



Research article

Leishmania tarentolae novel responses to Bi³⁺-doped strontium aluminum oxyfluorides

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ABSTRACT

Novel therapeutics for the treatment of leishmaniasis are of interest as the disease not only is becoming more prevalent, but drug resistance is increasing in certain regions of the world. Reported here is the use of Bi³⁺-doped strontium aluminum oxyfluoride phosphors and protease inhibitors to test *in vitro* inhibitory activity against cultured promastigote *Leishmania tarentolae* and effects on *L. tarentolae* secreted acid phosphatase (SAP) activity. Cell viability did not significantly decrease in the presence of 50 μM anti-perovskite compounds, implying limited cytotoxicity. Yet SAP activity did increase in the cell free preparations with time in the presence of strontium compounds. Of interest was the observation that cell free SAP activity did not increase in the presence of protease inhibitors with or without added strontium compounds. Since secreted proteases may play a role in the maturation of *Leishmania* SAP and thus be involved with parasite-host infection establishment, this is in further need of evaluation. Nitric oxide production on day 4 post-addition of the strontium compounds was evaluated and showed an approximately 50% decrease in NO production in the presence of two test compounds relative to DMSO control cells. This is the first report of anti-perovskite compound inhibition of NO production by *Leishmania*.

1. Introduction

Leishmania is a genus of trypanosome parasitic protozoans that cause the neglected tropical disease leishmaniasis in many vertebrate species [1]. Phlebotomine sandflies serve as the main vector of transmission, where the parasite is transferred into a host organism through the sandfly bite. Roughly 2 million new cases occur each year and the current therapeutics for treatment consists of pentavalent antimonials, miltefosine, and amphotericin B [2]. All three of these are not without side effects to the patient, however, and pentavalent antimonials are beginning to show less efficacy in some regions due to parasite resistance. The development of novel therapeutics for treatment of leishmaniasis is of interest in order to combat this growing drug resistance and provide alternative routes of treatment to patients. It is, therefore, important to understand various basic biochemical parameters that may have influential roles in parasite-host interactions and basic research involving modulation of parasite enzymes, such as proteases [3] and secreted acid phosphatases [4], as well as other enzymes. There have been some important reviews on the cell biology involved in *Leishmania* infections addressing these molecules and their involvement in infection [5, 6].

Strontium is a group 2 alkaline earth metal and is the 15th most abundant element on earth. It is found in seawater, soil, and in a variety of foods [7]. Strontium is reported to substitute for calcium especially in bones [8]. Since calcium ions have a number of important biological roles, both in *Leishmania* and host organisms, evaluation of the substitution of strontium ion for calcium ion is of interest and may give insight into the complex interactions of parasite and host. This is especially of interest in light of the role of calmodulin (a Ca²⁺ signal transduction protein) that activates nitric oxide synthase and thus modulates production of nitric oxide [9]. We hypothesized that displacement of calcium ions with strontium ions should affect production of nitric oxide. The U.S. Department of Health and Human Services reported that there are no harmful effects of stable strontium in humans at the levels typically found in the environment [10]. However, no reports of interactions with *Leishmania* were found. The correlation of strontium concentrations in water and/or soil in certain regions of the world relative to *Leishmania* infection in said region could provide a unique perspective for how the prevalence and uptake of strontium in populations affects the infectivity of the parasite. The potential of Bi³⁺-activated phosphors of the general composition Sr_{3-x}Bi_{2x/3}AlO₄F (0 ≤ x ≤ 0.1) to negatively affect the *Leishmania* parasite, especially nitric oxide production and secreted acid phosphatase activity

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(as a negative control enzyme that is not reported to be affected by calcium ions), were investigated in this study. We have used the non-human parasite, *L. tarentolae*, to develop this model test system.

