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ORIGINAL PAPER

Synergistic effect of inhibitors of fatty acid desaturases on *Trypanosoma* parasites

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Abstract The pathway for unsaturated fatty acid biosynthesis is essential in trypanosomatid parasites and has been a key target in our work on the discovery and analysis of several inhibitory compounds. Here, we show the effect of novel inhibitors of stearoyl-CoA desaturase (SCD) and oleate desaturase (OD), alone and in combination, on the growth rate of parasite cultures. GS-456332, an inhibitor of human Δ9 desaturase, efficiently inhibited growth of both *Trypanosoma cruzi* epimastigotes and *Trypanosoma brucei* bloodstream form cells, with EC_{50} values of 136.9±24.2 and 9.4±3.1 nM, respectively. This effect was specific for SCD. Stearolic acid (9-octadecynoic acid) was also able to arrest *T. cruzi* and *T. brucei* growth by specific inhibition of their OD, with EC_{50} values of 1.0±0.2 μM and 0.1±0.01 μM, respectively. When these compounds were administered simultaneously, a clearly synergistic effect was observed for both *Trypanosoma* species, with EC_{50} values in the low nanomolar range. These results demonstrate the feasibility of using combinations of drugs, inhibiting different enzymes on the same metabolic pathway, for the development of more efficient chemotherapeutic strategies against neglected diseases caused by these parasites.

Introduction

Trypanosomatids are parasitic flagellated protists that cause several diseases in humans, animals and plants. *Trypanosoma cruzi* is the etiological agent of Chagas' disease in America and *Trypanosoma brucei* subspecies are responsible for sleeping sickness in humans and nagana in cattle, in many locations of sub-Saharan Africa. Additionally, *Leishmania* spp. are the causative agents of different types of leishmaniasis, all of them focused in tropical and subtropical regions throughout the world. Together, these vector-transmitted parasites cause diseases that threaten over 400 million people, with 30 million currently being infected, mainly distributed in underdeveloped countries. These diseases are often fatal if not treated. Moreover, there are no vaccines to prevent these serious infections and merely a few drugs are currently available, most of them noxious and sometimes little efficacious as parasites may generate drug resistance (Barrett et al. 2003). Consequently, there is an urgent need for both the development of new drugs -more efficacious and safe- and the identification of new targets for appropriate drug intervention.

Another motivation for drug development is that parasite transmission continues, despite intensive attempts to curb it. Efforts to control parasite-carrying insects, involving measures such as spraying of infested houses and outbuildings with insecticides, have eliminated the vectors in some countries or states, but there are still many areas where the eradication was not successful or could not directly be accomplished. Moreover, some of the insect hosts have evolved resistance to the insecticides used to control them (Germano et al. 2010).

We have previously shown that both stearoyl-CoA desaturase (SCD) and oleate desaturase (OD) from *T. cruzi* and *T. brucei* are essential for parasite development and have validated them as plausible drug targets (Alloatti et al. 2010 and 2011). As a result of chemical (by means of different inhibitors) and genetic approaches, a decrease in both desaturases activities was always correlated with a significant growth retardation of cultured parasites. Indeed, recently published results showed that feeding *T. brucei* infected mice with Isoxyl, a SCD inhibitor, decreased parasitemia in the blood, and augmented mice survival (Alloatti et al. 2011).

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Synergistic interactions of different drugs have been extensively studied in parasitic diseases (Williamson et al. 1982; Santa-Rita et al. 2005; López-Muñoz et al. 2010; Parquet et al. 2010). The serial—sequential—inhibition of a common biochemical pathway is one of the mechanisms responsible for antimicrobial synergism against diverse microorganisms (Pillai et al. 2005). *T. brucei* and *T. cruzi* present SCD and OD as consecutive enzymes within the same metabolic pathway. Hence, as both enzymes have proved to be essential for the parasites, their simultaneous inhibition is likely to show a synergistic interaction between their inhibitors.

In this paper, we present novel compounds capable of inhibiting *T. cruzi* and *T. brucei* SCD and OD and consequently parasite growth. In addition, synergy between SCD and OD inhibitors was evaluated. Concurrent treatment with inhibitors of each of these consecutive enzymes within the same metabolic pathway exhibited significant synergism.

