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Herbicides and Protozoan Parasite Growth Control: Implications for New Drug Development

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1. Introduction

Modern chromalveolates were originated through a series of events of endosymbiosis of microalgae by an eukaryote, leading to the retention of its plastid by this new host. These complex events, which took place multiple times during the course of evolution, originated new organisms that either lost or retained parts of the metabolisms present in their ancestral microalgae symbionts, achieved by transferring some of their genes into the nucleus of the host cell or even maintaining a relic organelle, a plastid circular DNA. The presence of this relic DNA was discovered in some apicomplexas, and thus named apicomplast. It was later found that a large number of parasites belonging to Alveolata also contained a relic non-photosynthetic plastid, homologous to the chloroplast of plants and algae, thus expanding the apicomplast concept outside the apicomplexas. During the 90's, some important metabolic pathways just known to occur in plants, algae, fungus and some bacteria were also identified in a series of important human parasites like *Plasmodium falciparum* and *Toxoplasma gondii,* the agents of malaria and toxoplasmosis respectively, opening a new era in drug prevention/control. Some of those protozoan parasites are responsible for millions of disease cases worldwide and hence a major concern for human health. The growing understanding of the biology and biochemistry of protozoan parasites, which has considerably increased over the past two decades, has paved the way to the discovery of many potential targets for new parasite drugs. The decrypted genomes of several species and the new post-genomic tools considerably improved our ability to identify and study the different metabolic pathways at the molecular level, and consequently contribute to validate potential drug targets.

In 1998 the journal Nature published the discovery of a group of protozoan parasites that shared a metabolic pathway (shikimate pathway) essential for their survival with many plants, fungi and bacteria, but not found in mammals. Furthermore, researchers demonstrated that the herbicide glyphosate, known to interfere with this pathway in plants, could be used successfully to inhibit the *in vitro* growth of these parasites, opening the possibility of using available herbicides as a start point to develop new drugs to control parasite growth.

Various studies have since then highlighted the importance of plastid or algae-like pathways for other parasite metabolisms such as fatty-acid FAS II, heme and isoprenoid

biosynthesis pathway, and since they are both essential for parasite survival and not present in their hosts, they have attracted considerable attention as targets for therapeutics. Furthermore, since they are already known as targets of existing herbicides, this should significantly reduce the time and cost of specific drug development.
Herbicides are a class of compounds known to produce a wide range of toxic side effects, thus posing a threat to several organisms including humans. However, and despite this toxicity, which represents the negative side of their use, we have to acknowledge that the development and use of pesticides and herbicides over the past decades has played an important role in increasing agricultural productivity and in controlling potential carriers of human diseases (Table 1). In the near future, the same herbicides may become the precursors of new drugs against protozoan parasite diseases.

2. Metabolic pathways as drug targets

Parasite cells as well as their corresponding hosts have hundreds of metabolic pathways that are vital for their normal function. Therefore, there is always a need to study host pathways and compare them with those of the parasite. Each pathway has a number of enzyme reactions that catalyze its different steps. Enzyme activity at every step is regulated to ensure that the final product of the pathway provides for the needs of the cell. With the recent completion of many genome projects, it became possible to provide provisional maps of the proteome of several parasites. A large portion of predicted or confirmed open reading frames result in unique proteins not found in the corresponding hosts, and these are good news when it comes to drug development since compounds that inhibit whatever function of these proteins are potentially less likely to cause severe side-effects to the host. The challenge resides in showing that a particular protein or pathway is essential for the survival of the parasite and thus if that protein can be a potential drug target. To find an answer to this question we either have high-throughput methods or can use a more traditional approach, based on some prior knowledge of candidate metabolic pathways or cellular processes.

2.1 Shikimate pathway

Biosynthesis of aromatic amino acids in plants, in many bacteria, and in microbes relies on the enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, a good target for several drugs including herbicides. Because the shikimate is absent in more complex organisms, EPSP synthase is an attractive target for the development of new antimicrobial agents effective against bacterial, parasitical, and fungal pathogens. A valuable lead compound and an exemple in the search for new drugs and herbicides is glyphosate. Glyphosate is a successfully used herbicide, being the active ingredient of the widely used weed control agent Roundup, and was shown to inhibit the growth of the parasites *P. falciparum*, *T. gondii*, *C. parvum* (Roberts et al. 1998) and *P. olseni* (Elandalloussi et al. 2008), among others.

