Antimicrobial Section / Original Paper

Chemotherapy

Chemotherapy 2015–16;61:249–255
DOI: 10.1159/000443496

Received: October 8, 2015
Accepted: December 16, 2015
Published online: March 8, 2016

Potential of Casiopeínas® Copper Complexes and Antituberculosis Drug Combination against *Mycobacterium tuberculosis*

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Key Words
Mycobacterium tuberculosis · Casiopeínas® · Resistance · Metal-based drugs · Copper · Synergism

Abstract
New compounds with antituberculosis activity and their combination with classic drugs have been evaluated to determine possible interactions and antagonism. The aim of this study was to evaluate the in vitro activity of Casiopeínas® copper-based compounds (CasIIIia, CasIIIEa, and CasIIgly) alone and combined with isoniazid (INH), rifampicin, or ethambutol (EMB) against resistant and susceptible *Mycobacterium tuberculosis*. Seventeen clinical *M. tuberculosis* isolates (5 multi-drug resistant and 2 resistant to INH and/or EMB) were subjected to determination of the minimal inhibitory concentration (MIC) by the resazurin microtiter assay and combination assessment by the resazurin drug combination microtiter assay. The Casiopeínas® alone showed a remarkable effect against resistant isolates with MIC values from 0.78 to 12.50 μg/ml. Furthermore, a synergistic effect mainly with EMB is shown for both resistant and susceptible clinical isolates. Casiopeínas® are promising candidates for future investigation into the development of antituberculosis drugs, being one of the first examples of essential metal-based drugs used in this field.

Introduction

Tuberculosis (TB) is an infectious disease with a high incidence worldwide. In 2014, approximately 9 million people were diagnosed with TB, which led to an estimated 1.5 million deaths/year. The treatment of TB is based on a regimen with isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide. Long-term combination therapy is necessary to prevent disease relapse and

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the development of drug resistance [1]. The co-infection with human immunodeficiency virus and the emergence of multidrug-resistant (MDR) and extensively drug-resistant TB strains have hampered treatment and reduced the options of drugs available on the market [2].

In recent years, bioinorganic chemistry has seen impressive advances, especially regarding medicinal chemistry [3]. The versatility afforded by coordination compounds is the product of the large number of geometries that these compounds can adopt and the modulation of their physicochemical properties that results from the modification of the metal coordination sphere. Many efforts have been made in medicinal chemistry to develop metal-based drugs for the treatment of several diseases, such as AIDS [4], cancer [5], metabolic syndrome [6], tropical neglected diseases (e.g. Chagas' disease, leishmaniasis, malaria, sleeping sickness, and amebiasis) [7, 8] and bacterial diseases, such as TB [9, 10].

Many transition elements, especially on the second and third row of the periodic table of the elements, have been used in the production of these drugs. However, recent investigations in this area have focused on metal-based compounds of essential ions, such as copper. Copper is an essential trace element important for the function of several enzymes involved in energy metabolism, respiration, and DNA synthesis in the cell [11].

The major functions of biologically active copper compounds involve redox reactions, in which copper reacts directly with molecular oxygen or hydrogen peroxide to produce free radicals (i.e. reactive oxygen species), displace other metal ions, participate in lipid peroxidation, and directly cleave DNA and RNA [12].

All of these properties were considered with advances in the design of copper(II) coordination compounds that have been recorded and patented [13] under the name Casiopeínas® (Trademark Casiopeína Reg. 407543 SECOFI; 1992, Re. 2002, 2012). The general formulas of these compounds are $[Cu(N\text{-}N)(N\text{-}O)]NO_3$ and $[Cu(N\text{-}N)(O\text{-}O)]NO_3$, in which N-N = nonsubstituted and substituted 2,2′-bipyridine or 1,10-phenanthroline, N-O = α-aminoacidate or peptides, and O-O = acetylacetonate or salicylaldehydate.

These copper complexes have been developed primarily for their action against cancerous tumors [14–16] in in vitro and in vivo models. Although the action mechanism is not known in detail, at the molecular level, several results support that these compounds are able to inhibit cell proliferation mainly by apoptotic pathways [15, 16], that participate in redox reactions that produce reactive oxygen species [15, 16] and interact directly with DNA and its constituents [17].