Materials and methods

Materials

Stearate, linoleate, oleate and sodium methoxide were obtained from Sigma (Sigma-Aldrich, St. Louis, MI, USA). All organic solvents were purchased from Merck (Whitehouse Station, NJ, USA). Isoxyl was from Cayman Chemical Company (Ann Arbor, MA, USA). GS-456332 was kindly provided by Gilead Sciences, Inc. Stearolic acid was purchased from Alpha Aesar (Lancaster).

Trypanosomatids, culture conditions, and inhibition assays

Epimastigotes of *T. cruzi*, CL Brener strain, were grown at 28 °C in brain–heart infusion tryptose medium (Cazzulo et al. 1985). Bloodstream form (BSF) of *T. brucei* Lister 427, cell line 90-13 (Brun et al. 1979) was cultured in HMI-9 medium at 37 °C under water–saturated air with 5 % CO_2 (Wirtz et al. 1999). In all cases culture media were supplemented with 10 % fetal bovine serum and hemin. Cultures were always harvested in the exponential growth phase, i.e., at densities lower than 2×10^6 cells/ml for *T. brucei* BSF, and 2×10^7 cells/ml for *T. cruzi*, by centrifugation at 1,000×*g* for 10 min.

Isoxyl, stearolic acid, and GS-456332 were added to the cultures as solutions in dimethyl sulfoxide. The final concentration of this solvent in the cultures was always adjusted to 0.1 %. Cultures were split every 48 h by adding one volume of fresh medium with the corresponding concentrations of compounds. Cells were counted immediately before splitting the cultures using a Neubauer chamber. Although no significant effect of dimethyl sulfoxide was seen on parasite growth, for each experiment we performed a "control" in which only the solvent was added to the culture. The growth curves were highly reproducible. EC_{50} indicates the concentration of compound required to cause 50 % inhibition of the growth rate. For the compounds studied, the values obtained for this parameter were highly reproducible, irrespective of the day of treatment to determine them.

Analysis of interaction of compounds in in vitro cultured parasites

Classical isobolograms were constructed by plotting compound concentrations that either alone or in combination inhibited by 50 % the proliferation of the tested trypanosomatids. Fractional inhibitory concentrations (FICs) (Canfield et al. 1995) were calculated using the following formula: $FIC_{50}A=EC_{50}$ of drug A in combination/EC_{50} of compound A alone, and $FIC_{50}B=EC_{50}$ of compound B in combination/EC_{50} of compound B alone. The $\Sigma FIC_{50}=FIC_{50}A+FIC_{50}B$ (Berenbaum 1978). Isobolograms were constructed by plotting a pair of FICs for each compound combination. The straight diagonal line ($\Sigma FIC_{50}=1$) on the isobologram indicates a theoretical additive effect. ΣFIC_{50} between 0.5 and 4 indicates no interaction. A concave curve below the line ($\Sigma FIC_{50}\leq0.5$) indicates significant combinatorial synergy, whereas a convex curve above the line ($\Sigma FIC_{50}>4$) indicates antagonism.

Statistics

EC_{50} and growth rate calculations were analyzed by non-linear regression. When necessary, data were also analyzed through two-way ANOVA. The compound combination treatments were analyzed by isobologram constructions through sum-of-squares plus Fisher test. Values of $p\leq0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism 5.0 software. Results presented correspond to means±SD from at least three independent experiments.

Fatty acid analysis results correspond to means±SD from at least three independent experiments. Significance of differences was validated by ANOVA analysis.

Fatty acid analysis

Cells (2×10^8) from cultures grown for 4 to 8 days (*T. cruzi*) and 48–72 h (*T. brucei*) in the late logarithmic phase of growth were collected by centrifugation and the pellets washed twice with 8 ml of isotonic saline solution. Lipids were extracted according to Bligh and Dyer (1959). The organic phase was treated as described previously to obtain the FA derivatives and analyze them by GC-MS (Alloatti et al. 2009). The retention time and mass spectrum of any new peak obtained was compared with those of standards (Sigma) and those available in the database NBS75K (http://www.nist.gov/srd/analy.htm) (National Bureau of Standards, USA). Percentages

of FAs were calculated after integration of the chromatogram peaks. The sum of C20 and C22 FAs represents, in all cases, 9–11 % of total FAs and was not taken in consideration.