2.2 Isoprenoid biosynthesis pathway

Isopentenyl diphosphate (IPP) is the central intermediate in the biosynthesis of isoprenoids, the most ancient and diverse class of natural products. Two distinct routes of IPP biosynthesis occur in nature, the mevalonate pathway and the recently discovered deoxyxylulose 5-phosphate (DOXP) pathway. The evolutionary history of the enzymes involved in both routes and the phylogenetic distribution of their genes across genomes suggests that the mevalonate pathway is unique to archaebacteria, as the DOXP is for

Herbicides	Organism	Target	References
Norflurazon, fluridone	*Plasmodium falciparum*	Carotenoid synthesis	US Patent 5859028
Diclofop, sethoxydim, tralkoxydim, alloxydim, clethodim and cycloxydim	*Plasmodium falciparum*	Fatty acid synthesis	US Patent 5877186
Fosmidomycin	*Plasmodium falciparum*	Isoprenoids pathway	(Lichtenthaler 2000)
Trifluralin, oryzalin and amiprophos-methyl	*Plasmodium falciparum*	Microtubule inhibitor	(Fennell et al. 2006)
Ethalfluralin, oryzalin and trifluralin	*Toxoplasma Gondii*	Microtubule inhibitor	(Stokkermans et al. 1996)
Dinitroaniline herbicides	*Trypanosoma cruzi*	Microtubule inhibitor	(Traub-Cseko et al. 2001)
Dinitroaniline herbicides	*Cryptosporidium parvum*	Microtubule inhibitor	(Arrowood et al. 1996)
Dinitroaniline and phosphorothioamidate herbicides	*Plasmodium falciparum*	Microtubule inhibitor	(Fennell et al. 2006)
Trifluralin, pendimethalin, oryzalin, and benfluralin (dinitroaniline herbicides)	*Plasmodium berghei*	Microtubule inhibitor	(Dow et al. 2002)
Dinitroaniline herbicides	*Entamoeba histolytica*	Microtubule inhibitor	(Makioka et al. 2000)
2,4,5 trichlorophenoxy acetic acid	*Tetrahymena pyriformis*	Mitochondria	(Silberstein and Hooper 1977)
Aryloxyphenoxypropionate herbicides	*Toxoplasma Gondii*	Plastid acetyl-CoA carboxylase	(Zuther et al. 1999)
Clodinafop-propargyl	*Babesia equi* and *B. caballi*	Plastid acetyl-CoA carboxylase	(Bork et al. 2003)
Flufenacet	*Perkinsus marinus*	PUFA pathway	(Venegas-Caleron et al. 2007)
Glyphosate	*Plasmodium falciparum*	Shikimate pathway	(Roberts et al. 1998)
Glyphosate	*Perkinsus olseni*	Shikimate pathway	(Elandalloussi et al. 2008)

Table 1. Herbicide derivatives used to control parasite proliferation, their metabolic targets and corresponding references

eubacteria, and that eukaryotes have inherited their genes for IPP biosynthesis from prokaryotes. The occurrence of genes specific to the DXP pathway is restricted to plastid-

bearing eukaryotes, indicating that these genes were acquired from the cyanobacteria ancestor of plastids (Lim and McFadden 2010). The non-mevalonate isoprenoid biosynthesis pathway is essential for many protozoan parasites survival, since DOXP inhibitors like herbicide fosmidomycin strongly inhibit their *in vitro* proliferation (Lichtenthaler 2000; Wiesner et al. 2002), and thus is effective in managing the clinical symptoms of malaria that are associated with the intra-erythrocytic phase of the parasite cell cycle (Jomaa et al. 1999). Hence, all herbicides which are inhibitors of this pathway in plants are also potential drugs against all parasites bearing the same pathway.

