survey of chemotherapeutic agents for re-purposing to treat amoebic gill disease caused by *Neoparamoeba perurans*

Cyril Pierre Frédéric Henard

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Institute of Aquaculture

Immunology & Vaccinology Group

University of Stirling

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Declaration

I Cyril Henard, hereby certify that this thesis has been written by me, that it is the record of the work carried out by me and that it has not been submitted in any previous application for a higher degree.

Cyril Henard

Date

« *Primum non nocere*, *deinde curare* »

Hippocrate, 410 B.C.

Acknowledgements

I would like to begging my acknowledgements by a wish. I wish that, through this work, my family will at last recognise my value despite the fact that I was unable in the past to achieved a scientific baccalaureate as they have desired it for me.

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Abstract

Amoebic gill disease (AGD) is an important disease affecting marine fish including Atlantic salmon. The aetiological agent of AGD is *Neoparamoeba perurans*, an amoeba notably characterised by the presence of an intracellular kinetoplastid endosymbiont which belongs to the genus *Perkinsela*. This disease can cause high mortality and economic losses to salmon farming have been estimated at US$12.55 million in Norway in 2006 and US$81 million in Scotland in 2013. Current treatments include freshwater bathing and hydrogen peroxide but these methods are relatively impractical, effective against only mild cases, can stress the fish, and may represent 10-20% of present production costs. As a result, there exists an opportunity to develop a new chemotherapeutic intervention to treat AGD because such an approach could be beneficial with regard to cost-effectiveness, high treatment efficacy, improvement in fish welfare, and offering a long-lasting protective effect. This present study aimed to identify and prioritise existing drugs with efficacy against pathogens related to AGD that could be evaluated further as potential new chemotherapeutants for treating AGD. To this end, literature searching was performed to identify diseases of humans, animals and plants caused by amoebic and kinetoplastids parasites and the drugs used for treatment. In total, seven major relevant diseases were found to be caused by amoebae and kinetoplastid parasites, including amoebiasis and trypanosomiasis. A list of 118 drugs related to these diseases was established. From these 118 drugs, 222 drug targets were listed. Initially each drug was scored according to the amount of information available publicly that would be useful for seeking regulatory authorisation for use against AGD. Then, prioritisation was performed based on evidence for the presence of the target of the drug in AGD by use of iPath and BLAST software to investigate metabolic pathways and target proteins, respectively. This new bibliographic-based approach has highlighted numerous potential chemical candidates to assist in the development of new cost-effective and practical chemotherapeutic solutions for the Atlantic salmon farming industry to mitigate against AGD.

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Chapter 1: Introduction

1.1. Importance of aquaculture

Aquaculture is a fast-growing industry with mean annual growth of 5.8% between 2001 and 2016 (Food and Agricultural Organisation [FAO]., 2018). Aquaculture production provided only 7% of the fish for human consumption in 1974 but this increased to 26% in 1994, 39% in 2004 and was more than 50% in 2014 (FAO., 2016). In 2016, the production of aquatic animals reached 80 million tonnes with an estimated value of US$ 231.6 billion (FAO., 2018). Among this production, finfish represent 54.1 million tonnes with an estimated value of US$ 138.5 billion (FAO., 2018). Two-thirds of aquaculture production is inland and dominated by the production of cyprinids, whereas marine aquaculture is represented mainly by salmonids, and worldwide demand for Atlantic salmon (*Salmo salar*) is growing steadily (FAO., 2016). The production of salmon increased greatly from 347,931 tonnes in 1994 to 2,326,288 tonnes in 2014 (Figure 1.1). Nevertheless, for the first time in recent years, production has decreased significantly; indeed, 2016 saw a decline of 5.6% in output compared to 2015 (Figure 1.1). The major producer countries are Norway, Chile, the United Kingdom (almost exclusively Scotland) and Ireland. Between 1996 and 2016, Scottish Atlantic salmon production almost doubled from 83,121 tonnes to 162,817 tonnes and reached 189,707 tonnes in 2017 (Figure 1.2). Even if the salmon industry seems to show good health, the 2016 figures show that production can be hindered by parasitic pathogens such as *Neoparamoeba perurans*, the aetiological agent of amoebic gill disease (AGD), and sea lice (*Lepeophtheirus salmonis*), as well as a number of viral and bacterial diseases. The incidence of parasitic diseases has increased with the development of cage culture (Nowak., 2007). The virulence of a parasite could be amplified in a context of low genetic diversity of the host, which leads the parasite to specialise to a specific genotype found in a homogeneous population like the strains of Atlantic salmon cultured in fish farms nowadays (Nowak., 2007).

1.2. Amoebic Gill Disease

1.2.1. Emergence and discovery of the pathogen

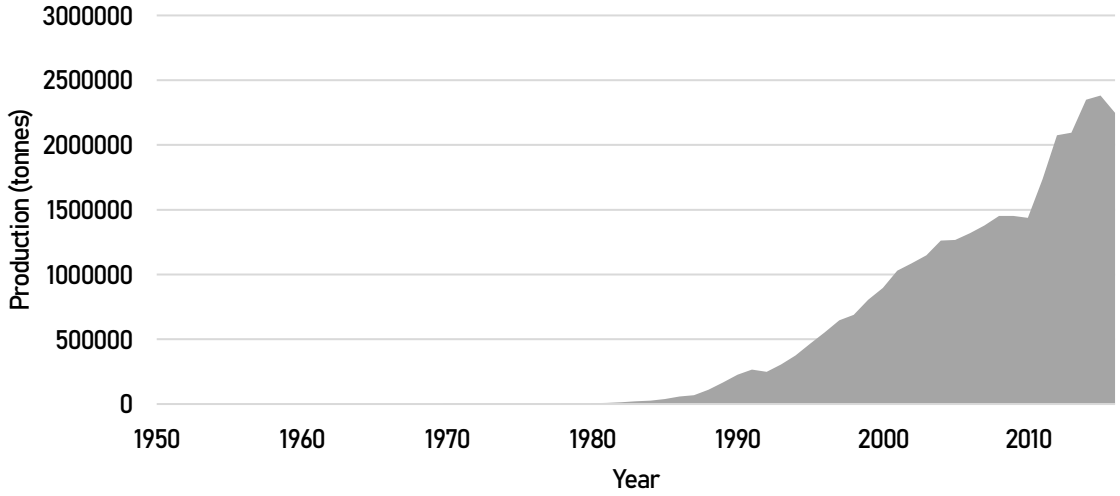

Figure 1.1. World annual salmon production (tonnes) between 1950-2016 (FAO, FishstatJ).

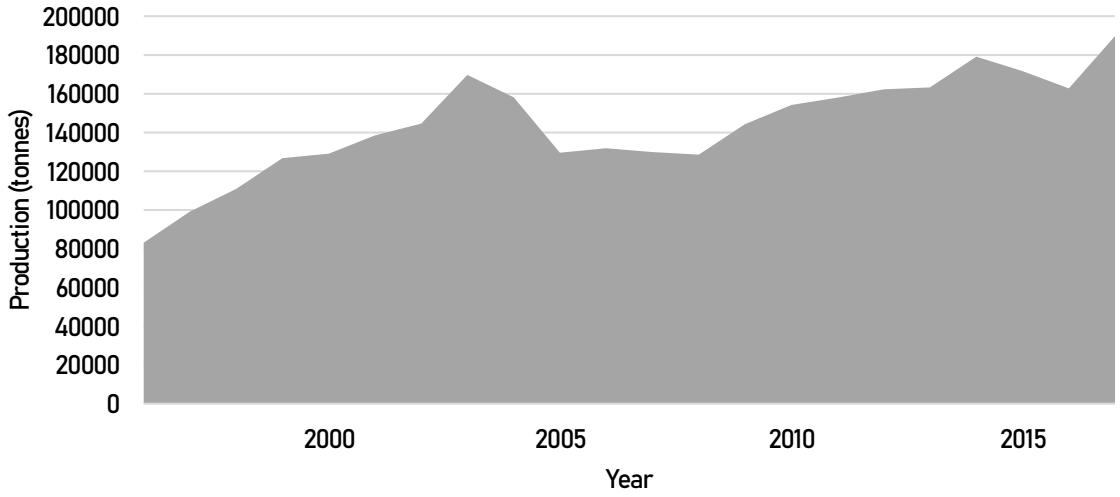

Figure 1.2. Scottish annual salmon production (tonnes) between 1996-2017 according to the Scottish fish farm production survey report 2017.

AGD is a relatively well-studied gill disease in salmon farming (Buchmann., 2015). Other gill diseases such as nodular gill disease, likely caused by an amoeba also, have received far less attention compared to AGD (Nowak et al., 2014). The first record of an amoebic gill disease in salmon was reportedly caused by *Neoparamoeba pemaquidensis* in Coho salmon (*Oncorhynchus kisutch*) reared in sea cages in Washington, USA and in tank facilities in California, USA (Kent et al., 1988). Mortalities in sea cages were about 25% in 1985 and the disease was still present in 1986 and 1987 (Kent et al., 1988). At the same time, *N. pemaquidensis* was reported to attach to Atlantic salmon gills in Tasmania and was associated with hyperplasia of the gills (Roubal et al., 1989). In 1995, AGD was confirmed in Ireland in a fresh gill smear after histological examination (Rodger & McArdle., 1996). As a result of the Kent et al. (1988) report, *N. pemaquidensis* was identified initially as the aetiological agent of AGD in Atlantic salmon, Coho salmon and Chinook salmon (*Oncorhynchus tshawytscha*) from Australia (Tasmania), Ireland, USA and the United Kingdom (Wong et al., 2004). However, some researchers questioned *N. pemaquidensis* to be the primary agent of AGD in Irish salmonid culture due to the low density of cells in association with gill pathology (Bermingham & Mulcahy, 2004). Indeed, experimental infection with *N. pemaquidensis* failed to elicit AGD and, though this amoeba was detected in the mucus, no clinical signs were present (Morrison et al., 2005; Villavedra et al., 2007). Finally, molecular-based approaches led to the identification of a different species with possible association with AGD, *N. perurans* (Young et al., 2007). Some years later *N. perurans* was confirmed finally as the aetiological agent of AGD by fulfilment of Koch's postulates (Crosbie et al., 2012).

1.2.2. Economic impact

Many fish species cultured in aquaculture can be affected by AGD such as coho salmon, chinook salmon, Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*), ayu (*Plecoglossus altivelis*), seabass (*Dicentrarchus labrax*), turbot (*Scolphtalmus maximus*), sharpsnout seabream (*Diplodus puntazzo*) and lumpfish (*Cyclopterus lumpus*) (Fiala & Dyková., 2003; Hauglandet al., 2017; Buchmann., 2015; Munday et al., 2001). Parasitic diseases, such as AGD, increase production costs through direct mortalities of fish and

by adding costs associated with treatment and handling (Nowak ., 2007). AGD is estimated to represent 10 to 20% of the production costs of Atlantic salmon in Tasmania (Munday et al., 2001), and it can cause mortality of up to 50% in cage culture (Parsons et al ., 2001). In 1982, a disease similar to AGD affected a sea-reared rainbow trout fish farm and cumulative losses were up to 50% of the stocks. In Norway, in 2006 82% of mortalities were reportedly due to AGD in an Atlantic salmon fish farm and this was considered to be a record high (Steinum et al. 2008). The same year, losses to the Norwegian salmon sector due to AGD were estimated to be US$ 12.55 million while in 2013 the impact of the disease to Scottish salmon production was estimated to be US$ 81 million, representing approximately 8% of total production (Shinn et al., 2015).

1.2.3. Symptoms, clinical signs and disease progression

AGD is characterized by three different stages (Adams & Nowak., 2003). The first stage involves attachment of the trophozoite, followed by proliferation of the amoeba leading to oedema of the host tissue and localised epithelial desquamation (Adams & Nowak., 2004). The second stage is the activation of the host innate immune system (notably migration of immunoregulatory cells) and initial focal hyperplasia of undifferentiated epithelial cells (Adams & Nowak., 2003). The third and final stage is characterized by squamation and stratification of epithelia at the surface of lesions, lesion expansion and variable recruitment of mucous cells to these regions (Adams & Nowak., 2003). Amoeba densities on the gill correlate positively with the phase of the disease and this amoebic density on the gill show a decrease with advancing gill pathology (Bermingham & Mulcahy., 2004). Amoeba preferentially colonize the lesion margins during the third stage of the disease (Adams & Nowak., 2003). In the dorsal region of the second-gill arch, the number of lesions and pathological severity is greater than observed in the ventral region (Adams & Nowak., 2001). Nevertheless, there is no difference in terms of severity between proximity of the lesion to the gill arch and gill area in terms of lesion size (Adams & Nowak., 2001). The disease is characterized by a decrease of feeding, respiratory distress and lethargy (Rodger & McArdle., 1996).

1.2.4. Detection

Three main techniques are used to detect AGD with greater or lesser accuracy: gross assessment, histology, and real-time PCR. The most widely used gross assessment tool to evaluate the severity of the disease is the Gill Score (GS) (Table 1.1) (Taylor et al., 2009). GS is a good indicator of mortality rate if the disease is not treated (Taylor et al., 2009). Gross assessment shows moderate to good agreement with histological assessment (Adams et al., 2004). Gross assessment can be used as a farm-monitoring tool for the detection of AGD, but the stage of the disease or the interaction with other pathogens present at any one time may distort the diagnosis. Additionally, minor variations in interpretations by different users may limit its application in comparative studies. High specificity primers have been designed to detect specifically the 18S rRNA gene of *N. perurans* for rapid detection and quantification purposes, and this permits formal identification of the pathogen (Bridle et al., 2010).

1.2.5. Distribution, epidemiology and risk factors

N. perurans is a cosmopolitan parasite that has been detected in Australia (Tasmania), Ireland, USA, UK, Spain, France, Chile, North America and South Africa (Rodger., 2014; Mouton et al., 2014; Munday et al., 2001; Young et al., 2008). While *N. perurans* is known to impact cultured fish species, other marine species could be colonised and act as vectors to transmit the amoeba (Nowak., 2007). Several biotic and abiotic risk factors could influence the appearance of AGD outbreak in Atlantic salmon fish farm (Table 1.2). It has been suggested that sea lice (*Lepeophtherius salmonis*) may have a role in the transmission of *N. perurans* in Atlantic salmon (Bustos et al. 2011), and ballan wrasse (*Labrus bergylta*) may represent a risk to infection at Atlantic salmon farms as these fish are co-cultured with the salmon to reduce sea lice infestations (Karlsbakk et al. 2013). Lumpfish are more resistant to AGD than Atlantic salmon and can act as a passive carrier (Haugland et al., 2017; Hellebø et al., 2017). Some wild fish such as saithe (*Pollachius virens*) migrate between salmon farms and could also spread AGD outbreaks (Hellebø, Stene, and Aspehaug 2017). Moreover, biofouling communities may

Table 1.1. Gill score system rating (Taylor et al., 2009).

Infection Level	Gill score	Gross description
Clear	0	No sign of infection and healthy red colour
Very light	1	1 white spot, light scarring or undefined necrotic streaking
light	2	2/3 spots / small mucus patch
Moderate	3	Established thickened mucus patch or spot
Advanced	4	Established lesions covering up to 50% of the gill area
Heavy	5	Extensive lesions covering most of the gill surface

Table 1.2. List of biotic and abiotic risk factors influencing AGD.

Biotic factor	Reference
Ballan wrasse (*Labrus bergulta*)	Karlsbakk et al., 2013
Biofouling communities	Hellebø et al., 2017
Lumpfish (*Cyclopterus lumpus*)	Hellebø et al., 2017
Psychroserpens sp.	Bowman & Nowak., 2004
Saithe (*Pollachius virens*)	Hellebø et al., 2017
Sea lice (*Lepeophtherius salmonis*)	Bustos et al., 2011
Winogradskyella sp.	Embar-Gopinath et al., 2005
Abiotic factor	**Reference**
Sea water temperature	Nowak., 2007
Sea water salinity	Nowak., 2007

contribute to disease outbreaks and should be considered as a further risk factor (Tan et al., 2002). This fact could be location-dependent and a PCR survey in Norway showed opposite results regarding biofouling organisms that led the authors to conclude these organisms seem not to be reservoirs of *N. perurans* (Hellebø, Stene, and Aspehaug 2017). Nevertheless, seawater and plankton disseminated by ocean currents may provide a source of amoeba and elicit AGD outbreaks. Still, there remains a lack of knowledge of AGD regarding epidemiology and risk factors, particularly in Europe (Rodger., 2014). Roubal et *al*. (1989) hypothesised that the presence of gill-colonising bacteria could exacerbate AGD (Embar-Gopinath et al., 2005). A 16S rRNA gene-based study identified the bacteria at the gills in AGD-affected salmonids and demonstrated that the gills were colonized predominantly by *Winogradskyella* spp., a genus belonging to the *Flavobacteriaceae* (Embar-Gopinath et al., 2006). This study showed that while the presence of *Winogradskyella* sp. did not increase AGD severity, the physiological effects of this bacteria on Atlantic salmon were unknown (Embar-Gopinath et al., 2006). Another study concluded that *Winogradskyella* sp. could enhance the ability to *Neoparamoeba* sp. to infect the gill filaments and cause AGD (Embar-Gopinath, Butler, and Nowak 2005). Interestingly, *N. pemaquidensis* reportedly associates with marine bacteria of the genus *Psychroserpens* (*Flavobacteriaceae*, *Bacteriodetes*) in cases of AGD (Bowman & Nowak., 2004).

There is some evidence suggesting a seasonal influence on *Neoparamoeba* sp. virulence. (Crosbie et al., 2005). In Tasmania, a bimodal pattern of AGD prevalence exists, with a large peak of cases in summer and a second smaller increase in autumn, though the timing of this second peak is more difficult to predict (Clark & Nowak., 1999). Temperature seems to be an aggravating factor of AGD outbreaks and most severe cases occur in summer in Tasmania (Nowak., 2007). AGD prevalence is most likely directly related with environmental factors such as salinity and water temperature (Nowak., 2007). After a transfer of smolts to marine/estuarine cage structure sites, AGD appears after around 18-19 weeks, thus implicating the presence of the pathogen in saline environment (Adams & Nowak., 2003). AGD has been recorded in Australia (Tasmania) at temperatures as low as 10.6°C and salinity as low as 7.2 ppt (Adams & Nowak., 2003). AGD cases were reported in Norway at four distant fish farms for 7-12 weeks in late autumn

2006 and, importantly, seawater temperatures were 3.5°C higher than recorded before the outbreaks (Steinum et al. 2008). Temperature could become the main risk factor in years to come due to global warming leading to increased seawater temperatures in salmon-farming areas (Oldham et al., 2016). The effect of temperature on AGD outbreaks is thought to be more due to the effects on fish physiology rather than on the amoeba (Douglas-Helders et al., 2005), as pathological changes to the gills can be observed before amoeba are observed microscopically in significant numbers (Bermingham & Mulcahy., 2004). Furthermore, environmental changes predispose salmon to colonisation by ciliates and amoeba (Bermingham & Mulcahy., 2004).

Cage conditions restrict the ability of the fish to demonstrate their natural behaviour against parasites, such as avoidance and leaping (Nowak., 2007). *Neoparamoeba* sp. shows the ability to colonise Atlantic salmon carcasses (Douglas-Helders et al., 2000) and the amoeba from dead fish can colonize uninfected carcasses to create an additional pathogen reservoir (Douglas-Helders et al., 2000). As caged fish would be unable to move away from such sources of infection it is imperative that dead fish are removed regularly inform the cage and that other measures are taken to minimise infection pressures. Copper treated net-cages have significantly higher *Neoparamoeba* sp. abundance and AGD prevalence, probably because these structures may irritate the fish gills (Douglas-Helders et al., 2003). Fish ploidy (i.e. triploid) does not affect AGD in terms of severity, manifestation and gill score (Chalmers et al., 2017). Stocking densities have a significant impact on AGD-morbidity, and salmon stocked at 5 kg/m^3 show mortalities at 23 days post-challenge compared to fish stocked at 1.7 kg/m^3 which start to die at 29 days (Crosbie et al., 2010).

1.3. *Neoparamoeba perurans*, the aetiological agent of AGD

1.3.1. Amoeba as pathogens

Amoeba are unicellular eukaryotes studied since the very beginning of microscopy, with *Amoeba proteus* (measuring 1-5 mm) being one of the first amoeba observed using light microscopy (Siddiqui & Khan., 2012). Some amoebae are the aetiological agents of widespread and virulent diseases affecting humans.

Amebiasis, also called amoebic dysentery, is caused by *Entamoeba histolytica*, a pathogen formally described by Lösh in 1875 and initially named *Amoeba coli* (Reed., 1992). This disease may be the amoebic disease known for the longest time, as the first presumed report was by Mateo Aleman in 1611 (Brandt & Tamayo., 1970). The most common form of the disease is intestinal amoebiasis, characterised by 4 clinical forms (ameboma of the colon, bloody diarrhoea, amoebic appendicitis and fulminating colitis), but *E. histolytica* is able to infect other organs as well (extraintestinal amoebiasis), notably the liver (amoebic liver abscess) (Espinosa-Cantellano & Martínez-Palomo., 2000; Reed., 1992). This disease is transmitted between people the via faecal-oral route thanks to a cyst stage, and this infection is widespread in developing countries (Botero., 1978; Haque et al., 2003). It is presumed that amoebiasis is the second leading cause of death from parasitic disease in the world, with 40,000-100,000 people dying each year (Stanley., 2003).

More recently, others diseases caused by amoeba have been reported. Some species of amoeba belonging to the genus *Acanthamoeba* are known to cause amoebic keratitis (AK) and granulomatous amoebic encephalitis (GAE) (Martinez & Visvesvara., 1997). AK was first reported in 1974 and associated with soft contact lens solution contaminated with species from the genus *Acanthamoeba* (e.g., *A. polyphaga*, *A. castellani*) (Larkin et al., 1992; Moore et al., 1985). Even if AK is characterised by inconvenient symptoms (photophobia, pain, eyes watering), an appropriate treatment relying on antimicrobial (e.g. chlorhexidine) and disinfectant (e.g. polyhexanide also known as polyhexamethylene biguanide) compounds and keratoplasty (i.e. a corneal graft) are sufficient to treat this disease (Lorenzo-Morales et al., 2015; Wright et al., 1985). GAE is a disease affecting the central nervous system (CNS) (Marciano-Cabral & Cabral., 2003). The primary amoebic meningoencephalits (PAM) caused by *Naegleria fowleri* and the balamuthia amoebic encephalitis (BAE) caused by *Balamuthia mandrillaris* are also involved in infections of the CNS (Seidel et al., 1982; Siddiqui & Khan., 2008). CNS infections in humans are characterised by high death rate (73-100%) but can be treated with broad spectrum drugs if administered early in infection (Deetz et al., 2003; Marciano-Cabral & Cabral., 2003; Martinez et al., 1973; Orozco et al., 2011; Sell et al., 1997).

1.3.2. Biology of *Neoparamoeba perurans*

There is a need to gain a better understanding of *N. perurans* biology and its relationship with its environment (Oldham, Rodger, and Nowak 2016). The presence or absence of micro-scales on the cell surface of amoebae have been used to discriminate species in the *Paramoeba* and *Neoparamoeba* genera (Page., 1987). However, recent findings based on microscopic observations and phylogenetic analysis suggest that micro-scales are not in fact a distinguishing feature and so it has been suggested that *Paramoeba* and *Neoparamoeba* are synonymous (Sibbald et al. 2017). The genus *Neoparamoeba* (Amoebozoa, Discosea, Dactylopodia, Vexilliferidae) contains species that are widespread in the marine environment (Page., 1987), but only two species have been formally described: *N. eilhardi* and *N. atlantica* (Kudryavtsev et al., 2011; Nowak & Archibald., 2018). Nevertheless, the species taxonomically recognized for the genus *Neoparamoeba* are: *N. eilhardi* (Schaudinn, 1896), *N. aesturiana* (page, 1970), *N. atlantica* (Kudryavtsev et al., 2011), *N. branchiphila* (Dyková et al., 2005), *N. invadens* (Jones, 1985), *N. pemaquidensis* (Page, 1970), *N. perniciosa* (Sprague et al., 1969), *N. perurans* (Yong et al., 2007) and *N. schaudinni* (de Faria et al., 1922) (Feehan et al., 2013). Other species from the genus can infect marine organisms, such as *N. invadens*, which causes sea urchin mortalities (*Strongylocentrus droebachiensis*), and *N. perniciosa*, which causes outbreaks of “grey crab disease” in the blue crab (*Callinectes sapidus*) (Feehan et al., 2013; Sprague et al., 1969). These species of amoebae appear star-shaped due to pseudopodia and measure 15 to 20 µm in diameter (Rodger & McArdle., 1996). *Neoparamoeba* spp. are also characterized by a thick glycocalyx of about 10 nm in thickness (Dyková et *al*., 2000) and by a direct life cycle that lacks a cyst stage (Nowak., 2007). Almost nothing is known on the ecology of *N. perurans*, but it seems to be an opportunistic free-living parasite with many reservoirs in the environment (Karlsbakk et al. 2013). Indeed, this cosmopolitan amoeba lacks host specificity and has been detected in various marine organisms including Chordata (e.g. *Ciona sp*.), Cnidaria (e.g. *Obelia geniculata*) and Mollusca (e.g. *Mytilus edulis*) (Hellebø, Stene, and Aspehaug 2017). Water chemistry is a key component for the survival of *N. perurans* in freshwater and especially concentrations of Mg^{2+} and Ca^{2+} (Powell & Clark., 2003). Salinity is also major factor for the survival of *Neoparamoeba*

spp. which influence its morphology (Douglas-Helders et al., 2005). To adapt to changes in salinity, *N. perurans* use contractile vacuole for osmotic regulation (Lima et al., 2016). This organelle allows quick adaptation in coastal areas that can undergo considerable daily variations in salinity (Lima et al., 2016). Furthermore, water depth seems to be one of the most important parameters affecting amoeba abundance with greatest concentrations of the cells at the surface (0 to 4 meters (Wright et al., 2015). The water column distribution of *N. perurans* in sea water surface could be explained by its life history or by relationships (competitor, prey-predator) with other organism (plankton or neuston) (Wright et *al*., 2015). Another explanation regarding the distribution of *N. perurans* in the water column is the behaviour of farmed Atlantic salmon in sea cage which can lead them to neighbour the surface on several occasions (feeding, leaping, swallow air) and thus concentrate the amoeba close to the sea surface (Wright et al., 2015). Greatest abundances of amoeba (*N. pemaquidensis* and *N. perurans*) are observed in early autumn (Wright et al., 2015).

In the marine environment, bacteria are the major source of food for amoeba (Horn & Wagner., 2004.). In laboratory conditions, *Neoparamoeba* sp. was successfully cultured using heat-killed *E. coli* as a nutrient source (Morrison et al. 2005). However, some bacteria can survive in amoebic organisms, acting like trojan horse which constitutes a risk for bacterial dissemination (Horn & Wagner., 2004.). As an example, certain bacteria associated with *Acanthamoeba* spp. cannot survive outside of the amoeba and resist to encystation performed by the amoeba which indicates an adaptation to this internal environment (Horn & Wagner., 2004.). Eradication of the bacterial symbionts from the amoeba with antibiotics has never been reported meaning that wiping-out internal symbionts out for treatment could represent a challenging task (Horn & Wagner., 2004.). The main group of the bacteria which are endosymbionts of free-living amoeba belong to the order of *Chlamydiales* (Horn & Wagner., 2004). The putative role or function of these bacteria within the amoeba is unknown and not well studied (Horn & Wagner., 2004.). Moreover, bacterial organisms living in amoeba represent only a part of endosymbiont diversity observed in amoebae.

Endosymbiotic perkinsela-like organisms (PLOs) (nowadays called *Perkinsela* sp.) maintain a mutualistic symbiosis with the host (Dyková et al., 2008; Tanifuji et al., 2017).

A co-evolution between *Neoparamoeba* spp. and PLOs is suspected due to the localisation of the symbiont, which is found close to the amoeba nucleus (Figure 1.3) (Dyková et al., 2008). Indeed, comparison of phylograms between PLOs and *Neoparamoeba* spp. confirms the hypothesis of co-evolution thanks to a high level of congruence of phylograms of these two organisms (Dyková et al., 2008). The data available supports the assumption that PLOs and *Neoparamoeba* spp. share a strictly hereditary relationship (Young et al., 2014). The nature of the symbiosis between *Neoparamoeba* sp. and PLOs is considered mutualistic symbiosis, which means that the symbiotic association formed is obligatory and if separated both host and symbiont die which is critical information to consider when identifying chemotherapeutic targets (Dyková et al., 2008).