Preliminary studies of the antibacterial and antifungal actions of Casiopeínas® were performed by Onawumi et al. [18] using *Escherichia coli, Pseudomonas aeruginosa, Candida albicans*, and other microorganisms. Recently, Becco et al. [19] explored the in vitro anti-*Trypanosoma cruzi* activity of these compounds, and found that Casiopeínas® have shown similar IC_{50} to that of the reference drug nifurtimox.

Knowing that these complexes have various levels of hydrophobicity, the structures of which may favor entry into the mycobacterial cell, the aim of the present study was to evaluate the in vitro anti-*M. tuberculosis* activity of Casiopeínas® alone and combined with INH, RIF and EMB.

Materials and Methods

Bacterial Samples

The *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) reference strain and 17 *M. tuberculosis* clinical isolates (5 MDR, 2 INH resistant, 1 EMB resistant and 10 susceptible) from the collection of the Medical Bacteriology Laboratory, Laboratory of Teaching and Research in Clinical Analyses (LEPAC), Department of Clinical Analyses and Biomedicine, State University of Maringá, Brazil, were tested.

Antimycobacterial Agents and Casiopeínas®

Stock solutions of INH, EMB and RIF (Sigma, St. Louis, Mo., USA) and CasIIIia [Cu(4,4′-dimethyl-2,2′-bipyridine)(acetylacetonate)H_2O]NO_3, CasIIIEa [Cu(4,7-dimethyl-1,10-phenanthroline)(acetylacetonate)H_2O]NO_3, and CasIIgly [Cu(4,7-dimethyl-1,10-phenanthroline)(glycinate)H_2O]NO_3 (synthesized at the Faculty of Chemistry, Universidad Nacional Autónoma de México following the reported patent) were firstly prepared at concentrations of 4,000, 512, 4,000, 200, 200, and 200 μg/ml, respectively, and stored at –20°C. Upon use, they were diluted in Middlebrook 7H9 (Difco Laboratories, Detroit, Mich., USA) supplemented with OADC (oleic acid, albumin, dextrose, and catalase; BBL/Becton-Dickinson, Sparks, Md., USA) prepared according to the manufacturer's instructions.

Determination of the Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) of each antimicrobial and Casiopeínas® for *M. tuberculosis* was determined by 3 independent assays in triplicate using the resazurin microtiter assay plate as described by Palomino et al. [20]. Bacterial growth and sterility controls were performed in all assays. The MIC was defined as the lowest concentration that resulted in resazurin color change from blue to pink, which indicates its reduction by bacterial growth. Isolates with MIC ≤0.25 μg/ml [20], 0.5 μg/ml [21] and 2 μg/ml [22] were considered susceptible to INH, RIF and EMB, respectively.

Synergism Assay

The interactions between the drugs were tested by 3 independent assays in triplicate using the resazurin drug combination microtiter assay [22], a modified checkerboard method. The assay was performed in 96-well microplates (Kartell, Milan, Ita-

Table 1. Susceptibility and MIC for INH, RIF, EMB and Casiopeínas® (CasIIIia, CasIIIEa, and CasIIgly), determined by the resazurin microtiter assay, for the *M. tuberculosis* H_{37}Rv reference strain and susceptible and resistant clinical isolates