2.3 Fatty acid biosynthesis

It was previously thought that apicomplexan parasites were incompetent for *de novo* fatty acid synthesis (Holz 1977; Matesanz et al. 1999), but recent work showed the presence of nuclear-encoded apicomplast-targeted genes for all enzymes of the fatty acid biosynthesis pathway in several apicomplexa parasites and this finding provided strong arguments in favor of the presence of a *de novo* fatty acid biosynthesis in this organelle (Surolia et al. 2004). The presence of highly conserved proteins known as Type II fatty acid synthase explains the susceptibility of *T. gondii* and *P. falciparum* to herbicides targeting plastidic Acetyl-CoA carboxylase, like the aryloxyphenoxypropionates. This pathway is seen as a promising drug target, mostly because it is structurally and functionally distinct from its equivalent pathway present in the vertebrate hosts (Goodman and McFadden 2008).

3. Perkinsus, a protozoa parasite of interest for pharmaceutical testing

Protozoa represents one of the earliest branches of eukaryotic organisms and the key to understand early global evolution. Inside protozoa, alveolata represents one of the classes better studied due to the presence, within this class, of the apicomplexa, which include parasites like the agents of malaria and toxoplasmosis (leading opportunistic infections often associated, among others, with AIDS and with congenital neurological birth defects), responsible for the infection of man and cattle, thus making this phylum particularly important for medical and veterinary reasons. Most of these organisms are a major cause of disease worldwide, but many of them have received little attention from pharmaceutical industry. This scenery is now changing due to the completeness of genome sequence of some of the most important protozoan parasites within this phylum. Comparative genomics using growing information provided by multiple genome sequencing efforts can now be used to help identify parasite-specific targets for drug development. However, and despite the increasing variety of genomes already sequenced, many possible applications resulting from their analysis are most likely not yet unveiled since, very often, knowledge of the genome alone is not sufficient to provide answers to many of the existing questions, and the unveiling of both transcriptome and/or proteome are also required.

3.1 The genus Perkinsus

The microorganisms of the genus Perkinsus are protist parasites responsible for important mortalities in different mollusc species. It was first described in 1946 as a spherical unknown organism found in moribund *Crassostrea virginica* oysters but not in healthy ones in Louisiana (USA) (Mackin et al. 1950). For the past two decades a severe mortality is affecting bivalve molluscs particularly in Portugal and Spain (Leite et al. 2004). This mortality was first associated with the parasite *Perkinsus* (P.) *atlanticus* in 1989 (Azevedo

1989). Recently, phylogenetic studies and the use of molecular data have shown that *P. atlanticus* and *P. olseni* are, in fact, the same organism (Murrell et al. 2002). On the other side of the Atlantic, oyster's mortalities are related with a parasite from the same genus, *P. marinus*. This parasite was first classified as a fungus, then as an Apicomplexa and now, a new taxonomic class (Perkinsea) was created inside Alveolata to place all Perkinsus.

Some authors suggest that Perkinsus represents an early branch between dinoflagellates and apicomplexa. These two groups of organisms are quite different, each containing unique characteristics, like the absence of histones and presence of photosynthesis in dinoflagellates and the presence of a circular DNA within a plastid in some apicomplexa like *P. falciparum* (Gardner et al. 1991) and *T. gondii*. Furthermore, it was recently suggested that Perkinsus also possesses both a non-photosynthetic plastid reminiscent of the apicomplexan relic plastid organelle, the apicomplast, (Teles-Grilo et al. 2007) and additional specific cell compartments (Fernández-Robledo et al. 2008) raising questions about the nature and origin of putative relic plastid/compartments in Perkinsus species.

Although the analysis of Perkinsus genes has only started very recently, some notorious findings are being made, in terms of characterization of metabolic pathways susceptible to play a critical role for drug targeting. Approaches to begin unveil Perkinsus transcriptome were already made by our group like the usage of Suppression Substractive Hybridization (SSH) to identify genes differentially expressed by *P. olseni* when exposed to hemolymph from Ruditapes decussatus (Ascenso et al. 2007) or the use of differential transcriptomic analysis to unveil Perkinsus transcripts resulting from gene transcription under differential conditions (Leite el al., unpublish data). But at present, as more and more genome sequence information for Perkinsus becomes available, the use of more high throughput techniques/tools such as microarrays is possible. There is currently ongoing a project conducting P. marinus genome (almost complete) sequencing by The Institute for Genomic Research (TIGR)/Center for Marine Biotechnology (COMB) (http://www.tigr.org/tdb/e2k1/pmg/). The strategy chosen for sequencing *P. marinus* was whole genome shotgun sequencing (8x coverage).

