# Anti-*toxoplasma gondii* effect of metalocomplex compounds N0414 and N5814

# Efeito anti-*toxoplasma gondii* do composto metalocomplexo N0414 e N5814

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**ABSTRACT**
*Toxoplasma gondii*, toxoplasmosis agent, is a obligate intracellular protozoan that is able of infecting a broad spectrum of vertebrate's cells. Toxoplasmosis is a pathology related to severe damages to immunocompromised hosts and its current chemotherapy is quite restricted, being more used the combination of sulfadiazine and pyrimethamine, which is a therapy associated with adverse reactions. This fact highlights the importance of the study of new drugs against *Toxoplasma gondii*. Has been studied the biological effect of new metallocomplexe compounds, which are inorganic compounds that present promising biological activity as fungicide, bactericide and antiviral. The metallocomplexes, dinuclear ferric compounds N0414 (Fe alfanaftol BMPA) and N5814 (Fe beta-naphthol BMPA) showed activity against *Toxoplasma gondii in vitro* and it was nontoxic to LLC-MK2 cells, being able to reduce the activity of crucial antioxidant enzymes for the defense of the parasite. In this project, it will be investigated the activities of compounds of the metallocomplexes family as the compounds coordinated to sulfadiazine as the nucleus compound of ferric N0414 and N5814, which showed anti-*Toxoplasma gondii* activities and were able to eliminate the infection in almost all host cells. In further steps, we will investigate what kind of death the parasite undergoes after the treatment with the compounds through the ultrastructure analysis and the usage of specific markers by fluorescence microscopy. The compounds will also be used *in vivo* tests with mouse models in the acute phase of toxoplasmosis to prove the efficacy of these compounds.

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

**RESUMO**
*Toxoplasma gondii*, o agente da toxoplasmose, é um protozoário intracelular obrigatório que é capaz de infectar um amplo espectro de células de vertebrados. A toxoplasmose é uma patologia relacionada a danos graves aos hospedeiros imunocomprometidos e sua quimioterapia atual é bastante restrita, sendo mais utilizada a combinação de sulfadiazina e pirimetamina, que é uma terapia associada a reações adversas. Este fato destaca a importância do estudo de novos medicamentos contra o *Toxoplasma gondii*. Tem sido estudado o efeito biológico de novos compostos metalocomplexos, que são compostos inorgânicos que apresentam atividade biológica promissora como fungicida, bactericida e antiviral. Os compostos metalocomplexos, férricos dinucleares N0414 (Fe alfanaftol BMPA) e N5814 (Fe beta-naftol BMPA) mostraram atividade contra o *Toxoplasma gondii in vitro* e não foi tóxico para células LLC-MK2, sendo capaz de reduzir a atividade de enzimas antioxidantes cruciais para a defesa do parasita. Neste projeto, serão investigadas as atividades de compostos da família metalocomplexos como os compostos coordenados à sulfadiazína como o núcleo composto da N0414 e N5814, que apresentaram atividades anti-*Toxoplasma gondi*i e foram capazes de eliminar a infecção em quase todas as células hospedeiras. Em outras etapas, investigaremos que tipo de morte o parasita sofre após o tratamento com os compostos através da análise de ultraestrutura e o uso de marcadores específicos por microscopia de fluorescência. Os

compostos também serão utilizados em testes *in vivo* com modelos de camundongos na fase aguda da toxoplasmose para comprovar a eficácia desses compostos.

**Palavras-Chave:** *Toxoplasma gondii*, toxoplasmose, metalocomplexos, quimioterapia.

## 1 INTRODUCTION

The forms of *Toxoplasma gondii* that are capable of infecting hosts are: tachyzoites, present in the acute phase of toxoplasmosis; bradyzoites (inside tissue cysts) that 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 intestine of felines, definitive hosts (TENTER et al., 2000; HILL & DUBEY, 2005). As an adaptive immune response, the host weakens, tissue cysts rupture and release bradyzoites through a still 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: the ingestion of food or water contaminated with oocysts eliminated 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).

*Toxoplasma gondii* is a mandatory intracellular parasitic protozoan, belongs to the phylum Apicomplexa and class Sporozoa, in addition to being 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; DE CARVALHO et al., 2021).