The kinoteplastids are named due to a prominent disk-shaped mass of DNA the "kinetoplast" inside their mitochondrion (Tanifuji et al. 2017). The best studied kinetoplastids include the parasitic flagellates *Trypanosoma cruzi* and *Leishmania* spp. and the fish pathogens belonging to the genus *Cryptobia* and *Ichthyobodo* (Tanifuji et al. 2017; Stuart et al. 2008). The *Perkinsela* sp. symbiont of *Neoparamoeba* spp. has a reduce proteome and genome compared to other kinetoplastids due to adaptation to intracellular life showed by a reduced intergenic distance (515 bp) and reduced size of untranslated regions (100 nucleotides) (Tanifuji et al., 2017). Kinetoplastids can undergo some common changes like the reduction or loss of pathways that are subsequently provided or augmented by the host (Cenci et al. 2016). Arginine and proline related enzymes involved in these metabolic pathways are encoded by the host while purine metabolism got related protein encoded by the organism (Tanifuji et al. 2017). *Perkinsela* sp. has lost during evolution all the genes needed to maintain and construct a flagellum and the tubulins associated with flagellum function (Tanifuji et al., 2017). Another characteristic of *Perkinsela* sp. is the presence of a glycosome-like organelle in the residual cytoplasm, which performs several metabolic reactions (Tanifuji et al., 2017). There are lots of metabolic pathway that take place in the putative glycosome/peroxisome (notably glycolysis) (Tanifuji et al., 2017). The *Perkinsela* sp. lives in the cytoplasm, is non-photosynthetic and provides no apparent energetic benefit to its amoeba host (Tanifuji et al. 2017). However, *N. pemaquidensis* and *Perkinsela* sp. cannot be cultured separately, notably because of shared metabolic pathways and

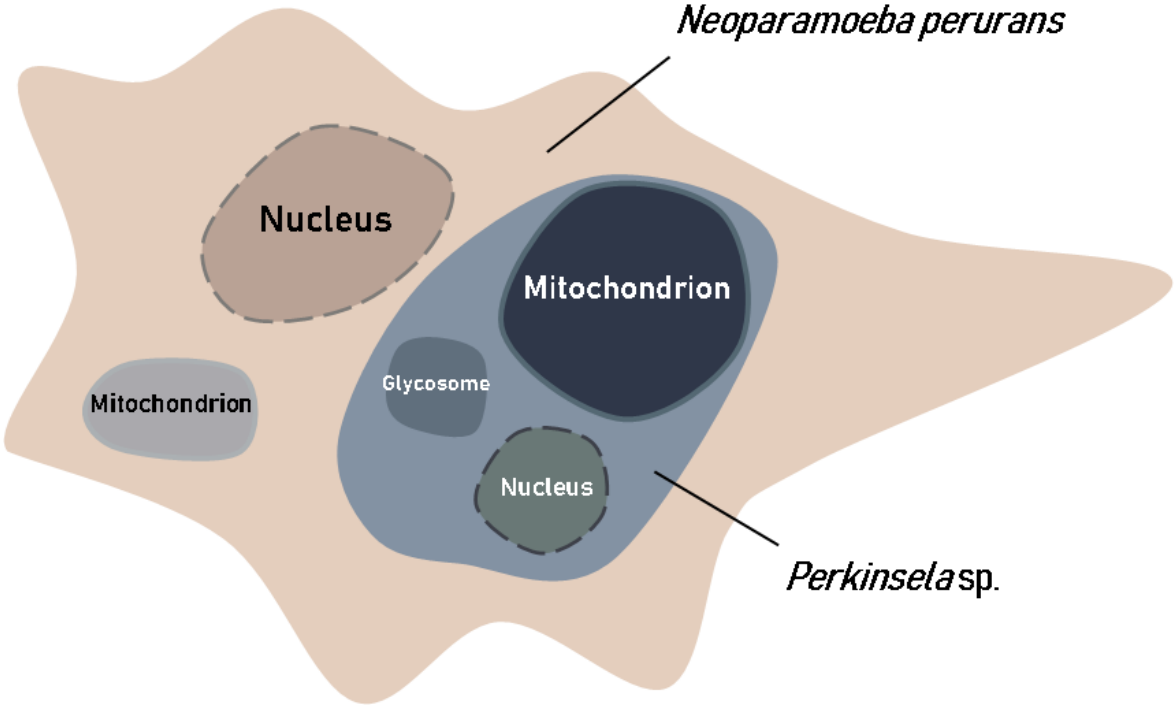

Figure 1.3. Schematic representing the symbiotic association between *Neoparamoeba pemaquidensis* and *Perkinsela* sp (modified from Tanifuji et al., 2017).

enzymes involved in trypanothione, heme and ubiquinone metabolisms (Tanifuji et al., 2017). Indeed, the heme pathway in some parasitic species is incomplete or totally absent and essential metabolites are up taken from their hosts or by bacterial endosymbionts (Cenci et al. 2016). Both organisms seem to communicate with each other by endocytosis and exocytosis (Tanifuji et al., 2017).

In laboratory conditions, *N. perurans* shows differential antigen expression on agar and in liquid and over time (Villavedra et al., 2005). Freshly isolated parasite can be infectious for up to 14 days (Villavedra et al., 2007) but shows a loss of virulence during three years in clonal culture, likely caused by a lack of attachment to the gills or absence of necessary extracellular products (Bridle et al., 2015). In addition to Villavedra et al. (2007), Bridle et al. (2015) found that *N. perurans* was still virulent after 70 days of clonal culture.

1.3.3. Virulence factors

In AGD, the key virulence factors are associated with attachment, growth on the gills and immunosuppression (Collins et al. 2017). The amoeba adheres to normal gill epithelium but cannot attach to proliferative epithelial tissues produced during recovery from an epithelial abrasion and this thereby limits the occurrence of re-infection (Adams & Nowak., 2004). *Acanthamoeba* spp., responsible for corneal infection in humans, has a 400-kDa mannose-binding protein (MBP) as a major virulence factor (Valdenegro-Vega et al., 2014), and specific antibodies against the *Acanthamoeba* MBP inhibits parasite adherence (Valdenegro-Vega et al., 2014). *N. perurans* possess MBP homologs and thus could play a role in attachment and virulence (Valdenegro-Vega et al., 2014). Glycoprotein subunits of *N. perurans* are resistant to degradation by PNGase F, PNGase A, O-glycosidase, 4-galactosidase, β-N-acetylglucosaminidase and neuraminidase, which could be considered as another virulence factor. Parasite suppression of the host immune system has been demonstrated for human and fish parasites (*Cryptosporidium molnari*, *Nanophyetus salmincola*) (Sitjà-Bobadilla., 2008). Extracellular products (ECPs) are virulence factors for *Acanthamoeba* spp. and *Entamoeba* spp. and this could also be the case for AGD (Nowak & Archibald., 2018). However, when the *N. perurans* is lysed, the

lysate does not elicit any immunological reaction from the host, suggesting that toxins do not play a major role in AGD infection (Adams & Nowak., 2004).

1.4. Fish defence against AGD

1.4.1. Innate immunity

In teleost fish, the immune system is complex and integrates many components (Figure 1.4) (Uribe et al., 2011). Studies regarding the immune response of AGD-infected Atlantic salmon are numerous and focus on many different aspects. An important proportion of the Atlantic salmon produced is triploid and a study investigated this putative effect in the case of AGD response. Ploidy has a significant effect on lysozyme activity at 20 days post-infection in the case of AGD challenge (Chalmers et al., 2017). Variable such as ploidy, infection or time do not affect complement and anti-protease activity (Chalmers et al., 2017). Therefore innate immune response seems to have a limited role in protecting salmon from *N. perurans* (Chalmers et al., 2017). Another study focused on proteins present on the gill and in mucus (Valdenegro-Vega et al., 2014). A total of 186 and 322 proteins were identified in gill and mucus respectively in AGD affected salmon and control group (Valdenegro-Vega et al., 2014), and 52 salmon gill and 42 salmon skin mucus proteins were differentially expressed between these two groups (Valdenegro-Vega et al., 2014). Some of these proteins have a role in innate immunity and belong to cell signalling and inflammation pathways such as granulin, angiogenin-1, cathepsin, apolipoprotein and C-reactive protein (Valdenegro-Vega et al., 2014). Importantly, protein markers in mucus in AGD infection have been described such as gradient-2 protein, complement C3 factor and annexin A-1 (Valdenegro-Vega et al., 2014).

The interleukin-1β is a cytokine which plays a major role in the inflammatory and immune response of Atlantic salmon and, in the case of infection with AGD, the expression of the associated receptor inteurleukin-1β receptor type 1 (IL-1RI), but not IL-1RII (inteurleukin-1β receptor type 2), is modulated in AGD-affected gill tissue (Morrison et al., 2012). IL-1R1 is a compensatory mechanism to dampen the effects of chronic IL-1β

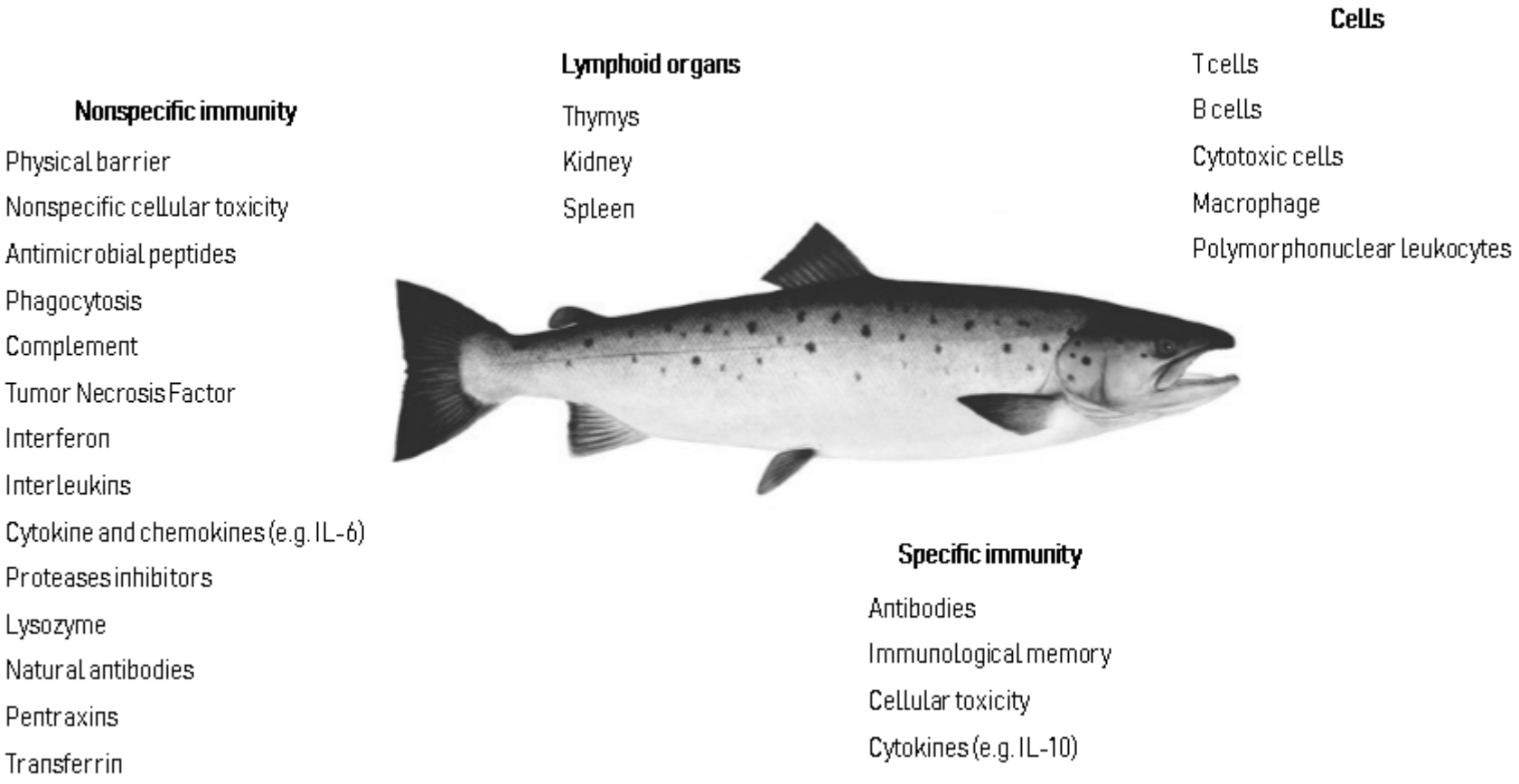

Figure 1.4. Diagram representing the different component of the immune system in teleost fish (modified from Uribe et al., 2011).

over expression in AGD (Morrison et al., 2012). In Atlantic salmon, up-regulation of IL-1β occurs in AGD-affected gill tissue without changes in the expression of either iNOS (inducible Nitric Oxide synthase) or TNF-α (Alvarez-Pellitero., 2008). However, naïve rainbow trout infected with *Neoparamoeba* sp. showed that at both 7 and 14 days post-infection iNOS and IL-1β1 gene expression were up-regulated in the gill (Bridle et al., 2006). At 7 days post-infection, interleukin-8 (IL-8), a cytokine which is involving is immune cell chemotaxis, was up-regulated in the liver (Bridle et al., 2006). In the early AGD infection stages, when the GS increases from GS1 to GS2, several immune parameters work towards a decrease of their activities and/or abundance such as immunoglobulin M (activate the complement system), peroxidase (enzyme), lysozyme (antimicrobial enzyme), esterase (enzyme) and protease (enzyme) (Marcos-López et al. 2017).

1.4.2. Adaptive immunity

Heritability of resistance against AGD in Atlantic salmon families is moderate (Nowak., 2007). This finding was supported in a large scale study involving 1,500 Atlantic salmon challenged with AGD, host resistance to AGD was reported to be moderately heritable ($h^2 \approx 0.25 - 0.3$) while using gill damage and amoebic load as indicator traits (Robledo et al., 2018). However, it appears that repeated infection of Atlantic salmon with AGD results in a resistance to re-infection (Vincent et al., 2006). In an experimental trial involving Atlantic salmon challenged with AGD, at 35 days post infection 73% of the salmon previously exposed survived a further AGD challenge, meanwhile only 26% of naive fish survived (Vincent et al., 2006). Serum and skin mucus IgM are able to bind formalin-fixed *N. perurans* after injection of recombinant r22C03, a *N. perurans* MBP-like protein (Valdenegro-Vega et al., 2014). This same study also concluded that IgM could potentially reduce the attachment of the parasite (Valdenegro-Vega et al., 2014). Nevertheless, there is no evidence that antibodies recognising wild-type *N. perurans* confer significant protection against AGD infection (Taylor et al., 2010). The expression of signature cytokines in Atlantic salmon (Th1, Th17 and Treg related pathway) has been investigated after challenge with AGD (Benedicenti et al., 2015). Th1, Th17 and Treg related

pathways were down-regulated, mainly in gill samples from fish treated with 5000 cells/l of *N. perurans* (Benedicenti et al., 2015). However, IL4/13 expression, involved potentially in the Th2 pathway, showed up-regulation in the gills of AGD-infected fish (Benedicenti et al., 2015). The absence of IgM-positive cells and the lack of evidence for macrophage alternative activation pathway lead to the assumption that immune evasion is performed by the parasite (Benedicenti et al., 2015). The systemic and mucosal IgM levels in AGD-infected Atlantic salmon are not affected after infection with *N. perurans* (Valdenegro-Vega et al., 2015). Peroxiredoxin 1 (Prx 1), also known as natural killer enhancing factor A (NKEFA), is implicated in the immune response of both mammals and fish (Loo et al., 2012). In the Atlantic salmon liver, after infection with *N. perurans*, *Prx 1* gene expression is down-regulated in Atlantic salmon displaying symptoms of AGD (Loo et al., 2012). AGD-affected gills of Atlantic salmon display an increase in mRNA expression of cellular markers of immune cells, most notably professional antigen-presenting cells (MHC-II, CD4), B cells (IgM, IgT, MHC-II) and T cells (TCR, CD4, CD8) (Pennacchi et al., 2014). T cells within AGD affected gills are mainly constituted of CD8+ cells (Pennacchi et al., 2014). The infiltration of T cells highlights the importance of CD8+ T cells, which are professional antigen presenting cells, in AGD infections suggests the possible involvement of gill intraepithelial lymphoid tissue in AGD (Pennacchi et al., 2014). In teleosts, the Interbranchial Lymphoid Tissue (ILT) is a part of Gill Associated Lymphoid Tissue (GIALT) formed by an accumulation of lymphoid T cells (Norte dos Santos et al., 2014). *N. perurans* infection in Atlantic salmon elicits changes in the ILT as lymphocyte density increases up to 7 days post-infection, probably due to hyperplasia, before then decreasing (Norte dos Santos et al., 2014). There is currently no evidence of a coordinated adaptive and innate immune response in AGD affected Atlantic salmon and this could explain the high susceptibility of Atlantic salmon in AGD (Alvarez-Pellitero., 2008).

1.5. Existing measures to reduce the impact of AGD

1.5.1. Prevention

Current control strategies for AGD are selection for resistant strains and prophylactic measures (Buchmann., 2015). Among the prophylactic measures, AGD is

monitored in Scotland and Ireland through weekly examination of the gills of 5 to 10 fish per pen (Rodger., 2014). Prompt removal of dead fish in the pen and cage can limit the reservoir of amoebae and thus reduce the risk of appearance of AGD outbreaks in non-infected farms and reduce the magnitude of the disease in AGD-infected farms (Douglas-Helders et al., 2000). Greater densities of salmon in sea cages can facilitate the onset and progression of AGD in fish farms (Crosbie et al., 2010). Another control method is the co-culture of blue mussels (*Mytilus edulis*) with Atlantic salmon in integrated multi-trophic systems, which may have a beneficial effect thanks to the quick removal of the parasite by filtration, as has been demonstrated in experimental challenge trials (Rolin et al., 2016). Another precautionary principle to avoid AGD is the screening by PCR of cleaner fish such as lumpfish, which develop the disease slowly and can be asymptomatic carriers of AGD (Haugland et al. 2017).

The snorkel lice barrier (Figure 1.5) is a device which places sea cage facilities deeper (by 10 m) in order to avoid the surface waters where both sea lice and *N. perurans* are found at greatest concentrations (Wright et al., 2017). In addition to this device, freshwater is spread at the surface to create a layer of brackish water (of 4-5 m in depth) that repels both sea lice and *N. perurans* (Wright et al., 2017).

1.5.2. Treatment

Freshwater bathing treatment (2-3 h duration on average) provides effective protection against AGD for 3 weeks and a second bath has a longer lasting effect likely by stimulating the non-specific immune response and restoring the fish immune status (Clark & Nowak., 1999; Marcos-López et al., 2017). Field and laboratory experiments have shown that soft freshwater (19,3-37,4 mg/l CaC03) is more efficient than hard fresh water (173-236,3 mg/l CaC03) for treating AGD (Roberts & Powell., 2003). An *in vitro* experiment has showed that freshwater remains the most effective treatment, with only 6% of viable amoeba seen after 24 h and no viable amoeba observed by microscopy after 48 h (Florent et al., 2010). Freshwater bathing reduces significantly the appearance of characteristic white mucoid patches on the gills and also the presence of amoeba in gill smears and the number of amoeba per lesion (Parsons et al. 2001). After 2 h of bathing, only 27% of

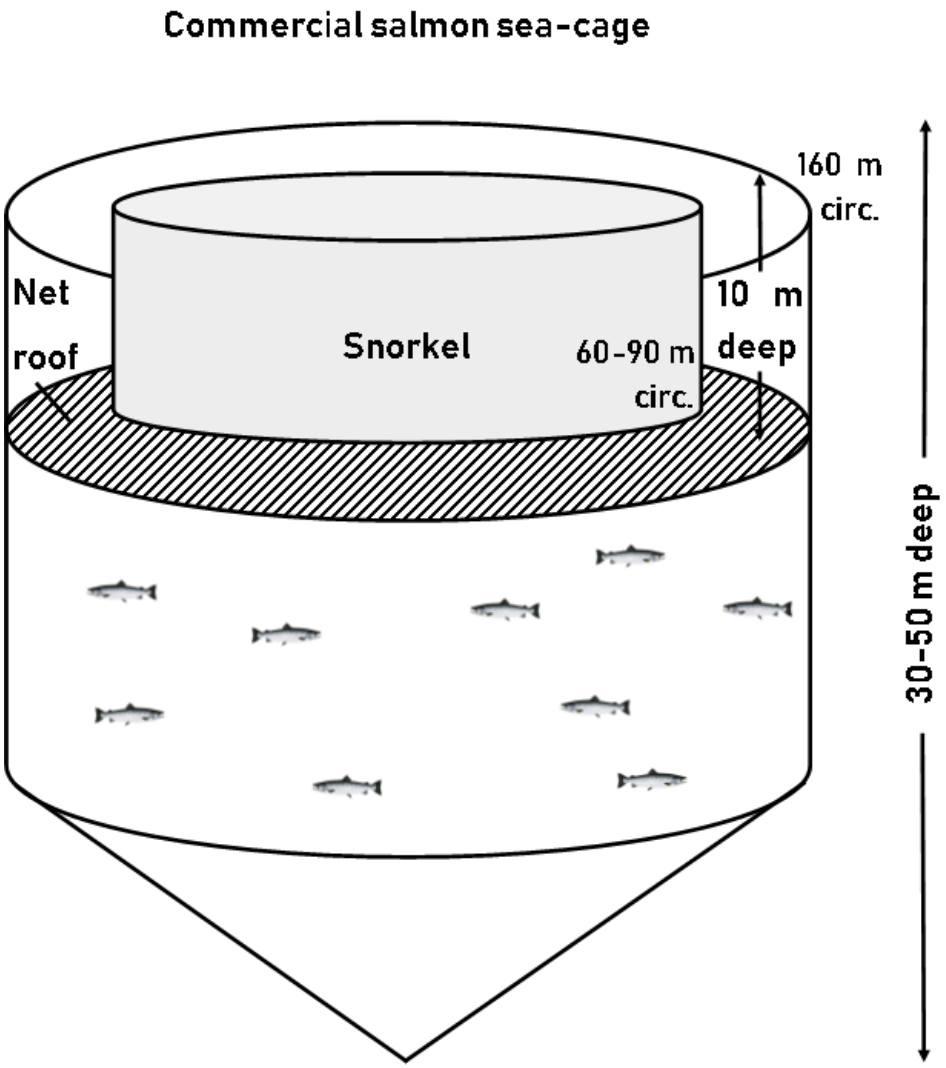

Figure 1.5. Schematic of a commercial snorkel cage (modified from Wright et al., 2017).

the amoebae present on gill smears are still alive (Parsons et al. 2001). Freshwater bathing is effective for removing most amoeba cells, but alternative treatments are still needed for their total removal to reduce production costs and the stress caused to the fish by such treatment (Rodger., 2014).

1.5.3. Chemotherapeutic products

Scottish and Irish salmon farms use hydrogen peroxide to control AGD outbreaks with dosages ranging from 1000 to 1400 mg/l for 18 to 22 min (Rodger., 2014). However, this process causes stress to the salmon through handling and the toxicity of the chemical itself (Rodger., 2014). Hydrogen peroxide (1250 mg/l for 15 min) has the same effect for treating light cases of AGD (9% of salmon gill filament affected by lesion) compared to freshwater bathing (Adams et al., 2012). Moreover, hydrogen peroxide is more efficient for removing amoeba at low temperature (8°C) and can also delay re-infection by several weeks (Martinsen et al., 2018).

1.6. New treatment and control solutions

1.6.1. Vaccine

Vaccines based on both live or killed *N. perurans* and DNA have been tested without success (Buchmann., 2015). Attachment proteins of other amoebae are also considered to be potential vaccine candidates (Valdenegro-Vega et al., 2014), and *N. perurans* MBP has been produced as a recombinant protein (Valdenegro-Vega et al., 2014). Indeed, an MBP-like factor (R22C03) of *N. perurans* was tested as a vaccine candidate (Valdenegro-Vega et al., 2015). Immunisation provided an IgM response from 8 to 12 weeks after primary exposure and further analysis testified that these IgM molecules could bind formalin-fixed *N. perurans* showing that there is promise to pursuing a protective vaccine (Valdenegro-Vega et al., 2014). However, the IgM response to R22C03 was elicited

in serum and not in mucus, which correlates with the findings of another study, and supports that serum IgM could have potential neutralising activity against *N. perurans* (Valdenegro-Vega et al., 2015).

1.6.2. Chemotherapeutic product

Successful elimination of endoparasites, such as nematodes and cestodes, has been achieved using oral administration of antiprotozoals including levamisole and praziquantel in fish (Florent et al., 2007). In the case of AGD, several chemical products have been tested as possible treatments (Table 1.3). Bithionol and bithionol sulfoxide are anthelmintics known to act on the mitochondrial respiratory chain to suppress ATP production via uncoupling of oxidative phosphorylation (Florent et al., 2010). These two chemicals have been used as anti-infective agents against cestode and trematode infestations in humans (Florent et al., 2010). Bithionol was tested *in vitro* and *in vivo* as bath treatment for other salmonids parasites (*Gyrodactylus* sp., *Ichtyobodo necator*) (Florent et al., 2007). Concentrations of bithionol of 1, 5 and 10 mg/l for 1 h in a seawater bath shows efficiency comparable to freshwater bathing in Atlantic salmon against AGD (Florent et al., 2007). However, bithionol was toxic above 25 mg/l in freshwater and seawater for both Atlantic salmon and rainbow trout (Florent et al., 2007). Bithionol, when fed for 2 weeks as a prophylactic or therapeutic treatment at 25 mg/kg of feed, delayed the onset of AGD and reduced the percentage of gill filaments with lesions (Florent et al., 2009). *In vitro* toxicity of bithionol and bithionol sulfoxide demonstrated at all concentrations (0,1 to 10mg/l over 72h) a toxic effect on *Neoparamoeba* sp. (Florent et al., 2010). Moreover, bithionol and bithionol sulfoxide at a dose superior to 5 mg/l for 72 h showed toxicity comparable to freshwater bathing in *Neoparamoeba* sp. (Florent et al., 2010). Bithionol and bithionol sulfoxide are not currently licensed in Australia for fish use as therapeutics and they are forbidden for use in USA after being used in deodorants and shampoo because it can cause photocontact sensitisation (U.S. Food & Drug Administration, 2002).

Oral supplementation with levamisole in feed (500 mg/kg for 20 days) or β-glucan (1 g/kg for 31 days) do not prevent mortalities when Atlantic salmon naïve to AGD are

Table 1.3. Summary of experimental treatments tested for efficacy against AGD.

Product	Dose	Study	Reference
Levamisole	2.5–5 mg/l	*In vivo*	Clark & Nowak,, 1999
Levamisole	1.25–5 mg/l	*In vivo*	Zilberg et al., 2000
Chlorine dioxide	0–50 mg/l	*In vivo*	Powell & Clark,, 2004
Chloramine-T	0–50 mg/l	*In vivo*	Powell & Clark,, 2004
CPG-ODNs	50 µg/fish	*In vivo*	Bridle et al.,2003
Hydrogen peroxide	0–100 µl/l	*In vivo*	Powell & Clark,, 2004
L-cysteine ethyl ester	0–200 µg/ml	*In vivo*	Roberts & Powell,, 2005
Chloramine-T	10 mg/l	*In vivo*	Leef et al., 2007
Chloramine-T	10 mg/l	*In vivo*	Leef et al., 2007
Bithionol	0–35 mg/l	*In vivo*	Florent et al., 2007
Garlic extract	20 µl/well	*In vitro*	Peyghan et al., 2008
Metronidazole	0.1–100 mg/l	*In vitro*	Peyghan et al., 2008
Bithionol	25 mg/kg feed	*In vivo*	Florent et al., 2009
Bithionol	0.1–10 mg/l	*In vitro*	Florent et al., 2010
Bithionol sulfoxide	0.1–10 mg/l	*In vitro*	Florent et al., 2010

then exposed to this pathogen (Zilberg et al., 2000). Indeed, addition of levamisole in freshwater baths (2.5 and 5 mg/l) does not show any effect on *N. perurans* compared to the untreated control (Clark & Nowak., 1999). Levamisole is not the only substance to have been tested in feed. Commercial β-glucan can stimulate the respiratory burst activity of Atlantic salmon head kidney macrophage *in vitro* and thus was tested in feed to see if this compound could enhance the resistance of Atlantic salmon against AGD (Bridle et al., 2005). However, oral administration of β-glucan does not elicit *in vivo* respiratory burst activity in head kidney macrophage or serum lysozyme production in Atlantic salmon (Bridle et al., 2005).