3.2 *In vitro* parasite cultures as tools for drug screening

In vitro cultures have been extensively used for screening chemotherapeutic agents. This technique is less costly than animal screening but depends on the ease of establishing laboratory cultures. The relationship between the parasite and the host is very intricate and in most cases disruptions of the host-parasite relationship *in vitro* leads to gradual death of the parasite. A frequent difficulty to establish continuous parasite cultures is their usually complex life cycle, in addition to some of them having intermediate hosts. An attempt to provide the parasite with a specific culture medium which is the most similar to its *in vivo* environment has been conducted for each parasite that can be cultured in vitro (Allen *et al*. 2005).

Various *in vitro* cultures of the protozoan parasite of genus *Perkinsus* (Figure 1) have already been developed (Gauthier et al. 1995; Robledo et al. 2002; Casas et al. 2008), and have the advantage of not requiring the presence of a host enhancing the capability of assessing the effects of drugs on parasite growth (Gauthier and Vasta 1994; Elandalloussi et al. 2005; Leite et al. 2008). The effects of a given drug can thus be determined by analyzing the survival of the parasites as a function of time in culture in the presence or absence of the drug to be tested (Figure 2) and analyzing the data using specific software (Figure 3).

Fig. 1. *Perkinsus olseni* cells under culture

3.3 Perkinsus proliferation can also be affected by herbicides derivatives

To demonstrate the advantages of the usage of Perkinsus as an alternative for the screening for new drug targets affecting viability/proliferation of the parasite and thus identification of possible drug precursors among known herbicides, a test using the most common herbicides was developed. Ten herbicides were chosen between the most frequently used worldwide (see Table 3). Other aspect taken into consideration was the choice of a non dinitroaniline, phosphorothioamidate or aryloxyphenoxypropionate herbicide because their mode of action is already known for several protists. The only exception to this criteria was the presence of Pendimethalin, which was used as positive control. A brief description of the main usages of the selected herbicides is presented below:

- 2,4-Dichlorophenoxyacetic acid (2,4-D), a broadleaf herbicide have been commercially available for over 50 years and are a widely used family of phenoxy herbicides worldwide, being a case study on agricultural chemicals. Now mainly used in a blend with other herbicides, it is the most widely used herbicide in the world, third most commonly used in the United States. It is an example of synthetic auxin (plant hormone) (Kennepohl et al. 2001).
- Atrazine, a triazine based herbicide is used in corn and. The low cost/good performance on a broad spectrum of weeds common in the U.S. is explains the widely usage of this pesticide. It is also commonly used with other herbicides to lower potential groundwater contamination. It is a photosystem II inhibitor (Lim et al. 2009).
- Dicamba another example of a synthetic auxin is a benzoic acid herbicide that acts by mimicking the effects of auxins (i.e., natural plant growth hormones), causing enhanced but uncontrolled growth rates, alterations in plant function homeostasis, and death. Dicamba is often combined with one or several other herbicidal agents including 2,4-D, 2,4-DP, atrazine, glyphosate, imazethapyr, ioxynil, and mecoprop. It is used to control a wide spectrum of annual and perennial broadleaf weeds and is effective in both pre- and post-emergence applications. A primary agricultural use is weed reduction in grain/cereal cropsand maintenance of pastures, forest lands, fence rows, and transportation and utility rights-of-way. (Harp et al. 2001).

