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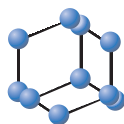
Sulfonamides as Inhibitors of *Leishmania* – Potential New Treatments for Leishmaniasis

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Abstract: **Introduction:** Leishmaniasis is an endemic disease caused by the protozoan parasite *Leishmania*. Current treatments for the parasite are limited by cost, availability and drug resistance as the occurrence of leishmaniasis continues to be more prevalent. Sulfonamides are a class of compounds with medicinal properties which have been used to treat bacterial and parasitic disease *via* various pathways especially as antimetabolites for folic acid.

Methods: New derivatives of sulfonamide compounds were assessed for their impact on *Leishmania* cell viability and potential pathways for inhibition were evaluated. *Leishmania tarentolae* (ATCC Strain 30143) axenic promastigote cells were grown in brain heart infusion (BHI) medium and treated with varying concentrations of the new sulfonamide compounds. Light microscopy and viability tests were used to assess the cells with and without treatment.

Discussion: A non-water soluble sulfonamide was determined to have 90-96% viability inhibition 24 hours after treatment with 100 μM final concentration. Because *Leishmania* are also autotrophs for folate precursors, the folic acid pathway was identified as a target for sulfonamide inhibition. When folic acid was added to untreated *Leishmania*, cell proliferation increased. A water soluble derivative of the inhibitory sulfonamide was synthesized and evaluated, resulting in less viability inhibition with a single dose (approximately 70% viability inhibition after 24 hours with 100 μM final concentration), but additive inhibition with multiple doses of the compound.

Results: However, the potential mechanism of inhibition was different between the water-soluble and non-water soluble sulfonamides. The inhibitory effects and potential pathways of inhibition indicate that these compounds may be new treatments for this disease.

1. INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*. Leishmaniasis is transmitted by sandflies and is often found in areas of Africa, Central and South America, the Mediterranean regions, Central and East Asia, and Southeastern Europe. The parasite can be transmitted when a female sandfly carrying the parasite bites a host, either human or other animal, for a blood meal. Leishmaniasis is endemic in over eighty countries worldwide and is considered a dangerous public health concern with an incidence of 1.3 - 2 million affected each year with approximately 310 million at risk of infection [1]. The disease can present in three main ways: cutaneous, mucocutaneous, or visceral leishmaniasis [2].

Treatments exist today for these diseases, but, due to the infections primarily occurring in the developing countries with weak infrastructure and impoverished economies, access to these treatments is costly and complicated and advancement of known and new treatments is largely ignored [2]. Medical professionals agree that with a recent increase in cases worldwide, it is important that the focus is placed on finding cost effective, readily available treatment options.

One possible treatment option is the development of new sulfonamides that target parasite cell function to inhibit cell viability and proliferation while causing minimal negative side effects for the host. Sulfonamides became popular antibacterial agents in the 1940s, and studies have identified a primary pathway of inhibition in bacteria as competitive inhibitors of the enzymes dihydropteroate synthase (DPS) or dihydrofolate reductase (DHFR), enzymes involved in folate biosynthesis [3]. Bacteria and *Leishmania* both have specific enzymes to metabolize folate and pterines differently from humans who must take in the vitamin folate through their diet. Because of this, sulfonamides have also been tested to treat leishmaniasis [4]. However, due to a more complex pathway of folate production than bacteria, *Leishmania*, when present in the amastigote form typically found *in vivo,* has been seen to overcome the inhibitive capabilities of other known medicinal sulfonamides. This

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is believed to be due to a second enzyme, pteridine reductase 1 (PTR-1), that can reduce folates and unconjugated pteridines. Thus, PTR-1 can act as a metabolic bypass of DHFR inhibitory drugs solely targeting tetrahydrofolate production by the bifunctional DHFR-TS (thymidylate synthase) enzyme and halting single carbon metabolic pathways [5].