These previously synthesized anti-perovskite compounds chosen for this study were $\text{Sr}_{2.976}\text{Bi}_{0.016}\text{AlO}_4\text{F}$, $\text{Sr}_{2.952}\text{Bi}_{0.032}\text{AlO}_4\text{F}$, and $\text{Sr}_{2.928}\text{Bi}_{0.048}\text{AlO}_4\text{F}$, as their composition, structure and correlated photoluminescence are thoroughly characterized [11]. Previous structural characterization studies of these polycrystalline non-molecular extended solids via powder X-ray diffraction (PXRD) and neutron powder diffraction (NPD) have shown them to exhibit anti-perovskite related structures crystallizing in the tetragonal $I4/mcm$ space group with density field theory (DFT) calculations presenting a strong case for preferential occupation of the ten-coordinate Sr(1) crystallographic site by Bi^{3+} . Due to the volatile tendency of bismuth, the composition of these samples was verified using inductively coupled plasma-optical emission spectroscopy (ICP-OES). Optical characterization showed these materials absorb light in the UV region of the spectrum (between 240–326 nm) and emit blue light in the range 446.5–455 nm, varying slightly with Bi^{3+} -concentration. The composition $\text{Sr}_{2.976}\text{Bi}_{0.016}\text{AlO}_4\text{F}$ yielded the maximum emission intensity observed [11]. For ease of labelling, these Bi^{3+} -activated phosphors are referred to in this report by the fraction of Bi in their molecular formula (e.g. $\text{Bi}_{0.016}$ = compound 16). Strontium carbonate was also evaluated for comparison with the bismuth containing compounds.

2. Results

2.1. Incubation effects on *L. tarentolae* growth

No major differences were observed via light microscopy during the *Leishmania* promastigote growth curve (data not shown) in either the morphology or motility of *L. tarentolae* over a 120-hr incubation time-frame. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay [12] was used to evaluate cell viability with and without the compounds. Initial MTT values (day zero) were not statistically different ($p < 0.05$; one-way ANOVA with Tukey's post hoc test) implying the same number of cells in each flask prior to incubation with the compounds. MTT values from cells treated with test compounds stayed very close to the DMSO control throughout the 5-day incubation (Figure 1) and exhibited no statistical differences ($p < 0.05$).

2.2. Incubation of standard pool of secreted acid phosphatase (SAP) enzyme with compounds with or without co-incubation with protease inhibitors

Cell free supernatant containing SAP activity was incubated with test compounds (dissolved in DMSO) or only DMSO (control) for 5 days showed modest changes in enzyme activity over the 5-day period, with

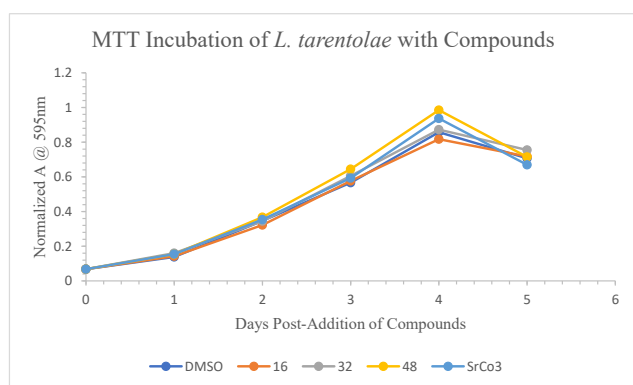


Figure 1. MTT values at 24 h intervals post addition of 50 μM of each compound or DMSO control. Compounds were added immediately after the day 0 MTT evaluation. Values are the mean \pm SD for $n = 4$ independent replicates and SD values are less than 5 % so error bars are not visible.

the largest change detected in the presence of SrCO_3 (15% decrease relative to control cells on day 4; $p < 0.05$). Protease inhibited samples showed significant reduction in cell free SAP activity relative to uninhibited samples on every day (Figure 2; $p < 0.05$). This implies that when proteases are functional, they may activate the SAP activity with time. Others have reported that *Leishmania* secrete proteases [13] but have not indicated an effect of these on secreted acid phosphatase activity.