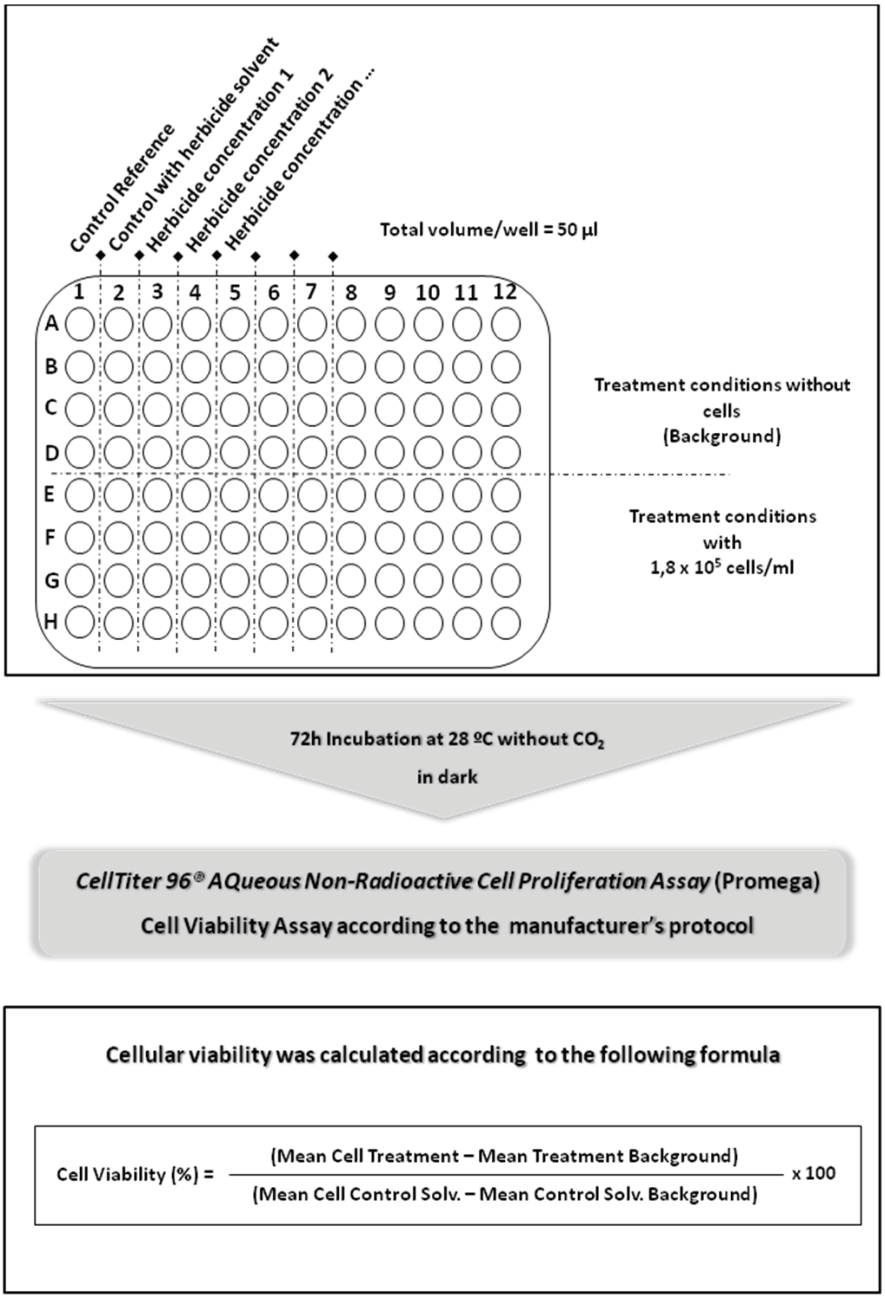

Fig. 2. Method to screen the effect of compounds in the proliferation of *Perkinsus olseni* using *in vitro* cultures (Elandalloussi et al. 2005; Leite et al. 2008).