Strain/ isolates	Susceptibility/ resistance	MIC, μg/ml					
		INH	RIF	EMB	CasIIIia	CasIIIEa	CasIIgly
H_{37}Rv	susceptible	0.03 (0.21)	0.12 (0.14)	2 (9.78)	6.25 (14.03)	3.13 (7.07)	3.13 (6.94)
20	susceptible	0.03 (0.21)	0.06 (0.07)	1 (4.89)	3.13 (7.02)	1.56 (3.52)	1.56 (3.46)
22	susceptible	0.01 (0.07)	0.03 (0.03)	1 (4.89)	6.25 (14.03)	3.13 (7.07)	6.25 (13.87)
24	susceptible	0.03 (0.21)	0.06 (0.07)	2 (9.78)	6.25 (14.03)	3.13 (7.07)	3.13 (6.94)
TB27	susceptible	0.01 (0.07)	0.01 (0.01)	1 (4.89)	6.25 (14.03)	1.56 (3.52)	1.56 (3.46)
TB46	susceptible	0.06 (0.43)	0.01 (0.01)	1 (4.89)	6.25 (14.03)	3.13 (7.07)	3.13 (6.94)
TB49	susceptible	0.12 (0.87)	0.12 (0.14)	2 (9.78)	6.25 (14.03)	3.13 (7.07)	3.13 (6.94)
TB57	susceptible	0.12 (0.87)	0.06 (0.07)	1 (4.89)	6.25 (14.03)	3.13 (7.07)	3.13 (6.94)
TB80	susceptible	0.06 (0.43)	0.06 (0.07)	1 (4.89)	6.25 (14.03)	3.13 (7.07)	6.25 (13.87)
4851	susceptible	0.01 (0.07)	0.03 (0.03)	2 (9.78)	6.25 (14.03)	1.56 (3.52)	3.13 (6.94)
13638	susceptible	0.01 (0.07)	0.03 (0.03)	2 (9.78)	3.13 (7.02)	1.56 (3.52)	1.56 (3.46)
18	INH, RIF, EMB	16 (116.66)	62.50 (76.02)	4 (19.57)	3.13 (7.02)	1.56 (3.52)	3.13 (6.94)
40	INH, RIF	8 (58.33)	200 (243.16)	2 (9.78)	3.13 (7.02)	0.78 (1.76)	0.78 (1.73)
73A	INH, RIF, EMB	2 (14.58)	128 (155.69)	4 (19.57)	6.25 (14.03)	3.13 (7.07)	6.25 (13.87)
91	INH	8 (58.33)	0.25 (0.30)	2 (9.78)	6.25 (14.03)	3.13 (7.07)	6.25 (13.87)
97S	INH, RIF, EMB	4 (29.16)	8 (9.73)	16 (78.31)	12.50 (28.07)	3.13 (7.07)	6.25 (13.87)
3408	INH, RIF, EMB	16 (116.66)	200 (243.16)	32 (156.62)	6.25 (14.03)	3.13 (7.07)	3.13 (6.94)
3614	INH, RIF, EMB	8 (58.33)	200 (243.16)	16 (78.31)	12.50 (28.07)	6.25 (14.12)	6.25 (13.87)

Figures in parentheses give concentrations in micromolars.

ly), in which the combinations were tested two-dimensionally (INH combined with CasIIIia, CasIIIEa, and CasIIgly; RIF with CasIIIia, CasIIIEa, and CasIIgly; EMB with CasIIIia, CasIIIEa, and CasIIgly). The concentrations ranged from 0.120 to 64 μg/ml for INH, 0.003 to 500 μg/ml for RIF, 0.120 to 64 μg/ml for EMB, and 0.390 to 25 μg/ml for CasIIIia, CasIIIEa, and CasIIgly. Mycobacterial inoculum was standardized using 1 McFarland turbidity scale, diluted 1:20 in OADC-supplemented Middlebrook 7H9 medium and inoculated (100 μl) into well plates that contained different drug concentrations and controls. Medium, drug sterility, and bacterial growth controls were used in all assays. The microplates were covered, sealed, and incubated in a normal atmosphere for 7 days at 35°C. The MIC readings were performed after the addition of 30 μl of freshly prepared 0.01% resazurin solution (Acros, Morris Plains, N.J., USA) to each well, and incubation for 24–48 h at 35°C. The change of blue to pink, based on the reduction of resazurin, was considered as a sign of the presence of bacterial growth. To evaluate possible synergistic effects, the fractional inhibitory concentration index (FICI) was calculated: FICI = (MIC A + B/MIC A) + (MIC B + A/MIC B), in which MIC A + B is the MIC of drug A combined with drug B, MIC B + A is the MIC of drug B combined with drug A, MIC A is the MIC of drug A tested alone, and MIC B is the MIC of drug B tested alone. The effects of the combinations were classified as synergistic (FICI ≤0.5), additive or indifferent (FICI >0.5–4.0), and antagonistic (FICI >4.0) [22].