Results

Novel inhibitor of *Trypanosoma* spp. oleate desaturases

We have previously investigated the growth-inhibitory effect of some inhibitors of trypanosome desaturases. Thiastearic acids (10-TS, 12-TS, and 13-TS) and Isoxyl were shown to be deleterious for parasite growth, exerting their effect with different efficacies (Alloatti et al. 2009). For instance, 12- and 13-TS specifically inhibited *T. cruzi* OD with a concomitant growth arrest with EC_{50} values of 50 and 10 μM, respectively. These concentrations were 1 or 2 orders of magnitude higher than the ones achieved when testing SCD inhibitors. We hypothesized that 12- and 13-TS, in order to effectively inhibit OD, must be first converted into their thia-oleoyl derivatives, introducing a double bond between carbons 9 and 10 by the action of the SCD. This metabolic step seems to be crucial as trypanosome ODs present a ν+3 regioselectivity (Alloatti and Uttaro 2011), implying the necessity for the thiastearic acid to undergo this desaturation in order to be recognized by OD's catalytic site. As a consequence of this metabolic requirement, the exogenous amount of 12- and 13-TS added to the cultures is higher than the effective inhibitory concentration.

To bypass this situation, we assayed several drugs and found 9-octadecynoic acid (stearolic acid, SA), a fatty acid capable of inhibiting OD without the need for prior activation (Fig. 1a). This compound was tested on cultures of *T. cruzi* epimastigotes and *T. brucei* BSF. We found SA to be deleterious for parasite growth, with an EC_{50} value of 1.0±0.2 μM and 0.1±0.01 μM, respectively.

In order to confirm the target of this inhibitor, we extracted lipids from membranes of *T. cruzi* and *T. brucei* cultures grown in the presence of SA. We focused our analysis on C16 and C18 species, which can be desaturated by SCD and OD, as we have previously shown (Alloatti and Uttaro 2011). Relative concentrations of C20 and C22 lipid moieties remained invariant and are not included in the figure. The data presented in Fig. 2a provide evidence that OD is the target of SA: the level of 18:1 in cultures of treated *T. cruzi* increased approximately 17 % when compared with those of non-treated parasites, whereas 18:2 decreased correspondingly. In addition, conversion of palmitic acid (16:0) into palmitoleic acid (16:1) remained unaffected when the parasites were treated with SA. These data strongly suggest that the inhibition is specific for OD. A similar lipid profile was observed in treated *T. brucei* cultures (Fig. 2b).

Our previous works with 12- and 13-TS showed analogous results, with total unsaturated fatty acids (total UFA) being invariant, and exerting a lytic effect on both trypanosome species, just as reported here after SA treatment or after knocking down the expression of *T. brucei* OD by RNA interference (Alloatti et al. 2009 and 2010).

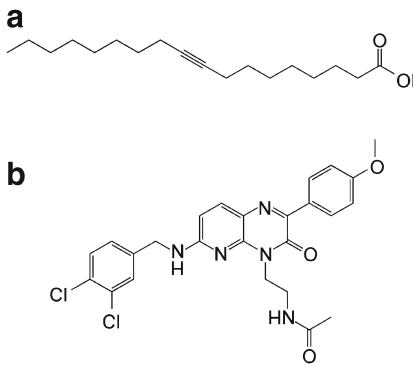

Fig. 1 Structure of the compounds assayed in this work. **a** Stearolic acid (9-octadecynoic acid). **b** GS-456332, a novel inhibitor of the mammalian Δ9 desaturation