The promastigote form of *Leishmania* is transferred to an alternative host upon feeding by an infected sandfly [6]. The cells are phagocytized by a macrophage or other phagocytic cells in the immune system. Once in the phagocytic cell, *Leishmania* transforms to the amastigote form, which is characterized microscopically by a more spherical shape and loss of flagellum. Amastigotes replicate in the phagocytic cell until rupture and the release of amastigotes, thus can infect other cells. The amastigote form of the *Leishmania* has several characteristics different from promastigote form, including enzymatic changes that allow the cells to survive in the acidic environment of a phagocytic cell. One proposed difference between promastigotes and amastigotes is substrate selectivity of the PTR-1 enzyme at lower pH, like the environment expected in phagocytic cells. The change in enzyme selectivity is believed to play a role in the cell's ability to resist inhibition by current sulfonamide treatments [4].

The purpose of these current studies was to test new sulfonamides as inhibitors of the promastigote form of *Leishmania tarentolae in vitro*. The general structure of the tested sulfonamides is shown in Fig. (**1**), where X signifies different functional groups. For this study, X is -S-CH$_3$, -O-CH$_3$, or -CH$_3$. Differences in substituent electronegativity, size, and/or reactivity could affect inhibitory function and were, therefore, evaluated.
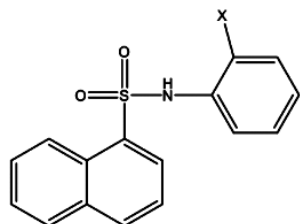

Fig. (1). Generic structure of sulfonamides of interest.

Due to the non-polar structure of these compounds, solubility in aqueous solutions was a major concern. Because of this, and the potential impact solubility could have on cellular uptake and consequential inhibitory performance, compounds with the addition of a carboxylate group para to the amide, as seen in Fig. (**2**), were synthesized and evaluated.
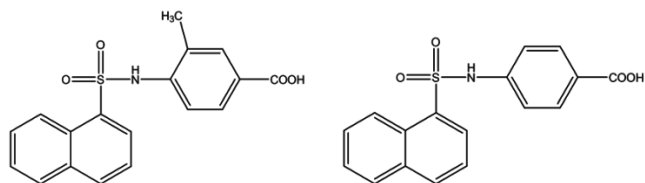

Fig. (2). Compounds of interest with added carboxylate group to improve water solubility.

Cellular inhibition was measured quantitatively using the MTT cell viability assay to compare the cells treated with the new sulfonamides to those not treated [7]. The cells were also evaluated qualitatively with microscopy, assessing cells for shape, motility, and clumping (a sign of cell age/stress).

Using the MTT viability assay, a typical culture growth curve is represented in Fig. (**3**); dimethyl sulfoxide (DMSO) was used to solubilize the sulfonamides being tested and was, therefore, included as part of the control growth curve.
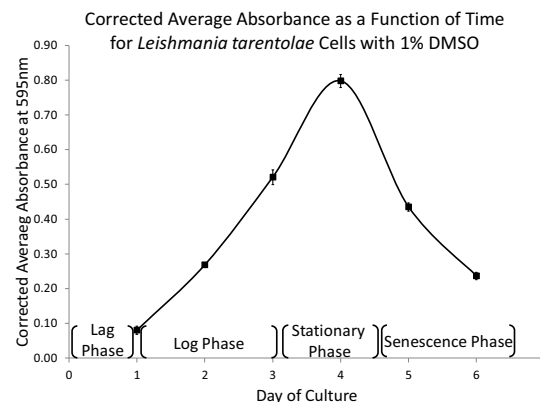

Fig. (3). Typical growth curve of *Leishmania tarentolae* promastigotes: Mean ± standard deviation for n = 4 replicates

Healthy *Leishmania* promastigote cells have a specific shape in the log and stationary phases of their culture life cycle and typically move around using their flagella. When they die or enter senescence, they can become spherical and lack motility. Cell clumping is a normal phenomenon seen with *Leishmania* cells as they age or are stressed, with typically more and larger clumps of cells forming later in the culture growth curve. Examples of these phenomena can be seen by light microscopy (Fig. **4**), where A indicates an individual cell and B shows an example of cell clumping.
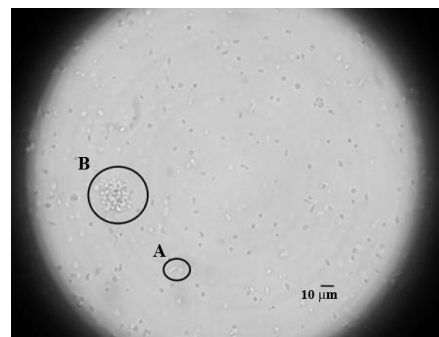

Fig. (4). *Leishmania tarentolae* promastigotes - 400x magnification. Bar indicates 10 μm.