Macrophage Viability Assays

Macrophages were derived from human peripheral blood monocytes isolated from blood samples of a healthy individual with written consent. Monocytes were isolated in a Ficoll gradient, and then placed in Petri dishes with supplemented RPMI 1640 medium at 37°C under 5% CO_2 for 5 days. Changing of medium was carried out every 48 h for monocyte differentiation into macrophages. Macrophages were sorted by flow cytometry using a specific F4/80 antibody.

The effect of Casiopeínas® (CasIIIia, CasIIIEa and CasIIgly) on macrophage viability was determined by 3 independent assays in triplicate. For each experiment, 1×10^5 macrophages/well were placed in 96-well plates with 100 μl of supplemented RPMI 1640 and the corresponding Casiopeínas® at final concentrations of 1, 10, 100 and 1,000 μM in each well. The treated cultures were incubated for a further 60 h, taking an aliquot every 12 h for determining viability employing two different markers: (a) vital marker trypan blue and (b) carboxyfluorescein diacetate and propidium iodide using a hemocytometer. In brief, 100 μl trypan blue 0.4% or 1 μl of 5 μM carboxyfluorescein diacetate (Invitrogen, USA) and 1 μl of 1.5 μM propidium iodide were added to samples of 100 μl containing 1×10^4 macrophages. Final solutions were mixed and incubated at room temperature for 15 min. Both markers were counted using the fluorescent microscope Olympus BX51.

Results

The MIC of INH, RIF and EMB ranged from 0.01 to 0.12 μg/ml (0.07–0.87 μM), 0.03 to 0.12 μg/ml (0.03–0.14 μM) and 1 to 2 μg/ml (4.89–9.78 μM) for susceptible iso-

Table 2. FICI of Casiopeínas® (CasIIIia, CasIIIEa, and CasIIgly) with INH, RIF and EMB against the *M. tuberculosis* $H_{37}Rv$ reference strain and clinical isolates

Strain/ isolates	INH/ CasIIIia	INH/ CasIIIEa	INH/ CasIIgly	RIF/ CasIIIia	RIF/ CasIIIEa	RIF/ CasIIgly	EMB/ CasIIIia	EMB/ CasIIIEa	EMB/ CasIIgly
$H_{37}Rv$	2.00	2.00	2.00	1.25	1.00	1.00	*0.24*	*0.37*	*0.37*
20	1.00	2.00	2.00	1.00	1.00	1.00	*0.37*	*0.37*	*0.50*
22	0.75	1.00	2.00	1.50	1.00	1.00	0.62	*0.50*	*0.50*
24	1.00	1.00	2.00	1.00	0.75	2.00	*0.50*	*0.37*	*0.37*
TB27	2.00	2.00	2.00	1.00	1.50	2.00	*0.31*	1.12	0.62
TB46	1.00	2.00	2.00	1.00	1.00	1.00	*0.37*	*0.37*	*0.50*
TB49	2.00	1.00	1.50	1.00	1.00	0.75	*0.12*	*0.37*	*0.50*
TB57	1.00	1.00	2.00	2.00	0.75	2.00	*0.50*	*0.25*	*0.37*
TB80	1.00	1.00	1.00	1.50	1.00	1.00	*0.25*	*0.31*	*0.31*
4851	2.00	2.00	1.00	1.00	1.00	1.00	*0.50*	*0.25*	*0.37*
13638	1.00	2.00	2.00	1.00	1.50	1.00	*0.50*	*0.31*	*0.37*
18	2.00	1.00	1.00	1.00	1.00	1.00	*0.50*	*0.50*	0.75
40	*0.50*	2.00	1.00	1.00	2.00	1.00	*0.37*	0.61	0.67
73A	1.00	1.00	1.00	*0.37*	1.00	*0.50*	*0.50*	*0.50*	*0.50*
91	1.00	1.50	1.00	1.50	1.00	1.00	*0.50*	*0.50*	*0.37*
97S	1.00	1.00	1.00	0.75	1.00	1.00	*0.31*	0.75	1.00
1193	1.00	1.00	2.00	1.00	2.00	2.00	*0.50*	*0.37*	*0.31*
3408	1.00	*0.50*	0.75	1.00	1.50	1.00	*0.50*	*0.37*	*0.50*
3614	1.00	1.00	1.50	1.00	1.00	1.00	*0.25*	*0.28*	*0.25*