In immunocompetent organisms, infections by *Toxoplasma gondii* are rarely serious, being asymptomatic, about 90% of them (KRAVETZ & FEDERMAN, 2005). In this case, tachyzoites differ in bradyzoites and, subsequently, in 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 seizures (MCAULEY et al., 1994). Pneumonia and myocarditis can also occur in these individuals. In congenitally infected children, 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).

There are some reports in the literature showing that compound coordination can be an interesting alternative to antiparasitic therapy. For example, compounds containing copper or cobalt ions bound to the HmtpO linker, where HmtpO is {5-methyl-1,2,4-triazole [1,5-a] pyrimidine-7 (4H) -um}, strongly affects metabolism energy of *Leishmania infantum* and *Leishmania braziliensis* 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* in concentrations similar to compounds commonly used in clinical therapy, such as benznidazole; however, with reduced toxicity to the host cell and better selectivity index. In addition, in *in vivo* tests the compounds caused a reduction in the parasitic load in relation to the treatment with benznidazole (CABALLERO et al., 2011). There are some reports in the literature showing that compound coordination can be an interesting alternative to antiparasitic therapy. For example, compounds containing copper or cobalt ions bound to the HmtpO linker, where HmtpO is {5-methyl-1,2,4-triazole [1,5-a] pyrimidine-7 (4H) -um}, strongly affects metabolism energy of *Leishmania infantum* and *Leishmania braziliensis* 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* in concentrations similar to compounds commonly used in clinical therapy, such as benznidazole; however, with reduced toxicity to the host cell and better selectivity index. In addition, in *in vivo* tests the compounds caused a reduction in the parasitic load in relation to the treatment with benznidazole (CABALLERO et al., 2011).

The objective of this work is to evaluate the anti-Toxoplasma effect of new coordinated metallocomplex compounds *in vitro*, in order to evaluate their possible mechanisms of action, including effects in tachyzoite form of the parasite.

## 2 MATERIAL AND METHODS

### *2.1 TOXOPLASMA GONDII*

Cepa RH - Tachyzoites from 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, pH 7.3) 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 resuspended in DMEM medium and diluted in fixative (4% formaldehyde in PBS) for quantification through the Neubauer chamber under the optical microscope Axioplan - ZEISS.

## 2.2 HOST CELLS

The lineage Rhesus monkey kidney epithelial cells (Macaca mulatta), LLC-MK2 (ATCC CCL7, Rockville, MD / USA) were kept in 25cm$^3$ plastic bottles (CORNING / USA), containing RPMI medium and 5% of SFB (GIBCO-Life Technologies, Rockville, MD). The pH of the medium was maintained in an atmosphere with 5% $CO_2$ in a $CO_2$ oven at a temperature of 37ºC (Culture $CO_2$ Incubator, model CCL-170B-8, Sigapore). Twenty-four hours before each experiment, 2x10$^5$ cells were plated in twenty-four well plates containing glass coverslips, for the interaction experiments.

## 2.3 METALOCOMPLEXES

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

## 2.4 CELL CYTOTOXICITY

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

## 2.5 EVALUATION OF ANTI-PROLIFERATIVE ACTIVITY OF COMPOUNDS

Parasites resuspended in RPMI medium were allowed to interact for 1h at 37° C with LLC-MK2 epithelial cells, using a parasite: cell ratio of 3:1 to check the antiproliferative effects, 10:1 for electron microscopy experiments. After the interaction, the cells were washed with PBS (pH 7.3) to remove extracellular parasites. The compounds were added after 1 h of interaction for up to 48 h for the tests of growth curves and obtaining the IC 50 of the compounds. After 1h of interaction, and 24h and 48h in contact with the drugs, the cells were fixed in a solution of nascent formaldehyde 4% in

PBS (pH 7.3), stained with Giemsa dye 10% (Merk) 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 Entellan drops. The finished slides were observed under the optical microscope Axioplan - ZEISS. The proliferation rate was determined by analyzing a total number of 100 infected cells in duplicate for each condition of the experiment. The results were representative of three different experiments, using the Student's t test as a statistical tool. For the calculation of the Selectivity Index (IS) of the compounds, the host cells were treated in concentrations of 20 to 500 µM for 48 hours and later the percentage of viable cells was determined after incubation in trypan blue or rhodamine 123. The is 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): IS = IC50 host cells / IC50 parasites.

