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EVALUATION OF THE *ANTI-Toxoplasma gondii* EFFECT OF METALLOCOMPLEXES COORDINATED OR NOT WITH SULFADIAZINE

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Abstract: *Toxoplasma gondii*, the agent of toxoplasmosis, is an obligate intracellular protozoan capable of infecting a wide range of vertebrate cells. Toxoplasmosis is a pathology related to severe damage to immunosuppressed hosts and its current chemotherapy is quite restricted, with the combination of sulfadiazine and pyrimethamine being used more often, a therapy associated with adverse reactions. This fact highlights the importance of studying new drugs against *Toxoplasma gondii*. Currently, the biological effect of new compounds has been studied, drugs that use a metallic core, metallocomplexes, inorganic compounds that show promising biological activities such as fungicide, bactericide and antiviral. The metallocomplexes, the dinuclear ferric compounds N0414 (Fe alpha-naphthol BMPA) and N5814 (Fe beta-naphthol BMPA) showed activity against *Toxoplasma gondii in vitro* and were not toxic to LLC-MK2 cells, being able to reduce the activity of antioxidant enzymes crucial to the parasite's defense. In this project, we investigated the activity of compounds from the metallocomplex family, such as the ferric core compounds N0414 and N5814, which showed *anti-Toxoplasma gondii* activity and were able to eliminate the infection from almost all host cells. Some of the advantages of these drugs is that they have increased lipophilicity, making it easier for the compound to pass through biological membranes and thus requiring lower concentrations to obtain the necessary biological activity, as well as reversing the resistance profiles of the target cell. In future stages, we will investigate what type of death the parasite suffers after treatment with the compounds through ultrastructure analysis and the use of specific markers by fluorescence microscopy. Due to the promising results found *in* this work, to prove their efficacy, the compounds will also be used *in in vivo* tests with murine models under the acute phase of toxoplasmosis.

Keywords: *Toxoplasma gondii*; toxoplasmosis; metallocomplexes; chemotherapy.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasitic protozoan, belonging to the phylum Apicomplexa and class Sporozoa, and is the etiologic agent of toxoplasmosis, with worldwide distribution in warm-blooded animals, including humans (LEVINE et al., 1980; LYONS & JOHNSON, 1995; LUDER *et al*, 2001).

The forms of *Toxoplasma gondii* that are capable of infecting hosts are: tachyzoites, present in the acute phase of toxoplasmosis; bradyzoites (inside tissue cysts) which are usually found in the brain and skeletal muscle in the chronic phase of infection; sporozoites, present inside oocysts produced during the sexual cycle that occurs in the intestines of cats, the definitive hosts (TENTER et.al, 2000; HILL & DUBEY, 2005). As an adaptive immune response, the host weakens, tissue cysts rupture and release bradyzoites through an as yet unknown mechanism. These recurrent infections allow the parasite to convert to the rapidly dividing tachyzoite stage and produce significant morbidity, including toxoplasmic encephalitis (FERGURSON et al., 1989; SULLIVAN et al., 2009).

The most relevant routes of transmission in humans are: ingestion of food or water contaminated with oocysts shed by cats; ingestion of raw or undercooked meat with tissue cysts; and congenitally, when the mother acquires the infection for the first time during pregnancy (TENTER et al., 2000).

The cycle begins when felids ingest cysts present in meat. The cyst wall is dissolved by enzymes in the stomach and small intestine and the parasite is released from the cyst, penetrates the cells of the animal's intestinal mucosa and multiplies asexually, giving rise to tachyzoites. A few days after this infection,

the process of sexual reproduction begins and the merozoites formed during asexual reproduction give rise to gametes (GOULART & RESENDE, 2015).

These gametes, male and female, descended from the same parasite or from two different ones, fuse to form the egg or zygote, which after secreting the cystic wall gives rise to the oocyst. This is expelled with the animal's feces after nine days, with each cat expelling more than 500 million oocysts with each defecation. Once outside the host, the oocyst undergoes sporulation - meiotic division - again after a few days, forming two sporocysts, each with four sporozoites, a highly resistant form, which ensures that the parasite can survive for years outside the intracellular environment. These sporozoites are activated into tachyzoites if they are ingested by another animal (intermediate host) and can infect and replicate in all mammalian cells, with the exception of red blood cells. Once attached to a host cell, the parasite penetrates it and forms a parasitophorous vacuole, inside which it divides. This replication of the parasite continues until the number of parasites inside the cell reaches an amount that causes the cell to rupture, releasing parasites that will infect other adjacent cells (GOULART & RESENDE, 2015).