Once sulfonamide inhibition performance had been evaluated, the mechanism of inhibition related to the folic acid pathway was investigated. Identification of potential mechanisms of inhibition included investigating protective effects of metabolic compounds and measurement of cell viability in the presence and absence of those sulfonamides with inhibitory effects.

2. MATERIALS AND METHOD

2.1. Cell Growth

Leishmania tarentolae promastigotes (ATCC Strain 30143) were grown sterilely in brain heart infusion (BHI) medium supplemented with penicillin/streptomycin (100 units/0.1 mg per mL) and hemin (10 μM) in flasks following the method of Mor-

genthaler *et al.* [7]. Promastigote forms of the *Leishmania* cells were transferred during the log phase of the growth curve to maintain cell cultures. The study with promastigotes was performed in a sterile hood to prevent contamination.

2.2. MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate promastigote viability at different points in the growth curve and after treatments. The MTT assay is a colorimetric assay which assesses cell metabolic activity based on the activity of oxidoreductase enzymes [8]. These enzymes reduce the yellow tetrazolium reagent to an insoluble, purple formazan. One hundred microliters of cells were transferred to the wells of a 96 well plate; 4 replicates were allowed for calculation of mean and standard deviation, then 10 µL of MTT reagent was added to each well. The cells and reagent were allowed to incubate for 1 hour before the addition of a stopping agent. A 96 well plate reading spectrophotometer (BIO RAD, iMark Microplate Reader) was then used to obtain absorbance data at 595 nm. BHI medium, without cells, treated with dimethyl sulfoxide (DMSO) or the same concentration of sulfonamide in DMSO being tested were used as blanks as appropriate. Corrected absorbance at 595 nm is correlated to the concentration of viable cells present.

2.3. Evaluation of Sulfonamide Treatment of Promastigotes

The new sulfonamides were dissolved in the DMSO and added to promastigotes grown in BHI medium. A large volume (60 mL) of cells were grown and transferred to smaller flasks (5 mL or 10 mL) for treatment. This was done to try to reduce variation in the number and age of cells in each flask. Promastigotes were treated with varying concentrations of the sulfonamides (between 10 µM to 100 µM) dissolved in the DMSO, ensuring that the cells were exposed to no more than 1% (v/v) DMSO, at different points in the growth curve for the cells. Untreated cells and cells treated only with 1% volume DMSO were used as controls for these studies. MTT assays were used to quantitatively evaluate cell viability, while light microscopy (JENCO, Model CP-2A1) was used to qualitatively assess cells for shape, clumping and cell motility. Each experiment was performed at least three times to confirm repeatability and a Students t-test was used to determine statistical significance for the results (p < 0.05 was considered significantly different). Cultures determined to be severely inhibited were transferred to fresh media to determine if they were in early or late senescence phase and could recover. Those without recovery over 3 days were considered fully inhibited (dead).

2.4. Synthetic Procedures

2.4.1. General Synthetic Information

Starting materials were obtained from commercial sources and used as received. Compounds A, B, and C (Fig. **5**) were synthesized by reaction of 1-naphthalene sulfonyl chloride with the appropriate aniline in pyridine as reported elsewhere (manuscript in preparation). NMR Spectra were recorded at 500 MHz in DMSO-*d*6. Compounds D and E (Fig. **8**) were synthesized as indicated below.

2.4.2. Synthesis of N-(4'-carboxy-2'-methylphenyl)-1-naphthalene sulfonamide (Compound D)

A 30 mL solution of 1 *M* sodium carbonate containing 2.200 g (9.710 mmol) of 1-napthalenesulfonyl chloride and 1.528 g (10.11 mmol) 3-methyl-4-aminobenzoic acid was prepared in a 250 mL Erlenmeyer flask. The solution was allowed to stir for 24 hours and then acidified with 6 *M* hydrochloric acid. The precipitate was filtered and washed with water. The precipitate that formed was collected by vacuum filtration, washed with water, and dried to yield 1.765 g (47.33%) of Compound D as a light pink solid. ^{1}H NMR (500 MHz, $(CD_3)_2SO$): 12.73 (br s, 1H, COO*H*); 10.16 (s, 1H, N*H*); 8.73 (d, 1H, aromatic); 8.23 (d, 1H, aromatic); 8.09 (m, 2H, aromatic); 7.69 (m, 2H, aromatic); 7.59 (m, 3H, aromatic); 7.20 (d, 1H, aromatic); 1.95 (s, 3H, -C*H₃*) (Fig. **S1**).