## 2.6 ELECTRONIC TRANSMISSION MICROSCOPY

In order to evaluate the ultrastructure of the parasites, in the absence and presence of the different compounds, experiments for transmission electron microscopy, with different interaction times were prepared. The samples were fixed in a solution containing 2.5% glutaraldehyde (Merk) in 0.1 M sodium cacodylate buffer (Merk), pH 7.4. The cells were washed in the same buffer and post-fixed for 40 minutes in the dark in 1% osmium tetroxide solution, 1.25% potassium ferrocyanide and 5mM $CaCl_2$ (Merk) in 0.1 M sodium cacodylate buffer (Merk), pH 7.4. The cells were washed in the same buffer, dehydrated in acetone and soaked in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed using the FEI SPIRIT 120 Kvolts Transmission Electron Microscope.

## 2.7 DATA ANALYSIS

Means were analyzed by Two-way ANOVA with Bonferroni multiple comparison post-test, using GraphPad Prism Software, Version 5.0 (San Diego, CA, USA).

## 3 RESULTS

1) Metalocomplexes:

The compounds N0414 and N5814 (Figure 3) appear, among others, as candidates due to their potential effects against the parasite in low concentrations and, at the same time, maintain low toxicity against host cells.

**Figure 1:** A- N5814 (Fe beta-naftol BMPA); B- N0414 (Fe alfanaftol BMPA).

Figure 1A

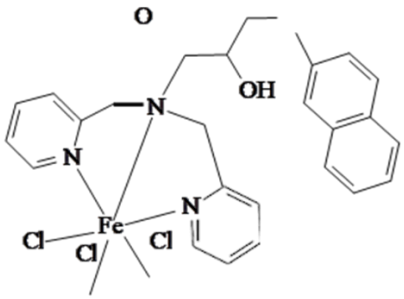
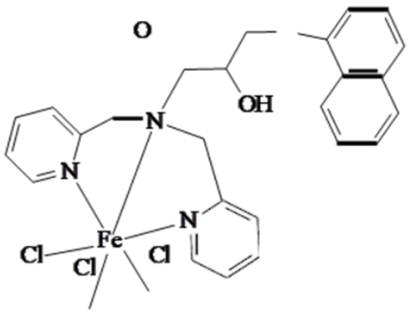