In immunocompetent organisms, *Toxoplasma gondii* infections are rarely serious and are often asymptomatic, around 90% of them (KRAVETZ & FEDERMAN, 2005). In this case, the tachyzoites differentiate into bradyzoites and then into tissue cysts as a form of resistance, generating the chronic phase of the disease (DUBEY *et al.,* 1998; TENTER et.al, 2000).

On the other hand, in immunocompromised individuals, the most common condition is encephalitis, whose symptoms include headache, disorientation, lethargy, hemiparesis, altered reflexes and convulsions (MCAULEY et al., 1994). Pneumonia and myocarditis can also occur in these individuals.

In congenitally infected children, the tachyzoite invades the brain and retina, resulting in potentially serious consequences, including decreased visual acuity, mental retardation, intracranial calcifications and also hydrocephalitis (MCAULEY et al., 1994). Recently, associations have been made between infection with the parasite and neurological disorders such as schizophrenia (KAMELAR & DAVIS, 2012).

Toxoplasma gondii

The ultrastructure of *Toxoplasma gondii* is made up of numerous organelles and specific structures, such as the nucleus, apical rings, polar rings, conidia, rostria, micronemes, micropore, mitochondria, subpellicular microtubules, rough and smooth endoplasmic reticulum, Golgi system, ribosomes, dense granules and apicoplast (Figure 2), which is reminiscent of the classic structure of a eukaryote (DUBEY *et al*., 1998).

The probing of the host cell and its subsequent invasion takes place through one of the characteristics present in the ultrastructure of the parasite mentioned above, the conoidal end. Subsequently, the rhopters and micronemes secrete proteins inside the host cell which guarantee the initial infection and its permanence, and thus form the mobile junction and sequentially the parasitophorous vacuole, by moving the parasite from the anterior to the posterior direction of its body, between the membranes of the parasite and the host cell (DUBREMETZ *et al.*, 1998; MORDUE *et al*, 1999).

This vacuole will contain components of both the host cell and the parasite, which means that it will not be identified by the immune system and will not fuse with the host cell's lysosomes. In this way, the parasite will develop and replicate by asexual reproduction, called endodiogeny, within the vacuole (DUBEY *et al*., 1998).

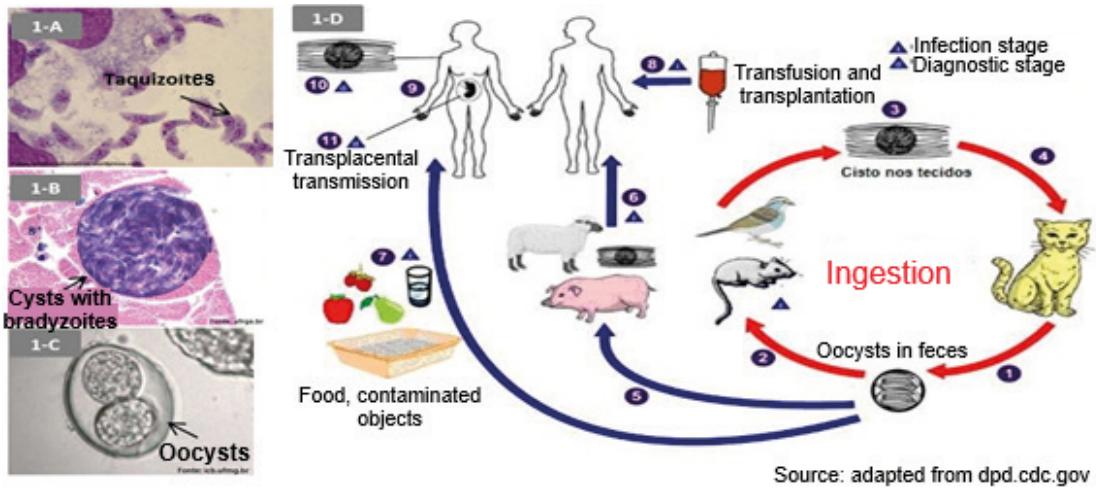


Figure 01: Infective forms of T. gondii (1A-1C) and parasite transmission cycle (1D).