Figures in italics show synergistic effects.

lates and from 2 to 16 μg/ml (14.55–116.45 μM), 62.5 to 200 μg/ml (63.79–243.03 μM) and 4 to 32 μg/ml (19.57–156.62 μM) for resistant *M. tuberculosis* clinical isolates, respectively. The MIC of CasIIIia, CasIIIEa, and CasIIgly for susceptible and resistant clinical isolates ranged from 3.13 to 12.50 μg/ml (7.03–28.07 μM), 0.78 to 6.25 μg/ml (1.73–13.87 μM), and 0.78 to 6.25 μg/ml (1.76–14.12 μM), respectively (table 1).

For the *M. tuberculosis* $H_{37}Rv$ strain, Casiopeínas® had a synergistic effect only in combination with EMB. In clinical isolates, a synergistic effect was observed with the combinations INH-CasIIIia (1/18; 5.55%) INH-CasIIIEa (1/18; 5.55%), RIF-CasIIIia (1/18; 5.55%) and RIF-CasIIgly (1/18; 5.55%). For EMB combined with CasIIIia, CasIIIEa, and CasIIgly, the synergistic effect was observed for the most susceptible (10/10, 100%; 9/10, 90%; 9/10, 90%) and resistant (8/8, 100%; 6/8, 75%; 5/8, 62.5%) isolates, respectively (table 2).

The maximum effect of Casiopeínas® on human macrophage viability was observed after 12 h of exposure with the highest concentration employed (1 mM). The viability decrease was 11, 14 and 20% for CasIIIia, CasIIIEa and CasIIgly, respectively, compared with the control culture. During the following 48 h, the viability still decreased with a time-dependent behavior reaching final viability values of 79.5, 75.77 and 72.07%, respectively, as shown in figure 1. The same behavior was observed for the lower doses.

Discussion

Drug combinations are already used in conventional treatment of TB due to their better outcome than with a single drug treatment. In this way, new compounds with anti-TB activity and their combination with old and classically used drugs should be evaluated to determine possible interactions or antagonism. Such interactions should also be evaluated in targeting to reduce the MICs of classic therapeutic drugs and consequently reduce adverse effects for the patients, which is a strong factor that hinders successful treatment. In this sense, medicinal inorganic chemistry can contribute to the development of new compounds that can be used alone or in combination with classic anti-TB drugs.

Considering the arguments and knowing that Casiopeínas® are able to directly interact with DNA, participate in reactive oxygen species production reactions and

induce cellular death by apoptotic mechanisms in tumor cells, and since they have shown antiparasitic activity in *T. cruzi* cultures, in this study the activity of Casiopeínas® (CasIIIia, CasIIIEa, and CasIIgly) to produce a growth inhibition effect on resistant and susceptible *M. tuberculosis* clinical isolates was evaluated besides their synergistic, additive or antagonistic interactions with INH, RIF and EMB.

Casiopeínas® showed activity against *M. tuberculosis* isolates studied with similar MIC values for susceptible and resistant isolates (table 1) that were comparable to some second-line anti-TB drugs. Additionally, Casiopeínas® showed MIC values lower than INH, RIF and EMB for resistant isolates, with the exception of isolate 91 that had a MIC value of 0.25 μg/ml (0.30 μM) for RIF. The present results are consistent with those of Gu et al. [23] who indicated that a good anti-*M. tuberculosis* drug candidate should have MIC values of at least 64 μg/ml for pure compounds. These results show that Casiopeínas® could be considered as an important candidate for new studies in anti-TB drugs.

Besides the focused development of metal-based compounds for the treatment of TB, other compounds that already have shown cytotoxic activity in other systems through oxidative stress induction or DNA interaction have been studied.

One example can be found in the Mn(II) compounds with 2-acetylpyridine-N(4)-R-thiosemicarbazones as ligands which have shown anti-TB activity. Some of these compounds have shown high selectivity for *M. tuberculosis* due to the low cytotoxicity observed in Vero cells and the murine macrophage cell line J774A.1. In this study the most active compound also possesses the higher oxidation value for Mn(II)/Mn(III) transformation, which suggests that redox processes might also be related to the biological activity [10].