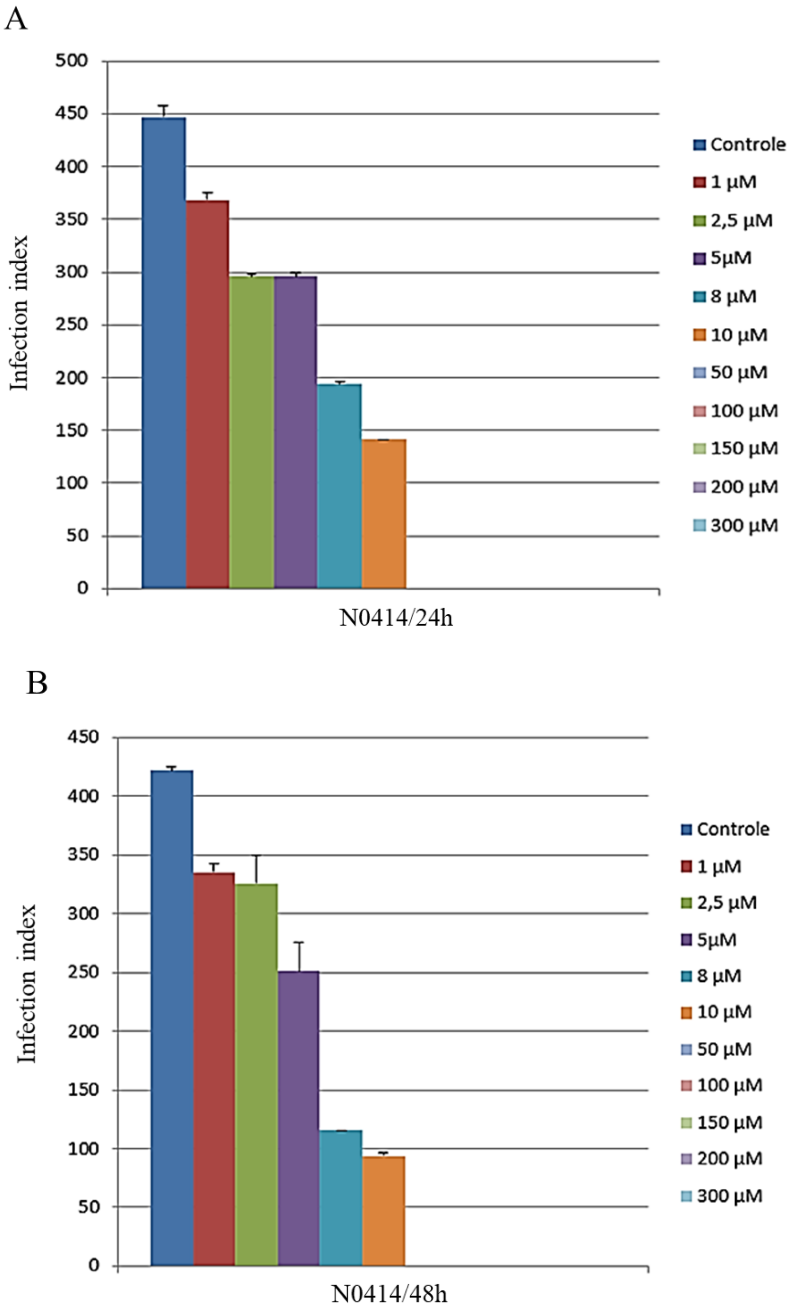

Figure1B




2) Effect of metalocomplex N0414 on diferente concentrations

It can be seen in graph 2A different concentrations used to evaluate the degree of toxicity needed for compound N0414 in 24 hours so that it does not damage the host cell, but reaches the parasite inside it. It is also noted that after 10µM the compound generates loss of cell viability. Graph 2B shows the different concentrations used to assess the degree of toxicity required for compound N0414 in 48 hours to reach the parasite inside the host cell, but does not affect it. It can also be noted that after 10µM the compound causes loss of cell viability.

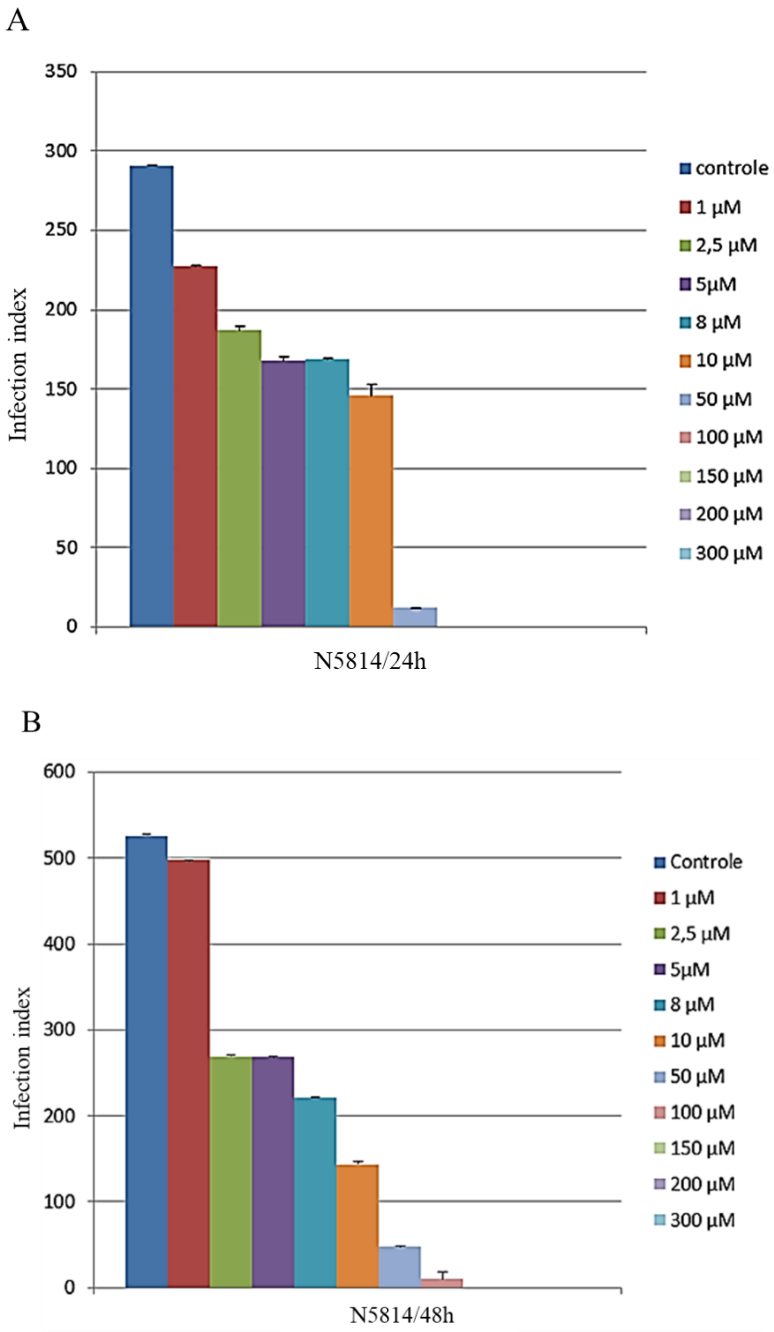
**Figure 2:** A- Different concentrations of compound N0414 in 24h and B- for 48h.