2.4.3. Synthesis of N-(4'-carboxyphenyl)-1-naphthalene sulfonamide (Compound E)

A 25 mL solution of 1 *M* sodium carbonate in a 125 mL Erlenmeyer flask was charged with 1.371g (10.00 mmol) of anthranilic acid and 2.269 g (10.01 mmol) 1-naphthalenesulfonyl chloride. The solution was stirred for 20 hours and acidified with 6 *M* hydrochloric acid. The precipitate that formed was collected by vacuum filtration, washed with water, and dried to yield 2.588 g (74.01%) of Compound E as a white solid. ^{1}H NMR (500 MHz, $(CD_3)_2SO$): 13.90 (br s, 1H, COO*H*); 8.62 (d, 1H, aromatic); 8.31 (d, 1H, aromatic); 8.19 (d, 1H, aromatic); 8.04 (d, 1H, aromatic); 7.79 (d, 1H, aromatic); 7.69 (t, 1H, aromatic); 7.63 (m, 2H, aromatic); 7.34 (m, 2H, aromatic); 6.88 (t, 1H, aromatic) (Fig. **S2**).

3. RESULTS AND DISCUSSIONS

3.1. Sulfonamide Inhibition of *Leishmania tarentolae* Promastigotes

Promastigote forms of *Leishmania tarentolae* were grown to the log phase of the growth curve (day 2) and treated with 100 µM concentrations of three sulfonamides (Fig. **5**): N-(2'-methylthiophenyl)-1-napthalne sulfonamide (Compound A), N-(2'-methoxyphenyl)-1-napthalene sulfonamide (Compound B), and N-(2'-methylphenyl)-1-napthalene sulfonamide (Compound C).
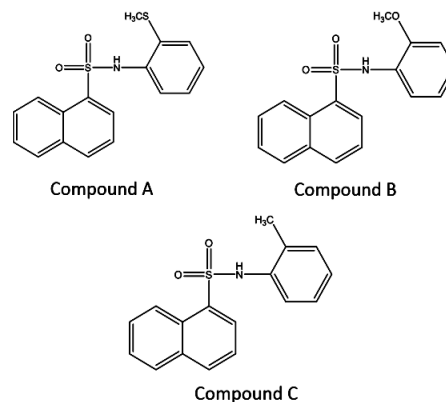
Fig. (5). Chemical structures of N-(2'-methylthiophenyl)-1-napthalene sulfonamide (Compound A), N-(2'-methoxyphenyl)-1-napthalene sulfonamide (Compound B), and N-(2'-methylphenyl)-1-napthalene sulfonamide (Compound C)

Upon addition to cells grown in BHI, water solubility issues were seen at this concentration for the three sulfonamides with crystals forming within 24 hours after addition. Significant inhibitory effects on cell viability were seen with a 100 µM addition of Compound C, but were not seen with the other sulfonamides (Fig. **6**). Within 24-48 hours after addition of Compound C, an 85-95% reduction in cell viability was observed. Cells transferred to fresh medium were unable to recover over three days, which was an indication of cell death.
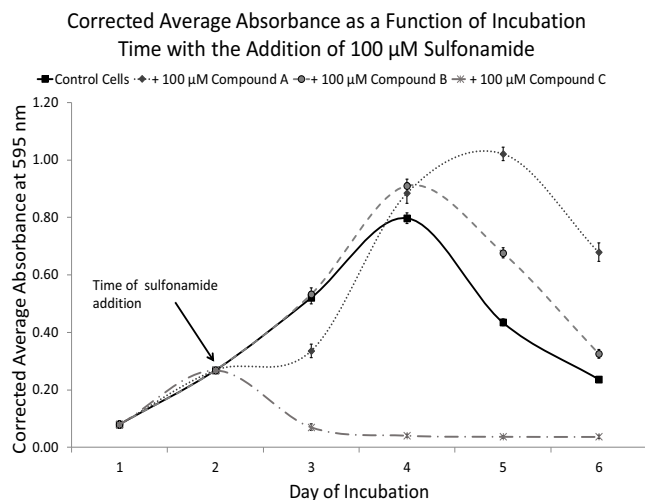
Fig. (6). MTT cell viability results in the presence of 100 µM sulfonamides. Mean ± standard deviation for n = 4 replicates.