Chloramine-T as a treatment of AGD demonstrates a role of moderator of some metabolic effects on Atlantic salmon *in vivo* such as oxygen consumption (Leef et al., 2007). Chloramine-T (10-25 mg/l) causes a significant reduction by 50% of amoebae on Atlantic salmon gills when added to freshwater (Powell & Clark., 2004). Chloramine-T impairs carbon dioxide excretion by eliciting hypersecretion of branchial mucus (Powell & Clark., 2004). Use of chlorine dioxide (25 mg/l) can remove up to 50% of amoeba cells from the gills of Atlantic salmon (Powell & Clark., 2004). In the same study, hydrogen peroxide was tested as a freshwater bath additive but there was no clear evidence of efficacy for hydrogen peroxide below 100 µl/l to reduce amoebae numbers (Powell & Clark., 2004). However, a high concentration (100 µl/l) of hydrogen peroxide is more toxic than both chloramine-T and chlorine dioxide and which may limit its potential for commercial use (Powell & Clark., 2004).

L-cystein ethyl esther (LCEE) is a mucolytic drug that reduces mucus hypersecretion and mucus thickening (Roberts & Powell., 2005). LCEE may delay the progression of AGD after 2 weeks of administration in feed at 50 mg/kg fish/day (Roberts & Powell., 2005). Three days after removing LCEE from the feed, all physiological parameters (whole body ionic flux, mucus viscosity, plasma analysis) returned to their normal expected range, indicating a quick recovery of the fish after treatment (Roberts & Powell., 2005).

Oligodeoxynuvleotides (ODNs) containing cytosine-phosphodiester-guanine (CpG) motifs are powerful immunostimulants which activate mammalian immune cells and may be used in Atlantic salmon to increase its resistance against AGD (Bridle et al., 2003).

The CpG-ODNs are recognised by the Atlantic salmon immune system and induce immune cell proliferation and cytokine production (Bridle et al., 2003). Intraperitoneal injection of CpG (50 µg/fish) increased salmon resistance against AGD by about 30% compared to the control at 16 days post-infection (Bridle et al., 2003). Therefore, CpG-ODNs could be used in disease treatment or as a vaccine adjuvant (Bridle et al., 2003).

An *in vitro* study investigated the effect of garlic (*Allium stavium*) extract and metrinodazole, an antiprotozoal, against *N. perurans* (Peyghan et al., 2008). Exposure of *N. perurans* to garlic extract (10 g/L) for 8 h can kill the amoeba, whereas metronidazole at <50 mg/L had no effect after 24 h exposure and only affected survival at 100 mg/L after 6 days exposure (Peyghan et al., 2008).

1.7. Development of new chemotherapeutants

In view of current solution to treat AGD, new ways to prevent and treat AGD in salmon fish farming are needed. Indeed, three major scales have to be taken in account which are the drug producer, the fish farmer and the environment. In order to provide a sustainable and effective solution to treat AGD, many elements have to be considered (Table 1.4). Even if AGD is one of the most economically impacting diseases for the marine aquaculture, no specific drugs have been developed to treat this disease compared to other pathogens (Figure 1.6).

1.8. Aim of the thesis

The aim of this thesis is to identify candidates a new chemotherapeutic treatment for AGD administered by bath or in feed by using knowledge of related diseases and existing drugs used as treatments. To do this, the following steps will be addressed:

1. Identify diseases caused by amoebae and kinetoplastids that affect humans, animals and plants.
2. Identify past and present drug treatments used against AGD-like diseases.
3. Identify drugs active against pathogenic amoebae and kinetoplastids causing AGD-like disease.

Table 1.4. Considerations before developing a new chemotherapeutant.

	Drug producer	Fish farm	Environment
Cost effectiveness	■	■	
Ease to manufacture and/or acquire	■		
Efficient against all AGD strain			■
Lack of existing resistance mechanism			■
Long lasting protection		■	
Long term storage		■	
Metabolised and excreted quickly			■
Narrow spectrum			■
No environemental impact	■	■	■
No off target toxicity			■
Novel mechanism	■		
Patentable compound and/or mechanism	■		
Resistance impossible to evolve			■
Safe to manufacture, transport and administer	■	■	
Works in all fish species		■	■

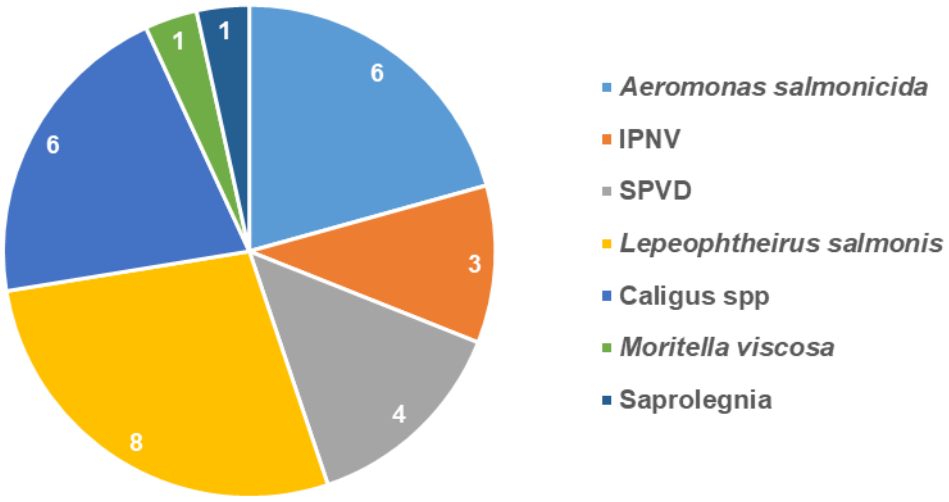

Figure 1.6. Number of veterinary drugs currently authorized for salmon in UK by pathogens type (Veterinary Medicine Directorate., 2018).

4. Identify drugs closely related chemically to the ones active against amoebic and kinetoplastid pathogens organisms.

5. Compile information on each drug that is important for subsequent regulatory approval as a new anti-AGD agent and score each drug for information availability.

6. Investigate drug target presence in AGD according to drug targets information compiled.

7. Prioritise drugs according to drug targets presence in AGD.

8. Speculate on rational drug combination therapy for proposing a treatment for AGD.

Chapter 2: Identification of diseases related to AGD and the drugs used for treatment

2.1. Introduction

Amoebic gill disease (AGD) is a serious growing threat in the salmon industry for two main reasons. First, this disease is related to warmer water temperatures and climate change is leading to increased seawater temperatures in salmon-farming areas (Johnson-Mackinnon et al., 2016). Second, genetic selection in the salmon industry has focussed for many years on fish growth rate without taking into account a balance with disease resistance (Nowak., 2007; Oldham et al., 2016), although efforts have been made recently to incorporate AGD resistance genes as part of wider programmes of genetic selection. The current existing solutions to AGD are bath treatments of freshwater and hydrogen peroxide (Rodger., 2014) with freshwater bath treatments representing a significant part of the production cost (i.e. 10-20%) in Tasmania and, more recently, millions of USD in Norway, Scotland and Ireland (Munday et al., 2001; Shinn et al., 2015). Therefore, it is desirable to extend the therapeutic arsenal against AGD particularly through identification of new drugs, as administration of medicines (fed or bath) in aquaculture is a practical means of disease control. Furthermore, until genetically resistant hosts are produced on a large scale and the development of vaccines against AGD are developed and licenced, there will be a need for chemotherapeutants to treat outbreaks.

The aim of this Chapter was to identify, prioritise and propose drugs used previously against non-fish amoebic and kinetoplastid infections for possible re-purposing to treat AGD using publicly-available information in various bibliographic databases. The following objectives were proposed:

1. Identify related diseases affecting humans, animals and plants caused by similar organisms to the aetiological agent of AGD, the amoeba *Neoparamoeba perurans* and its kinetoplastid endosymbiont, *Perkinsela* sp.).

2. Identify drugs previously and currently used to treat these diseases.

3. Identify drugs related to those identified in objective 2.

4. Develop a scoring system that incorporates available information on each compound to inform the potential of each compound to be developed further for use as a medicine in aquaculture.

2.2. Materials and Methods

2.2.1. Database and bibliographic inclusion criteria

All the bibliographic research was performed using information gathered mainly from Google Scholar (https://scholar.google.com), PubMed (www.ncbi.nlm.nih.gov), Wiley Online Library (https://onlinelibrary.wiley.com) and Science Direct (https://www.sciencedirect.com) databases. Drug research was performed using Anatomical Therapeutic Chemical (ATC: https://www.whocc.no/atc_ddd_index/) and Kyoto Encyclopedia of Genes and Genomes (KEGG: https://www.genome.jp/kegg/drug/) using U.S. Pharmacopeia (USP) classification. Drug information was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov), DrugBank (https://www.drugbank.ca), Food and Drug Administration (https://fda.gov) and the British National Formulary (BNF, 2011). Bibliographic research was performed in English and only articles published in English between 1900 to 2018 were included because this language represents the most common for scientific literature. Database searches were performed for the "exact phrase" (only available for Google Scholar) and search terms in the titles and abstracts of the articles. From the databases, the types of peer-reviewed articles taken into account in our study were: book chapters, brief communications, brief reports, case reports, correspondence, editorial comments, invited reviews, meta-analyses, mini-reviews, perspectives, rapid communications, research articles, reviews, seminars, short papers and symposia. Patent literature was excluded from the analysis to avoid potential infringements in the absence of "freedom to operate" analysis and because molecules identified and patented do not necessarily include extensive additional data beyond initial demonstration of effect on the target organism (*i.e* they may not have been tested for user, environmental and target animal safety, residue data, which is a prerequisite of the scoring system planned for use in this study). For each document fulfilling our inclusion criteria, each abstract was read to determine its relevance for inclusion in the

study. The bibliographic research strategy was based on chronological research for each disease which means from earliest to most recent studies. The aim of this approach was to see the evolution of effective treatments over time for all the considered diseases and provide an exhaustive list of drugs for re-purposing in the case of AGD (Figure 2.1).

2.2.2. Information on related diseases

The databases cited above were searched to identify literature on amoebic and kinetoplastid organisms causing diseases using the keywords searches: “amoebic”, “kinetoplastid”, “disease”, “human” and “animals” in different combinations using Boolean search terms (i.e. “amoebic AND disease AND human”, “amoebic AND disease AND animals”, “kinetoplastid AND disease AND human”, “kinetoplastid AND disease AND animal”). Once the name of the disease or the name of a pathogenic organism was found, respectively the binomial name was included in subsequent searches of the same databases to reveal similar diseases or similar organisms causing diseases. For example, the research terms “amoebic AND disease AND human” led to identification of “amoebic keratitis” to be caused by *Acanthamoeba* sp. Thus, the query “*Acanthamoeba* sp. AND disease AND human” was performed subsequently to search each database again, which in turn led to identification of ‘granulomatous amoebic encephalitis’ and so on (Table 2.1). The searches of amoebic diseases were performed between April and May 2018, while and the kinetoplastid research was performed between May and June 2018. As a result, any studies published or uploaded after June 2018 are excluded.

2.2.3. Literature on treatment of related diseases

The research of treatments for related diseases caused by amoebae and kinetoplastids was performed using the same databases as Section 2.2.2. The keywords used were the name of the disease and/or the name of the aetiological agent followed by “treatment” and/or “drug” (i.e. “Amoebiasis treatment”, “*Entamoeba histolytica* treatment”, “Amoebiasis drug”, “*Entamoeba histolytica* drug”, “Amoebic Keratitis (AK)

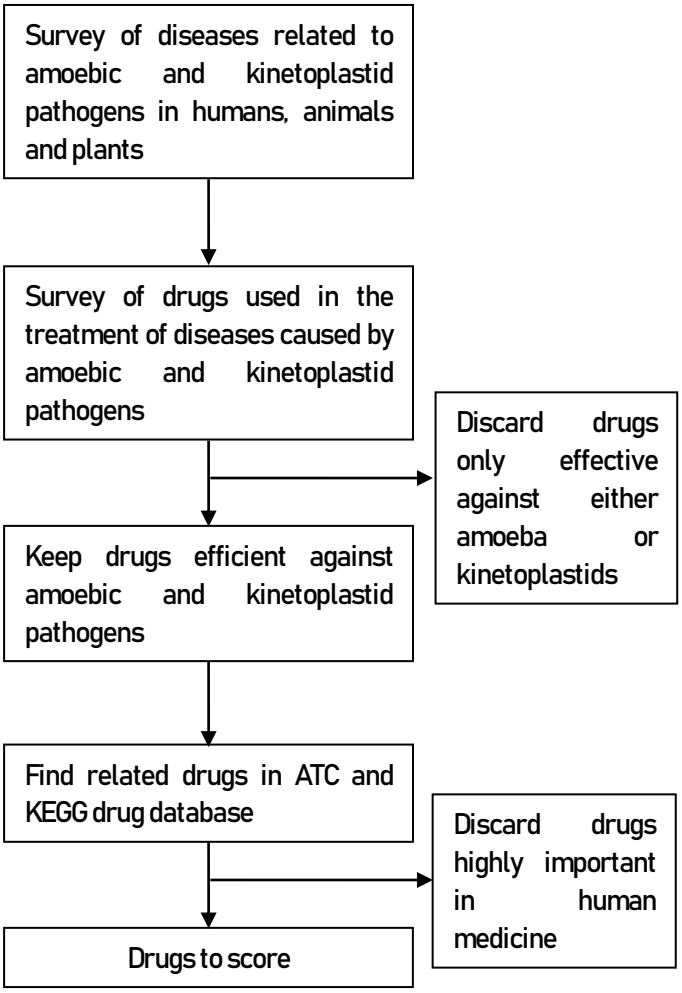

Figure 2.1. Diagram representing the drug identification process in the study from early stage (disease considered) to late stage (drug to score). ATC: Anatomical therapeutic chemical. KEGG: Kyoto encyclopedia of genes and genomes.

Table 2.1. Overview of diseases caused by amoebic and kinetoplastid organisms in non-fish hosts.

Disease	Aetiological agent(s)	Host	Source
Amoeba			
Amoebiasis			
	Entamoeba histolytica	Human	Stanley., 2003
		Non-Human primates	Stanley., 2003
Amoebic keratitis			
	Acanthamoeba castellanii	Human	Moore et al., 1985
	Acanthamoeba culberstoni	Human	Martinez and Visvesvara., 1997
	Acanthamoeba griffini	Human	Marciano-Cabral & Cabral., 2003
	Acanthamoeba hatchetti	Human	Martinez and Visvesvara., 1997
	Acanthamoeba lugdunensis	Human	Marciano-Cabral & Cabral., 2003
	Acanthamoeba polyphaga	Human	Moore et al., 1985
	Acanthamoeba quina	Human	Marciano-Cabral & Cabral., 2003
	Acanthamoeba rhysodes	Human	Martinez and Visvesvara., 1997
Balamuthia amoebic encephalitis			
	Balamuthia mandrillaris	Colobus monkey	Foreman et al., 2004
		Dog	Foreman et al., 2004
		Gibbon	Foreman et al., 2004
		Gorilla	Kinde et al., 1998
		Horse	Kinde et al., 1998
		Human	Visvesvara et al., 1990
		Mandrill	Visvesvara et al., 1990
		Orangutan	Foreman et al., 2004
		Sheep	Kinde et al., 1998

Granulomatous amoebic encephalitis

Acanthamoeba castellanii	Human	Seijo Martinez et al., 2000
Acanthamoeba culberstoni	Dog	Bauer et al., 1993
	Horse	Kinde et al., 2007
	Human	Bauer et al., 1993

Kinetoplastids

Leishmaniasis

Leishmania amazonensis	Human	Weigle and Saravia., 1996
Leishmania braziliensis	Human	Herwaldt et al., 1992
Leishmania donovani	Human	Sundar., 2001
Leishmania guyanensis	Human	Weigle and Saravia., 1996
Leishmania infantum	Dog	Adler and Tchernomoretz., 1946
	Human	Chappuis et al., 2007
Leishmania lainsoni	Human	Santos et al., 2008
Leishmania major	Human	Alrajhi et al., 2002
Leishmania mexicana	Human	Herwaldt et al., 1992
Leishmania naiffi	Human	Santos et al., 2008
Leishmania panamensis	Human	Weigle and Saravia., 1996
Leishmania peruviana	Human	Weigle and Saravia., 1996
Leishmania pifanoi	Human	Santos et al., 2008
Leishmania shawi	Human	Santos et al., 2008
Leishmania tropica	Human	Palumbo., 2009
Leishmania venezuelensis	Human	Weigle and Saravia., 1996

Primary amoebic meningoencephalitis

Naegleria fowleri	American tapir	Lozano-Alarcón et al., 1997
	Human	Martinez et al., 1973
	Mouse	Martinez et al., 1973

Trypanosomiasis

Trypanosoma brucei	Cat	Thomas., 1905a
	Cattle	Losos and Ikede., 1972
	Dog	Thomas., 1905a
	Goat	Losos and Ikede., 1972
	Guinea-pig	Thomas., 1905a
	Horse	Losos and Ikede., 1972
	Monkey	Losos and Ikede., 1972
	Mouse	Merchant., 1947
	Rabbit	Thomas., 1905a
	Rat	Thomas., 1905a
	Sheep	Losos and Ikede., 1972
Trypanosoma brucei brucei	Human	Brun et al., 2010
Trypanosoma brucei gambiense	Cat	Thomas., 1905a
	Cattle	Losos and Ikede., 1972
	Dog	Thomas., 1905a
	Goat	Losos and Ikede., 1972
	Guinea-pig	Thomas., 1905a
	Horse	Losos and Ikede., 1972
	Human	Pearce., 1921
	Monkey	Losos and Ikede., 1972
	Rabbit	Thomas., 1905a
	Rat	Thomas., 1905a
	Sheep	Losos and Ikede., 1972
Trypanosoma brucei rhodesiense	Cat	Losos and Ikede., 1972
	Cattle	Losos and Ikede., 1972
	Dog	Losos and Ikede., 1972
	Goat	Losos and Ikede., 1972
	Horse	Losos and Ikede., 1972
	Monkey	Losos and Ikede., 1972
	Rodent	Losos and Ikede., 1972
	Sheep	Losos and Ikede., 1972

Trypanosoma congolense	Cat	Losos and Ikede., 1972
	Cattle	Losos and Ikede., 1972
	Dog	Andrews., 1912
	Goat	Losos and Ikede., 1972
	Horse	Andrews., 1912
	Human	Brun et al., 2010
	Monkey	Losos and Ikede., 1972
	Mule	Andrews., 1912
	Rodent	Losos and Ikede., 1972
	Sheep	Andrews., 1912
Trypanosoma cruzi	Cat	Thomas., 1905a
	Dog	Thomas., 1905a
	Guinea-pig	Thomas., 1905a
	Human	Stuart et al., 2008
	Rabbit	Thomas., 1905a
	Rat	Thomas., 1905a
Trypanosoma dimorphon	Cat	Thomas., 1905a
	Dog	Thomas., 1905a
	Guinea-pig	Thomas., 1905a
	Rabbit	Thomas., 1905a
	Rat	Thomas., 1905a
Trypanosoma equinum	Cat	Thomas., 1905a
	Dog	Thomas., 1905a
	Guinea-pig	Thomas., 1905a
	Human	Pearce., 1921
	Rabbit	Thomas., 1905a
	Rat	Thomas., 1905a
Trypanosoma equiperdum	Cat	Thomas., 1905a
	Dog	Thomas., 1905a
	Guinea-pig	Thomas., 1905a
	Human	Pearce., 1921

	Rabbit	Thomas., 1905a
	Rat	Thomas., 1905a
Trypanosoma evansi	Cat	Thomas., 1905a
	Dog	Thomas., 1905a
	Guinea-pig	Thomas., 1905a
	Human	Pearce., 1921
	Rabbit	Thomas., 1905a
	Rat	Thomas., 1905a
Trypanosoma hippicum	Mouse	Merchant., 1947
Trypanosoma musculi	Mouse	Evans and Brightman., 1980
Trypanosoma vivax	Cat	Losos and Ikede., 1972
	Cattle	Losos and Ikede., 1972
	Dog	Losos and Ikede., 1972
	Goat	Losos and Ikede., 1972
	Horse	Losos and Ikede., 1972
	Monkey	Losos and Ikede., 1972
	Rodent	Losos and Ikede., 1972
	Sheep	Losos and Ikede., 1972

treatment", "*Acanthamoeba* sp. treatment", "AK drug", "*Acanthamoeba* sp. drug", "*Balamunthia* Amoebic Encephalitis (BAE) treatment", "*Balamunthia mandrillaris* treatment", "BAE drug", "*Balamunthia mandrillaris* drug", "Granulomatous Amoebic Encephalitis (GAE) treatment", "*Acanthamoeba* sp. treatment", "GAE drug", "*Acanthamoeba* sp. drug", "Leishmaniasis treatment", "*Leishmania* sp. treatment", "Leishmaniasis drug", "*Leishmania* sp. drug", "Primary Amoebic Meningoencephalitis (PAM) treatment", "*Naegleria fowleri* treatment", "PAM drug", "*Naegleria fowleri* drug", "Trypanosomiasis treatment", "*Trypanosoma* sp. treatment", "Trypanosomiasis drug", "*Trypanosoma* sp. drug"). The types of considered treatments were currently used clinical treatments, *in vitro* studies, *in vivo* studies and clinical trials (phase I, II and III). Only successful treatments were taken into account so to focus only on drugs with proven efficacy.

2.2.4. Research of related drugs

Drugs with proven efficacy against amoebic and kinetoplastid pathogens were the most important for this present study because they are more likely to exert activity against both *N. perurans* and the intra-amoebic *Perkinsela* sp. symbiont, as there are likely to be shared target pathways within these organisms. The drugs with activity against amoebic and kinetoplastid pathogens were researched in ATC and KEGG databases in July 2018. The ATC classification system classifies active compounds at five levels, notably: anatomical main group, therapeutic subgroup, pharmacological subgroup, chemical subgroup and chemical substance. The U.S. Pharmacopeia classification system is based on the drug biological activities (e.g. analgesics, antibacterials, antineoplastics). These two classification systems are complementary because one is based mostly on chemical family while the other is based on biological activities. The use of two different classification systems confers benefit from providing more results and allowing for confirmation of the results obtained from one database to the other.

2.2.5. Characteristics of related drugs

To evaluate the potential of the related drugs for application to the treatment of AGD, it was first necessary to collect information about the basic characteristics of each compound, specifically: molecular formula, molecular weight, class (Medical Subject Headings (MeSH) Pharmacological Classification), physical description, solubility, melting point (°C), and LogP (also called Log *Kow*). Thereafter, in the context of possible application of the compounds in a fish farm environment, information on some other criteria were also collected, specifically: current legislative status with respect to approval for medicinal use in humans and/or animals according to two administrative authorities, the *FDA* and *BNF*. Information relating to the biological actions of each drug was investigated with respect to the following criteria: drug target and broad action (i.e. substrate, inhibitor, agonist, antagonist, binder), pharmacodynamics, specific mechanism of action, bioavailibility/absorption, half-life, and route of administration (e.g. topical, oral, intravenous). Finally, potential environmental and financial considerations were also researched: safety and hazards, toxicity, and price (USD/g).

2.2.6. Scoring of drugs

The scoring system was designed on the presence or absence of certain information for each drug. In total, 13 criteria were used to achieve a score for each drug between 1 and 13, and the following criteria were selected for inclusion in the scoring scheme because of their relevance for subsequent application as potential anti-AGD compounds at salmon fish farms: *FDA* status (approved or unapproved), *BNF* status (licensed or unlicensed), target/action, pharmacodynamics, mechanism of action, solubility, melting point (°C), LogP, half-life, administration, use, safety and hazards, and toxicity. Presence of information relating to each criterion represented 1 point, and this meant that the maximum achievable score was 13. Thereafter, drugs were ranked from high information availability (13/13) to no information availability (0/13).

2.3. Results

In total, 184 studies were identified that contained information useful to the identification of amoebic and kinetoplastid diseases of humans, animals and plants related to the causative agent of AGD. Among the 184 studies, 107 concerned amoebic pathogens while 77 focused on kinetoplastid pathogens (Figure 2.1). The diversity of documents (i.e. book chapter, brief communication, brief report, case report, correspondence, editorial comment, invited review, meta-analysis, minireview, perspective, rapid communication, research article, review, seminar, short paper and symposium) was greater for amoebic diseases compared to kinetoplastid diseases (Figure 2.2). Research articles were the most represented type of study in the case of amoebic and kinetoplastid diseases, with *n*=48 and *n*=50 respectively. In the case of amoebic diseases, the next most represented type of study were case reports (*n*=20) and reviews (*n*=18), whereas in the case of kinetoplastid diseases it was reviews (*n*=17) and case reports (*n*=4) (Figure 2.2). The five selected diseases of human and animals caused by amoebic organisms are: amoebiasis, amoebic keratitis (AK), granulomatous amoebic encephalitis (GAE), balamuthia amoebic encephalitis (BAE) and primary amoebic meningoencephalitis (PAM). The two kinetoplastid diseases of mammals identified were leishmaniasis (caused by *Leishmania* sp.) and trypanosomiasis (caused by *Trypanosoma* sp.). However, some diseases were not taken in account in this present study. Specifically, the amoebic diseases, *Sappinia diploidea* and *Paravalkampfia francinae*, were not considered as only one report case exists for each pathogen (in 1998 and 2009, respectively; (Sathornsumetee., 2017; Schuster & Visvesvara., 2004). In the case of kinetoplastid diseases, *Ichtyobodo necator*, a fish external parasite was also not included when researching the treatments for this disease because most of the treatments for this disease are already well-known (e.g. acetic acid, betadine, formaldehyde, copper sulphate, permanganate potassium and salt) in aquaculture and the goal of this present study was to highlight compounds for re-purposing in treating AGD (Kayis et al., 2009).

The number of studies or drugs differ considerably between the different diseases (Figure 2.3). Trypanosomiasis, leishmaniasis and amoebiasis have the most published studies (27, 20 and 17 studies, respectively), which may reflect the economic importance

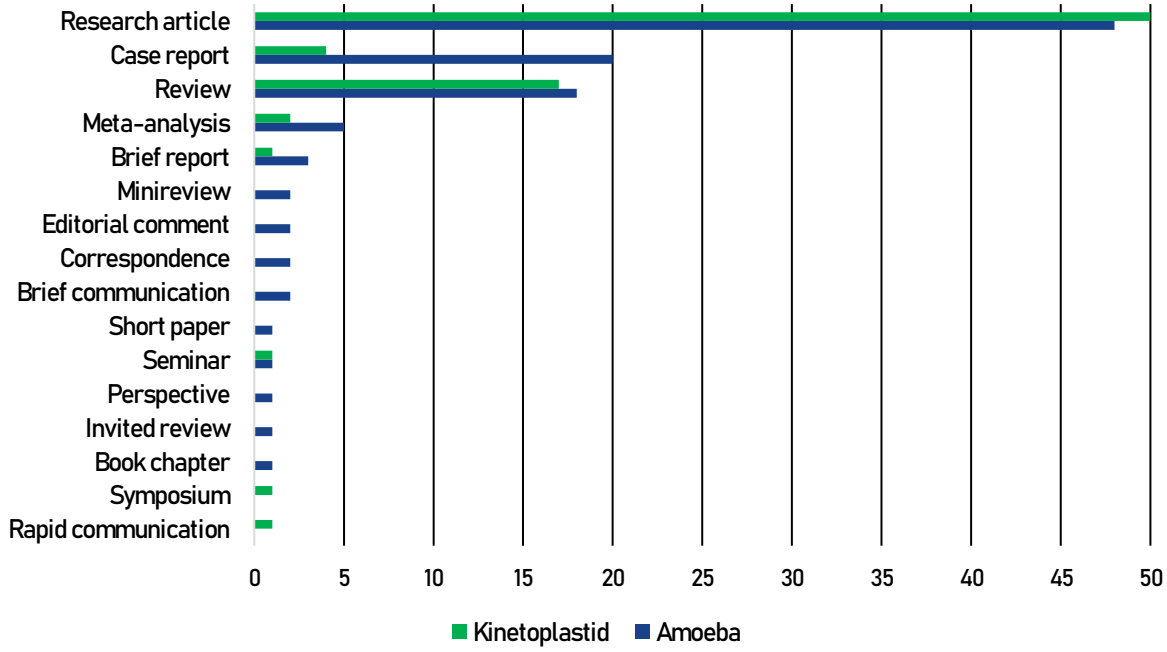

Figure 2.2. Bar graph of abundances of types of studies retrieved from *Google Scholar* (https://scholar.google.com), *PubMed* (www.ncbi.nlm.nih.gov), *Wiley Online Library* (https://onlinelibrary.wiley.com) and *Science Direct* (https://www.sciencedirect.com) databases regarding amoebic (blue, *n*: 107) and kinetoplastid (green, *n*: 77) pathogen organisms.