The protease inhibitor treated samples (indicated by 'p' in legend of Figure 2) show no enzymic increase in cell free SAP activity over the 5-day incubation period, which is in contrast to the trend shown with the no protease addition samples. Over the time frame from day 1 to day 5, the apparent activity of the cell free SAP preparation increases, in general, about 3-fold when protease inhibitors are not added. This implies that endogenous *Leishmania* protease activity is modulating the activity of the SAP. There are no statistical differences between control and experimental values in the protease inhibitor addition group indicated by 'p' before compound name in legend. Also, only on days 4 and 5 are the values in the non-protease inhibitor addition samples with the SrCO_3 , compound 16, and compound 48 incubations (day 4) or SrCO_3 , compound 32, and compound 48 incubations on day 5 different from control values ($p < 0.05$). This is the first report of this interaction of parasite protease and secreted activity phosphatase.

2.3. Nitric oxide detection post-incubation with a specific fluorescent probe, DAF-FM

Detection of nitric oxide (NO) in the *Leishmania* was possible through the use of a NO specific fluorescent probe used in a single experiment. Incubations of cells with either DMSO or SrCO_3 show approximately the same proportion of fluorescent cells (shown in Table 1). However, incubation of cells with the Sr-complexes 32 and 48 resulted in a decrease (of approximately 50%) in the proportion of NO positive cells especially as the Bi^{3+} concentration of the complexes increased (as seen with compounds 32 and 48). To our knowledge there have been no reports of effects of these strontium compounds on nitric oxide synthase, the enzyme responsible for biosynthesis of nitric oxide. It is of interest that the two compounds (32, 48) with the highest proportion of bismuth (Bi) appeared to decrease the amount of detectable nitric oxide; others (Andrews, et al., 2011) have reported anti-leishmanial promastigote activity of bismuth (III) complexes of non-steroidal anti-inflammatory drugs [14]. Clearly more research using bismuth complexes is needed.

3. Discussion

Although the test compounds lack any observed cytotoxicity, the modest decrease in SAP activity in the presence of SrCO_3 and decreased

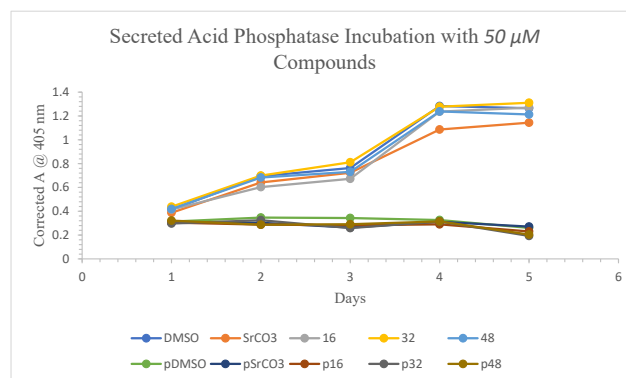


Figure 2. Cell free supernatant SAP activity over 5-day incubation period with and without test compounds and with and without protease inhibitors. Protease inhibited samples are denoted by "p" before compound name in legend. Values are the mean \pm SD for $n = 3$ independent replicates. The SD values are less than 5 % so error bars are not visible.

Table 1. Proportion of *Leishmania* Cells Fluorescing Green (indicative of detection of nitric oxide) as a Function of Additions.