Microscopy was also used to evaluate the cells in the presence of the sulfonamides. As can be seen in Fig. (**S3**), 100 µM Compound C caused cells to maintain typical shape, but, per the MTT cell viability assay, lose viability. This is different than typical senescence phase cell death, which is visually characterized by cells becoming spherical in shape. This effect on cell shape was noted for each 100 µM Compound C experiment. Compounds A and B did not negatively affect viability or motility, whereas cells lost motility in the presence of Compound C.

Because of the water solubility issues with Compound C, it was not possible to know the effective concentration of Compound C that caused negative cell responses. To determine if lower concentrations could provide similar results 10 µM or 50 µM concentrations of Compound C were added to cells and cells were evaluated for viability. As can be seen in Fig. (**7**), lower concentrations of Compound C did not have the same inhibitory effect without recovery over time. The 50 µM concentration appeared to have some effect on cell viability, but crystal formation was still seen within 24 hours of addition, meaning the cells were likely not exposed to the full 50 µM concentration. The water solubility issues also prevented the ability for multi-day compound additions.

3.2. Water Soluble Sulfonamide Investigation

To address the water solubility concerns, two water soluble compounds were synthesized (Fig. **8**). Because the group ortho to the amide in Compounds A, B, and C differentiated between cellular inhibition, a derivative of Compound C with a carboxyl group para to the amide was synthesized (Com-

pound D) and a second compound without the functional group at the 2'-phenyl position but with a carboxyl para to the amine (Compound E) was also synthesized and characterized.
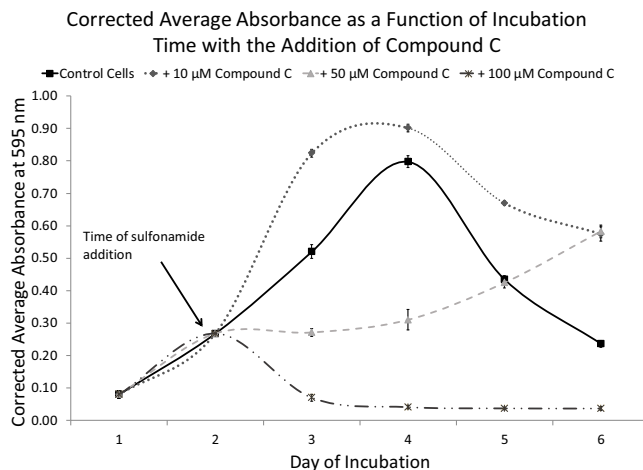
Fig. (7). MTT cell viability of cells with 10 µM, 50 µM, or 100 µM additions of Compound C. Mean ± standard deviation for n = 4 replicates.
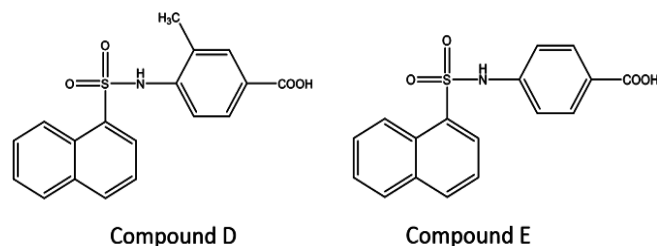

Fig. (8). Chemical structures of N-(2'-methylphenyl)-(4-carboxyl)-1-napthalene sulfonamide (Compound D) and N-(4-carboxyl)-1-napthalene sulfonamide (Compound E).

Compounds D or E were added to cells at 100 µM concentrations and cell viability was evaluated with an MTT assay. The results in Fig. (**9**) were plotted as a percentage of control cells, and show Compound E had only modest inhibition of cell viability and was only tested for 2 days after addition, while Compound D had some inhibitory effect on cell viability in days 1-3 after addition (approximately 70% inhibition) with viability recovery four days after addition. Where results were compared to Compound C, less inhibition and more apparent cell recovery was observed over the 4 days, unlike results with Compound C (approximately 90-96% inhibition without recovery). However, because Compound D is water soluble, multiple additions of the compound can be applied to the cells.