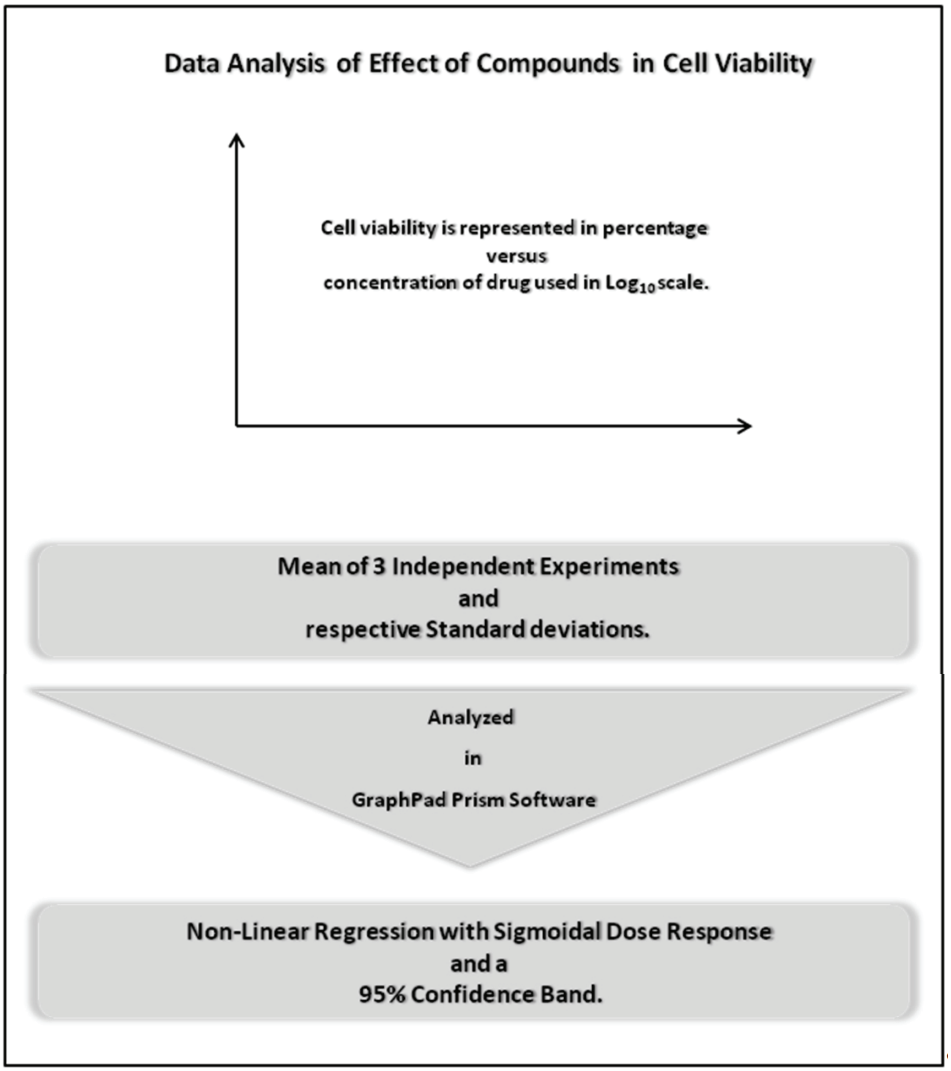

Fig. 3. Data analysis of *Perkinsus olseni* antiparasitic compounds screening

- Glufosinate ammonium, a broad-spectrum contact herbicide and is used to control weeds after the crop emerges or for total vegetation control on land not used for cultivation. It is a structural analogue of glutamate and acts in plants by inhibiting glutamine synthetase, thereby blocking synthesis of glutamine from glutamate and thus assimilation of NH_4 (Manderscheid and Wild 1986; Hack et al. 1994).
- Fluroxypyr, a systemic, selective herbicide is used for the control of broad-leaved weeds in small grain cereals, maize, pastures, range land and turf. It is a synthetic auxin. In cereal growing, fluroxypyr's key importance is in the control of cleavers, Galium aparine. Other key broad-leaved weeds are also controlled (Wu et al. 2009).

- Imazapyr is a non-selective herbicide used for the control of a broad range of weeds including terrestrial annual and perennial grasses and broadleaved herbs, woody species, and riparian and emergent aquatic species (Hess et al. 2010).
- Linuron is a non-selective herbicide used in the control of grasses and broadleaf weeds. It works by inhibiting photosynthesis (Snel et al. 1998).
- Metolachlor is a pre-emergent herbicide widely used to control annual grasses in corn and sorghum; it has partially replaced atrazine in these uses (Heydens et al. 2010).
- Pendimethalin, is a pre-emergent herbicide widely used to control annual grasses and some broadleaf weeds in a very wide range of crops, including corn, soybeans, wheat, cotton, many tree and vine crops, and many turfgrass species (Heydens et al. 2010).
- Picloram, is a pyridine herbicide mainly used to control unwanted trees in pastures and edges of fields. It is another synthetic auxin (Grossmann 2010).

All the herbicides were ordered from Sigma and the methodology followed was identical to that described in Figures 1 and 2. In order to test the range of concentrations to be used, a preliminary assay using all these herbicides in three different concentrations (1, 100 and 500μM) was conducted (table 2). Preliminary resulted suggested that only four herbicides demonstrated some effect on Perkinsus proliferation (Fig. 4) and thus a more extended test was performed using only these selected ones.