| Addition To Cells | Green Cells Counted | Non-Green Cells Counted | Total Cells Counted | % Cells Green |
|--|---------------------|-------------------------|---------------------|---------------|
| DMSO | 374 | 769 | 1143 | 33 |
| SrCO ₃ | 398 | 1034 | 1432 | 28 |
| Sr _{2.976} Bi _{0.016} AlO ₄ F | 241 | 866 | 1117 | 22 |
| Sr _{2.952} Bi _{0.032} AlO ₄ F | 293 | 1672 | 1965 | 15 |
| Sr _{2.928} Bi _{0.048} AlO ₄ F | 218 | 1350 | 1581 | 14 |

proportion of cells (by about 50%) exhibiting detectable NO levels are of interest. We have previously reported that *Leishmania tarentolae* are able to synthesize nitric oxide [15]. This is especially of interest since there are reports that both nitric oxide production and secreted acid phosphatase are linked to successful parasite-host interactions [15, 16]. We can thus speculate that a modest decrease in parasite virulence is likely in regions richer in strontium and perhaps bismuth. Future work in this direction should consist of using a Ca²⁺ specific probe to track mobilization of this ion and evaluate if the presence of strontium is able to displace the calcium ions and thus affect function.

The protease inhibitor-mediated reduction in SAP enzyme activity has many implications. Secreted proteases have been shown to be virulence factors for *Leishmania* in establishing an infection [17], yet their many roles are not entirely known. Our work suggests that parasite secreted proteases have a role in boosting SAP activity so that these different enzymes could work in concert to establish infection. The protease inhibitors could have inhibited the SAP enzyme directly; however, the consistent level with time of the SAP activity is more likely due to reduced activity of the proteases. We hypothesize that the SAP exists in an immature form and requires protease cleavage of a specific portion of the peptide to allow increased activity. Of additional consideration, the proteases could destroy a separate, inhibitory peptide that binds to SAP and prevents activity. Research in these potential interactions should be carried out. Future work should also include use of the *Leishmania* amastigote form as well as mammalian cell lines.

4. Materials and methods

To generate uniform test cultures, *Leishmania tarentolae* (*L. tarentolae* strain 30143 from ATCC, Manassas, VA) were cultured, in 60 mL of medium in the dark in 250 mL CELLTREAT Suspension Culture Flasks (ThermoFisher Scientific, Waltham, MA, USA) at 22 °C. Although this species is not a human parasite, these cells have previously been established as a good model system for testing novel compounds [18] especially to establish proof of concept. Brain heart infusion media (BHI from Sigma Aldrich) supplemented with streptomycin, penicillin, and hemin (all from Sigma Aldrich) was used for *L. tarentolae* culturing following the method of Morgenthaler et al. [18]. To set up test cells from these stock cells, equal volumes of early log-phase *L. tarentolae* were mixed well then aliquoted (total volume of 4.95 mL) into 25 mL Falcon culture flasks. Test compounds were dissolved in dimethylsulfoxide (DMSO) to generate 5.0 mM stock solutions. Fifty μ L of each compound or DMSO was added to the flasks yielding a final 1% (v/v) DMSO concentration with or without 50 μ M compound concentration. Cell viability was determined using the MTT assay reported by Mendez et al. [12]. From a homogeneous suspension, *L. tarentolae* (100 μ L) were loaded, in quadruplicate, into Falcon® 96 well plates; following incubation with the MTT reagent (5 mg/mL water), A 595 nm values were determined using the Bio-Rad iMark Microplate Reader plate reader. Values were normalized by subtraction of the A 595 nm value from the cell free BHI from the values obtained from test cells and reported per 24 h of incubation time [4].

A *Leishmania* secreted acid phosphatase assay [12] was used to determine enzyme activity from cell-free supernatant as a function of compound incubation and time. SAP activity was assayed using as a