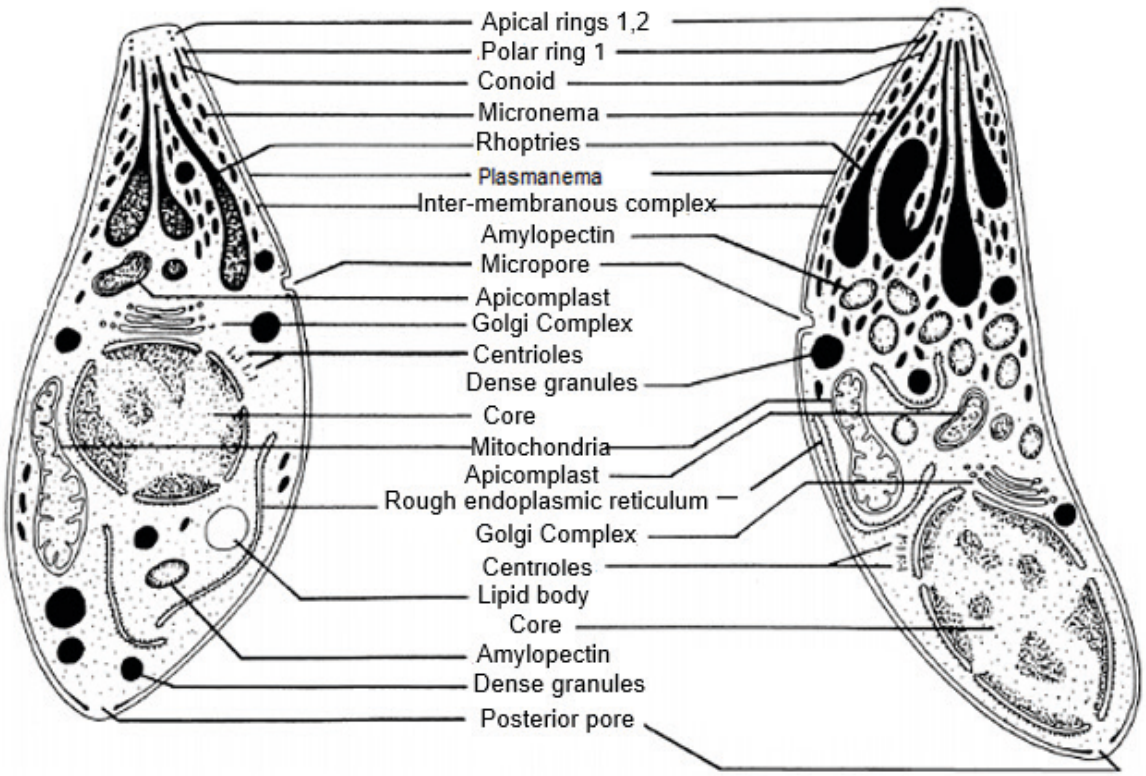


Figure 02: Schematic drawing showing the ultrastructural differences between tachyzoites (left) and bradyzoites (right) (Taken from Dubey *et al*., 1998).

Although there is knowledge about the biology of *Toxoplasma gondii*, treatment against toxoplasmosis is still limited to a few available therapies, which are toxic to the host. Currently, the most effective therapy against toxoplasmosis is the administration of antifolate compounds, such as the combination of sulfadiazine and pyrimethamine. Sulphonamides act synergistically with pyrimethamine by blocking the metabolic pathway related to folic and folinic acid synthesis (GEORGIEV, 1994), thus affecting thymidine synthesis and parasite replication.

Patients being treated with pyrimethamine should also receive concomitant administration of folinic acid to prevent adverse hematological effects caused by the drug. In cases of sulfa intolerance, replacing sulfa with clindamycin is recommended, as the pyrimethamine-clindamycin combination is as effective as primethamine-sulphadiazine during the acute phase of the disease (DANNEMANN et al., 1992; KATLAMA et al., 1996).

Although effective, this therapy is often associated with many side effects, mainly observed in patients with Acquired Immune Deficiency Syndrome (AIDS). These adverse effects can include bone marrow suppression and hematological toxicity caused by pyrimethamine, as well as causing teratogenesis and therefore being contraindicated for pregnant women, and/or hypersensitivity and allergic skin reactions caused by sulfadiazine (HAVERKOS, 1987; LEPORT et al., 1988; GEORGIEV, 1994; GOMELLA et.al, 2013).

Due to the medical importance of toxoplasmosis, the development of new therapies for the parasitic disease is urgent. However, the main drawback in this field is the delay in developing compounds that are able to reach the protozoan inside the host cell at a concentration that is both toxic to it and safe for it

There are some reports in the literature showing that coordination compounds can be an interesting alternative for antiparasitic therapy. For example, compounds containing copper or cobalt ions linked to the HmtpO ligand, where HmtpO is {5-methyl-1,2,4-triazole [1,5-a] pyrimidine-7 (4H)-one}, strongly affect the energy metabolism of *Leishmania infantum* and *Leishmania braziliensis* by altering the membrane structure of organelles and inducing cell death (RAMIREZ-MACIAS et al., 2011).