Likewise, ruthenium(II) compounds, denominated as SCAR compounds, have shown in in vitro and in vivo assays that these showed good activities against susceptible and resistant *M. tuberculosis* [9]. SCAR compounds have shown MICs <10 μM with low and middle acute toxicity according to in vivo assays. The action mechanism of these compounds is still unknown, but it is reported that the activity can be related to cell wall biosynthesis besides a direct DNA interaction [9].

Preceding discussion has established several crucial links between cancer cells and parasites. Both share an important feature of living and multiplying in a host organism. *M. tuberculosis*, which is an intracellular pathogen that at least initially occupies the phagosomal com-

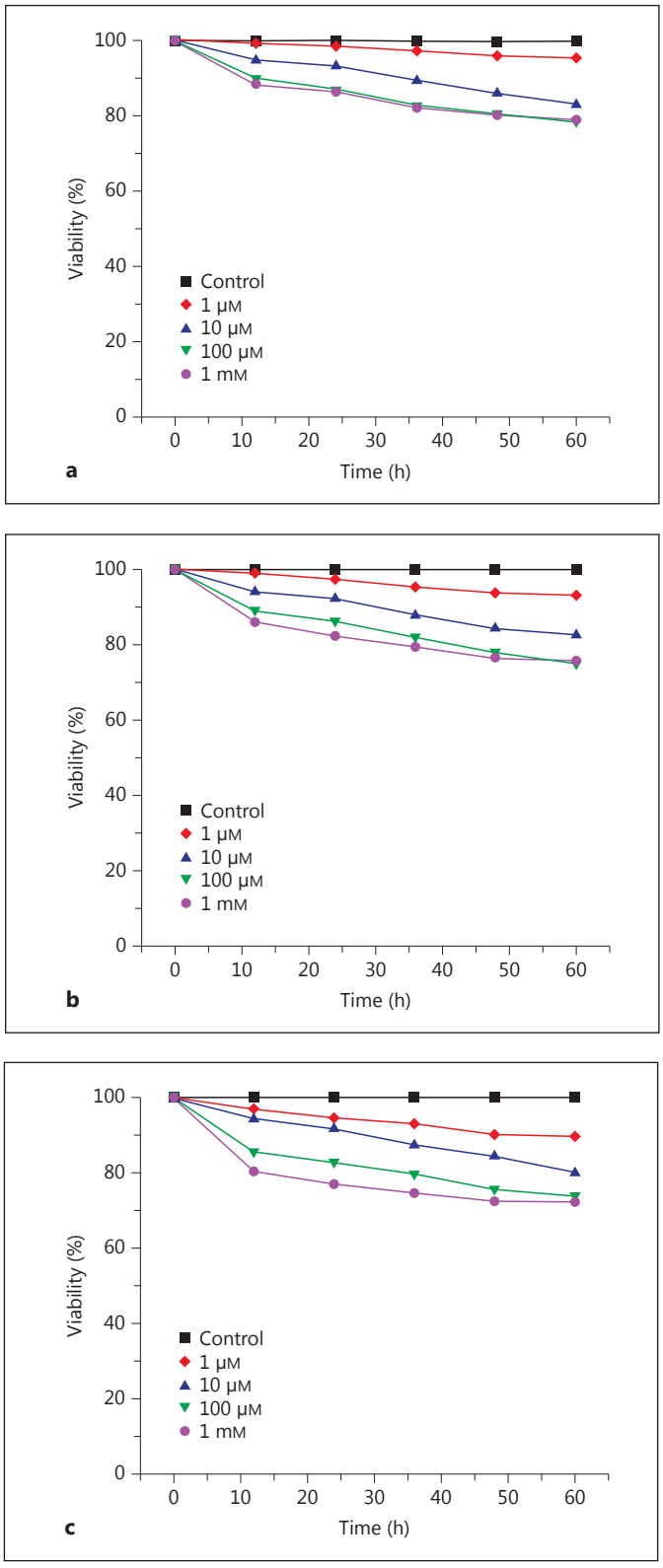

Fig. 1. Macrophage viability after exposure to different concentrations of CasIIIia (**a**), CasIIIEa (**b**) and CasIIgly (**c**). Determinations were made every 12 h, up to 60 h.

partment of host macrophages, is subject to oxidative and nitrosative stress [24].