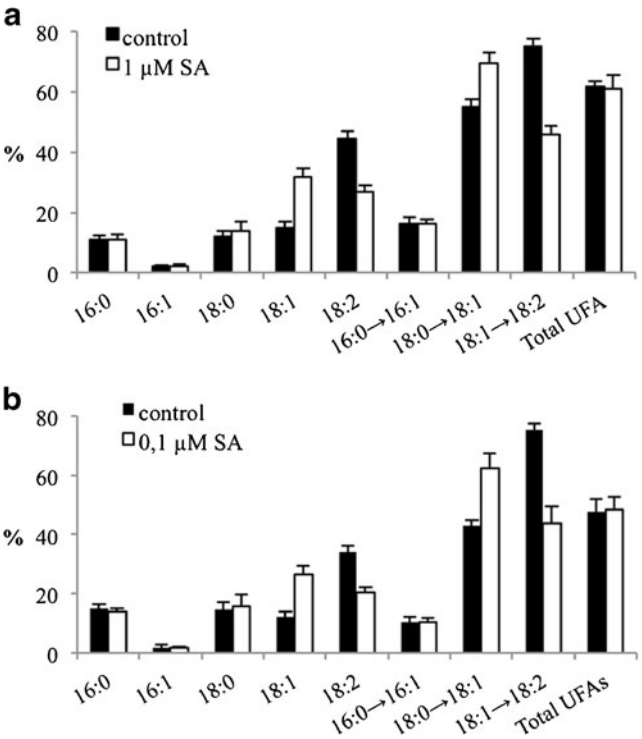

Fig. 2 Fatty acid profile of **a** *T. cruzi* epimastigote cultures grown in the presence of 1 μM stearolic acid for 4 days, and **b** *T. brucei* BSF cultures treated with 0.1 μM and untreated (control) grown for 36 h. The abundance of each fatty acid is presented as percentage of the total fatty acids. *C16:0* palmitate; *C16:1* palmitoleate; *C18:0* stearate; *C18:1* oleate, and *C18:2* linoleate. *16:0→16:1* means percentage of conversion of palmitic acid to palmitoleic acid; *18:0→18:1* percentage of conversion of stearic acid to oleic acid; *18:1→18:2* percentage of conversion of oleic acid to linoleic acid; *Total UFA* percentage of total unsaturated fatty acids (sum of 16:1, 18:1, and 18:2). *Control* untreated culture. The results are means±SD of three independent experiments

A potent inhibitor of human stearoyl-CoA desaturase is effective against trypanosomes

We also wanted to analyze different SCD inhibitors in in vivo experiments, particularly those that were more efficacious than the previously tested Isoxyl, in order to determine if lower amounts of these compounds could be administered to rodents as a preliminary experimental curative assay for the diseases. Recently—mostly due to the discovery of the involvement of human SCD in some human diseases (Flowers and Ntambi 2009; Igal 2010)—several SCD selective small-molecule inhibitors have been developed. The compound known as GS-456332 [*N*-(2-(6-((3,4-dichlorobenzyl)amino)-2-(4-methoxyphenyl)-3-oxopyrido[2,3-*b*]pyrazin-4(3*H*)-yl)ethyl)acetamide] (Fig. 1b), has shown to be a potent and specific inhibitor of rat microsomal and HepG2 cell Δ9-desaturation (Koltun et al. 2009). Therefore, we tested the compound on cultures of trypanosomes. EC_{50} values of 136.9±24.2 nM for *T. cruzi* and 9.4±3.1 nM for *T. brucei* were attained.

Fatty acid profiles of *T. cruzi* and *T. brucei* cultures treated with GS-456332 were analyzed (Fig. 3a and b, respectively). In both parasites, a drop in levels of 16:1, 18:1, and 18:2 was observed when treated. Nevertheless, the conversion of 18:1 to 18:2 remained unmodified indicating unaffected OD activity, whereas conversion of 16:0 to 16:1 and 18:0 to 18:1 decreased approximately 35 % in both *Trypanosoma* species. Therefore, GS-456332 acts as a specific SCD inhibitor with an apparent trypanostatic effect, like that observed after treatment with Isoxyl and 10-TS, or following RNA interference of *T. brucei* SCD expression (Alloatti et al. 2009 and 2011).