3) Effect of metalocomplex N5814 on diferente concentrations

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 reach the parasite within it. It can also be seen that after 50µM the compound generates loss of more cell viability. The following graph shows different concentrations used to evaluate the degree of toxicity of compound N5814 in 48 hours for the host cell, but at the same time reach the parasite within it. It can also be seen that after 100µM the compound generates loss of more cell viability.

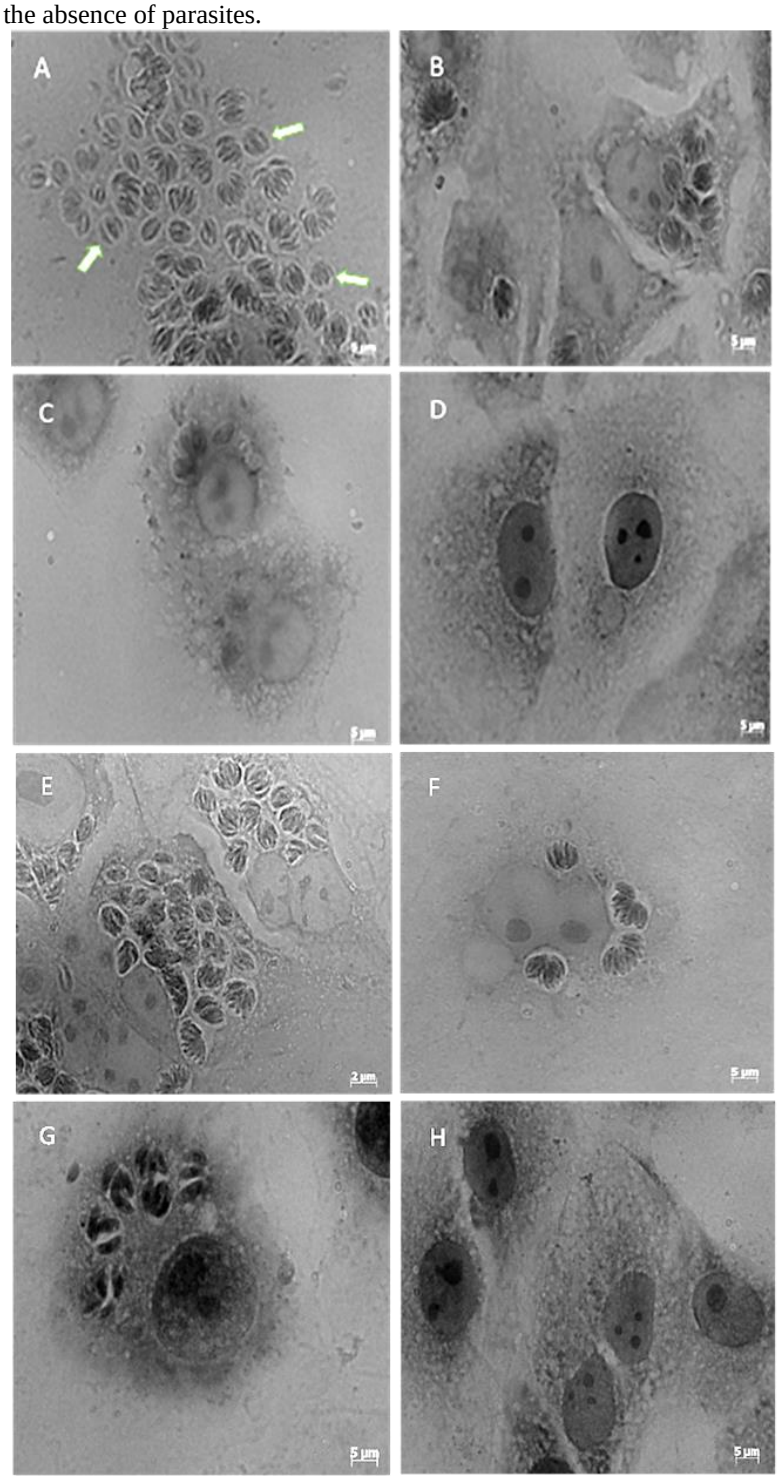
**Figure 3**: Different concentrations of compound N5814 in A- 24h and B- for 48h.