Coumpound	Chemical Formula	Group (herbicides)	Observed inhibition (Perkinsus)	IC50 (μM)
2,4-D	$C_8H_6Cl_2O_3$	phenoxy herbicides	Yes	ND
Atrazine	$C_8H_{14}ClN_5$	chlorotriazine	No	-
Dicamba	$C_8H_6Cl_2O_3$	benzoic acid	No	-
Fluroxypyr	$C_7H_5Cl_2FN_2O_3$	pyridine	No	-
Glufosinate-ammonium	$C_5H_{15}N_2O_4P$	organophosphorus	No	-
Imazapyr	$C_{13}H_{15}N_3O_3$	imidazolinone	No	-
Linuron	$C_9H_{10}Cl_2N_2O_2$	phenylurea	Yes	391,3
Metolachlor	$C_{15}H_{22}ClNO_2$	chloroacetanilide	Yes	193,2
Pendimethalin	$C_{13}H_{19}N_3O_4$	dinitroaniline	Yes	396,6
Picloram	$C_6H_3Cl_3N_2O_2$	pyridine	No	-
Glyphosate	$C_3H_8NO_5P$	organophosphorus	Yes	3400
Fosmidomycin	$C_4H_{10}NO_5P$	-	No	-

Table 2. Compound names and corresponding chemical formulas of herbicides derivatives shown to affect Perkinsus proliferation.

From the panel of herbicides tested, four of them had some effect on *Perkinsus olseni* proliferation, namely 2,4-D, Linuron, Metolachlor and Pendimethalin (Figure 4).

- The effect of Pendimethalin on Perkinsus proliferation was expected since an effect was already observed for trypanosomatids and Plasmodium falciparum (Chan and Fong 1994; Dow et al. 2002). It works by inhibiting microtubule disruption during parasite development.
- Metolachlor belongs to the class of chloroacetanilides herbicides responsible for inhibition of very-long-chain fatty acids (VLCFA) biosynthesis in plant and algal cells

(Böger et al. 2000; Trenkamp et al. 2004). It is already described in the literature that *Perkinsus marinus* possess some genes related with VLCFA like FAE-1. *P. marinus* FAE1-like elongating activity is also sensitive to the herbicide flufenacet, in accordance to some higher plant 3-ketoacyl-CoA synthases (Venegas-Caleron et al. 2007) and explain the results abtained with Metolachlor.

- The primary effect of linuron is the inhibition of photosystem II electron flow (Snel et al. 1998), resulting in damage and plant weakness. This result can be surprise but due to the lack of photosystem II in Perkinsus, but it can be related to the inhibition of electron flow in other systems.
- 2,4-D also revealed some effect but even at higher concentrations (500 μM) it inhibit less that 20% of the proliferation. Together with the pattern of the proliferation graphic, the results suggest that this herbicide has some cytotoxic effect on Perkinsus may be due to contaminations present in 2,4-D formulation.