These compounds were also active *in vitro* against trypomastigote and amastigote forms of *Trypanosoma cruzi* at concentrations similar to those of compounds commonly used in clinical therapy, such as benznidazole; however, with reduced toxicity to the host cell and a better selectivity index. Furthermore, *in in vivo* tests the compounds caused a reduction in parasite load compared to treatment with benznidazole (CABALLERO et al., 2011).

Currently, the biological effect of metallocomplexes, inorganic compounds that show promising biological activities such as fungicide, bactericide and antiviral, has been studied (SINGH et al., 2000; NATH et al., 2001). Horn et al. (2005) showed that the HPClNOL ligand [1-(bis-pyridin-2-ylmethyl-amino)-3-chloropropan-2-ol] is a promising ligand for the development of metallopharmaceuticals, provided that its copper and iron complexes have promising biological activities. The copper complex [Cu(HPClNOL)Cl] showed nuclease activity and was cytotoxic to leukemia cells (FERNANDES et al., 2006). Iron complexes with the same ligand were also tested biologically and it was observed that the mononuclear compound [Fe(HPClNOL)(Cl)2] was able to protect *Saccharomyces cerevisiae* from oxidative stress, as it mimicked superoxide dismutase and catalase (HORN JR. et al., 2010).

These same compounds and their derivatives, Fe alpha-naphthol BMPA and Fe beta--naphthol BMPA were able to accelerate DNA hydrolysis by more than 108 times when compared to the rate of spontaneous DNA cleavage, revealing an impressive nuclease activity. However, their activities on cancer cells were modest and they showed very low toxicity to normal human peripheral blood mononuclear cells (HORN JR. et al., 2013). The absence of toxicity to host cells is one of the attractions for using these inorganic compounds to evaluate their activity in therapy involving the protozoan *Toxoplasma gondii*, since the main disadvantage of current antiparasitic therapy is that it does not preserve their viability.

OBJECTIVES

GENERAL OBJECTIVE

To evaluate the *anti-Toxoplasma* effect of new coordinated metallocomplex compounds *in vitro*, in order to assess their possible mechanisms of action, including effects on the tachyzoite form of the parasite.

SPECIFIC OBJECTIVES

- *To* evaluate the in vitro interactions of LLCMK2 cells infected with *Toxoplasma gondii* treated with metallocomplexes using optical microscopy;
- *To* evaluate the ultrastructure of cells treated or not during interaction with *Toxoplasma gondii* by transmission electron microscopy;
- To evaluate a possible cytotoxic effect of the compound on the host cell;

METHODOLOGY

PARASITE MAINTENANCE

RH strain - Tachyzoites of the RH strain were maintained by intraperitoneal passages in Swiss mice (CF-1). After 48 hours of infection, the parasites were collected in phosphate-buffered saline (PBS) by peritoneal lavage. The peritoneal lavage was centrifuged (100g; ;5'4°C), and the collected supernatant was centrifuged (1000g; ;10'4°C) to obtain the tachyzoite forms.

The parasites contained in the pellet were resuspendedem meio DMEM and diluted in fixative (4% formaldehyde in PBS) for quantification using a Neubauer chamber under an Axioplan - ZEISS optical microscope.

CULTIVATION AND MAINTENANCE OF THE HOST CELL

Rhesus monkey (*Macaca mulatta*) kidney epithelial cells, LLC-MK2 (ATCC CCL7, Rockville, MD/USA) were kept in 25cm plastic bottles[3] containing RPMI medium and 5§ SFB. The pH of the medium was maintained in a 5% CO_2 atmosphere in a CO_2 oven at 37°C. Twenty-four hours before each experiment, $2x10^5$ cells were plated in 24-well plates containing glass coverslips for the interaction experiments.

METALLOCOMPLEX COMPOUNDS

In this project, metallocomplex compounds (N0414 and N5814) were tested (synthesized and provided by Dr. Adolfo Horn Jr. and collaborators from the Chemical Sciences Laboratory, UENF).

CYTOTOXICITY OF COMPOUNDS

In order to assess the cytotoxic effect of the different compounds on the host cell, control experiments were carried out in which the cells were incubated in medium containing the compounds at different concentrations for 48 h. The cells were then incubated in Rhodamine 123 (10 µg/ml;) or Trypan blue (0.4§;). After this period, the cells were incubated in Rhodamine 123 (10 µg/ml; ;20'37°C**) or** Trypan blue (0.4§;1') and the non-viable cells were quantified after observation by light microscopy.