4) Light microscopy of LLCMK2 interactions with *T. gondii* treated or not with compound N5814 for 24h and 48h

The interactions of both 24h (4A, B, C and D) and 48h (4E, F, G and H) show a great reduction in the number of intracellular parasites when compared to the control, in the different concentrations (8, 10 and 50 µM) of treatment.

**Figure 4**: Light microscopy of LLC-MK2 cells infected with *T. gondii*. A, B, C and D 24h of interaction; E, F, G and H 48h of interaction. A and E without treatment. Note the large number of parasites in A. B and F treatment with 8 µM of N5814. C and G treatment of 10 µM of N5814. D and G treatment of 50 µM of N5814. Note the absence of parasites.

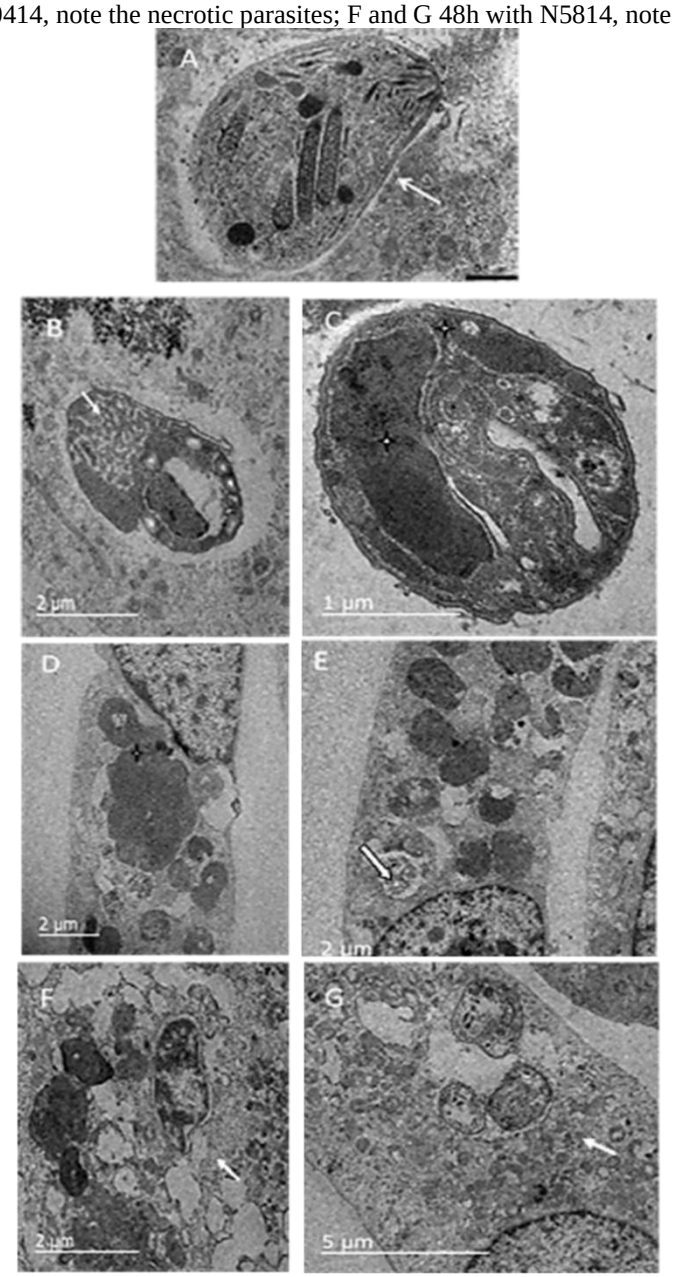



5) Analysis by electronic microscopy of cell transmission LLC-MK2 treated or not with metalocomplexes N0414 and N5814