standard cell free supernatant enzyme preparation. This was obtained by centrifuging 100 mL of cell incubation (2000 \times g, 10 min) to remove cells. Ten enzyme incubations were set up, consisting of 6.93 mL of cell free supernatant, and 70 μ L of test compound was added to each tube. Only DMSO was added to the control tubes. To five of the tubes protease inhibitors were also added (indicated as 'p' tubes in Figure 2). The protease inhibitors (Sigma-Aldrich, P2174; Sigma-Aldrich, P4265) added were AEBSF (30 μ M), aprotinin (5 nM), bestatin (2 μ M), E-64 (200 nM), leupeptin (15 nM), EDTA (15 μ M), and pepstatin (10 μ M). These ten reaction tubes were incubated, in the dark, at room temperature for 5 days and assayed for SAP activity each day. To assess enzyme activity each day, a reaction mix of 450 μ L of supernatant was mixed with 450 μ L of sodium acetate buffer (0.5 M, pH 4.5) in a 1.5 mL microcentrifuge tube with each condition performed in triplicate. One hundred μ L of a 5 mg/mL (14.7 mM) aqueous solution of *para*-nitrophenyl phosphate disodium salt hexahydrate (pNPP, Sigma Aldrich) was added to each sample to begin the enzymic reaction. Reactions proceeded for 24 h after which they were stopped using 100 μ L of 10 M sodium hydroxide. SAP enzymic activity produces *para*-nitrophenol which, upon the addition of sodium hydroxide, is converted into the *para*-nitrophenolate anion having an absorbance at 405 nm [12]. Reactions were evaluated using an Agilent Hewlett Packard 8453 UV-visible spectrophotometer. Samples were averaged per condition for $n = 3$ independent replicates, corrected for BHI background, and normalized per 24 h incubation time.

Nitric oxide detection (NO) assay was performed using DAF-FM diacetate (a compound that fluoresces after reaction with nitric oxide; from Millipore Sigma, Darmstadt, Germany) at a final concentration of 20 μ M and 1% DMSO in saline. Cells were centrifuged (10,000 rpm, 60 s, Eppendorf Centrifuge 5415C) and resuspended in this DAF-FM diacetate solution for 1 h before re-centrifuging, which was followed by washing the cell pellet with 500 μ L saline. Cells were resuspended in a final total volume of saline of 100 μ L. Prior to imaging, 10 μ L of each sample was loaded onto a glass slide within a silicon grease ring and then covered with a cover slip. Sample slides were placed in a BZX-810 Keyence Fluorescent Microscope for imaging using a green fluorescent protein filter (GFP; 470 nm: 525 nm excitation-emission coupling). Images were captured at a 0.5 s exposure time and overlays of fluorescent cells with the brightfield images were produced using the analyzer software. Cells were then counted for number of green fluorescent cells (indicating production of nitric oxide) and number of non-green cells. A total of 7238 cells were counted using this methodology. The percentage of green cells (and thus detection of nitric oxide producing cells) varied with addition; the lowest percentage of detectable nitric oxide producing cells (by a factor of 2) was in the presence of compounds 32 and 48 which are the ones with the highest proportion of bismuth.

When applicable, all statistics performed consisted of a one-way ANOVA followed by Tukey Post Test to evaluate statistical difference relative to control cells; a $p < 0.05$ is considered significantly different. Number of independent replicates is indicated in the appropriate graphs and tables.

5. Conclusions

The need for new therapeutics to treat both human and domestic animal leishmaniasis should be further pursued and a novel avenue for potential research was pursued here in this first report of the use of strontium-containing Bi³⁺-doped oxyfluorides. Worldwide trends in strontium uptake by specific populations may influence the virulence of *Leishmania* and needs assessment. A modest decrease in SAP activity upon incubation with SrCO₃ is here reported and this should be further investigated. This is especially of interest since secreted acid phosphatase has been strongly implicated in host-parasite interactions [19]. Also, this appears to be the first report of the effect of co-incubation of protease inhibitors on the potential interactions of parasite secreted proteases and secreted acid phosphatase. Nitric oxide production with these compounds appears to moderately decrease in axenic *Leishmania*. Future

work should involve testing these compounds with the amastigote form as well as with macrophages, the generally accepted host cell for *Leishmania* infections. These compounds should also now be tested in human parasitic *Leishmania* species.

Declarations

Author contribution statement

C. Fiore Apuzzo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Eirin C. Sullivan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

David C. Platt: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ian Seger-Held: Performed the experiments; Analyzed and interpreted the data.

Marjorie A. Jones: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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