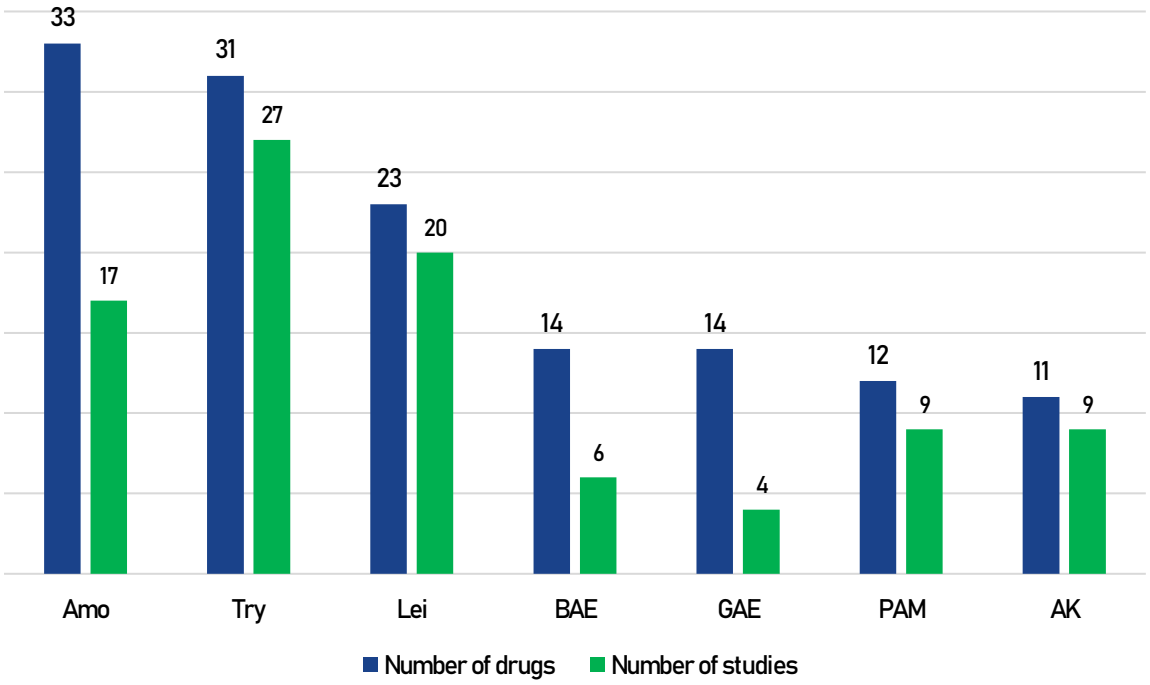

Figure 2.3. Bar graph representing the number of drugs (blue) used effectively to treat amoebic and kinetoplastid diseases and the number of studies containing this information (green). Amo (Amoebiasis); Try (Trypanosomiasis); Lei (Leishmaniasis); BAE (Balamuthia amoebic encephalitis); GAE (Granulomatous amoebic encephalitis); PAM (Primary amoebic meningoencephalitis); AK (Amoebic

of these disesaes and because they have been studied since the beginning of the 20th century (Figure 2.4). The other diseases, PAM, AK, BAE and GAE, constitute a more homogenous group regarding number of studies (9, 9, 6 and 4 studies, respectively) and the literature on these diseases commenced more recently (mid to late 20th-century) (Figure 2.4). Leishmaniasis and trypanosomiasis are the two diseases that possess the greatest number of pathogens able to cause the diseases, with 15 and 13 species, respectively (Table 2.1). AK and GAE are caused by 8 species and 2 species respectively from the genus *Acanthamoeba* (Table 2.1). In contrast, amoebiasis, BAE and PAM are caused by a single pathogen.

In total, 120 drugs were identified to be efficacious in the treatment of these seven diseases. Of these 120 drugs, 68 were used to treat amoebic diseases and 52 were for kinetoplastid diseases (Table 2.2). Amoebiasis, trypanosomiasis and leishmaniasis are the diseases for which the number of drugs used was the highest (33, 31 and 23 drugs, respectively) (Figure 2.3). In all other diseases considered (BAE, GAE, PAM and AK), this number was reduced and more homogenous (14, 14, 12 and 11 drugs, respectively). Nine drugs have been used effectively to treat both amoebic and kinetoplastid diseases, specifically amphotericin B, fluconazole, itraconazole, ketoconazole, metronidazole, miltefosine, paromomycin, pentamidine and tinidazole (Figure 2.5).

Starting from the nine drugs used against both amoebic and kinetoplastid disease, 142 related drugs were found, of which 102 were from the ATC database and 66 were from the KEGG database using UPS classification system (parameter selected in KEGG) (Figure 2.6; Figure 2.7). This process has led to overlapping results from one database to the other which explains why the sum of the results of each database was superior (168) compare to the actual number of drugs identified which is 142 (Figure 2.6; Table 2.3; Table 2.4). The drugs which possess the most related structures are metronidazole (n=59), miltefosine (n=56), ketoconazole (n=50), fluconazole (n=49), amphotericin B (n=46), itraconazole (n=31), tinidazole (n=30), paromomycin (n=17) and pentamidine (n=14) (Figure 2.6). Among these 142 drugs, 24 drugs were discarded before the scoring process because of their high importance in human medicine (Collignon et

al. 2016); indeed, these drugs are essential in human medicine according to the World Health

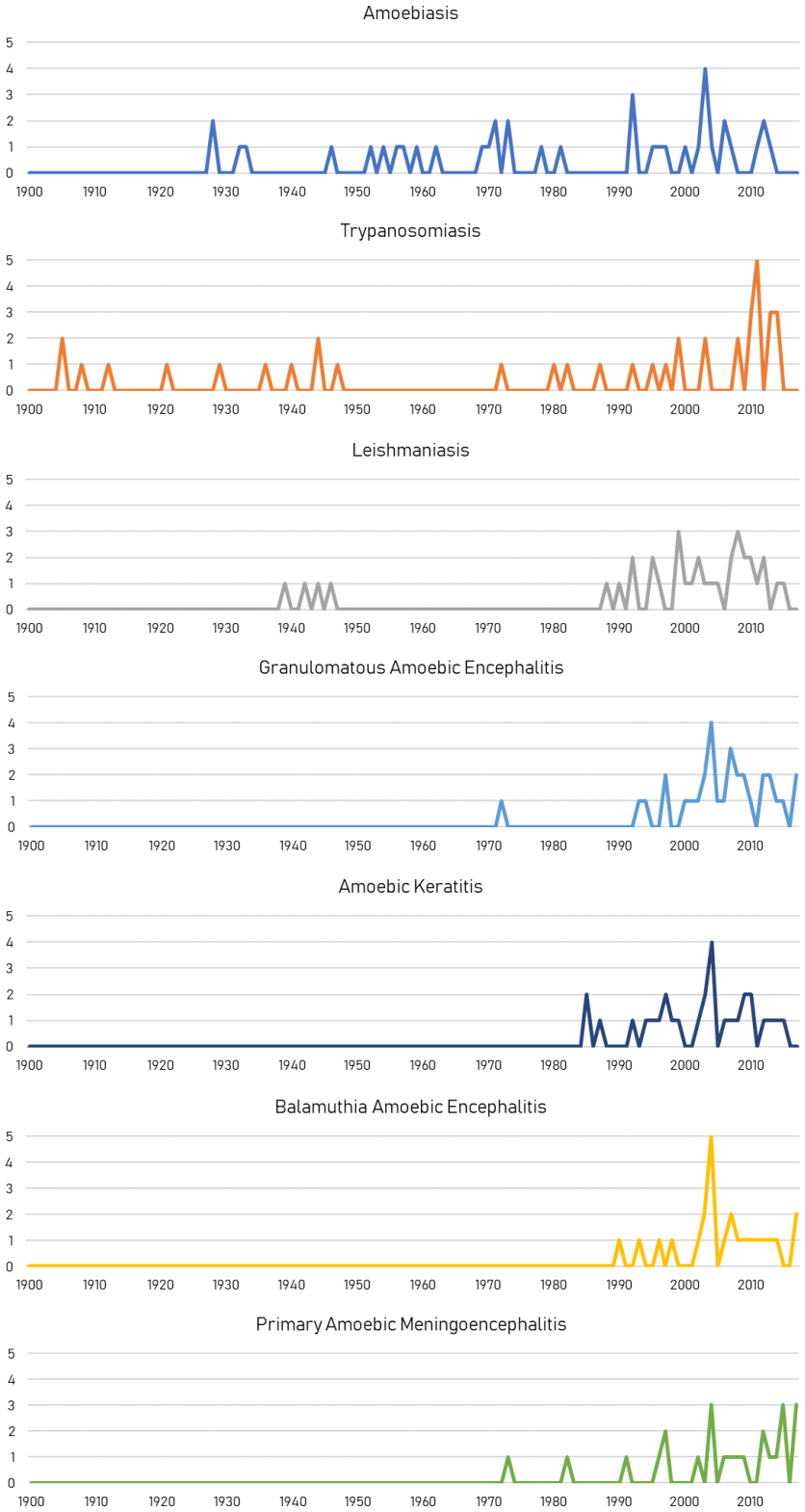

Figure 2.4. Line graph representing the number of peer review articles (in ordinate) published overtime (in abscissa) regarding the amoebic and kinetoplastid diseases considered in the study.

Table 2.2. Overview of the drugs used over time against amoebic and kinetoplastid diseases affecting humans, non-fish animals and plants.

Disease	Drug	Model	Source
Amoebiasis			
	Acetarsol	Human	Gilles and Tompkins., 1962
	Azithromycin	Human	Khaw and Panosian., 1995
	Camoform	Dog-Hamster-Human-Rat	Bustamente and Rivero., 1957
		Human	Woodruff et al., 1956
	Carbarsone	Human	Hamilton., 1954
		Human-Rat	Jones., 1946
	Chiniofon	Human-Rat	Jones., 1946
	Chloramphenicol	Human	Woodruff et al., 1956
	Chlorbetamide	Human	Powell., 1969
	Chloroquine	Human	Hamilton., 1954
	Clefamide	Human	Botero., 1978
	Clioquinol (Iodochlorhydroxyquin)	Human	Hamilton., 1954
	Difretarsone	Human	Powell., 1969
	Diloxanide	Human	Marie and Petri., 2013
	Diloxanide furoate	Human	Blessmann et al., 2006
		Human	Botero., 1978
		Human	Haque et al., 2003
		Human	Khaw and Panosian., 1995
		Human	McAuley and Juranek., 1992
		Human	Reed., 1992
		Human	Wolfe., 1973
	Emetine	Human	Khaw and Panosian., 1995
	Emetine Bismuth Iodide	Human	Woodruff et al., 1956
	Emetine HCL	Rat	Jones., 1946
	Etofamide	Human	Botero., 1978

Fumagillin	Human	Killough et al., 1952
Glaucarubin	Human	Woodruff et al., 1956
Glycobiarsol	Human	Hamilton., 1954
Iodoquinol	Human	Khaw and Panosian., 1995
	Human	McAuley and Juranek., 1992
	Human	Reed., 1992
Mebendazole	Human	Davila-Gutierrez et al., 2002
Mepacrine (Quinacrine)	Human	Hamilton., 1954
Metronidazole	Human	Blessmann et al., 2006
	Human	Haque et al., 2003
	Human	Khaw and Panosian., 1995
	Human	Reed., 1992
Niridazole	Human	Powell., 1969
Nitazoxanide	Human	Davila-Gutierrez et al., 2002
Ornidazole	Human	Reed., 1992
Paromomycin	Human	Blessmann et al., 2006
	Human	Haque et al., 2003
	Human	Khaw and Panosian., 1995
	Human	McAuley and Juranek., 1992
	Human	Reed., 1992
Quinfamide	Human	Davila-Gutierrez et al., 2002
Secnidazole	Human	Salles et al., 2007
Stovarsol	Human-Rat	Jones., 1946
Teclozan	Human	Botero., 1978
Thiacetarsamide	Human	Powell., 1969
Thiocarbarsone	Human	Hamilton., 1954
Tinidazole	Human	Haque et al., 2003
	Human	Khaw and Panosian., 1995
	Human	Reed., 1992

Amoebic keratitis

Atropin	Human	Larkin et al., 1992
Chlorhexidine	Human	Kosrirukvongs et al., 1999
	Human	Lim et al., 2008
	Human	Lindquist., 1998
	in vitro	Seal et al., 1996
Clotrimazole	Human	Kosrirukvongs et al., 1999
Cyclopentaloate	Human	Larkin et al., 1992
Dexamethasone	Human	Larkin et al., 1992
	Human	Larkin et al., 1992
Dibromopropamidine	Human	Wright et al., 1985
Neomycin	Human	Kosrirukvongs et al., 1999
	Human	Larkin et al., 1992
	Human	Lindquist., 1998
Polyhexamethylene biguanide	Human	Larkin et al., 1992
	Human	Larkin et al., 1992
	Human	Lim et al., 2008
	Human	Lindquist., 1998
	Human	Martinez and Visvesvara., 1997
Prednisolone	Human	Wright et al., 1985
Propamidine	Human	Kosrirukvongs et al., 1999
	Human	Larkin et al., 1992
	in vitro	Seal et al., 1996
Propamidine isethionate	Human	Lindquist., 1998
	Human	Martinez and Visvesvara., 1997
	Human	Wright et al., 1985
Voriconazole	Human	Bang et al., 2010
Balamuthia amoebic encephalitis		
Albendazole	Human	Martínez et al., 2010
Azithromycin	Human	Deetz et al., 2003

	Human	Orozco et al., 2011
Clarithromycin	Human	Deetz et al., 2003
	Human	Jung et al., 2004
Fluconazole	Human	Deetz et al., 2003
	Human	Jung et al., 2004
	Human	Martínez et al., 2010
	Human	Orozco et al., 2011
Flucytosine	Human	Deetz et al., 2003
	Human	Orozco et al., 2011
Miltefosine	Human	Martínez et al., 2010
	Human	Orozco et al., 2011
	in vitro	Schuster et al., 2006
Pentamidine	Human	Deetz et al., 2003
	Human	Jung et al., 2004
	Human	Orozco et al., 2011
Sulfadiazine	Human	Deetz et al., 2003
	Human	Jung et al., 2004
	Human	Orozco et al., 2011
Thioridazine	Human	Deetz et al., 2003
Trifluoperazine	Human	Deetz et al., 2003
Voriconazole	*in vitro*	Schuster et al., 2006
Granulomatous amoebic encephalitis		
Chlorhexidine gluconate	Human	Martinez and Visvesvara., 1997
Fluconazole	Human	Seijo Martinez et al., 2000
Itraconazole	Human	Martinez and Visvesvara., 1997
Ketoconazole	Human	Martinez and Visvesvara., 1997
	Human	Singhal et al., 2001

Miconazole	Human	Martinez and Visvesvara., 1997
Miltefosine	Human	Webster et al., 2012
Pentamidine	Human	Martinez and Visvesvara., 1997
Rifampin	Human	Singhal et al., 2001
Sulfadiazine	Human	Seijo Martinez et al., 2000
Sulfametazine	Human	Martinez and Visvesvara., 1997
Trimethropim-sulfamethoxazole	Human	Singhal et al., 2001
Voriconazole	Human	Webster et al., 2012

Leishmaniasis

4:4'-diamidino stilbene	Human	Adler and Rachmilewitz., 1939
Allopurinol	Human	Monzote., 2009
	Dog	Noli and Auxilia., 2005
Amphotericin B	Human	Amato et al., 2007
	Human	Croft., 2008
	Human	Gradoni et al., 2003
	Human	Meyerhoff., 1999
	Human	Monzote., 2009
	Human	Murray., 2004
	Human	Santos et al., 2008
Fluconazole	Human	Alrajhi et al., 2002
	Human	Monzote., 2009
	Human	Palumbo., 2009
Hexadecylphosphocholine	Mouse	Kuhlencord et al., 1992
Imiquimod	Human	Croft., 2008
Itraconazole	Human	Monzote., 2009
Ketoconazole	Human	Monzote., 2009
Meglumine antimoniate	Human	Amato et al., 2007
	Human	Chulay et al., 1988

	Human	Croft., 2008
	Human	Gradoni et al., 2003
	Human	Monzote., 2009
	Human	Murray., 2004
	Dog	Noli and Auxilia., 2005
	Human	Palumbo., 2009
	Human	Santos et al., 2008
Miltefosine	Human	Croft., 2008
	Human	Dorlo et al., 2012
	Human	Jha et al., 1999
	Human	Monzote., 2009
	Human	Murray., 2004
	Human	Sundar et al., 2000
	Human	Sundar., 2001
Paromomycin	Human	Croft., 2008
	Human	Meyerhoff., 1999
	Human	Monzote., 2009
	Human	Murray., 2004
	Dog	Noli and Auxilia., 2005
	Human	Santos et al., 2008
	Human	Sundar., 2001
Pentamidine	Human	Amato et al., 2007
	Human	Croft., 2008
	Human	Humphreys., 1942
	Human	Meyerhoff., 1999
	Human	Monzote., 2009
	Dog	Noli and Auxilia., 2005
	Human	Palumbo., 2009
	Human	Santos et al., 2008
	Human	Sundar., 2001
Pentavalent antimony	Human	Badaro et al., 1990

Posaconazole	Human	Monzote., 2009
Sitamaquine	Human	Croft., 2008
	Human	Monzote., 2009
	Human	Moore and Lockwood., 2010
	Human	Sundar., 2001
Sodium stibogluconate	Human	Amato et al., 2007
	Human	Chulay et al., 1988
	Human	Croft., 2008
	Human	Monzote., 2009
	Human	Murray., 2004
	Human	Palumbo., 2009
	Human	Santos et al., 2008
Primary amoebic meningoencephalitis		
Amphotericin B	Human	Barnett et al., 1996
	Human	Brown., 1991
	Human	Brown., 1991
	in vitro	Kim et al., 2008
	Human	Martinez and Visvesvara., 1997
	Human	Seidel et al., 1982
	Human	Seidel et al., 1982
Azithromycin	Human	Linam et al., 2015
Bis-Benzimidazole	*in vitro*	Rice et al., 2015
Chlorpromazine	*in vitro*	Kim et al., 2008
Corifungin	Mouse	Debnath et al., 2012
Fluconazole	Human	Linam et al., 2015
Miconazole	Human	Barnett et al., 1996
	Human	Seidel et al., 1982
	Human	Seidel et al., 1982
Miltefosine	*in vitro*	Kim et al., 2008

	Human	Linam et al., 2015
	in vitro	Schuster et al., 2006
Rifampin	Human	Barnett et al., 1996
	Human	Brown., 1991
	Human	Linam et al., 2015
	Human	Seidel et al., 1982
Sulfixosazole	Human	Seidel et al., 1982
Voriconazole	*in vitro*	Schuster et al., 2006
Trypanosomiasis		
4:4'-diamidino stilbene	Human	McLetchie., 1940
Arsenophenylglycin	Cattle-Dog-Horse-Mule-Sheep	Andrews., 1912
Atoxyl	Cattle-Dog-Horse-Mule-Sheep	Andrews., 1912
	Cat-Dog-Guinea pig-Rabbit-Rat	Thomas., 1905a
Auranofin	*in vitro*	da Silva et al., 2014
Bayer 205 (suramin sodium)	Human	Brun et al., 2010
	Human	Croft., 2008
	Human	Docampo and Moreno., 2003
	Human	Maclean., 1929
	Human	McLetchie., 1940
	Human	Nok., 2003
	Human	Steverding., 2010
Benznidazole	Human	Barrett and Croft., 2012
	Human	Croft., 2008
	Human	Trunz et al., 2011
Benzoxaborole	Human-Mouse	Barrett and Croft., 2012
	Mouse	Eperon et al., 2014
	Dog	Eperon et al., 2014
DB829	Mouse	Wenzler et al., 2013

Eflornithine	Human	Brun et al., 2010
	Human	Croft., 2008
	Human	Docampo and Moreno., 2003
	Human	Eperon et al., 2014
	Human	Milord et al., 1992
	Human	Steverding., 2010
	Human	Taelman et al., 1987
Fexinidazole	Mouse	Barrett and Croft., 2012
	Mouse	Eperon et al., 2014
	Human	Tarral et al., 2014
	Human	Trunz et al., 2011
Melarsoprol	Human	Brun et al., 2010
	Human	Brun et al., 2010
	Human	Croft., 2008
	Human	Docampo and Moreno., 2003
	Human	Legros et al., 1999
	Human	Nok., 2003
	Human	Rhind et al., 1997
	Human	Steverding., 2010
Metronidazole	Human	Trunz et al., 2011
Neocryl	Mouse	Yorke et al., 1936
	Human	Yorke et al., 1936
Nifurtimox	Human	Barrett and Croft., 2012
	Human	Croft., 2008
	Human	Steverding., 2010
Novoflavin	Cattle-Dog-Horse-Mule-Sheep	Andrews., 1912
Oxaborole	Human	Tarral et al., 2014
Pafuramidine	Human	Brun et al., 2010
	Human	Croft., 2008
Pentamidine	Human	Brun et al., 2010

	Human	Croft., 2008
	Human	Docampo and Moreno., 2003
	Human	Nok., 2003
	Human	Saunders et al., 1944
	Human	Steverding., 2010
Phenamidine	Dog	Carmichael., 1944
Quinine	Cattle-Dog-Horse-Mule-Sheep	Andrews., 1912
Salicylhydroxamic acid	Mouse	Evans and Brightman., 1980
Salvarsan	Cattle-Dog-Horse-Mule-Sheep	Andrews., 1912
Sodium antimonyl tartrate	Rat	Plimmer and Thomson., 1908
Sodium arsenite	Cattle-Dog-Horse-Mule-Sheep	Andrews., 1912
Streptomycin	Mouse, chick embryo	Merchant., 1947
Tinidazole	Human	Trunz et al., 2011
Trypan blue	Cattle-Dog-Horse-Mule-Sheep	Andrews., 1912
Trypan red	Cat-Dog-Guinea pig-Rabbit-Rat	Thomas., 1905a
	Horse	Thomas., 1905b
Tryparsamide	Human	Maclean., 1929
	Human	McLetchie., 1940
	Human	Pearce., 1921

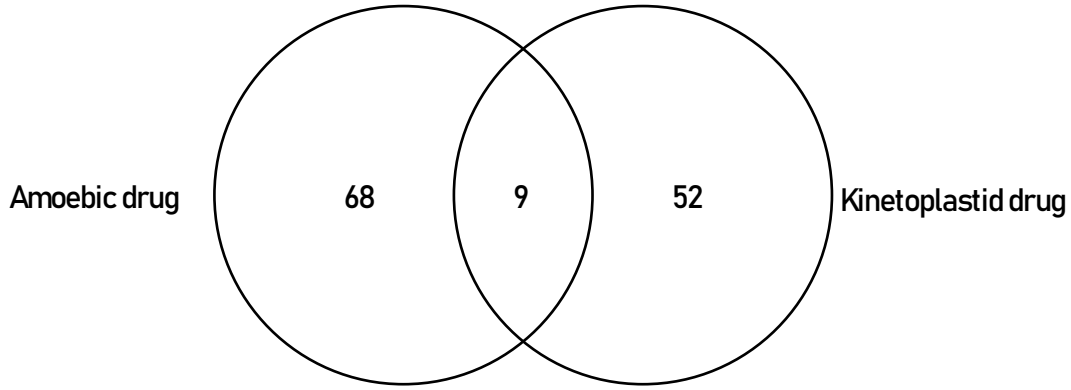

Figure 2.5. Venn diagram representing the number of drugs considered in the study of which drugs used in the case of amoebic diseases (n=68), drugs used in the case of kinetoplastids diseases (n=52), drugs effective in the case of both amoebic and kinetoplastids diseases (n=9).

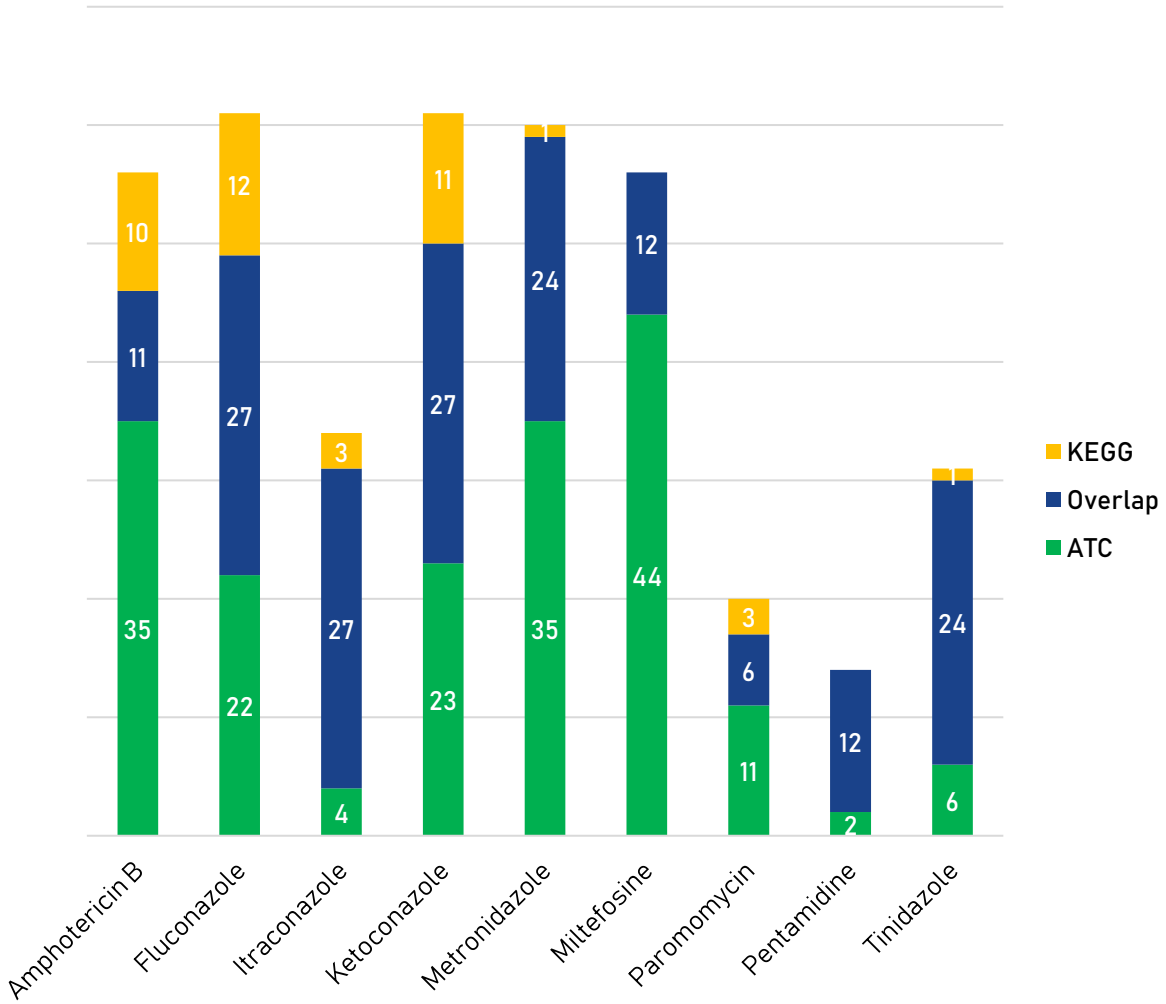

Figure 2.6. Bar graph representing the number of related drugs after researching with anatomical therapeutic chemical (ATC only; in green) database, Kyoto encyclopedia of genes and genomes (KEGG only; in yellow) and the overlapping of the results between the two databases (in blue).