With the analysis of the parasite's ultrastructure in untreated LLC-MK2 cells, it can be seen that there was no maintained parasitic integrity (Figure 16). The cells treated

with the metallocomplexes, on the other hand, underwent changes, such as amylopectin granules (Figure 5B), endodiogenic failure (Figure 5C), cytoplasmic extraction of the parasite (Figure 5E), altered parasite structure (Figure 5D) and presence of the necrotic parasite (Figure 5F and G).

**Figure 5**: Transmission electron microscopy of the LLC-MK2 interaction with T. gondi in the presence or absence of treatment with N0414 and N5814. A- Without treatment; B and C 24h treated with N0414. Note the presence of disorganization of the Endoplasmic Reticulum and the altered endodiogeny of the parasite; D and E 48h with N0414, note the necrotic parasites; F and G 48h with N5814, note the necrotic parasites.




## 4 DISCUSSION

*Toxoplasma gondii* is a mandatory intracellular parasite, of the phylum Apicomplexa and of the Sporozoa class, cosmopolitan and that puts at risk the health

mainly of immunocompromised individuals or children who congenitally acquire the disease (MCAULEY et al., 1994; MONTOYA et al., 2004).

The current treatment used for toxoplasmosis is a combined therapy based on pyrimethamine, sulfadiazine or folinic acid, however, many serious adverse effects are generated (GEORGIEV, 1994) and, therefore, other drugs are being studied to ensure cell integrity host and, concomitantly, destroy the parasite. Pyrimethamine and sulfadiazine act synergistically to treat toxoplasmosis by inhibiting the proliferation and survival of *Toxoplasma gondii* by inhibiting the metabolic pathway of folate synthesis (GEORGIEV, 1994; MONTOYA et al., 2004).

This work presents results obtained from the antiparasitic chemotherapy interaction of isolated form, that is, without coordination with the current therapy. The proposed treatment differs from the others by not coordinating these metallocomplexes with sulfadiazine or any other drug. From this, it can be assumed that the side effects previously generated to the host cell with the sulfa coordination were not observed without this coordination. Thus, the treatments of LLC-MK2 cells were carried out with different concentrations of each of the two compounds N0414 and N5814, both during 24h and 48h, to assess the level of toxicity sufficient to reach the parasite inside the host cell without that prevents their cell viability.

It appears that the first compound tested within 24 hours, N0414, reached its maximum level of toxicity without damaging the host cell at a concentration of 10µM. It was also possible to verify, through Transmission Electron Microscopy, that this metallocomplex generated both ultrastructural changes in the parasite, as well as its death. The same metallocomplex tested previously, although in 48h time, had its maximum degree of toxicity for the antiparasitic effect in order to guarantee the safety of the host cell at a concentration of 10µM. This compound obtained an indication of death of *Toxoplasma gondii* and failure in endodiogeny.

In the analysis of LLC-MK2 cells treated with the second compound tested - N5814 - within 24 hours, it was found that the maximum toxic level for the host cell and, concomitantly, antiparasitic action is the 50 µM concentration. Still in the analysis of the cells, it was noted that the indication of cell death occurred was the cytoplasmic extraction of *Toxoplasma gondii*. In the analysis during the time of 48h of this same compound, it was observed that the toxicity level above 100µM would affect the viability of the host cell, therefore this is the maximum concentration for which there is indication of parasitic

death ensuring cell integrity. Transmission electron microscopy could observe the death of the parasite by necrosis, as well as its structural alteration.

It is observed that the compound N0414 in the time of 24h was more effective in relation to that of 48h, because it killed the parasite with the same concentration, but in less time. The same conclusion can be drawn when comparing the first compound with N5814 in the same period, since the last one needed a higher concentration to show signs of parasitic death. Comparatively the compound N0414 in the time of 48h also obtained better result than the N5814 in the same time, since a much lower concentration was used to present damages to the parasite. Finally, the compound N5814/24h was better than that of 48h, since the second one needed twice the concentration to show necrosis and structural alteration. Thus, the compound that showed results for a more promising therapy was N0414 in the 24h period.

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

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