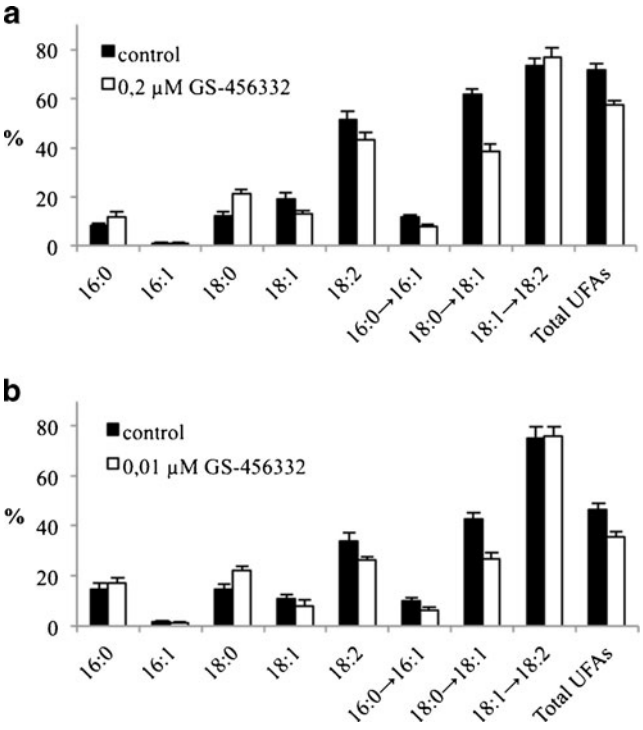

Fig. 3 Fatty acid profile of **a** *T. cruzi* epimastigotes cultures treated with 0.2 μM GS-456332 and untreated (control); and **b** *T. brucei* BSF cultures treated with 0.01 μM GS-456332 and untreated (control). The abundance of each fatty acid is presented as percentage of the total fatty acids. *C16:0* palmitate; *C16:1* palmitoleate; *C18:0* stearate; *C18:1* oleate; *C18:2* linoleate. *16:0→16:1* conversion of palmitic acid to palmitoleic acid; *18:0→18:1* conversion of stearic acid to oleic acid; *18:1→18:2* conversion of oleic acid to linoleic acid; *Total UFA* total unsaturated fatty acids (sum of 16:1, 18:1, and 18:2). *T. cruzi* cultures were grown for 4 days and *T. brucei* cultures were grown for 36 h prior to fatty acid analysis. The results are means±SD of three independent experiments

Effect of combined administration of GS-456332 and stearolic acid on trypanosomes

Subsequently, we wanted to determine if a synergic effect could be produced against parasites, when administering an inhibitor of SCD simultaneously with an inhibitor of OD. The rationale for performing this experiment was the fact that both enzymes are sequential components of the same biochemical pathway (Pillai et al. 2005). Although we did not succeed to completely abolish the enzymatic activity of the desaturases, we proved that a small decrease of each activity (by RNA interference or inhibition with chemical compounds) was sufficient to drastically retard the growth of the parasites (Alloatti et al. 2010 and 2011). With this in mind, our idea was to check whether the simultaneous inhibition of both enzymes—even a partial ablation—might cause any synergic effect on trypanosomes growth.

The synergistic effect of these two novel inhibitors of trypanosomatid desaturases, GS-456332 and SA, was studied by administering them simultaneously to the parasite cultures. To this end, we evaluated the effect of fixed SA concentrations at 0.5, 0.25, and 0.06 times its EC_{50} in combination with different amounts of GS-456332 (2, 1, 0.5, 0.25, and 0.06 times the EC_{50} of the compound). In addition, a combination of equimolar amounts of both compounds was considered. The EC_{50} values of the tested inhibitors—alone or in combination—are listed in Table 1.

The isobologram analysis of *T. cruzi* (epimastigote) and *T. brucei* (BSF) cultures treated with different combinations of GS-456332 and SA (Fig. 4) showed a clear synergism between both inhibitors for *T. brucei* and *T. cruzi*.

Therefore, GS-456332 and SA inhibited targets from the same metabolic pathway in *T. cruzi* and *T. brucei*, with as result that a significant synergism was observed in these parasites (ΣFIC_{50} value of 0.43±0.15 for *T. cruzi* and 0.50±0.09 for *T. brucei*).

When equimolar amounts of inhibitors were administered, a concentration of 3.8 nM of each compound was enough to inhibit *T. brucei* growth by 50 %, whereas 22.5 nM of GS-456332 and SA were sufficient to inhibit 50 % of *T. cruzi* growth. Equimolar amounts of Isoxyl and SA were also tested

Table 1 EC_{50} values of the different trypanocidal compounds (administered to cultured parasites, alone or in combination) used in the isobologram analysis. EC_{50} values±standard deviation are shown, when appropriate

EC_{50} for *T. cruzi* (epimastigote) (nM)		EC_{50} for *T. brucei* (bloodstream form) (nM)	
GS-456332	SA	GS-456332	SA
136.9±24.2	–	9.4±3.1	–
–	1036±220	–	107.3±10.0
7.0±1.2	500[a]	0.7±0.2	50[a]
14.4±2.4	250[a]	1.6±0.3	25[a]
39.4±2.9	62.5[a]	4.9±0.8	6.3[a]
22.5±4.0[b]	22.5±4.0[b]	3.8±0.6[b]	3.8±0.6[b]

[a] Fixed concentrations

[b] EC_{50} for equimolar amounts of the compounds

(in order to corroborate the previous results with an effective and tested compound): EC_{50} values of 5.8 and 5.0 nM of each compound were attained for *T. brucei* and *T. cruzi*, respectively.