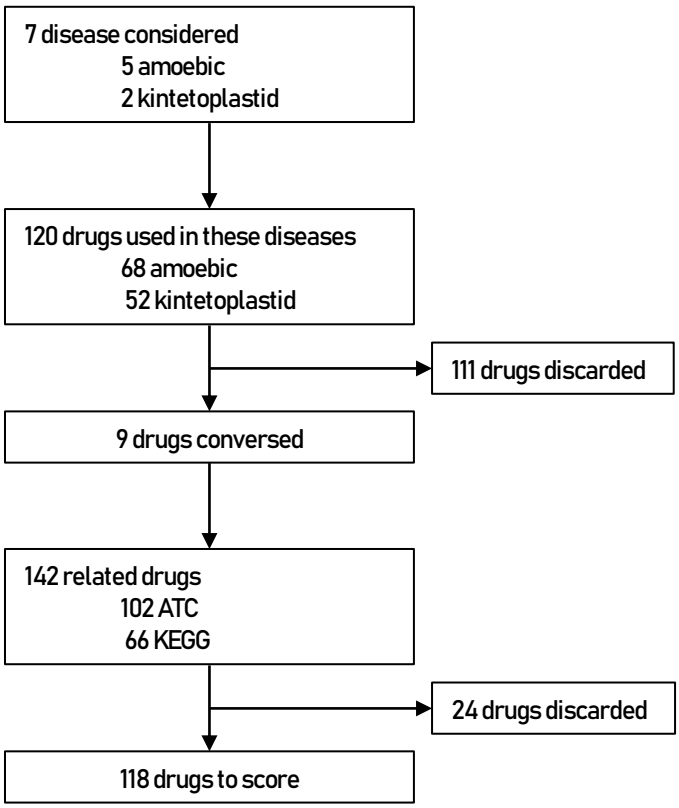

Figure 2.7. Diagram representing the drug identification process in the study from early stage (disease considered) to late stage (drug to score). ATC: Anatomical therapeutic chemical. KEGG: Kyoto encyclopedia of genes and genomes.

Table 2.3. Investigation performed in anatomical therapeutic chemical (ATC) database of the 9 drugs (amphotericin B, fluconazole, itroconazole, ketoconazole, metronidazole, miltefosine, paromomycin, pentamidine and tinidazole) affecting amoebic and kinetoplastid organism.

Drug name	ATC code	Anatomical main group	Chemical family	Number of related drugs
Amphotericin B				
	A01AB04	Alimentary tract and metabolism	Antiinfectives and antiseptics for local oral treatment	20
	A07AA07	Alimentary tract and metabolism	Antibiotics	13
	G01AA03	Genito urinary system and sex hormones	Antibiotics	11
	J02AA01	Antiinfectives for systemic use	Antibiotics	1
Fluconazole				
	D01AC15	Dermatologicals	Imidazole and triazole derivatives	18
	J02AC01	Antiinfectives for systemic use	Triazole derivatives	4
Itraconazole				
	J02AC02	Antiinfectives for systemic use	Triazole derivatives	4
Ketoconazole				
	D01AC08	Dermatologicals	Imidazole and triazole derivatives	18
	G01AF11	Genito urinary system and sex hormones	Imidazole derivatives	15
	J02AB02	Antiinfectives for systemic use	Imidazole derivatives	1
Metronidazole				
	A01AB17	Alimentary tract and metabolism	Antiinfectives and antiseptics for local oral treatment	20
	D06BX01	Dermatologicals	Other chemotherapeutics	1
	G01AF01	Genito urinary system and sex hormones	Imidazole derivatives	15
	J01XD01	Antiinfectives for systemic use	Imidazole derivatives	2
	P01AB01	Antiparasitic products, insecticides and repellents	Nitroimidazole derivatives	6
Miltefosine				
	L01XX09	Antineoplastic and immunomodulating agents	Other antineoplastic agents	48
Paromomycin				
	A07AA06	Alimentary tract and metabolism	Antibiotics	11
Pentamidine				

	P01CX01	Antiparasitic products, insecticides and repellents	Other agents against leishmaniasis and trypanosomiasis	2
Tinidazole				
	J01XD02	Antiinfectives for systemic use	Imidazole derivatives	2
	P01AB02	Antiparasitic products, insecticides and repellents	Nitroimidazole derivatives	6

Table 2.4. Investigation performed in Kyoto Encyclopedia of Genes and Genomes (KEGG) database of the 9 drugs (amphotericin B, fluconazole, itroconazole, ketoconazole, metronidazole, miltefosine, paromomycin, pentamidine and tinidazole) affecting amoebic and kinetoplastid organism.

Drug name	Code	Class	Related compound
Amphotericin B	D00203	Antibiotics	11
Fluconazole	D00322	Antifungals	27
Itraconazole	D00350	Antifungals	27
Ketoconazole	D00351	Antifungals	27
Metronidazole	D00409	Antibacterials	24
Miltefosine	D02494	Antiprotozoals	12
Paromomycin	D07467	Antibacterials	6
Pentamidine	D00834	Antiprotozoals	12
Tinidazole	D01426	Antibacterials	24

Organisation and were excluded to minimise the risk of resistance being exacerbated through their use in non-human therapy (Collignon et al. 2016).

The scoring was subsequently performed for the 118 drugs remaining at the end of the drug identification process (Figure 2.7). Among the 118 drugs scored, 60 of them obtained a score between 10 and 13 (Figure 2.8). The most represented scores were respectively, ten (18 drugs), twelve (17 drugs) and thirteen (17 drugs), which gives a great opportunity to work with drugs for which a lot of relevant information is available (Figure 2.8). According to the heat map (Figure 2.9), among the nine drugs researched in ATC and KEGG databases, amphotericin B (score=13), fluconazole (score=13), itraconazole (score=13), ketoconazole (score=13), pentamidine (score=13) and miltefosine (score=10) remain for further consideration.

2.4. Discussion

AGD is one of the most important gill-affecting diseases impacting the salmon farming industry. This is partially due to high-density farming systems, based mostly on the farming of one species (i.e. *Salmo salar*) which favours the emergence and expansion of diseases like AGD. The existing solutions for this major disease are either freshwater or hydrogen peroxide bath treatments, which provide short-term reductions in infection levels on fish but do not provide any residual protection to prevent re-infection. It is thus essential that medicines are developed or re-purposed to provide longer term protection and to provide suitable control measures to eradicate this disease in the aquaculture industry.

In this present study, the approach was performed relying on bibliographic research of similar pathogens to AGD, as well as their associated treatments, to identify related drugs that could be re-purposed for the treatment of AGD in a salmon farming context. Knowing that AGD results of a symbiotic association of an amoeba (*N. perurans*) and a kinetoplastid (*Perkinsela* sp.), we therefore researched amoebic and kinetoplastid diseases in the scientific literature. This led to studying the evolution of treatments over time for five key amoebic diseases (Amoebiasis, AK,

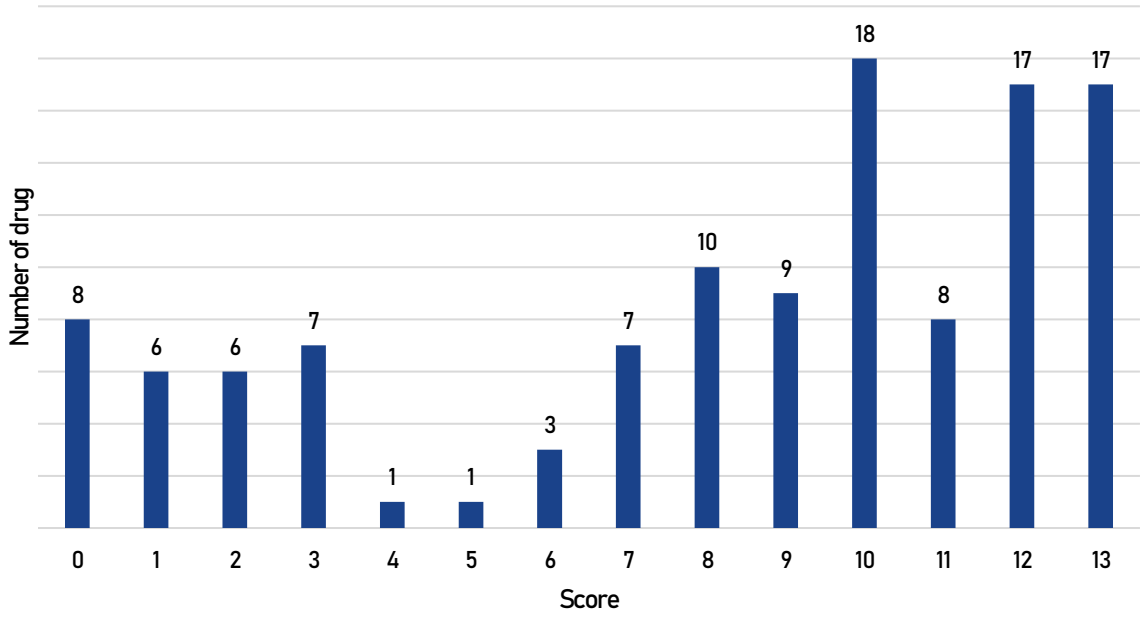

Figure 2.8. Diagram representing the distribution of scored drugs in our study from none information score (0) to high information score (13). The higher the score is, the more details we have on a given drug according to various criteria (status *FDA*, status *BNF*, target/action, pharmacodynamics, mechanism of action, solubility, melting point (°C), LogP, half-life, administration, utilisation, safety and hazards and toxicity).

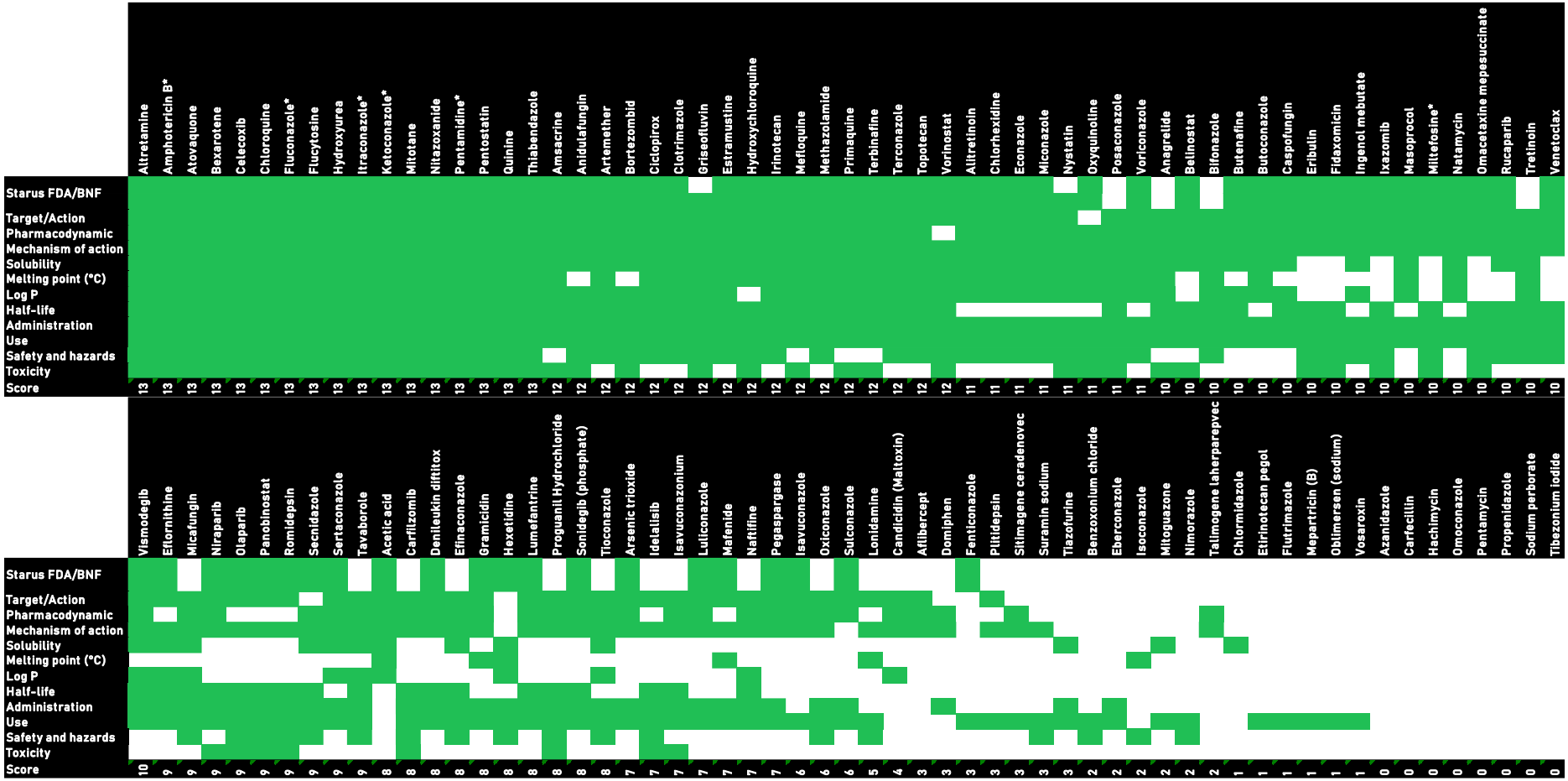

Figure 2.9. Heat map representing the amount of information available for the 118 scored drugs according to 13 criteria (displayed on the left of the figure). Green cell: information available. White cell: information unavailable. *: drug used to make the research of related drugs in anatomical therapeutic chemical (ATC) database and kyoto encyclopedia of genes and genomes (KEGG) database. The score which is reflecting the total amount of information for a given drug and is ranging from 13 (all information available) to 0 (none information available).

BAE, PAM) and two kinetoplastids diseases (leishmaniasis and trypanosomiasis). It is important to note some kinetoplastids from the genus *Phytomonas* are pathogens for plants (*Phytomonas satheli* causes “fatal wilt of coconut palm”, *Phytomonas leptovasorum* causes “coffee phloem necrosis” and *Phytomonas françai* causes “empty root syndrome”) (Jaskowska et al., 2015), but the current treatment for these diseases involves burning the infected area or removing of the infected part from the tree (Camargo., 1999), which is not helpful when pursuing as a new treatment for AGD. Among the considered diseases herein, 120 drugs were referenced in our research and among them, nine were used against both amoebic and kinetoplastid diseases. The interest in these drugs resides in their potential ability to act against both symbionts of AGD and thus increases the likelihood to find a new and effective treatment for this pathogen. In order to increase the probability of identifying a new treatment and also the variety of options, two drug databases (ATC and KEGG) were utilised to highlight similar compounds according to drug categories. This strategy has led to the potential number of drugs to be considered as 142, up from 9 originally identified through the literature searches. The next step was to select the most relevant drugs among these 142 to ensure no conflict with their use in human medicine. Antimicrobial resistance in bacteria is a major concern for human health and thus, in order to avoid any cross-resistance, all compounds considered as critically/highly important regarding human medicine were removed (Collignon et al. 2016). This present study aims for a sustainable solution so it is important to avoid the selection or enrichment of any potential drug-resistance by releasing these drugs more widely in the aquatic environment. At this stage, 118 drugs were still available to be processed by the scoring system, which relied on information availability relevant for development of any drug as a new treatment for AGD.

The desk-based approach taken in this present study was useful as it provides an evidence-based list of potential medicines that could be utilised to treat AGD in fish prior to conducting potentially more costly *in vitro and in vivo* studies and may reduce the overall number of non-successful animal experiments. Additionally, these kinds of desk-based studies can be used to monitor and reduce fatal adverse drug reactions (FADRs), inappropriate drug use (IDU) or adverse drug effects (ADEs) in human medicine (Cohen.,

2001; Johnell & Fastbom., 2008; Wester et al., 2008). The number of databases consulted and the keywords used in the present study, are relevant when compare to similar study (Boxall et al. 2011). Regarding the drug research databases selected for searches, according to the International Conference of Harmonisation (ICH), the World Health Organisation (WHO) is the most appropriate to set standards related to pharmaceutical and thus, using the ATC database created by the WHO was also a relevant choice (Lewis-Lettington et al., 2004). The scoring system set up in this present study is comparable to other studies which rely on calculation for prioritisation (Boxall et al. 2011). However, the scoring used is a step in the prioritisation of potential medicines for AGD.

The approach taken in this study has identified 118 potential compounds which could be used to combat AGD in salmon. The next iteration of this work will be to refine the list of candidate medicines by understanding the likely target pathways of the drugs and to propose a short-list of molecules that could be used to target both amoeba and its endosymbiont in a possible combination therapy approach.

Chapter 3: Prioritisation of drug candidates and proposal of possible drug combinations

3.1. Introduction

The present study aims to identify potential new treatments for AGD. In Chapter 2, 118 drugs with potential activity against AGD were scored according to the amount of information available on each of them and notably regarding drug targets, pharmacodynamics and mechanism of action and target categorisation (i.e. human, bacterial, fungal, protozoal and archaea). These drug candidates need to be prioritised such that the most promising ones can be evaluated further for their development as new chemotherapeutants for treating AGD notably by designing a rational combination therapy based on evidence of drug targets present in AGD. To do this, a target-based strategy of selection was performed in order to first differentiate drugs which are likely to affect targets in AGD and drugs without any evidence of drug target presence in AGD.

Drug research and target discovery have undergone remarkable changes over time and currently two main strategies are applied. The systems approach relies on disease study based on animal models and patients, identify targets through forward/reverse genetics and clinical sciences and validate targets by modulation in animal models (KO/transgenic mice) (Lindsay., 2003). The molecular approach relies on disease study based on cells and clinical samples, identify targets through genomics, proteomics, genetic association and forward genetics and validate targets by monitoring disease tissue expression as well as modulation in cell models (mRNA KO/protein overexpression) (Lindsay., 2003). Many diseases are treated using single compounds and such monotherapy is simple from a practical point of view because it needs just one approval from the appropriate health authorities (Woodcock et al., 2011). However, in some cases monotherapy leads to the evolution of resistance in the pathogen and a decrease in efficacy such as in the case for visceral leishmaniasis where pentamidine cure rate moved from 99% to 69-78% after approximatively a decade of use in India because of the emergence of resistant strains of *Leishmania* sp. to pentamidine (Sundar., 2001). Drug resistance to monotherapy has also been observed in the treatment of tuberculosis (*Mycobacterium tuberculosis*), invasive aspergillosis (*Aspergillus fumigatus*),

hospital-acquired infection (*Pseudomonas aeruguinosa*) and malaria (*Plasmodium falciparum*) (Amato et al., 2007; Kerantzas & Jacobs., 2017; Klastersky & Zinner., 1982; White., 2004). One way to slow or prevent the appearance of drug resistant strains is to increase the therapeutic dose, however, this may increase the extent of side effects (Sundar., 2001). One proposed solution to limit the appearance of drug resistance in monotherapy treatment is combination therapy. Synergy of antibiotics used in combination is defined by supra-additive interaction such as sequential blockade in the same metabolic pathway (e.g. trimethoprim and sulphonamides), inhibition of essential enzymes (e.g. clavulanic acid and penicillins) and potentiator effect such as permeabilization of bacterial membrane done by one drug which allow a better penetration of a second drug (e.g. penicillin and aminoglycosides) (Klastersky & Zinner., 1982). As an example, combination of antifungal drugs showed several advantages against invasive aspergillosis such as more rapid antifungal effect, lowered doses of toxic drugs, drug synergy, widened spectrum against more species that cause fungal disease, potency of drug activity and reduced risk of antifungal resistance (Steinbach et al., 2003). Therefore, combination therapy offers a possible solution and this approach is used to combat several diseases visceral leishmaniasis (e.g. amphotericin B and miltefosine), malaria (e.g. mefloquine and artesunate), community acquired pneumonia (β-lactam and macrolide) and tuberculosis (e.g. penicillin, streptomycin, arsphenamine and para-aminosalicylic acid) (Colombel et al., 2010; Möttönen et al., 1999; van Griensven et al., 2010; Kerantzas & Jacobs., 2017; Hutchinson et al., 1999; Rodrigo et al., 2013; Beshir et al., 2013). Some molecules like enzymes represent a widespread and well-known source of targets in the pharmaceutical industry and this is of key relevance in developing a novel combination therapy (Drews., 2000).

Currently, therapeutic options for AGD which rely on freshwater or hydrogen peroxide bath immersion are susceptible to attest treatment resistance from AGD over time (Rodger., 2014). Repeated exposure of *Neoparamoeba perurans* to freshwater or hydrogen peroxide may lead to the emergence of resistant strains and this could leave the Atlantic salmon farming industry without any options to treat AGD (Wright et al., 2018). Thus, it is important to take into account the potential for emergence of treatment

resistance in order to propose a reliable treatment which can sustain efficacy for a longer period, and a future combination therapy could provide an answer in the development of new treatments for AGD.

The present study aims to identify potential new treatments for AGD among 118 drugs previously scored in the Chapter 2. The main purpose of the Chapter 3 is to prioritise drugs according to the presence of drug targets in AGD. This approach relied upon two online and reliable tools which are Interactive Pathways Explorer (iPath) and Basic Local Alignment Search Tool (BLAST). The use of these two web-based tools provide information on the presence/absence of metabolic pathways and drug targets in *Neoparamoeba perurans* and *Perkinsela* sp. These two databases are complementary in terms of fulfilling the overall aim of this chapter to prioritise the scored drugs according to the identification of their targets in AGD and for including in possible combination therapies to treat AGD. To do this, the following objectives needed to be addressed:

1. Identify and list the known drug targets of the 118 scored drugs in Chapter 2.

2. Listing of drug targets Gene ID and FASTA sequence after investigation of their name in National Center for Biotechnology and Information (NCBI) Gene database.

3. Investigation drug target names in iPath.

4. BLAST the FASTA sequences of the drug targets using BLAST programs in order to identify drug targets presence in the genome of *Neoparamoeba pemaquidensis* and *Perkinsela* sp.

5. Use information of metabolic pathways targeted by the drugs to propose potential combination therapies targeting the same or different metabolic pathways.

3.2. Materials and methods

3.2.1. Interactive Pathways Explorer

Interactive Pathways Explorer (iPath; https://pathways.embl.de/) is a web-based tool that allows visualisation, customisation and analysis of metabolic pathways using

an interactive map. The information available derives from data in other databases, and in this present study the data from Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways, KEGG Reactions and KEGG Compounds, was the most useful. Both versions of iPath (*i.e* v2: pathways2.embl.de/iPath2.cgi#; v3: https://pathways.embl.de/ipath3.cgi?map=metabolic) were used herein because the main map "metabolic pathway" layout was changed for some of the metabolic pathways displayed from one version to the other. Thus, in order to benefit of the latest updates of iPath and also be able to compare metabolic map from previous version, iPath v2 and v3 were used in this study.

3.2.2. BLAST software

BLAST is a web-based tool that allows comparison of amino acid sequences or nucleotide sequences to sequences available in the database to identify similar sequences. The sequence query can be used to calculate the statistical significance of a possible match (i.e. their similarity according to the nucleotide or amino acid position in the sequence and the coverage of the sequence). This program can perform different kinds of comparisons and this present study relied on BLASTn (where a nucleotide sequence is compared to other nucleotide sequences) and BLASTx (where a 6-frame translated nucleotide sequence is compared to a protein). BLASTn parameters used were: program optimise for highly similar sequences (megablast), nucleotide collection (nr/nt) database for inter-species sequence comparisons and identification of nucleotidic sequences. However, as *N. pemaquidensis* and *Perkinsela sp.* could be genetically distant from the sequence of the reference organism's sequence (e.g. *Homo sapiens*, *Leishmania major*, *Acanthamoeba castellanii*) used because of their respective evolution over time, a BLASTx was also performed in order to identify potential protein encoded by a nucleotide sequence with the following parameters: organism search set (CCAP 1560/4 taxid: 1314962 for *Perkinsela* sp.; taxid: 180228 for *N. pemaquidensis*), non-redundant (nr) protein sequences database. The results obtained with BLASTn and BLASTx should present at least 80% of query cover (i.e. describes how much the query sequence is covered by the target

sequence) and 30% identity (i.e. describes how similar the query sequence is to the target sequence) in order to be deemed relevant according to established guidelines (Pearson., 2013). Finally, the E value (expected value) that gives an indication about the significance of the match should be as close as possible to 0.

3.2.3. KEGG Enzyme database

The KEGG ENZYME database was used to obtain information regarding the enzymes targeted by each of the 118 scored drugs (https://www.genome.jp/kegg/annotation/enzyme.html). The output from this analysis was a list of the names of the enzymes targeted by each of the 118 scored drugs.

3.2.4. Data set

The genome assembly of *N. pemaquidensis* and *Perkinsela* sp. as well as iPath metabolic map software outputs (Figure 3.1) used were from Tanifuji et al. (2017). The genomes of *N. pemaquidensis* and *Perkinsela* sp. have been deposited in GenBank under accession numbers MUHK00000000 and LFNC00000000, respectively (Tanifuji et al. 2017).

3.2.5. Target identification in iPath

iPath was used to identify the presence of targets of the 118 scored drugs identified in Chapter 2 in *N. pemaquidensis* and *Perkinsela* sp. metabolic pathways (Figure 3.1). In the first place, the list of the 222 names of molecules targeted by each of the 118 scored drugs were queried in the iPath search bar in the “metabolism” map. For each match, the displayed maps were compared to the metabolic map of *N. pemaquidensis* prepared by Tanifuji et al. (2017) in order to confirm the presence/absence of the match in *N. pemaquidensis* or/and *Perkinsela sp.* metabolic pathways (Figure 3.1).

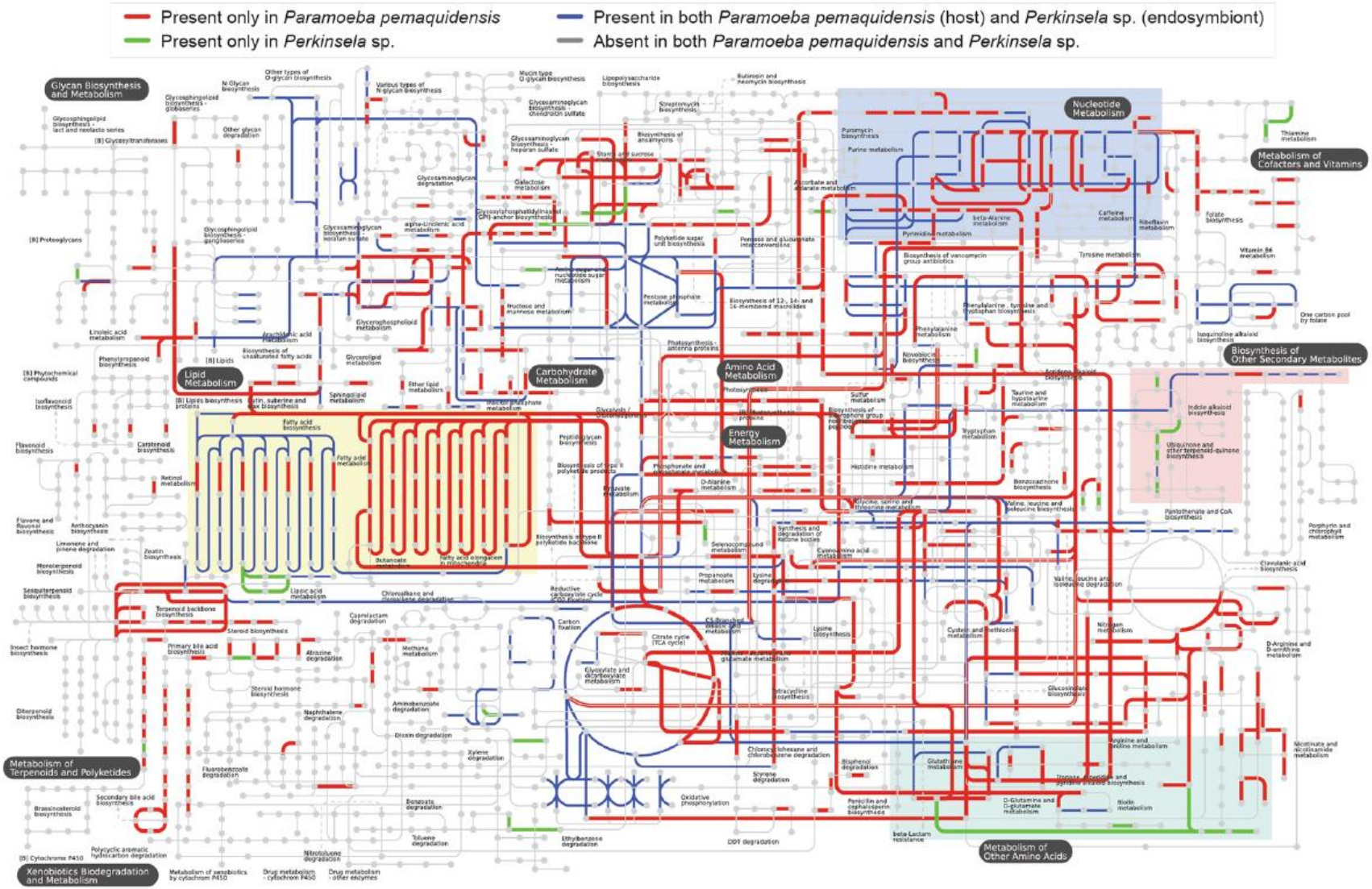

Figure 3.1. Metabolic map obtained with interactive Pathways Explorer 2 (iPath v2) displaying metabolic pathways identified in *Neoparamoeba pemaquidensis* (here called *Paramoeba pemaquidensis*) and *Perkinsela* sp. (Tanifuji et al., 2017).