SELECTIVITY INDEX

To calculate the Selectivity Index (S.I) of the compounds, the host cells were treated at concentrations of20 a 500 µM for 48 hours and then the percentage of viable cells was determined after incubation in trypan blue or rhodamine 123, as described in section 3.4. The I.S was calculated according to the formula below, where the IC50 corresponds to the inhibitory concentration of 50% of the growth of host cells (IC50 host cells) or parasites (IC50 parasites): I.S = IC50 host cells / IC50 parasites.

PARASITE-HOST CELL INTERACTION

Parasites resuspended in RPMI medium were allowed to interact for 1 h at37°C with LLC-MK2 epithelial cells, using a parasite:cell ratio of 3:1 to check for antiproliferative effects, 10:1 for electron microscopy experiments. After the interaction, the cells were washed with PBS to remove extracellular parasites. The compounds were added after 1 h of interaction for up to 48 h for growth curve assays and to obtain the IC50 of the compounds.

EVALUATION OF THE COMPOUNDS' ANTI-PROLIFERATIVE ACTIVITY

After 1 hour of interaction, and 24 and 48 hours in contact with the drugs, the cells were fixed in 4% nascent formaldehyde solution in PBS, stained with 10% Giemsa dye in distilled water, dehydrated in different concentrations of acetone-xylol: 1) 100% acetone; 2) 100% acetone; 3) 70% acetone and 30% xylol; 4) 30% acetone and 70% xylol; 5) 10% acetone and 90% xylol; 6) 100% xylol. After dehydration, the coverslips were mounted on drops of Entellan. The finished slides were observed under an Axioplan - ZEISS optical microscope. The proliferation index was determined by analyzing the total number of 100 infected cells in duplicate for each experimental condition. The results were representative of three different experiments, using the student's t-test as a statistical tool.

TRANSMISSION ELECTRON MICROSCOPY

In order to evaluate the ultrastructure of the parasites in the absence and presence of the different compounds, transmission electron microscopy experiments with different interaction times were prepared. The samples were fixed in a solution containing 2.5% glutaraldehyde in sodium cacodylate buffer0.1 M , pH 7.4. The cells were washed in the same buffer and post-fixed for 40 minutes in the dark in a solution of 1% osmium tetroxide, 1.25% potassium ferrocyanide and 5mM CaCl2 in sodium cacodylate buffer0.1 M , pH 7.4. The cells were washed in the same buffer, dehydrated in acetone and embedded in epoxy resin. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and observed under a FEI SPIRIT 120 Kvolt Transmission Electron Microscope.

RESULTS

Our group has tested different metallo-complex compounds due to their characteristics and the biological activities they have previously shown, such as *anti-Toxoplasma* activity. The compounds N0414 and N5814 (Figure 3) appear, among others, as candidates due to their potential effects against the parasite at low concentrations while maintaining low toxicity against host cells.

Figure 3A

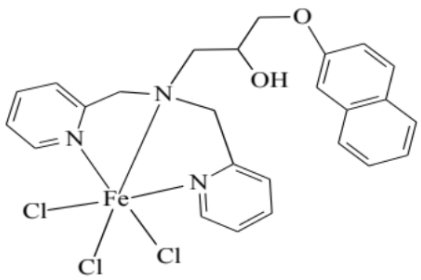
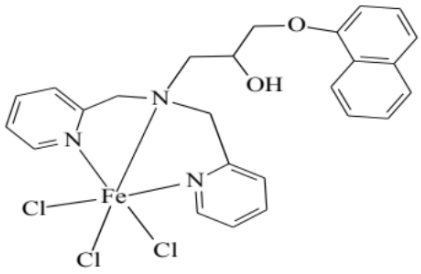


Figure 3B



Legend: Figure 03A: N5814 (Fe beta-naphthol BMPA); Figure 03B: N0414 (Fe alphanaphthol BMPA).

EFFECT OF THE N0414 METALLO-COMPLEX AT DIFFERENT CONCENTRATIONS OVER 24 HOURS

The graph shows the different concentrations used to assess the degree of toxicity required of compound N0414 in 24 hours for it not to damage the host cell, but to reach the parasite inside it. It can also be seen that from 10μM the compound causes a loss of cell viability.