To determine if inhibitory effects could be accumulative for Compound D, two sequential doses of 50 µM Compound D were added to cells and evaluated for effect on cell viability. As seen in Fig. (**10**), two sequential doses of 50 µM Compound D were equivalent to a single 100 µM addition of Compound D.

When evaluated with microscopy, the cells with Compound D had a different appearance than those treated with Compound C. This can be seen in Fig. (**S4**). Cells treated

with Compound C appeared to maintain a typical cell shape, though MTT cell viability results indicated cell inhibition, while cells treated with Compound D presented a cell shape more similar to typical senescence. The visual difference in cells after treatment implies different pathways of inhibition for the two sulfonamides.
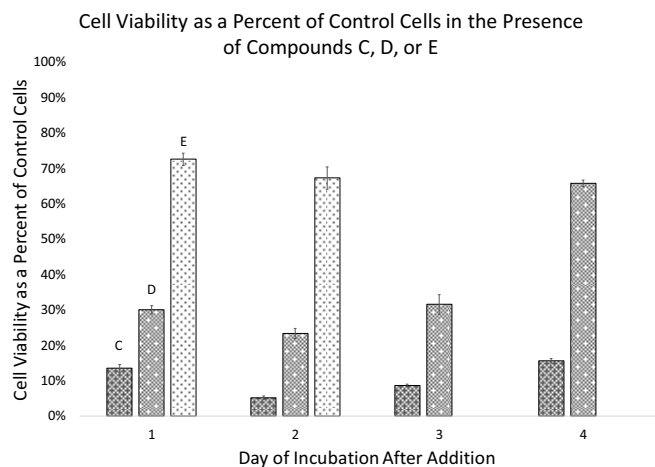
Fig. (9). Cell viability as a percent of control cells in the presence of 100 μM Compound C, D, or E. Mean ± standard deviation for n = 4 replicates.
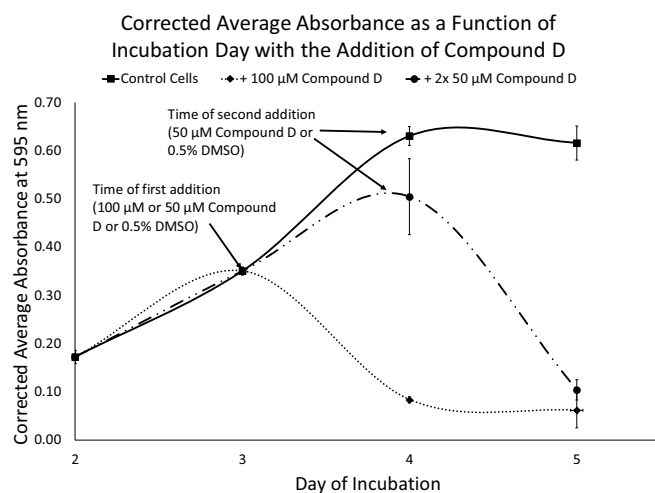
**Fig. (10).** Impact of two doses of 50 μM Compound D added on day 3 and day 4 on cell viability compared to single dose of 100 μM Compound D added on day 3. Mean ± standard deviation for n = 4 replicates.

3.3. Effects of Compound C or D in the Presence of Folate

Sulfonamides are known inhibitors of dihydrofolate reductase (DHFR), an enzyme in the folic acid cycle of some bacteria, preventing folate from being reduced to dihydrofolate and dihydrofolate from being reduced to tetrahydrofolate [4]. To determine if Compounds C or D could be inhibitors of DHFR, 100 μM folic acid and 100 μM Compound C or D were added to cells in the log phase and evaluated for cell viability compared to control cells.