3.2.6. Target identification in BLAST

The list of the names of the 222 targets targeted by the 118 scored drugs was queried in the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene/) to obtain the most similar gene sequences and FASTA files for each target (Table 3.1). For each target name, the gene sequence was investigated for kinetoplastids (i.e. *Leishmania* and *Trypanosoma*) and amoebic organisms (i.e. *Entamoeba* and *Acanthamoeba*) in the NCBI Gene database, as these are organisms most closely related to *N. perurans*. The gene sequence of each drug target in the genetically closest-related organism (i.e. *Leishmania* or *Trypanosoma* for kinetoplastids and *Entamoeba* or *Acanthamoeba* for amoebic organism) was selected for the gene sequence and FASTA file. Once all the target names had been queried in the NCBI database, the corresponding FATSA files were investigated using BLASTn and BLASTx. For each BLAST search, the investigation of the FASTA sequence was performed in *N. pemaquidensis* (taxid: 180228) and *Perkinsela* sp. (CCAP 1560/4 taxid: 1314962). The output of the analysis was the match of the FASTA sequence in the genome of *N. pemaquidensis* or *Perkinsela* sp. and all information related to the match (query cover, E value and identity) were indexed. The matches were considered if the query coverage was ≥80% and the identity ≥30% in both BLASTn and BLASTx.

3.2.7. Drug prioritisation and potential combination

The presence of drug target identified in *N. pemaquidensis* or *Perkinsela* sp. by iPath/BLAST was subsequently inquired into the 118 scored drugs from the Chapter 2 in order to identify related drug which have proven therapeutic activities against the investigated target. The drugs which have shown activities (e.g. inhibitor, substrate, antagonist) against identified targets in *N. pemaquidensis* and/or *Perkinsela* sp. were therefore considered as “prioritised” and were subsequently discussed for a combination therapy according to the involvement of their target in metabolic pathways. Information on the metabolic pathways containing enzymes targeted by the scored drugs was used to determine possible combinations of these. For combinations, two types were proposed: 1) combinations of drugs targeting the same metabolic pathway; or 2)

Table 3.1. List of the target names identified in NCBI Gene database, their origins and their Gene ID used to get the FASTA sequence investigated in the genome of *Neoparamoeba pemaquidensis* and *Perkinsela* sp. with BLASTn and BLASTx. Gene ID were obtained from NCBI Gene (https://www.ncbi.nlm.nih.gov/gene).

Target name	Organism	Gene ID
Gene ID used in *Neoparamoeba pemaquidensis*		
Human target		
11-hydroxylase	*Homo sapiens*	1584
17-hydroxylase	*Homo sapiens*	1586
3-phosphoinositide-dependent protein kinase 1	*Acanthamoeba castellanii*	14923178
60S ribosomal protein L3	*Acanthamoeba castellanii*	14920014
Acetylcholinesterase	*Acanthamoeba castellanii*	14923647
Adenosine Deaminase	*Acanthamoeba castellanii*	14911310
Adenosine receptor A2a	*Homo sapiens*	135
Adrenodoxin, mitochondrial	*Acanthamoeba castellanii*	14922966
Aldehyde oxidase	*Acanthamoeba castellanii*	14923076
Alpha-1-acid glycoprotein 1	*Homo sapiens*	5004
Androgen receptor	*Homo sapiens*	367
Apoptosis regulator Bcl-2	*Homo sapiens*	596
Arachidonate 5-lipoxygenase	*Homo sapiens*	240
Arylsulfatase A	*Homo sapiens*	410
ATP-binding cassette sub-family B member 5	*Homo sapiens*	340273
ATP-binding cassette sub-family G member 2	*Homo sapiens*	9429
ATP-sensitive inward rectifier potassium channel 11	*Homo sapiens*	3767
Beta-glucuronidase	*Acanthamoeba castellanii*	14918044
Bile salt export pump	*Homo sapiens*	8647
Calcium-activated potassium channel subunit alpha-1	*Homo sapiens*	3778
Calcium-activated potassium channel subunit beta-1	*Homo sapiens*	3779
Calcium-activated potassium channel subunit beta-2	*Homo sapiens*	10242
Calcium-activated potassium channel subunit beta-3	*Homo sapiens*	27094
Calcium-activated potassium channel subunit beta-4	*Homo sapiens*	27345
Canalicular multispecific organic anion transporter 1	*Entamoeba invadens*	14894231
Canalicular multispecific organic anion transporter 2	*Homo sapiens*	8714
Carbonic anhydrase 1	*Acanthamoeba castellanii*	14926610
Carbonic anhydrase 2	*Homo sapiens*	760
Carbonic anhydrase 3	*Homo sapiens*	761
Carbonic anhydrase 4	*Homo sapiens*	762
Carbonic anhydrase 6	*Homo sapiens*	765
Carbonic anhydrase 7	*Homo sapiens*	766

Carboxylesterase	*Acanthamoeba castellanii*	14924913
Catechol O-methyltransferase	*Homo sapiens*	1312
Cellular retinoic acid-binding protein 1	*Homo sapiens*	1381
Cellular retinoic acid-binding protein 2	*Homo sapiens*	1382
cGMP-inhibited 3',5'-cyclic phosphodiesterase A	*Homo sapiens*	5139
Cholesterol side-chain cleavage enzyme, mitochondrial	*Homo sapiens*	1583
Cholinesterase	*Acanthamoeba castellanii*	14926650
Cocaine esterase	*Cricetulus griseus*	100756666
Corticosteroid-binding globulin	*Homo sapiens*	866
Cyclin-dependent kinase inhibitor 1	*Homo sapiens*	1026
Cystic fibrosis transmembrane conductance regulator	*Homo sapiens*	1080
Cytochrome P450 11B1, mitochondrial	*Homo sapiens*	1584
Cytochrome P450 19A1	*Homo sapiens*	1588
Cytochrome P450 1A1	*Homo sapiens*	1543
Cytochrome P450 1A2	*Homo sapiens*	1544
Cytochrome P450 1B1	*Homo sapiens*	1545
Cytochrome P450 26A1	*Homo sapiens*	1592
Cytochrome P450 26B1	*Homo sapiens*	56603
Cytochrome P450 26C1	*Homo sapiens*	340665
Cytochrome P450 2A6	*Homo sapiens*	1548
Cytochrome P450 2B6	*Homo sapiens*	1555
Cytochrome P450 2C18	*Homo sapiens*	1562
Cytochrome P450 2C19	*Homo sapiens*	1557
Cytochrome P450 2C8	*Homo sapiens*	1558
Cytochrome P450 2C9	*Homo sapiens*	1559
Cytochrome P450 2D6	*Homo sapiens*	1565
Cytochrome P450 2E1	*Homo sapiens*	1571
Cytochrome P450 2J2	*Homo sapiens*	1573
Cytochrome P450 3A4	*Homo sapiens*	1576
Cytochrome P450 3A43	*Homo sapiens*	64816
Cytochrome P450 3A5	*Homo sapiens*	1577
Cytochrome P450 3A7	*Homo sapiens*	1551
Cytochrome P450 4A11	*Homo sapiens*	1579
Cytokine receptor common subunit gamma	*Homo sapiens*	3561
Dihydrofolate reductase	*Acanthamoeba castellanii*	14921939
Dihydroorotate dehydrogenase (quinone), mitochondrial	*Homo sapiens*	1723
Dimethylaniline monooxygenase [N-oxide-forming] 1	*Homo sapiens*	2326
Dimethylaniline monooxygenase [N-oxide-forming] 3	*Homo sapiens*	2328
DNA (cytosine-5)-methyltransferase 1	*Entamoeba histolytica*	3409584
DNA topoisomerase 1	*Acanthamoeba polyphaga mimivirus*	9924828
DNA topoisomerase 1, mitochondrial	*Homo sapiens*	116447
DNA topoisomerase 2-alpha	*Homo sapiens*	7153
Estrogen receptor alpha	*Homo sapiens*	2099
Estrogen receptor beta	*Homo sapiens*	2100
Extracellular calcium-sensing receptor	*Homo sapiens*	846

Folate transporter 1	*Homo sapiens*	6573
G protein-activated inward rectifier potassium channel 2	*Homo sapiens*	3763
G protein-activated inward rectifier potassium channel 3	*Homo sapiens*	3765
G1/S-specific cyclin-D1	*Homo sapiens*	595
Glutathione S-transferase A2	*Homo sapiens*	2939
Hematopoietic prostaglandin D synthase	*Homo sapiens*	27306
Hemoglobin subunit alpha	*Homo sapiens*	3039
Hexokinase-1	*Entamoeba histolytica*	3410328
Histamine N-methyltransferase	*Homo sapiens*	3176
Histone deacetylase	*Acanthamoeba castellanii*	14913131
Histone deacetylase 1	*Acanthamoeba castellanii*	14926624
Histone deacetylase 2	*Homo sapiens*	3066
Histone deacetylase 3	*Homo sapiens*	8841
Histone deacetylase 4	*Homo sapiens*	9759
Histone deacetylase 6	*Homo sapiens*	10013
Histone deacetylase 8	*Homo sapiens*	55869
Inhibitor of nuclear factor kappa-B kinase subunit beta	*Homo sapiens*	3551
Insulin-like growth factor-binding protein 3	*Homo sapiens*	3486
Interleukin-2 receptor subunit alpha	*Homo sapiens*	3559
Interleukin-2 receptor subunit beta	*Homo sapiens*	3560
Intermediate conductance calcium-activated potassium channel protein 4	*Homo sapiens*	3783
Keratin, type I cytoskeletal 12	*Homo sapiens*	3859
Keratin, type II cytoskeletal 7	*Homo sapiens*	3855
Lipocalin-1	*Homo sapiens*	3933
Liver carboxylesterase 1	*Acanthamoeba castellanii*	14916194
Microtubule-associated protein 1A	*Homo sapiens*	84557
Microtubule-associated protein 2	*Homo sapiens*	4133
Mitogen-activated protein kinase 1	*Homo sapiens*	5594
Mitogen-activated protein kinase 3	*Homo sapiens*	5595
Monocarboxylate transporter 1	*Homo sapiens*	6566
Multidrug and toxin extrusion protein 1	*Homo sapiens*	55244
Multidrug and toxin extrusion protein 2	*Homo sapiens*	146802
Multidrug resistance protein 1	*Homo sapiens*	5243
Multidrug resistance-associated protein 1	*Acanthamoeba castellanii*	14914395
Multidrug resistance-associated protein 4	*Acanthamoeba castellanii*	14915701
Nitric oxide synthase, endothelial	*Homo sapiens*	4846
Nitric oxide synthase, inducible	*Homo sapiens*	4843
Nuclear receptor subfamily 0 group B member 1	*Homo sapiens*	190
Nuclear receptor subfamily 1 group I member 2	*Homo sapiens*	8856
Nuclear receptor subfamily 1 group I member 3	*Homo sapiens*	9970
Odorant protein-binding 2a	*Homo sapiens*	29991
Ornithine decarboxylase	*Acanthamoeba castellanii*	14917597
Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic delta isoform	*Homo sapiens*	5290
Phospholipase d activity	*Acanthamoeba castellanii*	14917365
Placenta growth factor	*Homo sapiens*	5228
Platelet glycoprotein IX	*Homo sapiens*	2815

Poly [ADP-ribose] polymerase 1	*Homo sapiens*	142
Poly [ADP-ribose] polymerase 2	*Homo sapiens*	10038
Poly [ADP-ribose] polymerase 3	*Homo sapiens*	10039
Potassium voltage-gated channel subfamily A member 5	*Homo sapiens*	3741
Potassium voltage-gated channel subfamily D member 3	*Homo sapiens*	3757
Potassium voltage-gated channel subfamily H member 2	*Homo sapiens*	3752
Potassium voltage-gated channel subfamily H member 6	*Homo sapiens*	81033
Potassium voltage-gated channel subfamily H member 7	*Homo sapiens*	90134
Potassium voltage-gated channel subfamily KQT member 1	*Homo sapiens*	3784
Pregnancy-specific beta-1-glycoprotein 5	*Homo sapiens*	5673
Progesterone receptor	*Homo sapiens*	5241
Prostaglandin G/H synthase 1	*Homo sapiens*	5742
Prostaglandin G/H synthase 2	*Homo sapiens*	5743
Proteasome subunit beta type-1	*Acanthamoeba castellanii*	14918468
Proteasome subunit beta type-10	*Homo sapiens*	5699
Proteasome subunit beta type-2	*Homo sapiens*	5690
Proteasome subunit beta type-5	*Homo sapiens*	5693
Proteasome subunit beta type-8	*Homo sapiens*	5696
Proteasome subunit beta type-9	*Homo sapiens*	5698
Protein kinase C alpha type	*Homo sapiens*	5578
Protein kinase C delta type	*Homo sapiens*	5580
Protein PML	*Homo sapiens*	5371
Pyruvate dehydrogenase lipoamide kinase isozyme 4, mitochondrial	*Homo sapiens*	5166
RAC-alpha serine/threonine-protein kinase	*Acanthamoeba castellanii*	14914927
Retinal dehydrogenase 1	*Homo sapiens*	216
Retinal dehydrogenase 2	*Homo sapiens*	8854
Retinoic acid receptor alpha	*Homo sapiens*	5914
Retinoic acid receptor beta	*Homo sapiens*	5915
Retinoic acid receptor gamma	*Homo sapiens*	5916
Retinoic acid receptor responder protein 1	*Homo sapiens*	5918
Retinoic acid receptor RXR-alpha	*Homo sapiens*	6256
Retinoic acid receptor RXR-beta	*Homo sapiens*	6257
Retinoic acid receptor RXR-gamma	*Homo sapiens*	6258
Retinoic acid-induced protein 3	*Homo sapiens*	9052
Retinol-binding protein 4	*Homo sapiens*	5950
Ribonucleoside-diphosphate reductase large subunit	*Acanthamoeba polyphaga mimivirus*	9924930
Ribosyldihydronicotinamide dehydrogenase [quinone]	*Homo sapiens*	4835
Serum albumin	*Homo sapiens*	213
Sex hormone-binding globulin	*Homo sapiens*	6462
Small conductance calcium-activated potassium channel protein 1	*Homo sapiens*	3780

Small conductance calcium-activated potassium channel protein 2	*Homo sapiens*	3781
Small conductance calcium-activated potassium channel protein 3	*Homo sapiens*	3782
Smoothened homolog	*Homo sapiens*	6608
Sodium channel protein type 5 subunit alpha	*Homo sapiens*	6331
Sodium/potassium-transporting ATPase subunit alpha-1	*Homo sapiens*	476
Solute carrier family 22 member 1	*Homo sapiens*	6580
Solute carrier family 22 member 2	*Homo sapiens*	6582
Solute carrier family 22 member 3	*Homo sapiens*	6581
Solute carrier family 22 member 4	*Homo sapiens*	6583
Solute carrier family 22 member 5	*Homo sapiens*	6584
Solute carrier family 22 member 6	*Homo sapiens*	9356
Solute carrier family 22 member 8	*Homo sapiens*	9376
Solute carrier organic anion transporter family member 1A2	*Homo sapiens*	6579
Solute carrier organic anion transporter family member 1B1	*Homo sapiens*	10599
Solute carrier organic anion transporter family member 1B3	*Homo sapiens*	28234
Solute carrier organic anion transporter family member 2B1	*Homo sapiens*	11309
Squalene monooxygenase	*Homo sapiens*	6713
Steroid 21-hydroxylase	*Homo sapiens*	1589
Thioredoxin reductase 1, cytoplasmic	*Acanthamoeba castellanii*	14925107
Toll-like receptor 7	*Homo sapiens*	51284
Toll-like receptor 9	*Homo sapiens*	54106
Transcription factor AP-1	*Homo sapiens*	3725
tRNA (cytosine(38)-C(5))-methyltransferase	*Homo sapiens*	1787
Tubulin beta-1 chain	*Homo sapiens*	81027
Tumor necrosis factor	*Homo sapiens*	7124
UDP-glucuronosyltransferase 1-1	*Homo sapiens*	54658
UDP-glucuronosyltransferase 1-4	*Homo sapiens*	54657
UDP-glucuronosyltransferase 1-8	*Homo sapiens*	54576
UDP-glucuronosyltransferase 1-9	*Homo sapiens*	54600
Vascular endothelial growth factor A	*Homo sapiens*	7422
Vascular endothelial growth factor B	*Homo sapiens*	7423
Voltage-dependent L-type calcium channel subunit alpha-1C	*Homo sapiens*	775
Fungi target		
1,3-beta-d-glucan synthase activity	*Saccharomyces cerevisiae*	851055
1,3-beta-glucan synthase component FKS1	*Saccharomyces cerevisiae*	851055
Cytosolic leucyl-tRNA synthetase	*Acanthamoeba castellanii*	14912201
Ergosterol	*Acanthamoeba castellanii*	14920899
Lanosterol 14-aplha demethylase	*Acanthamoeba polyphaga mimivirus*	9925470
Lanosterol synthase	*Homo sapiens*	4047
Thymidylate synthase	*Acanthamoeba castellanii*	14914345
Tubulin alpha chain	*Acanthamoeba castellanii*	14922382

Tubulin beta chain	*Acanthamoeba castellanii*	14925210
Bacteria target		
16S rRNA	*Acanthamoeba castellanii*	1734019
Acetoin utilization protein	*Streptomyces coelicolor*	1098764
Chloramphenicol acetyltransferase	*Escherichia coli*	12657249
Cytochrome b	*Acanthamoeba castellanii*	1734035
Dihydroorotate dehydrogenase activity	*Escherichia coli*	945556
Fumarate reductase flavoprotein subunit	*Escherichia coli*	948667
Pyruvate-flavodoxin oxidoreductase	*Acanthamoeba castellanii*	14915700
RNA polymerase sigma factor	*Escherichia coli*	946839
Protozoa target		
Bifunctional dihydrofolate reductase-thymidilate synthase	*Acanthamoeba polyphaga mimivirus*	9925127
Fe(II)-protoporphyrin IX	*Homo sapiens*	213
Glutathione S-transferase	*Acanthamoeba castellanii*	14911711
Archaea target		
50S ribosomal protein L2	*Acanthamoeba castellanii*	1734043

Gene ID used in *Perkinsela* sp.

Human target		
11-hydroxylase	*Homo sapiens*	1584
17-hydroxylase	*Homo sapiens*	1586
3-phosphoinositide-dependent protein kinase 1	*Trypanosoma grayi*	20379852
60S ribosomal protein L3	*Leishmania major*	5656437
Acetylcholinesterase	*Leishmania major*	5649819
Adenosine Deaminase	*Leishmania major*	5654364
Adenosine receptor A2a	*Homo sapiens*	135
Adrenodoxin, mitochondrial	*Leishmania infantum*	5071629
Aldehyde oxidase	*Homo sapiens*	316
Alpha-1-acid glycoprotein 1	*Homo sapiens*	5004
Androgen receptor	*Homo sapiens*	367
Apoptosis regulator Bcl-2	*Homo sapiens*	596
Arachidonate 5-lipoxygenase	*Homo sapiens*	240
Arylsulfatase A	*Homo sapiens*	410
ATP-binding cassette sub-family B member 5	*Homo sapiens*	340273
ATP-binding cassette sub-family G member 2	*Homo sapiens*	9429
ATP-sensitive inward rectifier potassium channel 11	*Homo sapiens*	3767
Beta-glucuronidase	*Homo sapiens*	2990
Bile salt export pump	*Homo sapiens*	8647
Calcium-activated potassium channel subunit alpha-1	*Homo sapiens*	3778
Calcium-activated potassium channel subunit beta-1	*Homo sapiens*	3779
Calcium-activated potassium channel subunit beta-2	*Homo sapiens*	10242

Calcium-activated potassium channel subunit beta-3	*Homo sapiens*	27094
Calcium-activated potassium channel subunit beta-4	*Homo sapiens*	27345
Canalicular multispecific organic anion transporter 1	*Homo sapiens*	1244
Canalicular multispecific organic anion transporter 2	*Homo sapiens*	8714
Carbonic anhydrase 1	*Leishmania mexicana*	13450542
Carbonic anhydrase 2	*Homo sapiens*	760
Carbonic anhydrase 3	*Homo sapiens*	761
Carbonic anhydrase 4	*Homo sapiens*	762
Carbonic anhydrase 6	*Homo sapiens*	765
Carbonic anhydrase 7	*Homo sapiens*	766
Carboxylesterase	*Homo sapiens*	1066
Catechol O-methyltransferase	*Homo sapiens*	1312
Cellular retinoic acid-binding protein 1	*Homo sapiens*	1381
Cellular retinoic acid-binding protein 2	*Homo sapiens*	1382
cGMP-inhibited 3',5'-cyclic phosphodiesterase A	*Homo sapiens*	5139
Cholesterol side-chain cleavage enzyme, mitochondrial	*Homo sapiens*	1583
Cholinesterase	*Homo sapiens*	590
Cocaine esterase	*Cricetulus griseus*	100756666
Corticosteroid-binding globulin	*Homo sapiens*	866
Cyclin-dependent kinase inhibitor 1	*Homo sapiens*	1026
Cystic fibrosis transmembrane conductance regulator	*Homo sapiens*	1080
Cytochrome P450 11B1, mitochondrial	*Homo sapiens*	1584
Cytochrome P450 19A1	*Homo sapiens*	1588
Cytochrome P450 1A1	*Homo sapiens*	1543
Cytochrome P450 1A2	*Homo sapiens*	1544
Cytochrome P450 1B1	*Homo sapiens*	1545
Cytochrome P450 26A1	*Homo sapiens*	1592
Cytochrome P450 26B1	*Homo sapiens*	56603
Cytochrome P450 26C1	*Homo sapiens*	340665
Cytochrome P450 2A6	*Homo sapiens*	1548
Cytochrome P450 2B6	*Homo sapiens*	1555
Cytochrome P450 2C18	*Homo sapiens*	1562
Cytochrome P450 2C19	*Homo sapiens*	1557
Cytochrome P450 2C8	*Homo sapiens*	1558
Cytochrome P450 2C9	*Homo sapiens*	1559
Cytochrome P450 2D6	*Homo sapiens*	1565
Cytochrome P450 2E1	*Homo sapiens*	1571
Cytochrome P450 2J2	*Homo sapiens*	1573
Cytochrome P450 3A4	*Homo sapiens*	1576
Cytochrome P450 3A43	*Homo sapiens*	64816
Cytochrome P450 3A5	*Homo sapiens*	1577
Cytochrome P450 3A7	*Homo sapiens*	1551
Cytochrome P450 4A11	*Homo sapiens*	1579
Cytokine receptor common subunit gamma	*Homo sapiens*	3561
Dihydrofolate reductase	*Leishmania major*	5649109

Dihydroorotate dehydrogenase (quinone), mitochondrial	*Homo sapiens*	1723
Dimethylaniline monooxygenase [N-oxide-forming] 1	*Trypanosoma grayi*	20379836
Dimethylaniline monooxygenase [N-oxide-forming] 3	*Homo sapiens*	2328
DNA (cytosine-5)-methyltransferase 1	*Trypanosoma grayi*	20384364
DNA topoisomerase 1	*Leishmania major*	5651546
DNA topoisomerase 1, mitochondrial	*Homo sapiens*	116447
DNA topoisomerase 2-alpha	*Homo sapiens*	7153
Estrogen receptor alpha	*Homo sapiens*	2099
Estrogen receptor beta	*Homo sapiens*	2100
Extracellular calcium-sensing receptor	*Homo sapiens*	846
Folate transporter 1	*Leishmania mexicana*	13454710
G protein-activated inward rectifier potassium channel 2	*Homo sapiens*	3763
G protein-activated inward rectifier potassium channel 3	*Homo sapiens*	3765
G1/S-specific cyclin-D1	*Homo sapiens*	595
Glutathione S-transferase A2	*Homo sapiens*	2939
Hematopoietic prostaglandin D synthase	*Homo sapiens*	27306
Hemoglobin subunit alpha	*Homo sapiens*	3039
Hexokinase-1	*Leishmania major*	5651559
Histamine N-methyltransferase	*Homo sapiens*	3176
Histone deacetylase	*Leishmania major*	5651604
Histone deacetylase 1	*Homo sapiens*	3065
Histone deacetylase 2	*Homo sapiens*	3066
Histone deacetylase 3	*Homo sapiens*	8841
Histone deacetylase 4	*Homo sapiens*	9759
Histone deacetylase 6	*Homo sapiens*	10013
Histone deacetylase 8	*Homo sapiens*	55869
Inhibitor of nuclear factor kappa-B kinase subunit beta	*Homo sapiens*	3551
Insulin-like growth factor-binding protein 3	*Homo sapiens*	3486
Interleukin-2 receptor subunit alpha	*Homo sapiens*	3559
Interleukin-2 receptor subunit beta	*Homo sapiens*	3560
Intermediate conductance calcium-activated potassium channel protein 4	*Homo sapiens*	3783
Keratin, type I cytoskeletal 12	*Homo sapiens*	3859
Keratin, type II cytoskeletal 7	*Homo sapiens*	3855
Lipocalin-1	*Homo sapiens*	3933
Liver carboxylesterase 1	*Homo sapiens*	1066
Microtubule-associated protein 1A	*Trypanosoma grayi*	20379817
Microtubule-associated protein 2	*Homo sapiens*	4133
Mitogen-activated protein kinase 1	*Homo sapiens*	5594
Mitogen-activated protein kinase 3	*Homo sapiens*	5595
Monocarboxylate transporter 1	*Leishmania donovani*	13391366
Multidrug and toxin extrusion protein 1	*Homo sapiens*	55244
Multidrug and toxin extrusion protein 2	*Homo sapiens*	146802
Multidrug resistance protein 1	*Homo sapiens*	5243
Multidrug resistance-associated protein 1	*Leishmania major*	5654074