EFFECT OF THE N0414 METALLO-COMPLEX IN DIFFERENT CONCENTRATIONS OVER 48 HOURS

The following graph shows the different concentrations used to assess the degree of toxicity required of compound N0414 in 48 hours for it to reach the parasite inside the host cell, but not affect it. It can also be seen that from 10μM the compound causes a loss of cell viability.

EFFECT OF THE N5814 METALLO-COMPLEX IN DIFFERENT CONCENTRATIONS OVER 24 HOURS

The graph below shows the different concentrations used to assess the safe level of toxicity of compound N5814 in 24 hours for the host cell, but at the same time reaching the parasite inside it. It can also be seen that from 50μM the compound generates a loss of cell viability.

EFFECT OF THE N5814 METALLO-COMPLEX IN DIFFERENT CONCENTRATIONS OVER 48 HOURS

The following graph shows the different concentrations used to assess the degree of safe toxicity of compound N5814 in 48 hours for the host cell, but at the same time reaching the parasite inside it. It can also be seen that from 100μM the compound generates a loss of cell viability.

DISCUSSION

Toxoplasma gondii is an obligate intracellular parasite of the phylum Apicomplexa and class Sporozoa, which is cosmopolitan and puts the health of mainly immunocompromised individuals or children who congenitally acquire the disease at risk (MCAULEY et al., 1994; MONTOYA *et al.*, 2004).

The current treatment used for toxoplasmosis is a combination therapy based on pyrimethamine, sulfadiazine or folinic acid, however, many serious adverse effects are generated (GEORGIEV, 1994) and therefore

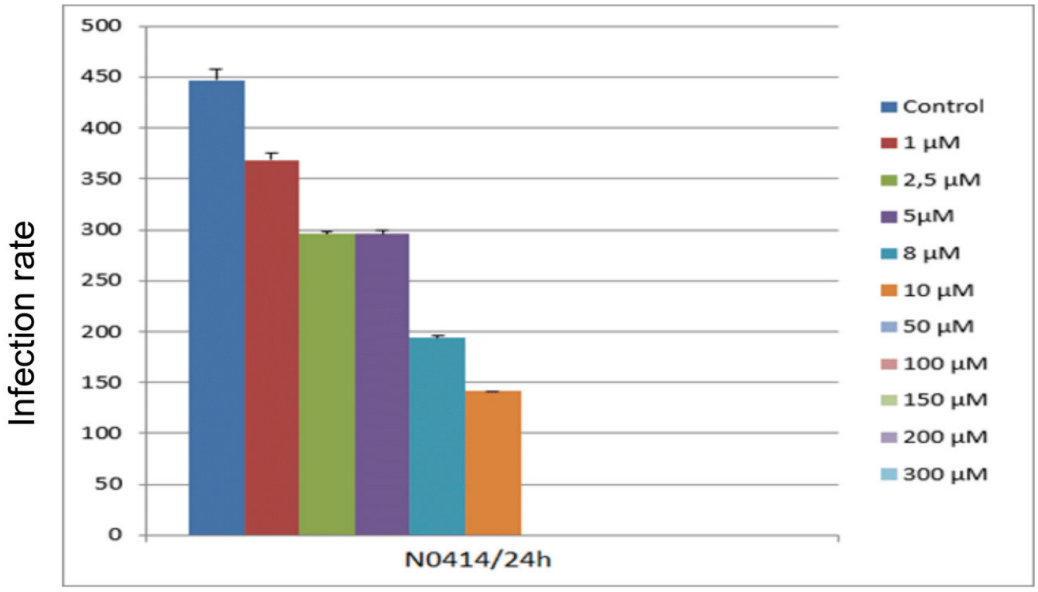


Figure 04: Representative graph of different concentrations of compound N0414 over 24 hours.