After the remarkable anti-*M.-tuberculosis* activity obtained with the Casiopeínas® in our study, mainly for resistant *M. tuberculosis* clinical isolates, we intended to determine their interaction with 3 anti-TB drugs (INH, RIF and EMB). The combination of Casiopeínas® with INH or RIF showed mainly an additive effect in susceptible and resistant isolates, while a synergistic effect was only observed in 3 MDR isolates with the combinations INH-CasIIIia for isolate 40, INH-CasIIIEa for isolate 3408 and RIF-CasIIIia and RIF-CasIIgly for isolate 73A. Bhusal et al. [25], working with new and old drugs that are already on the market (e.g. streptomycin and gatifloxacin, among others) in combination with RIF, obtained not very encouraging results. However, the lack of an alternative treatment for resistant TB demonstrates the high importance of studying synergism between new and old compounds for the purpose of having as soon as possible an alternative for treating resistant TB.

The best synergistic effects (FICI ≤0.5) were observed between Casiopeínas® (CasIIIia, CasIIIEa, and CasIIgly) and EMB for susceptible (10/10, 100%; 9/10, 90%; 9/10, 90%) and resistant (8/8, 100%; 6/8, 75%; 5/8, 62.5%) *M. tuberculosis* clinical isolates, respectively. Likewise, Ge et al. [26] tested the synergistic effects between oleanolic acid and INH, RIF and EMB and also found greater synergism in resistant isolates than in susceptible isolates.

Although the mechanism of action of Casiopeínas® has not been completely elucidated yet, the mechanism of action of EMB in *M. tuberculosis* is partially known. We can infer that the EMB may facilitate the action of Casiopeínas®. EMB inhibits the polymerization of cell wall arabinan of arabinogalactan, changing the permeability of the cell wall, which facilitates the uptake of Casiopeínas® that have various levels of hydrophobicity [27].

EMB is an important drug in the treatment of TB, especially in patients with MDR TB by causing suppression of growth of most bacilli that are resistant to INH and streptomycin. A side effect of EMB is its ocular toxicity, which is dose dependent [28]. The detection of synergism with EMB represents the possibility of reducing the concentration of this drug and its adverse effects, leading to increased patient adherence to therapy.

The selective toxicity of Casiopeínas® could be appreciated analyzing the effect of these compounds on the viability of human macrophages (fig. 1), where the highest viability decrease (30%) is obtained with the higher dose of coordination compounds employed, which is 1 mM. It is important to mention that the concentrations employed to decrease the macrophage population are 4 times higher than the concentration range used to produce the 90% growth inhibition of *M. tuberculosis* cultures (1.73–28.09 μM). These results are in agreement with the selective cytotoxicity observed for Casiopeínas® in several tumor cell lines and the lower effect observed in the viability of human lymphocytes. This selective toxicity may be the consequence of differences in biochemistry between normal and tumor cells. Tumor cells have a more active metabolism and proliferate faster, which increases the levels of oxidative stress leading to damage to this kind of cells.

These results show that Casiopeínas® could be used in susceptible and resistant isolates, and they seem to have a different action mechanism than that exerted by INH, RIF, and EMB and show synergism with EMB and an additive effect with INH or RIF combination, fulfilling some of the established criteria for the development of new drugs or as adjunctive anti-TB drug candidates.

In summary, Casiopeínas® (CasIIIia, CasIIIEa, and CasIIgly) have shown activity of growth inhibition in *M. tuberculosis* and no potential action in human macrophages. An interesting synergistic result was obtained when these compounds were combined with EMB, and an additive effect was observed in the combination with INH and RIF, which could be associated with a different action mechanism for these compounds. These results make the Casiopeínas® promising candidates for future investigations in the development of anti-TB drugs, being one of the first examples of essential metal-based drugs used in this field.

Acknowledgments

This work was financially supported by the São Paulo Research Foundation (FAPESP), Brazilian National Council for Scientific and Technological Development (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and PADC of the School of Pharmaceutical Sciences/UNESP, Brazil.

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