Multidrug resistance-associated protein 4	*Leishmania major*	5654058
Nitric oxide synthase, endothelial	*Homo sapiens*	4846
Nitric oxide synthase, inducible	*Homo sapiens*	4843
Nuclear receptor subfamily 0 group B member 1	*Homo sapiens*	190
Nuclear receptor subfamily 1 group I member 2	*Homo sapiens*	8856
Nuclear receptor subfamily 1 group I member 3	*Homo sapiens*	9970
Odorant protein-binding 2a	*Homo sapiens*	29991
Ornithine decarboxylase	*Leishmania major*	5649918
Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic delta isoform	*Homo sapiens*	5290
Phospholipase d activity	*Leishmania major*	5649176
Placenta growth factor	*Homo sapiens*	5228
Platelet glycoprotein IX	*Homo sapiens*	2815
Poly [ADP-ribose] polymerase 1	*Homo sapiens*	142
Poly [ADP-ribose] polymerase 2	*Homo sapiens*	10038
Poly [ADP-ribose] polymerase 3	*Homo sapiens*	10039
Potassium voltage-gated channel subfamily A member 5	*Homo sapiens*	3741
Potassium voltage-gated channel subfamily D member 3	*Homo sapiens*	3757
Potassium voltage-gated channel subfamily H member 2	*Homo sapiens*	3752
Potassium voltage-gated channel subfamily H member 6	*Homo sapiens*	81033
Potassium voltage-gated channel subfamily H member 7	*Homo sapiens*	90134
Potassium voltage-gated channel subfamily KQT member 1	*Homo sapiens*	3784
Pregnancy-specific beta-1-glycoprotein 5	*Homo sapiens*	5673
Progesterone receptor	*Homo sapiens*	5241
Prostaglandin G/H synthase 1	*Homo sapiens*	5742
Prostaglandin G/H synthase 2	*Homo sapiens*	5743
Proteasome subunit beta type-1	*Homo sapiens*	5689
Proteasome subunit beta type-10	*Homo sapiens*	5699
Proteasome subunit beta type-2	*Homo sapiens*	5690
Proteasome subunit beta type-5	*Homo sapiens*	5693
Proteasome subunit beta type-8	*Homo sapiens*	5696
Proteasome subunit beta type-9	*Homo sapiens*	5698
Protein kinase C alpha type	*Homo sapiens*	5578
Protein kinase C delta type	*Homo sapiens*	5580
Protein PML	*Homo sapiens*	5371
Pyruvate dehydrogenase lipoamide kinase isozyme 4, mitochondrial	*Homo sapiens*	5166
RAC-alpha serine/threonine-protein kinase	*Homo sapiens*	207
Retinal dehydrogenase 1	*Homo sapiens*	216
Retinal dehydrogenase 2	*Homo sapiens*	8854
Retinoic acid receptor alpha	*Homo sapiens*	5914
Retinoic acid receptor beta	*Homo sapiens*	5915
Retinoic acid receptor gamma	*Homo sapiens*	5916
Retinoic acid receptor responder protein 1	*Homo sapiens*	5918
Retinoic acid receptor RXR-alpha	*Homo sapiens*	6256

Retinoic acid receptor RXR-beta	*Homo sapiens*	6257
Retinoic acid receptor RXR-gamma	*Homo sapiens*	6258
Retinoic acid-induced protein 3	*Homo sapiens*	9052
Retinol-binding protein 4	*Homo sapiens*	5950
Ribonucleoside-diphosphate reductase large subunit	*Leishmania major*	5653298
Ribosyldihydronicotinamide dehydrogenase [quinone]	*Homo sapiens*	4835
Serum albumin	*Homo sapiens*	213
Sex hormone-binding globulin	*Homo sapiens*	6462
Small conductance calcium-activated potassium channel protein 1	*Homo sapiens*	3780
Small conductance calcium-activated potassium channel protein 2	*Homo sapiens*	3781
Small conductance calcium-activated potassium channel protein 3	*Homo sapiens*	3782
Smoothened homolog	*Homo sapiens*	6608
Sodium channel protein type 5 subunit alpha	*Homo sapiens*	6331
Sodium/potassium-transporting ATPase subunit alpha-1	*Homo sapiens*	476
Solute carrier family 22 member 1	*Homo sapiens*	6580
Solute carrier family 22 member 2	*Homo sapiens*	6582
Solute carrier family 22 member 3	*Homo sapiens*	6581
Solute carrier family 22 member 4	*Homo sapiens*	6583
Solute carrier family 22 member 5	*Homo sapiens*	6584
Solute carrier family 22 member 6	*Homo sapiens*	9356
Solute carrier family 22 member 8	*Homo sapiens*	9376
Solute carrier organic anion transporter family member 1A2	*Homo sapiens*	6579
Solute carrier organic anion transporter family member 1B1	*Homo sapiens*	10599
Solute carrier organic anion transporter family member 1B3	*Homo sapiens*	28234
Solute carrier organic anion transporter family member 2B1	*Homo sapiens*	11309
Squalene monooxygenase	*Leishmania major*	5650191
Steroid 21-hydroxylase	*Homo sapiens*	1589
Thioredoxin reductase 1, cytoplasmic	*Leishmania infantum*	5072181
Toll-like receptor 7	*Homo sapiens*	51284
Toll-like receptor 9	*Homo sapiens*	54106
Transcription factor AP-1	*Homo sapiens*	3725
tRNA (cytosine(38)-C(5))-methyltransferase	*Homo sapiens*	1787
Tubulin beta-1 chain	*Homo sapiens*	81027
Tumor necrosis factor	*Homo sapiens*	7124
UDP-glucuronosyltransferase 1-1	*Homo sapiens*	54658
UDP-glucuronosyltransferase 1-4	*Homo sapiens*	54657
UDP-glucuronosyltransferase 1-8	*Homo sapiens*	54576
UDP-glucuronosyltransferase 1-9	*Homo sapiens*	54600
Vascular endothelial growth factor A	*Homo sapiens*	7422
Vascular endothelial growth factor B	*Homo sapiens*	7423

Voltage-dependent L-type calcium channel subunit alpha-1C	*Trypanosoma grayi*	20380997
Fungi target		
1,3-beta-d-glucan synthase activity	*Saccharomyces cerevisiae*	851055
1,3-beta-glucan synthase component FKS1	*Saccharomyces cerevisiae*	851055
Cytosolic leucyl-tRNA synthetase	*Candida bubliniensis*	8049859
Ergosterol	*Trypanosoma grayi*	20380385
Lanosterol 14-aplha demethylase	*Leishmania major*	5649863
Lanosterol synthase	*Trypanosoma grayi*	20382041
Thymidylate synthase	*Leishmania major*	5649109
Tubulin alpha chain	*Leishmania infantum*	10966580
Tubulin beta chain	*Leishmania major*	5651759
Bacteria target		
16S rRNA	*Trypanosoma grayi*	20381413
Acetoin utilization protein	*Streptomyces coelicolor*	1098764
Chloramphenicol acetyltransferase	*Escherichia coli*	12657249
Cytochrome b	*Leishmania infantum*	5066231
Dihydroorotate dehydrogenase activity	*Escherichia coli*	945556
Fumarate reductase flavoprotein subunit	*Escherichia coli*	948667
Pyruvate-flavodoxin oxidoreductase	*Escherichia coli*	946587
RNA polymerase sigma factor	*Escherichia coli*	946839
Protozoa target		
Bifunctional dihydrofolate reductase-thymidilate synthase	*Leishmania major*	5649109
Fe(II)-protoporphyrin IX	*Homo sapiens*	213
Glutathione S-transferase	*Leishmania major*	5650353
Archaea target		
50S ribosomal protein L2	*Trypanosoma grayi*	20380895

combinations of drugs targeting different metabolic pathways. In the former case, the combination might provide an enhanced treatment by exerting a cumulative effect (also called supra-additive interaction) on the same metabolic pathway, while in the second case the combination provides a complementary effect (also called joint effect) by targeting different metabolic pathways.

3.3. Results

3.3.1. Target diversity

Of the 118 scored drugs identified in Chapter 2, a list of 222 names of molecules targeted by these drugs was generated (Figure 3.2). Of the 222 drug targets and according to the DrugBank target categorisation, 198 were classified as human targets (i.e. drugs with activity against a human-gene encoded target such as a receptor or an enzyme), 10 were bacterial targets, 9 were fungal targets, 3 were protozoan targets, 1 was an archaean target. DNA was also targeted by several of the 118 scored drugs but these drugs were discarded because it is shared by all living organisms and may result in non-target toxicity. Several other target names such as bacterial outer membrane (bacteria target) or gamma globulin (human target) were also investigated according our drug prioritisation process without any success because of no match in iPath or BLAST due to the lack of specificity devolved to their names. It was not possible to discriminate drug targets that act as part of the therapeutic effect from those targets that cause side effects after drug administration according to information available from PubChem and DrugBank.

3.3.2. Interactive Pathways Explorer

The web-based tool iPath (v2 and v3) confirmed the presence of 13 targets in the *N. pemaquidensis* or *Perkinsela* sp. map of Tanifuji et al. (2017) out of the total of 222 targets identified in the present study (Table 3.2). The remaining 209 targets were discarded from the prioritisation process using iPath because of no match or no apparent

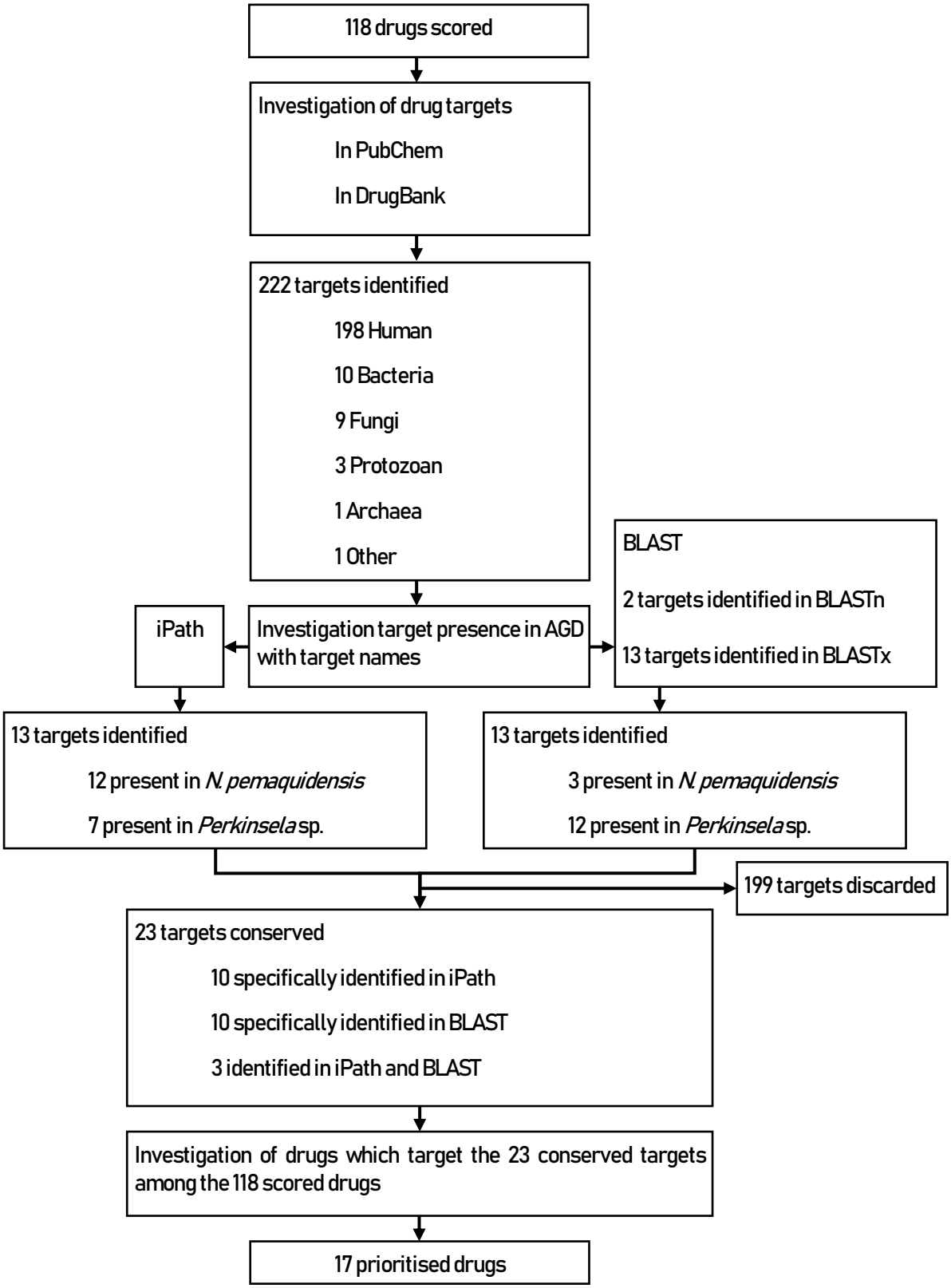

Figure 3.2. Diagram representing the drug prioritisation process used in the Chapter 3 in order to speculate on potential drug combination to treat AGD.

Table 3.2. Investigation of drug target names in iPath and their presence in *Neoparamoeba pemaquidensis* and/or *Perkinsela* sp. The 222 investigated drug target names originate from the 118 scored drugs in Chapter 2.

Molecular target	ID	Reaction number	Pathway	*N. pemaquidensis*	*Perkinsela sp.*
Adenosine deaminase	COG1816	R02556	Purine metabolism	Yes	No
	COG1816	R01560	Purine metabolism	Yes	No
Aldehyde oxidase	K00157	R01709	Vitamin B6 metabolism	Yes	No
	K00157	R02655-R02657	Tyrosine metabolism	Yes	No
	K00128-K00149-K00157-K14085	R04903-R04904	Tryptophan metabolism	Yes	No
	K00157	R08408	Nicotinate and nicotinamide metabolism	Yes	No
	K00157	R04085	Nicotinate and nicotinamide metabolism	Yes	No
Beta-glucuronidase	COG3250	R07818	Glycosaminoglycan degradation	Yes	Yes

Catechol O-methyltransferase	K00545	R02534	Tyrosine metabolism	Yes	No
	K00545	R02920	Tyrosine metabolism	Yes	No
	K00545	R04301	Tyrosine metabolism	Yes	No
	K00545	R04887	Tyrosine metabolism	Yes	No
	K00545	R03304	Tyrosine metabolism	Yes	No
	K00545	R04881	Tyrosine metabolism	Yes	No
Dihydrofolate reductase	COG0262-COG1028	R00936-R00939	Folate biosynthesis	Yes	Yes
	COG0262	R02235-R02236	Folate biosynthesis	Yes	Yes
	COG0262	R00937-R00940	Folate biosynthesis	Yes	Yes
Dihydroorotate dehydrogenase (quinone), mitochondrial	COG0044-COG0167-COG0543	R01867-R01868-R01869	Pyrimidine metabolism	Yes	No
DNA (cytosine-5)-methyltransferase 1	K00558-K17398-K17399-K17462	R04858-R10404	Cysteine and methionine metabolism	Yes	No
Glutathione S-transferase	K01830-K04097	R02266	Arachidonic acid metabolism	Yes	No
	K01800	R03181	Tyrosine metabolism	Yes	No
Lanosterol synthase	K01852	R03199	Steriod biosynthesis	Yes	No

Ornithine decarboxylase	K01581	R00670	Arginine, proline, glutathione metabolism	Yes	No
Phospholipase D	K01115-K16860-K17717	R01310	Glycerphospholipid metabolism	Yes	No
	K01115-K16860-K17717	R02051	Glycerphospholipid metabolism	Yes	No
Ribonucleoside-diphosphate reductase	K00524-K00525-K00526-K10807-K10808	R02017	Purine metabolism	Yes	Yes
	K00524-K00525-K00526-K10807-K10808	R02019	Purine metabolism	Yes	Yes
	K00524-K00525-K00526-K10807-K10808	R02024	Pyrimidine metabolism	Yes	Yes
	K04283-K10807-K10808-K11185-K11186	R03821-R08362-R08364	Glutathione metabolism	No	Yes
Thymidylate synthase	K00560-K13998	R02101	Pyrimidine metabolism	Yes	No

presence of the targets in *N. pemaquidensis* and *Perkinsela* sp. metabolic pathways. All the drug targets highlighted are enzymes of which some are involved in several reactions involved in metabolic pathways such as purine, valine or tyrosine metabolism (Table 3.2). The most represented type of target identified by iPath was human targets (10), then fungal targets (2), protozoan targets (2) and bacterial targets (1) showing that according to the initial distribution among these categories (Figure 3.2), iPath was not specifically selective towards non-human targets. Some targets like dihydrofolate reductase and dihydroorotate dehydrogenase are present in several organisms (i.e. respectively human/protozoa, and human/fungi) which explain the difference between the identified targets (n=13) and the type of target (n=14). Among all the drugs identified by the investigation using iPath, only one drug acts on two different targets. Indeed, flucytosine targets both the DNA (cytosine-5)-methyltransferase 1 and the thymidylate synthase which is something interesting to speculate for subsequent drug combination.

3.3.3. BLASTn

Among the 222 drug targets screened with BLASTn, only 2 drug targets showed significant results regarding the query coverage and identity thresholds (Table 3.3) in the genomes of *N. pemaquidensis* or *Perkinsela* sp. The tubulin alpha chain and beta chain gene sequences were identified in the genome of *Perkinsela* sp. and the sequence matched those from respectively *Leishmania infantum* and *Leishmania major*. It is interesting to note that these two molecules are targeted by the same drug, griseofulvin, which is as consequence an important drug for a potential combination.

3.3.4. BLASTx

Compared to the BLASTn, the BLASTx allowed a greater number of drug targets to be identified even if the query coverage and the identity were less striking compare to the BLASTn results (Table 3.4). According to the results in Table 3.4, three drug targets

Table 3.3. Investigation performed using BLASTn of the 222 drug targets FASTA sequence blasted in the genome of *Neoparamoeba pemaquidensis* (taxid: 180228) and *Perkinsela* sp. (CCAP 1560/4 taxid: 1314962).

Organism	Gene	Gene organism	Gene ID	Query cover	E value	Identity
Perkinsela sp.	Tubulin alpha chain	*Leishmania infantum*	10966580	89%	0	82%
Perkinsela sp.	Tubulin beta chain	*Leishmania major*	5651759	98%	0	84%

Table 3.4. Investigation in BLASTx of the 222 drug targets FASTA sequence blasted in the genome of *Neoparamoeba pemaquidensis* (taxid: 180228) and *Perkinsela* sp. (CCAP 1560/4 taxid: 1314962).

Organism	Gene	Gene organism	Gene ID	Query cover	E value	Identity
Neoparamoeba pemaquidensis	Tubulin beta chain	*Acanthamoeba castellanii*	14925210	83%	0.00E+00	66%
Neoparamoeba pemaquidensis	Cytochrome b	*Acanthamoeba castellanii*	1734035	94%	2.00E-116	51%
Neoparamoeba pemaquidensis	50S ribosomal protein L2	*Acanthamoeba castellanii*	1734043	96%	4.00E-43	39%
Perkinsela sp	50S ribosomal protein L2	*Trypanosoma grayi*	20380895	86%	7.00E-121	52%
Perkinsela sp	Cytosolic leucyl-tRNA synthetase	*Candida bubliniensis*	8049859	90%	0.00E+00	38%
Perkinsela sp	Fumarate reductase flavoprotein subunit	*Escherichia coli*	948667	90%	6.00E-119	38%
Perkinsela sp	Glutathione S-transferase	*Leishmania major*	5650353	90%	2.00E-83	47%
Perkinsela sp	Hexokinase-1	*Leishmania major*	5651559	91%	7.00E-77	36%
Perkinsela sp	Tubulin alpha chain	*Leishmania infantum*	10966580	91%	0.00E+00	85%
Perkinsela sp	60S ribosomal protein L3	*Leishmania major*	5656437	92%	0.00E+00	65%
Perkinsela sp	Bifunctional dihydrofolate reductase-thymidilate synthase	*Leishmania major*	5649109	95%	4.00E-166	50%
Perkinsela sp	Dihydrofolate reductase	*Leishmania major*	5649109	95%	4.00E-166	50%
Perkinsela sp	Thymidylate synthase	*Leishmania major*	5649109	95%	4.00E-166	50%

Perkinsela sp	Tubulin beta chain	*Leishmania major*	5651759	96%	0.00E+00	89%
Perkinsela sp	Ribonucleoside-diphosphate reductase large subunit	*Leishmania major*	5653298	99%	0.00E+00	63%

were identified in the genome of *N. pemaquidensis* and 12 drug targets were identified in the genome of *Perkinsela* sp.

3.3.5. Prioritised drug and targets

Among the 118 scored drugs and their respective 222 drug targets, 13 targeted enzymes (the same identified with iPath) present in *N. pemaquidensis* or *Perkinsela* sp. were found in the KEGG enzyme database (Table 3.5). More precisely, 10 enzymes were specifically detected in *N. pemaquidensis* and 3 were shared by *N. pemaquidensis* and *Perkinsela* sp. both symbionts (i.e. beta-glucuronidase, dihydrofolate reductase, ribonucleoside-diphosphate reductase). The identified enzymes belong to various enzyme classes according to KEGG ENZYME database which are: glycosidases (n=1), glycosylases (n=1), hydrolases (n=4), isomerases (n=2), lyases (n=1), methyltransferases (n=1), oxidoreductases (n=4) and transferases (n=4). The best represented metabolic pathways were glutathione metabolism (n=3), pyrimidine metabolism (n=3), purine metabolism (n=2) and tyrosine metabolism (n=2).

Overall, 23 drug targets were identified *N. pemaquidensis* and/or *Perkinsela* sp. according to the two research tools used in this study (i.e. iPatch and BLAST) from the initial 222 (Table 3.6). The glutathione s-transferase and the thymidylate synthase were identified by iPath and BLAST, which explains the difference between the total drug targets present in AGD count (n=23) and the sum of iPath and BLAST results (n=25).

The 23 identified drug targets in AGD, after the investigation of the information collected in Chapter 2, led to identification of 17 drugs of which 7 were specifically identified through iPath, 5 specifically identified through BLAST (i.e. BLASTn and BLASTx) and five identified in both web-based tools (Table 3.6). Among the 17 drugs, the most represented drug classes were: antifungal (n=5), antineoplastic (n=5), antimalarials (n=3), trypanocidal (n=2), antihelmintic (n=1) and polymerase inhibitors (n=1) (Table 3.6).

Table 3.5. Information obtained in KEGG ENZYME database (https://www.genome.jp/kegg/annotation/enzyme.html) relative to targeted enzymes identified in AGD in order to support a rational combination therapy.

Name	KEGG	Class	Pathway
Adenosine deaminase	ENZYME: 3.5.4.4	Hydrolases	Purine metabolism
Aldehyde oxidase	ENZYME: 1.2.3.1	Oxidoreductases	Valine, leucine and isoleucine degradation Tyrosine metabolism Tryptophan metabolism Vitamin B6 metabolism Nicotinate and nicotinamide metabolism Retinol metabolism
Beta-glucuronidase	ENZYME: 3.2.1.31	Hydrolases Glycosylases Glycosidases	Pentose and glucuronate interconversions Glycosaminoglycan degradation Porphyrin and chlorophyll metabolism Flavone and flavonol metabolism
Catechol O-methyltransferase	ENZYME: 2.1.1.6	Transferases Methyltransferases	Steroid hormone biosynthesis Tyrosine metabolism Betalain biosynthesis
Dihydrofolate reductase	ENZYME: 1.5.1.3	Oxidoreductases	One carbon pool by folate Folate biosynthesis
Dihydroorotate dehydrogenase	ENZYME: 1.3.5.2	Oxidoreductases	Pyrimidine metabolism
DNA (cytosine-5-)-methyltransferase 1	ENZYME: 2.1.1.37	Transferases	Cysteine and methionine metabolism
Glutathione S-transferase	ENZYME: 2.5.1.18	Transferases	Glutathione metabolism
Lanosterol synthase	ENZYME: 5.4.99.7	Isomerases	Steroid biosynthesis
Ornithine decarboxylase	ENZYME: 4.1.1.17	Lyases	Arginine and proline metabolism

			Glutathione metabolism
Phospholipase D	ENZYME: 3.1.4.4	Hydrolases	Glycerophospholipid metabolism
			Ether lipid metabolism
Ribonucleoside-diphosphate reductase	ENZYME: 1.17.4.1	Oxidoreductases	Purine metabolism
			Pyrimidine metabolism
			Glutathione metabolism
Thymidylate synthase	ENZYME: 2.1.1.45	Transferases	Pyrimidine metabolism

3.3.6. Combination therapy

The selection of potential combinations of drugs was performed using information from the KEGG enzyme database and the drugs known to target these enzymes (Table 3.5; Table 3.6; Figure 3.3). The combinations were evaluated for potential cumulative effects and complementary effects. First, it is important to notice that some drugs affect drug targets present in *N. pemaquidensis* and *Perkinsela* sp.: chloroquine, flucytosine, hydroxyurea, niraparib and proguanil hydrochloride. The association of these drug could lead to a promising combination knowing that they target both symbiont of AGD without targeting the same molecular target. Among all the drugs which could be relevant to be used in cumulative combination therapy, hydroxyurea (also known as hydroxycarbamide) is maybe the most interesting because this drug targets ribonucleoside-diphosphate reductase, which is involved in several metabolic pathways (glutathione, purine and pyrimidine) and thus, give the benefit to be more difficult for AGD to develop drug resistance (Table 3.5). In the case of a cumulative combination therapy, hydroxyurea could be combined with atovaquone, chloroquine, eflornithine, flucytosine and pentostatin, as these drug affects molecular targets present in the same metabolic pathway (Figure 3.3). Always in the case of a cumulative combination therapy, atovaquone and chloroquine are also two relevant drugs because they could be used in combination with flucytosine and hydroxyurea or eflornithine and hydroxyurea respectively according to the metabolic pathways they act on.

3.4. Discussion

AGD is an emerging disease that requires the development of additional and better therapeutic options than those currently used by the salmon farming and other industries.

In the present study, investigation avenues were followed to provide a new effective and sustainable treatment for AGD. The approach taken in this present study relied on the identification of drug targets in order to propose the most rational drug

Table 3.6. Overview of the drugs which affect identified drug targets in *Neoparamoeba pemaquidensis* and *Perkinsela* sp. IP: Interactive Pathways Explorer. BLASTn/x: drug targets identified by BLASTn and BLASTx. Drug class was obtained from DrugBank and PubChem (cf. Chapter 2).