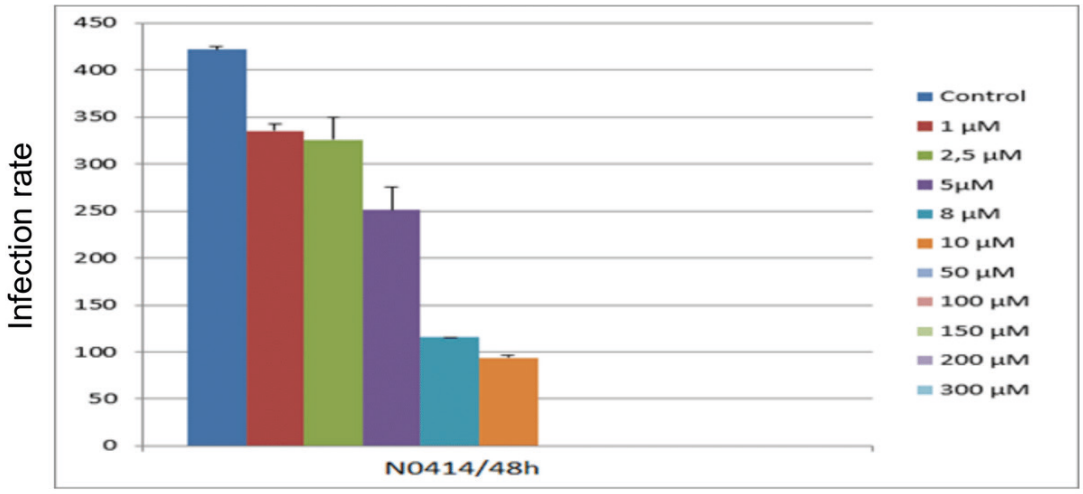


Figure 05: Representative graph of different concentrations of compound N0414 at 48h.

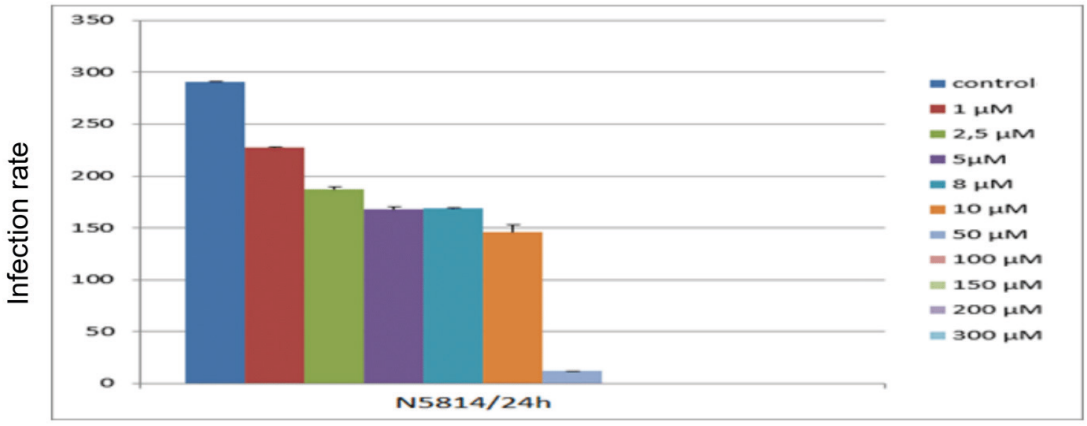


Figure 06: Representative graph of different concentrations of compound N5814 over 24 hours.

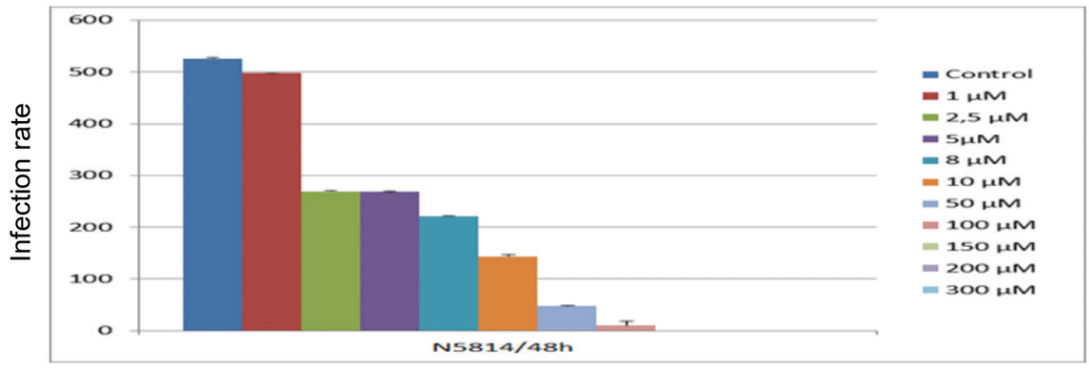


Figure 07: Representative graph of different concentrations of compound N5814 at 48h.

other drugs are being studied to ensure the integrity of the host cell and concomitantly destroy the parasite. Pyrimethamine and sulfadiazine act synergistically to treat toxoplasmosis by inhibiting the proliferation and survival of *Toxoplasma gondii* inhibiting the folate synthesis metabolic pathway (GEORGIEV, 1994; MONTOYA *et al*., 2004).