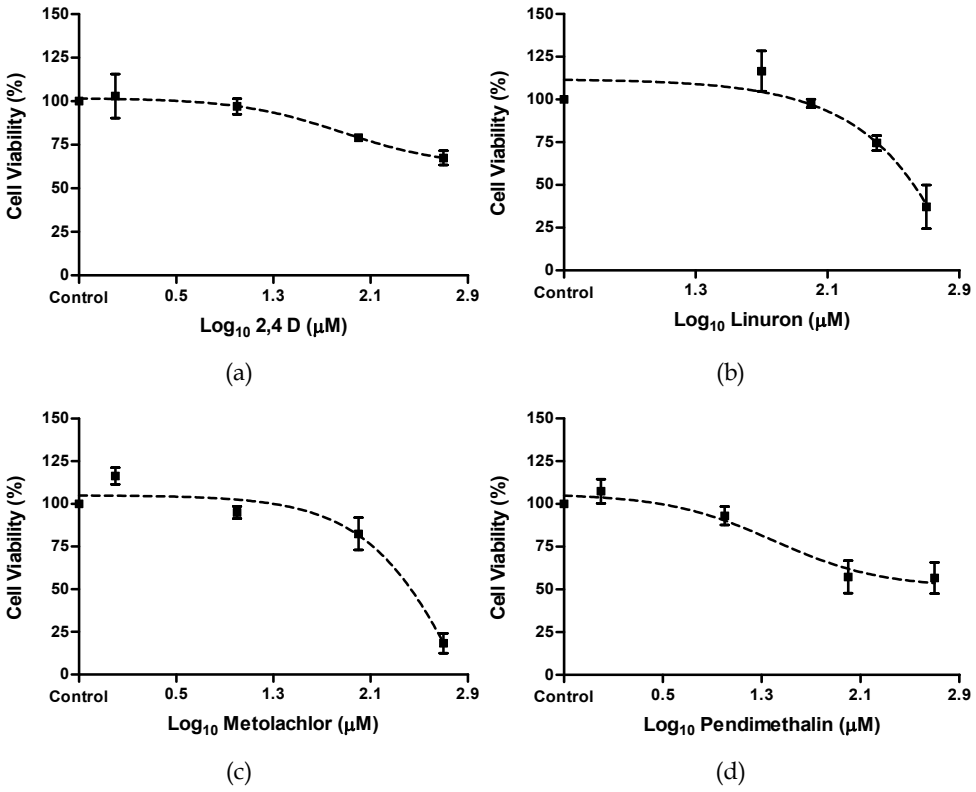

Fig. 4. Proliferation of *P. olseni* after 72 h of exposure upon different treatments.
Vertical axis stands for cell viability in percentage and horizontal axis for $\log_{10}$ concentration in μM (A) 2,4-Dichlorophenoxyacetic acid, (B) Linuron,(C) Metolachlor and (D) Pendimethalin. Percentage of proliferation is relative to normal (non-treated) conditions (100%).

4. Advantages of Perkinsus as model organism for new drug development against protozoa

Altogether, the available data suggest that Perkinsus can be a good alternative for herbicide screenings to detect potential drug precursors affecting parasite metabolic pathways not present in their hosts. Most of our currently used antiparasitic drugs have been identified as a result of random screening of a series of chemicals that are related to compounds with recognized therapeutic value. This is referred to as the empirical approach to drug discovery and remains a valid approach to the discovery of novel antiparasitic molecules. Despite not being comparable with true high-throughput screening, significant screening can be conducted against the parasite *in vitro* using direct approaches since all antiparasitic drugs must be tested against the parasite before advance to *in vivo* models (Woods and Knauer 2010).

Advantages of Perkinsus can be related to the fact that (i) it shares specific characteristics with algae, fungus and plants, in particular the presence of metabolic pathways that are not present in parasite hosts, (ii) these characteristic pathways are also present in several disease agents like plasmodium and toxoplasma and thus the response to specific drugs affecting these pathogenic parasites can first be tested in Perkinsus, (iii) it is harmless for mammals, (iv) it can be grown easily in vitro, (v) clonal cultures of the parasite have been developed and are available and (vi) it shares many similarities with highly pathogenic parasites, thus having the potential to became very useful for both the study of protozoan diseases and in pharmaceutical drug discovery and/or testing.

In addition, and because Perkinsus is an aquatic parasite and circulates and differentiates between the water column and the host, it can be an excellent biomarker of the degree of contamination by chemicals accumulated in the water or upon the host body and thus be very useful to detect effects of newly introduced chemicals and to be used to perform preliminary tests on aquatic bioaccumulation as quickly as possible, to avoid possible disasters.

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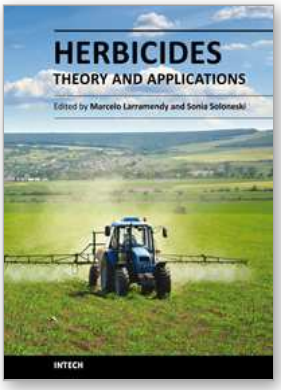

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The content selected in Herbicides, Theory and Applications is intended to provide researchers, producers and consumers of herbicides an overview of the latest scientific achievements. Although we are dealing with many diverse and different topics, we have tried to compile this "raw material" into three major sections in search of clarity and order - Weed Control and Crop Management, Analytical Techniques of Herbicide Detection and Herbicide Toxicity and Further Applications. The editors hope that this book will continue to meet the expectations and needs of all interested in the methodology of use of herbicides, weed control as well as problems related to its use, abuse and misuse.

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