Discussion

In this work, we have shown that the compound known as GS-456332, a reported potent inhibitor of mammalian Δ9 desaturation, was also able to inhibit *T. brucei* and *T. cruzi* SCDs with high efficacy. Fatty acid analysis of cultures of these parasites allowed us to confirm the specificity of GS-456332 for SCD, whereas OD was not affected.

We furthermore analyzed the effect of SA. It retarded parasite growth by means of specifically inhibition of ODs in *T. brucei* and *T. cruzi*, with EC_{50} values of 0.1 and 1 μM, respectively. Even though the compound showed only efficacy in the low micromolar range, it is the first reported inhibitor capable of inhibiting OD without requirement for prior metabolic modification.

Therefore, combinations of GS-456332 and SA were assayed in cultures of *T. cruzi* and *T. brucei*. These combinations showed a significant synergism for both *Trypanosoma* species when analyzed by isobolograms.

As mentioned, each compound inhibited a different trypanosome desaturase within the same metabolic pathway, with a concomitant synergistic effect that can be easily appreciated by isobologram analysis. Remarkably, the synergistic effect of GS-456332 and SA significantly decreased their respective EC_{50} values, both in *T. brucei* and *T. cruzi*, ranging from 3.8 to 22.5 nM when the two inhibitors were administered in equimolar quantities.

Simultaneous administration of drugs has been previously explored as chemotherapeutic strategies against diseases caused by protozoa. A classic example is the treatment with pyrimethamine, in combination with sulfonamide or nonsulfonamide compounds, against experimental toxoplasmosis, showing synergic effect (Beverley and Fry, 1957; Alder et al. 1994). Pyrimethamine–sulfadiazine or pyrimethamine–clindamycin is the current recommended therapy for human toxoplasmosis (Katlama et al. 1996). Combination of sulfadoxine and pyrimethamine is used in Africa as a first line treatment for non-severe *falciparum* malaria, and combination of multiple drugs (three or more) has been recently suggested to treat multi-drug resistant malaria. Several combinations have shown synergistic effects and, since these drugs act against targets on different pathways, they would delay the selection of parasites resistant to the three drugs, extending the useful therapeutic life of these compounds (Nduati and Kamau, 2006).

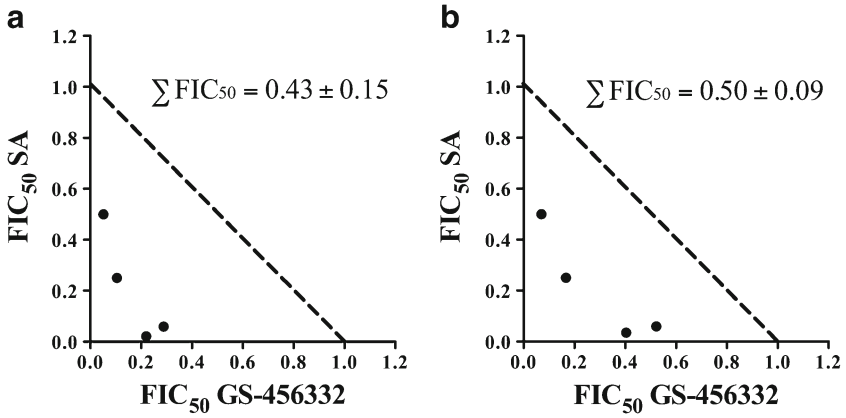

Fig. 4 Isobolograms describing the effect on parasite growth when compounds GS-456332 and SA are administered simultaneously to cultures of: **a** *T. cruzi* epimastigotes and **b** *T. brucei* BSF. The *numbers on the axes* represent the normalized FIC_{50}s. Mean fractional inhibitory concentration index (ΣFIC_{50})±standard deviation is shown for the combination in each parasite. The *diagonal (dashed) line* ($\Sigma FIC_{50}=1$) indicates the hypothetical additive compound effect. A *concave curve* ($\Sigma FIC_{50}\leq 0.5$) *below the diagonal line* indicates a synergistic effect of the combination, whereas a *convex curve* ($\Sigma FIC_{50}>4$) *above the diagonal line* indicates an antagonistic effect

We have shown that both OD and SCD are essential enzymes in trypanosome parasites. This manuscript builds upon these previous observations by providing strong support for the notion that synergy between inhibitors of two consecutive enzymes in the biosynthesis of trypanosomal PUFAs could and should be considered as an important mechanism in order to decrease drug concentrations in treatments.

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