Drug	Class	Target	Organism	Evidence
Atovaquone	Antimalarials	Dihydroorotate dehydrogenase (quinone)	*N. pemaquidensis*	IP
		Cytochrome b	*N. pemaquidensis*	Blastx
Chloroquine	Antimalarials	Glutathione S-transferase	*N. pemaquidensis*	IP
		Glutathione S-transferase	*Perkinsela sp.*	Blastx
Eflornithine	Trypanocidal	Ornithine decarboxylase	*N. pemaquidensis*	IP
Flucytosine	Antifungal	DNA (cytosine-5)-methyltransferase 1	*N. pemaquidensis*	IP
		Thymidylate synthase	*N. pemaquidensis*	IP
		Thymidylate synthase	*Perkinsela sp.*	Blastx
Griseofluvin	Antifungal	Tubulin alpha chain	*Perkinsela sp.*	Blastn/x
		Tubulin beta chain	*Perkinsela sp.*	Blastn/x
		Tubulin beta chain	*N. pemaquidensis*	Blastx
Hydroxyurea	Antineoplastic	Ribonucleoside-diphosphate reductase large subunit	*Perkinsela sp.*	Blastx
		Ribonucleoside-diphosphate reductase	*Perkinsela sp.*	IP
		Ribonucleoside-diphosphate reductase	*N. pemaquidensis*	IP
Idelalisib	Antineoplastic	Aldehyde oxidase	*N. pemaquidensis*	IP
Lonidamine	Trypanocidal	Hexokinase-1	*Perkinsela sp.*	Blastx
Micafungin	Antifungal	Catechol O-methyltransferase	*N. pemaquidensis*	IP
Miltefosine	Antineoplastic	Phospholipase D	*N. pemaquidensis*	IP
Niraparib	Polymerase Inhibitors	Beta-glucuronidase	*Perkinsela sp.*	IP
		Beta-glucuronidase	*N. pemaquidensis*	IP
Omacetaxine mepesuccinate	Antineoplastic	50S ribosomal protein L2	*N. pemaquidensis*	Blastx
		50S ribosomal protein L2	*Perkinsela sp.*	Blastx
		60S ribosomal protein L3	*Perkinsela sp.*	Blastx
Oxiconazole	Antifungal	Lanosterol synthase	*N. pemaquidensis*	IP
Pentostatin	Antineoplastic	Adenosine deaminase	*N. pemaquidensis*	IP

Proguanil Hydrochloride	Antimalarials	Dihydrofolate reductase	*Perkinsela sp.*	IP
		Dihydrofolate reductase	*N. pernaquidensis*	IP
		Bifunctional dihydrofolate reductase-thymidilate synthase	*Perkinsela sp.*	Blastx
		Dihydrofolate reductase	*Perkinsela sp.*	Blastx
Tavaborole	Antifungal	Cytosolic leucyl-tRNA synthetase	*Perkinsela sp.*	Blastx
Thiabendazole	Antihelmintics	Fumarate reductase flavoprotein subunit	*Perkinsela sp.*	Blastx

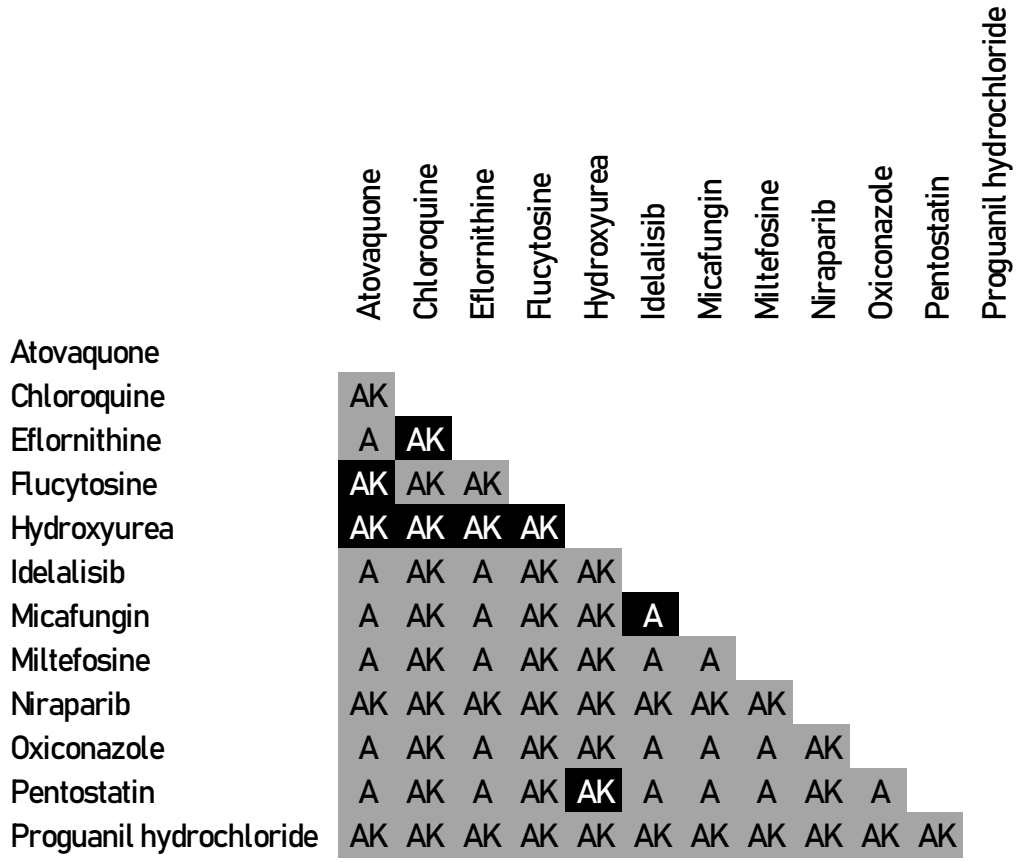

Figure 3.3. Possible combination of prioritised drugs identified in iPath. Black: drugs focusing on same metabolic pathway. Grey: drugs focusing on different metabolic pathways. A: the combination affects *Neoparamoeba pemaquidensis* (Amoeba). K: the combination affects *Perkinsela* sp. (Kinetoplastids) AK: the combination affects both *Neoparamoeba pemaquidensis* and *Perkinsela* sp.

candidates to pursue as a new AGD therapy among the 118 scored drugs in Chapter 2 and for possible combination therapy based on suspected cumulative or complementary effects. *In silico* research, as performed in this present study, is a widespread first step in drug discovery, and plays a major role regarding gene-expression analysis, prediction of gene function, prediction of drug-similarity, library design and virtual screening (Terstappen & Reggiani., 2001). Nevertheless, it was suggested that target-based drug discovery is partially responsible for a steady decline in the productivity of the pharmaceutical industry because the lack of knowledge regarding mechanisms involved in specific diseases which prevent the use of the most appropriate treatment for a given disease (Sams-Dodd., 2005). Yet, this was the most relevant approach in this present study considering that this could be achieved through a desk-based study, such as the current one. This approach is a preliminary work leading to *in vitro* analysis and the proposition of rational combinations including drugs that affect multiple targets in the same pathway.

Several tools were employed for this present study (iPath, BLASTn, BLASTx) and a number of databases (NCBI Gene, KEGG ENZYME) were used to prioritise the 118 scored drugs identified in Chapter 2. The drug prioritisation was based on the confirmation of the presence of drug target in AGD. The enzymes targeted by the 118 scored drugs were mainly investigated for potential combination because they represent a common and reliable source of drug targets and information regarding their involvement in several metabolic pathways are available (Drews., 2000). Regarding the BLAST findings, the only striking result concerns the highlight of griseofulvin by BLASTn which is a metabolic product produced by *Penicillium* spp. which is known to target both tubulin alpha and beta chain (Develoux., 2001). This result is even more important in that these targets were found in both organisms involved in AGD. Five drugs were highlighted by both iPath and BLAST (i.e. atovaquone, chloroquine, flucytosine, hydroxyurea and proguanil hydrochloride) for a total of 17 prioritised drugs from the Chapter 2.

The 17 prioritised drugs testify of a broad diversity regarding their application in specific disease and also their use in monotherapy or combination therapy (Table 3.7.) Among the 17 prioritised drugs, the drugs, hydroxyurea, atovaquone, chloroquine,

Table 3.7. Overview of the utilisation of the 17 prioritised drugs in monotherapy and combination therapy in various diseases.

Drug	Disease	Therapy	Reference
Atovaquone	Pneumocystis pneumonia (PJP) (*Pneumocystis jiroveci*)	Monotherapy	Baggish & Hill,, 2002
	Malaria (*Plasmodium falciparum*)	Combination with proguanil and tetracycline	Baggish & Hill,, 2002
	Babesiosis (*Babesia microti*)	Combination with azithromycin	Baggish & Hill,, 2002
	Toxoplasmosis (*Toxoplasma gondii*)	Monotherapy	Baggish & Hill,, 2002
Chloroquine	Rheumatoid arthritis	Combination with D-penicillamine	Gibson et al., 1987
	Porphyria cutanea tarda	Monotherapy	Kordać & Semrádová., 2006
	Malaria (*Plasmodium falciparum*)	Monotherapy	Pillai et al., 2001
	Chikungunya (alphavirus)	Monotherapy	De Lamballerie et al., 2008
	Malaria (*Plasmodium falciparum*)	Combination with doxycycline	Taylor et al., 2001
Eflornithine	Pneumocystis carinii pneumonia (PCP)	Monotherapy	Gilman et al., 1986
	Human african trypanosomiasis (HAT)	Monotherapy	Burri & Brun., 2002
	Human african trypanosomiasis (HAT)	Combination with nifurtimox	Franco et al., 2012
	Primary brain tumors	Combination with mitoguazone	Levin et al., 1987
	Gliomas	Monotherapy	Levin et al., 1992
Flucytosine	Chromoblastomycosis (*Phialophora verrucosa*, *Cladophialophora carrionii*)	Monotherapy	Mauceri et al., 1974
	Vaginitis (*Candida glabrata*)	Monotherapy	Sobel et al., 2003
	Cryptoccal meningitis (*Cryptococcus neoformans*; *Cryptococcus gattii*)	Combination with amphotericin b	Bennett et al., 1979
Griseofluvin	Shingles (*Herpes zoster*)	Combination with methisoprinol	Castelli et al., 1986
	Dermatophytoses (*Tricophyton mentagrphytes*)	Monotherapy	Aly et al., 1994
	Tinea capitis (*Trichophyton tonsurans*; *Microsporum canis*)	Monotherapy	Gan et al., 1987
Hydroxyurea	Essential thrombocythemia	Combination with anagrelide	Ahn et al., 2011
	Psoriasis	Combination with infliximab	Gach & Berth-Jones., 2003
	Sickel cell anemia	Monotherapy	Platt., 2008

Idelalisib	Chronic lymphocytic leukemia	Monotherapy	Khan et al. 2014
	Non-Hodgkin lymphoma	Monotherapy	Graf & Gopal., 2016
Lonidamine	Recurrent papillary carcinomas of the urinary bladder	Combination with adriamycin	Giannotti et al., 1984
	Chronic lymphocytic leukemia	Monotherapy	Tura et al., 1984
	Benign Prostatic Hyperplasia	Monotherapy	Ditonno et al., 2005
Micafungin	Invasive aspergillosis (*Aspergillus fumigatus*, *Aspergillus terreus*)	Combination with amphotericin b	Denning et al., 2006
	Candida albicans (*Candida albicans*)	Monotherapy	Rautemaa et al., 2008
	Invasive scedosporiosis (*Scedosporium apiospermum*, *Scedosporium boydii*)	Combination with posaconazole	Lackner et al., 2014
Miltefosine	Visceral leishmaniasis (*Leishmania major*, *Leishmania infantum*)	Monotherapy	Sundar et al., 2003
	Cutaneous metastase in breast carcinoma	Monotherapy	Clive et al., 1999
	Acanthamoeba keratitis	Combination with polyhexamethylene biguanide	Polat et al., 2014
Niraparib	Ovarian cancer	Monotherapy	Essel & Moore., 2018
	Colorectal cancer	Monotherapy	Vitiello et al., 2018
Omacetaxine mepesuccinate	Chronic Myeloid Leukemia	Monotherapy	Alvandi et al., 2014
Oxiconazole	Vaginal candidiasis	Combination with econazole	Gouveia & Jones da Silva., 1984
	Tropical dermatomycoses (*Epidermophyton floccosum*)	Monotherapy	Gugnani et al.,1993
Pentostatin	Hairy-cell leukemia	Monotherapy	Grever et al., 2003
Proguanil Hydrochloride	Malaria (*Plasmodium falciparum*)	Combination with atovaquone (Malarone)	Hutchinson et al., 1999
Tavaborole	Onychomycosis	Monotherapy	Elewski & Tosti., 2014
Thiabendazole	Toxocariasis (*Toxocara canis*)	Monotherapy	Stürchler et al., 1989
	Strongyloidiasis	Combination with mebendazole	Shikiya et al., 1990
	Pediculosis capitis	Monotherapy	Namazi., 2003

eflornithine, flucytosine and pentostatin are candidates for possible cumulative combination therapy because they target the same metabolic pathways. Because of appearance of resistance to basic treatments used in infectious diseases, combination therapy was investigated to face this problem. In the case of malaria caused by *Plasmodium falciparum*, atovaquone and proguanil are associated in combination therapy and in the case of late (or second) stage African trypanosomiasis caused by *Trypanosoma brucei gambiense*, nifurtimox and eflornithine are associated in combination therapy (Radloff et al., 1996; Priotto et al., 2009). These two examples reinforce the results of the approach adopted in this present study even if these two diseases could be considered to be distant from AGD. For some highlighted candidate drugs, it is possible to get one step further. Indeed, some of them are targeting more than one identified drug target such as atovaquone (dihydroorotate dehydrogenase (quinone) and cytochrome b), flucytosine (DNA (cytosine-5)-methyltransferase 1 and thymidylate synthase) and proguanil hydrochloride (dihydrofolate reductase and bifunctional dihydrofolate reductase-thymidilate synthase) (Table 3.6). These three drugs, even if they are already considered as prioritised according to the prioritisation process, present even more interests for a potential combination therapy to treat AGD.

Nowadays, rational drugs combination therapy can be obtained by isobologram analyses (dose-response curve), median-effect equations (Chou Talalay method) and even web applications such as SynergyFinder which is based on the study of dose-response matrix data (Chou., 2010; Ianevski et al., 2017). The main limit of the present study is the unavailability of the drug-response curve for each drug against AGD. Nevertheless, other limits of the present computational approach need to be highlighted. The quality of the match using BLAST and iPath is a reasonable interrogation. Indeed, iPath software only shows one version of a gene name and it is common to change the name of gene according to the creation of new gene families as example. The use of the BLAST program for the identification of drug target presence in AGD implies also some limits regarding the criteria of investigation (e.g. database, organisms) and the way that the results are sorted out. In this present study, drug targets were considered only if the folds of 80% of query cover and 30% of identity were reached. The choice in this study of drug

combination implies some drawbacks. Combination therapy can result in antagonism such as when itraconazole and amphotericin B are used in combination, notably because the lipophilic itraconazole interacts with the sterol components of the cell membrane at the cell surface and thus blocks the activity of amphotericin B (Steinbach et al., 2003). Moreover, speculation on possible combinations according to the properties of drugs, it is impossible to speculate about rational combinations providing cumulative effect unless *in vitro* tests are performed. Instead of rely on drug combination, another solution which could be investigated notably in a further study is the drug rotation in order to reduce the risk of emergence of drug resistance. The research approach used in this present study should lead on to the performance of *in vitro* and *in vivo* experiments on AGD and Atlantic salmon. This work has highlighted 17 prioritised drugs with evidence of drug targets present in AGD. In further work, once the prioritised drugs dose response curves have been obtained, subsequent and more accurate drug combinations could be investigated in order to propose an experimental AGD treatment for in vitro and *in vivo* testing.

Chapter 4: General Discussion

According to the FAO, one of main sources of animal proteins in the future will be provided by fish (FAO., 2016). Aquaculture, notably fish farming, represent a large proportion of fish supply with more than 50% of fish for human consumption derived from aquaculture. Modern marine fish farming of Atlantic salmon dates from around 1970 but there are still issues that limit the sustainable development and expansion of the industry. While genetic and nutritional aspects have been well understood and enhanced fish farming practices, diseases continue to affect Atlantic salmon, in particular those not covered by existing vaccinations, and these exert a heavy burden with dramatic and unpredictable economic impacts. Due to the proximity of the environment compared to other more enclosed methods of fish farming, treating farmed fish in the open environment is all the more difficult because of potential for direct environmental impact through release of drugs in the water column or to the benthic environment in feed or faeces. The variety of living organisms impacting the health of Atlantic salmon health is broad and includes viruses (e.g. infectious salmon anaemia virus) and parasites (e.g. amoebic gill disease (AGD) caused by *Neoparamoeba perurans*) (Wong et al., 2004; Rimstad & Mjaaland., 2002). AGD has been noted in farmed fish for over three decades as a disease of economic and welfare concern. Despite efforts by the industry and by the academic sector, to date limited effective treatments have been developed to reduce the impact of AGD.

It is possible to learn the lesson of disease management from other major Atlantic salmon affecting diseases in order to apply these teachings to AGD. As an example, so-called sea lice (*Lepeophtheirus* spp., *Caligus* spp.) that feed on mucus, blood and epidermal tissue of Atlantic salmon and other fish, can cause catastrophic losses to the fish farming industry in many places, such as Norway, Chile and Scotland (Overton et al., 2018). However, collaborative research between academics, producers and pharmaceutical companies has led to the development of several treatments in monotherapy to mitigate the effect of these parasites. These include, but are not limited to medicines and treatments such as dichlorvos, azamethiphos, emamectin benzoate, diflubenzuron, cypermethrin, deltamethrin and hydrogen peroxide (Overton et al., 2018).

Evidence of drug resistance in sea lice that have occurred mainly as a result of management decisions and of over-reliance on a small range of chemical classes and modes of action, have been noted. The cost of developing new medicines and the subsequent need to get a return on that investment by pharmaceutical companies means that investment in new molecule classes for diseases of concern in aquaculture can be limited. Furhtermore, these costs may not necessarily be borne by fish farm companies, at least in the short term, therefore exacerbating the lack of available treatments. Ironically, whilst AGD may be seen as both emerging and as an established disease, investment in developing therapies to combat the infection is limited by a lack of options and a lack of investment. Monotherapy, whilst useful when molecules are utilised on a rotational basis and when highly efficacious, can become problematic if these two criteria are not adhered to. As an example, the use of antimonial compounds overtime in the case of leishmaniasis has led to favour the selection of resistant strain against this drug and as a consequence, force to investigate other therapeutic options to treat this disease rely on nowadays amphotericin B or miltefosine notably in developed countries (Monzote., 2009).

AGD caused by *N. perurans* is a considerable threat for aquaculture worldwide production. Currently, knowledge regarding its general biology (reproduction, ecology, virulence mechanisms) are still missing although considerable research efforts continue to be undertaken to address these knowledge gaps. The regular monitoring of farmed fish by health managers and use of reactive intervention therapies such as bath treatments with freshwater and / or hydrogen peroxide can reduce the short-term impact of AGD. However, a long-term goal for the industry of a sustainable, effective and prophylactic solution which fits better with the needs of the fish farming industry is still required to solve this growing issue. As a result of the current treatment limitations, the present study aimed to provide an evidence-based approach to find additional options alongside those existing AGD treatments, particularly for Atlantic salmon farming. The approach of the present research project relied on identifying diseases that were taxonomically related to the AGD agent that affect other non-fish hosts and the treatments used for these diseases in order to evaluate these drugs as potential new

treatments for AGD through a scoring and a prioritisation process. This approach included thorough searches in the literature, use of key chemical and biological databases (e.g. DrugBank, PubChem, KEGG, ATC) and the use of bioinformatics tools (e.g. iPath, BLAST).

The main finding of the present study is the prioritisation of 17 drugs (i.e. atovaquone, chloroquine, eflornithine, flucytosine, griseofulvin, hydroxyurea, idelalisib, lonidamine, micafungin, miltefosine, niraparib, omacetaxine mepesuccinate, oxiconazole, pentostatin, proguanil hydrochloride, tavaborole and thiabendazole) (out of 262 initially identified) for follow-on studies to assess efficacy *in vitro* and *in vivo*. This result has been made possible thanks to strategic decisions. As example, in the drug scoring process performed in Chapter 2, drugs used in the case of amoebic and kinetoplastids were conserved (i.e. amphotericin B, fluconazole, itraconazole, ketoconazole, metronidazole, miltefosine, paromomycin, pentamidine and tinidazole) and later on in the same process, antimicrobial drugs considered as critically/highly important for human medicine (e.g. amoxicillin, colistin, doxycycline, lincomycin, metronidazole and tinidazole) were discarded. Another example which illustrate the strategic decisions that have led this work is the prioritisation process from Chapter 3 which rely on the availability of data relevant to drug development and the presence of the drug target in AGD. Regarding the evaluation of potential combination therapy, most of the prioritised drugs have already been used as components in drug combination treatment for various disease as shown in Chapter 3. However, there is no evidence that others (i.e. idelalisib, niraparib, omacetaxine mepesuccinate, pentostatin and tavaborole) have ever been used combination treatment. Drugs classified as antimalarial, antifungal, antihelmintic and trypanocidal were expected to be more likely included in the final analysis because of their use in several parasitic/infectious diseases. The most surprising results was the presence of five drugs (i.e. hydroxyurea, idelalisib, miltefosine, omacetaxine mepesuccinate and pentostatin) classified as antineoplastic drug, in DrugBank and PubChem database, among the 17 prioritised drugs. Antineoplastic drugs are generally designed for human use in the case of various cancer and thus, it was not expected to find this class of compound after scoring and

prioritisation process. Even if this result could seem counterintuitive, the alkyphosphocholine molecule, miltefosine, represents a good example of a molecule with the potential for multiple applications. Although miltefosine was originally developed as an antiprotozoal and antineoplastic drug, it has subsequently been approved as a local treatment for cutaneous metastases in patient affected by breast cancer as well as a treatment for leishmaniasis, trypanosomiasis and balamuthia amoebic encephalitis (Dorlo et al., 2012; Martínez et al., 2010) (Dorlo et al., 2012).

The potential toxic effects of drug towards the Atlantic salmon is a major concern of this study. The gene targets FASTA file used in the Chapter 3 in BLAST program (BLASTn and BLASTx) were researched in the genome of Atlantic salmon (taxid: 8030) with the exact same procedure performed with *Neoparamoeba pemaquidensis* (taxid: 180228) and *Perkinsela* sp. (CCAP 1560/4 taxid: 1314962) (Table 4.1). Unfortunately, almost all the researched drug targets were identified as functional targets in Atlantic salmon except one, the glutathione-S-transferase, which is classified as a protozoal target reported in *Plasmodium falciparum* (Srivastava et al., 1999). The drug demonstrating activity against this target is the chloroquine (Srivastava et al., 1999). Thus, among the drugs identified with the BLAST process (i.e. atovaquone, chloroquine, flucytosine, griseofulvin, hydroxyurea, lonidamine, omacetaxine mepesuccinate, proguanil hydrochloride, tavaborole and thiabendazole), chloroquine is an interesting candidate because of its potential non-toxic effects against Atlantic salmon.

The desk-based approach has permitted the identification of existing drugs with evidence of the drug targets present in AGD. However, there are several limitations with this kind of approach. First, the genome of *N. perurans* is not publicly available and so the project relied on information derived from a closely related non-pathogenic organism, *N. pemaquidensis*. However, specific genes (e.g. involved in virulence mechanisms) encoded by *N. perurans* but not by *N. pemaquidensis* have not been taken into consideration knowing that they could have been relevant targets. Second, it is impossible to predict if a drug will affect AGD *in vitro*, *in vivo*, or in the farm environment. Hence, even if the drug target is present in AGD, there is no certainty that the drug could reach this target to exert its therapeutic effect. Third, the design of the selection criteria

Table 4.1. Investigation in BLAST program of the drug targets FASTA sequence, used in the Chapter 3 and identified in AGD, in the genome of the Altantic salmon (taxid: 8030). n: BLASTn. x: BLASTx.

Organism	Molecular target name	Gene ID	Presence	BLAST	Query cover	E value	Identity
Perkinsela sp	50S ribosomal protein L2	20380895	No	nx	-	-	-
Neoparamoeba pemaquidensis	50S ribosomal protein L2	1734043	Yes	x	75%	6.00E-22	34%
Perkinsela sp	60S ribosomal protein L3	5656437	Yes	x	92%	3.00E-153	56%
Perkinsela sp	Bifunctional dihydrofolate reductase-thymidilate synthase	5649109	Yes	x	55%	4.00E-123	59%
Neoparamoeba pemaquidensis	Cytochrome b	1734035	Yes	x	94%	9.00E-128	53%
Perkinsela sp	Cytosolic leucyl-tRNA synthetase	8049859	Yes	n	98%	0.00E+00	47%
Perkinsela sp	Dihydrofolate reductase	5649109	Yes	x	55%	4.00E-123	59%
Perkinsela sp	Fumarate reductase flavoprotein subunit	948667	Yes	n	90%	6.00E-119	39%
Perkinsela sp	Glutathione S-transferase	5650353	No	nx	-	-	-
Perkinsela sp	Hexokinase-1	5651559	Yes	x	94%	1.00E-71	37%
Perkinsela sp	Ribonucleoside-diphosphate reductase large subunit	5653298	Yes	x	99%	0.00E+00	58%
Perkinsela sp	Thymidylate synthase	5649109	Yes	x	55%	4.00E-123	59%
Perkinsela sp	Tubulin alpha chain	10966580	Yes	n	85%	0.00E+00	77%
Neoparamoeba pemaquidensis	Tubulin beta chain	14925210	Yes	n	93%	0.00E+00	75%
Perkinsela sp	Tubulin beta chain	5651759	Yes	n	97%	0	79%

for scoring and prioritisation may have led to the discarding of drugs that could still be excellent candidates for developing as new agents for AGD, such as those of critical importance in human medicine, or those active only against amoebae or kinetoplastids and not both types of organism, or those with a lack of information relevant to drug development and regulatory approval. In total, 262 drugs were considered in the present study and, by the end, only 17 were prioritised. Moreover, the scoring process considered only the quantity of information was available and not the quality of the information, meaning no thresholds were established regarding the criteria (e.g. toxicity, solubility, production, prohibition) and this needs to be included in follow on studies. Another limitation was the fact that the seven infectious diseases considered in this present study were induced by systemic pathogens whereas AGD is an external pathogen. Therefore, the prioritised drugs may not be as efficacious as in their original application compared to possible use against AGD.

The combination therapy has some key advantages such as limitation of emergence of drug resistance were underlined (van Griensven et al., 2010). Nevertheless, it was suggested that in the case of malaria, if two drugs with different modes of actions are used in combination therapy, then the risk to develop a spontaneous mutation which leads to multidrug resistance is very unlikely (White., 2004). A study that contradict this hypothesis by showing that resistance of *Plasmodium falciparum* (i.e. aetiological agent of malaria) to the combination of artemisinin-piperaquine may be linked with polymorphism of K13-propeller gene in Africa and Southeast Asia, lessening the therapeutic option to face malaria (Huang et al., 2015). Some recent findings support these results and suggest that a reduced K13-propeller transcriptional response could represent one important step towards artemisinin tolerance or resistance in *Plasmodium falciparum* (Silva et al., 2019). The combination therapy also suffers from an institutional point of view. As example, for a combination of two drugs, 3 drug approvals may be necessary: one for each drug and one for the combination (Woodcock et al., 2011). Moreover, studies regulations around combination therapy constitutes also barrier. As an example, a study for a factorial two-drugs combination should have four groups to compare efficiency of the combination (i.e. a group for each component alone, a group

for the combination and a control group) (Woodcock et al., 2011). As a consequence, this fact will increase the cost of this kind of study compare to monotherapy. However, these potential increased costs associated with development and its subsequent uptake by the industry may be considered worthwhile if the impact of AGD on health and welfare of their hosts is minimised and if financial returns are sufficiently high.

Regarding the limitations inherent to the adopted approach in this project, several refinements to the process could be considered for future work. Primarily, it may have been useful to widen the focus of potential treatments on (all?) external parasite in human, animal and plants and their associated treatment rather than limit the research field of similar disease to amoebic and kinetoplastid organisms. As an example, malaria caused by *Plasmodium falciparum* was not considered in this study whereas some identified drugs through the process applied in the present study (scoring and prioritisation) identify antimalarial drugs (e.g. chloroquine, atovaquone). Secondly, the study may have been improved by conducting research of specific drug targets by using genome subtraction. This approach consists of comparing the genome of the host to that of the pathogens (i.e. in the case of AGD, compare the genome of Atlantic salmon to the genome of *N. perurans* and *Perkinsela* sp.), in order to identify genes involved specifically in the metabolism of the pathogens. These genes and encoded proteins could represent safe and relevant drug targets for the establishment of a treatment because of their specific presence in the pathogen and also aim to avoid toxic effects toward the host (Sakharkar et al., 2004). This approach was notably done in a study regarding at drug targets identification in *Pseudomonas aeruginosa* and has led to reveal 306 essential genes which could be considered as potential drug targets (Sakharkar et al., 2004).

The present study is a major milestone for the development of a new treatment to combat AGD outbreaks. However, prior to their approved use under commercial conditions a number of steps need to be undertaken. The first step that needs to be considered is an *in vitro* study. The 17 drugs prioritised in the selection process will need to be tested *in vitro* to confirm and quantify inhibitory activity against the amoeba, *N. perurans*. This study will be an important step to confirm the hypothetical drug activities against AGD and also to provide a solid basis to work on possible combination therapies

thanks to the ability to obtain dose-response curves that allow the plotting of isobolograms (i.e. diagram based on dose-response curve of two drugs in order to evaluate supra-additive or sub-additive effects against a given pathogens). This process could be applied more broadly to the 118 scored drugs even if only 17 of them have shown evidence of drug targets presence in AGD. After this *in vitro* study, an *in vivo* study would be performed with a subset of drugs showing efficacy and *in vivo* toxicity against several fish species known to be sensitive to AGD and which could be exposed with the proposed treatment such as Atlantic salmon, lumpfish and ballan wrasse. *In vivo* studies should include confirmation of effective dose, target animal safety, nature of residues as well as absorption, distribution, metabolism and excretion (ADME) studies. Data on environmental and non-target safety may need to be conducted if such data is not available in the public domain.

Another route of possible study is drug design against proteins in unique metabolic pathways that have been described in AGD such as heme metabolism, trypanothione metabolism and ubiquinone metabolism (Tanifuji et al., 2017). These metabolic pathways constitute possible targets for the development of drugs. Regarding the treatment speculated for AGD, the combination therapy has advantages and drawbacks. Once a suitable treatment (or combination of treatments) involving extant or specifically designed drugs against AGD are identified via the approach taken in this unique study, the route of administration (i.e. feed, injection, immersion) should be also evaluated in a parallel study to identify the most effective route of uptake by the host as well as minimising potential environmental impacts.

Amoebic gill disease caused by *N. perurans* is a significant risk for marine fish farming and the beginning of the path leading to the approval of a new treatment for AGD has been cleared by this present study with the prioritisation of 17 drugs.

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