In addition, current therapy brings with it a number of significant side effects for the patient, such as hematological toxicity, hypersensitivity and allergic skin reactions, bone marrow suppression and teratogenic effects (HAVERKOS, 1987; LEPORT et al., 1988; GEORGIEV, 1994; GOMELLA et.al, 2013)

In view of the problems presented by current therapy, the need for research into new forms of therapy is recognized. In this work, two metallocomplex compounds - N0414 and N5814 - were tested in order to analyze their maximum degree of toxicity *against Toxoplasma gondii* in a way that does not compromise the integrity of the host cell.

There are previous reports in other literature that have tested metallocomplex compounds, with a metal core other than ferric, against *Leishmania infantum* and *Leishmania brasiliensis* (RAMIREZ-MACIAS et al., 2011), as well as against *Trypanosoma cruzi* (CABALLERO et al., 2011) and have obtained significant results, generating little or no risk to the host cell. In addition, this type of compound is a promising antiparasitic, bactericidal, fungicidal and antiviral therapy (SINGH et al., 2000; NATH et al., 2001; RAMIREZ-MACIAS et al., 2011).

Therefore, due to the promising results presented in the literature, tests with the metallocomplex compounds N0414 and N5814 on LLC-MK2 cells were carried out in order to evaluate the anti-toxoplasma *gondii* effect and cell viability, since current antiparasitic therapy has been shown to be toxic to host cells.

This work aims to present results obtained from the interaction of antiparasitic chemotherapy in isolation, i.e. without coordination with current therapy. The proposed treatment differs from others in that these metallocomplexes are not coordinated with sulfadiazine or any other drug. From this, it can be assumed that the side effects previously generated in the host cell by coordination with sulfa were not observed without this coordination.

Thus, LLC-MK2 cells were treated with different concentrations of each of the two compounds - N0414 and N5814, both for 24h and 48h, in order to assess their level of toxicity sufficient to reach the parasite inside the host cell without impeding its cell viability.

The first compound tested at 24 hours, N0414, reached its maximum degree of toxicity without damaging the host cell at a concentration of 10µM. Transmission Electron Microscopy (TEM) also showed that this metallocomplex caused both ultrastructural changes in the parasite and its death.

The same metallocomplex tested earlier, albeit within 48 hours, had its maximum degree of toxicity for the antiparasitic effect to guarantee the safety of the host cell at a concentration of 10μM. This compound indicated death of *Toxoplasma gondii* and failure of endodiogenesis.

In the analysis of LLC-MK2 cells treated with the second compound tested - N5814 - at 24 hours, it was found that the maximum toxic level for the host cell and, concomitantly, antiparasitic action is at a concentration of 50μM. Also in the analysis of the cells, it was noted that the indication of cell death was the cytoplasmic extraction of *Toxoplasma gondii*.

In the 48-hour analysis of the same compound, it was observed that a toxicity level of more than 100μM would affect the viability of the host cell, so this is the maximum concentration to indicate parasite death, guaranteeing cell integrity. Transmission Electron Microscopy (TEM) showed the death of the parasite by necrosis, as well as its structural alterations.

It can be seen that compound N0414 at 24 hours was more effective than compound N0414 at 48 hours, as it killed the parasite at the same concentration, but in less time. The same conclusion can be drawn when comparing the first compound with N5814 over the same period, since the latter needed a higher concentration to show signs of parasite death.

Comparatively, compound N0414 at 48 hours also obtained better results than N5814 at the same time, since a much lower concentration was used to damage the parasite. Finally, compound N5814/24h proved to be better than the 48h compound, since the latter needed twice the concentration to show necrosis and structural alterations.

Thus, the compound that showed the most promising results for therapy was N0414 over the 24-hour period.

Regarding the low toxicity presented *in in vitro* models infection of LLC-MK2 cells and treated with compounds N0414 and N58140, the results show compatibility with those obtained by PORTES *et al.,* 2015.

Although there are satisfactory results for the two compounds evaluated, new tests on *in vitro* models of infection need to be carried out. *In* addition, *in vivo* models should also be taken into consideration for future tests. Further research to better understand and develop new chemotherapeutic treatments for toxoplasmosis is of great relevance, which is why it is so important to continue this study.

CONCLUSION

The antiproliferative effect of compounds N5814 and N0414 at different concentrations and times was effective against *Toxoplasma gondii in vitro*, reducing the infection rate in LLC-MK2 cells by killing the parasite. In addition to reducing the infection rate, the compounds also acted against the parasite by modifying its structure, cytoplasmic extraction and interference in parasite division.

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