As can be seen in Fig. (**11**), addition of an equivalent concentration of folic acid as Compound C provided approx-

imately 50% more cell viability than cells treated with Compound C alone. However, cells treated with an equivalent amount of folic acid and Compound D did not exhibit protection. This also indicates the likelihood of different pathways of inhibition for these two sulfonamides. A Students two tail statistical t-test where $p < 0.05$ confirmed mean cell viability as a percent of the control cells indicated statistically different responses with the addition of Compound C only or Compound C with 100 μM folic acid but there was no statistical difference with the addition of Compound D only or Compound D with 100 μM folic acid.
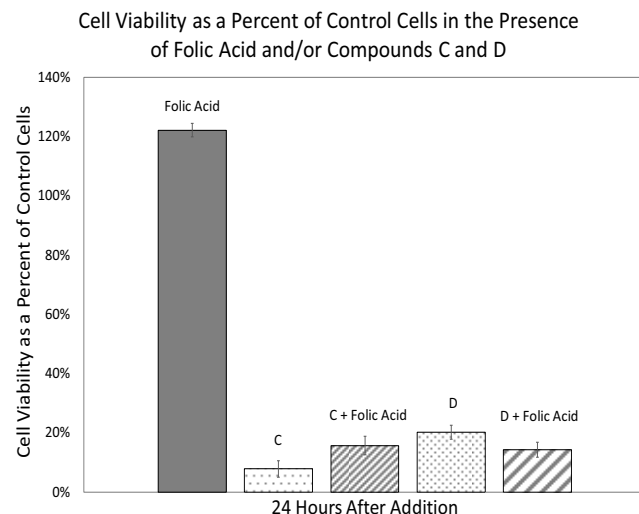
Fig. (11). Effects of folic acid or folic acid and sulfonamide on cell viability as a percent of control cells. Mean ± standard deviation for n = 4 replicates.

3.4. Sulfonamide Substituted Effects

Both Compounds C and D were effective at inhibiting *Leishmania* viability. Compound C was found to have a substantial inhibitory effect on cell viability when added at a 100 μM concentration, though due to water solubility issues it is difficult to determine the actual effective concentration for cell inhibition. Due to the significant difference in effect on cell viability compared to Compounds A, B, D and E, it can be interpreted that the small, non-polar methyl group ortho to the amide must play an important role in inhibition. We speculate this can involve direct enzyme inhibition in folate metabolism. Interestingly, the addition of the carboxyl group para to the amide on Compound C did not inhibit cell viability as well as the non-water soluble Compound C at the same concentration and cells that were inhibited did not have the same microscopic characteristics, especially cell motility. Thus we speculate that a different pathway may be affected with this carboxyl group modification to the compound, especially since folate addition did not have an apparent protective effect, and the carboxyl group addition (Compound E) is not sufficient in itself for substantial cellular inhibition.

Additionally, via microscopy, cell clumping seen late in the growth curve of healthy cells appeared to be interrupted with the addition of any concentration of Compound C, but not noticeably changed with the addition of Compound D. This is an interesting effect that could indicate an effect on cellular signaling and should be further studied.

CONCLUSION

Sulfonamides and water soluble derivatives were evaluated for inhibitory effects against *Leishmania tarentolae* promastigotes in culture. *Leishmania tarentolae* were used in these studies since this species has been shown to be a useful *in vitro* model for screening antileishmanial compounds [9]. Because the promastigote form of *Leishmania* is the infectious form of the cell, cellular inhibition of this cell type could be beneficial in disease treatment. Sulfonamides *N*-(2'-methylphenyl)-1-napthalene sulfonamide (Compound C) and *N*-(4'-carboxy-2'-methylphenyl)-1-naphthalene sulfonamide (Compound D) were found to substantially inhibit cells *in vitro* by 70-96%, dependent on compound and treatment concentrations, but likely by different mechanisms. With the primary difference between these two sulfonamides tested being the carboxyl group para to the amide, it can be interpreted that this position plays an important role in at least one inhibition mechanism. As seen with removal of the functional group in the ortho position, a non-polar, non-electronegative group appears critical for the sulfonamide to be an active inhibitor. These compounds should now be tested in the human infective species of *Leishmania*.

To better understand the pathway of inhibition, specific enzymatic targets of the folic acid pathway will be cloned and enzyme activity determined with and without addition of effective inhibitors. Additional derivatives of the basic sulfonamide structure with different non-polar groups para to the amide could also be synthesized in combination with the carboxyl group at the 4-position of the phenyl group to improve solubility while decreasing the effective dose.

CONFLICT OF INTEREST

ACKNOWLEDGEMENTS

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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