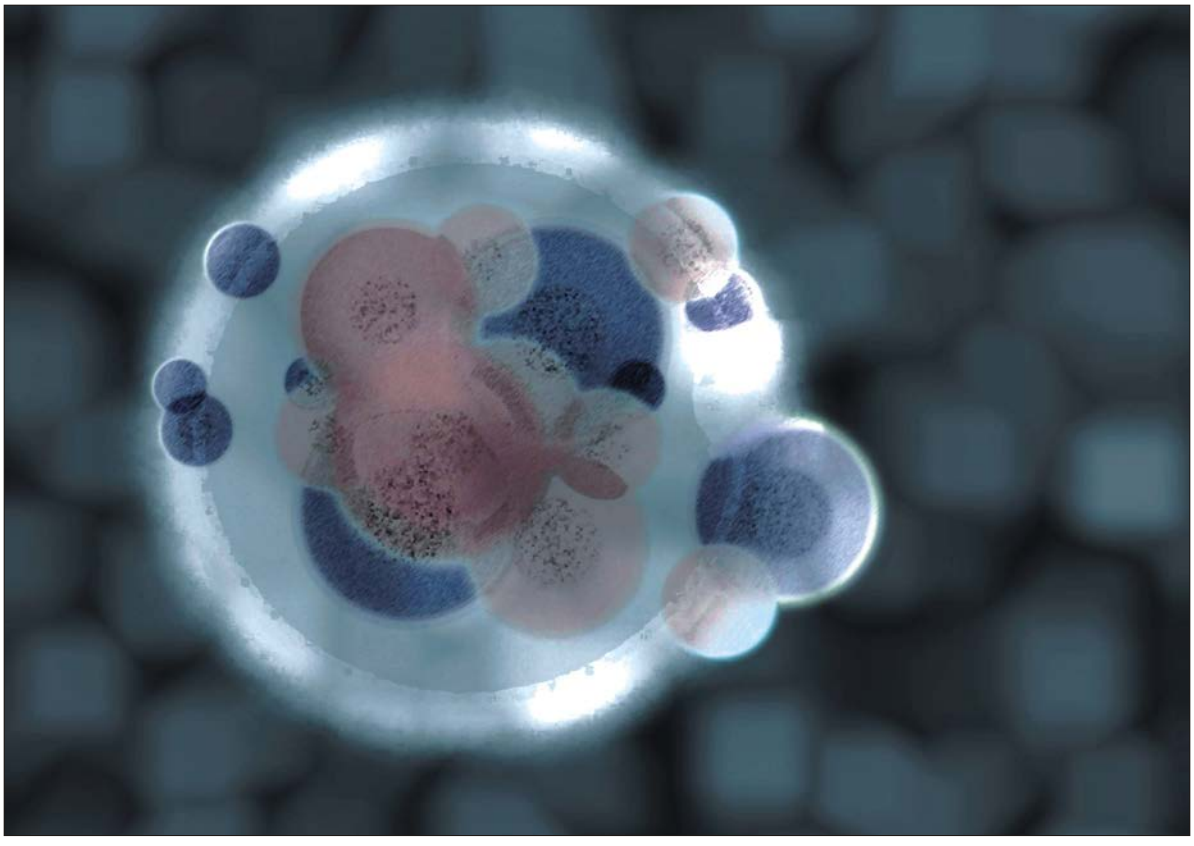

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Inhibition of Glutathione Synthesis as a Potential Therapeutic Strategy Against Chagas' Disease

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Abstract: Chagas' disease is a major parasitic cause of death and hardship, especially in the impoverished regions of Latin America. *Trypanosoma cruzi* is the etiologic agent of this disease. Characteristically, this hemoflagellate has an indirect life cycle with mammals as definitive hosts where attacks the heart, skeletal, smooth and cardiac muscles and the central nervous system. The drugs currently in use to treat this disease are nifurtimox and benznidazole. However, they have many side effects and are potentially toxic. Their mode of action includes free radical and electrophilic metabolites generation, which are toxic for the parasite. The parasite is more sensitive to the oxidative stress than the host. Gutathione and its spermidine derivative, trypanothione are the main antioxidative mechanisms in the parasite. Any modification in the parasite capacity to synthesise this thiols could induce a more susceptible environment to the action of the trypanocidal drugs. This effect may shorten treatment length or lower the required doses to treat this disease.

Key words: *Trypanosoma cruzi*, buthionine sulfoximine, nifurtimox, benznidazole, trypomastigotes, epimastigotes, amastigotes, glutathione, trypanothione

The disease and its agents. *Trypanosoma cruzi* is a hemoflagellate parasite[1], of the Kinetoplastida order and Trypanosomatidae family. This order is characterized by possessing one large mitochondria containing 15 to 30% of the cellular DNA. The disease extends from the southern part of the United States to the south of Chile and Argentina. Estimates are that the number of infected people in Latin America is 20 million, with mortality and morbidity rates of 0.4/1000 and 3.8/1000, respectively[2].

T. cruzi has an indirect life cycle, that comprises hematophagous insects (Triatomids) as intermediary hosts and mammals, including man, as definitive hosts[3]. The principal *T. cruzi* vectors in South America are *Triatoma infestans* (vinchuca), *Rhodnius prolixus* and *Panstrongylus megistus*. In Chile, *Mepraia spinolai* and *M. gajardoi* must be considered along with *T. infestans*.

Transmission of disease is mainly produced by the bite of a *T. cruzi* infected insect. Upon feeding on blood from a mammal, the insect deposits feces contaminated with infecting metacyclic trypomastigotes which then enter the blood stream through the skin, mechanism facilitated by scratching of the insect bite and by proteolytic enzymes present in the hematophagous insect's saliva[4]. Once in the blood stream, trypomastigotes enter macrophages and other leukocytes, differentiating into amastigotes, a form of obligatory cytoplasmic replication. After a certain number of divisions, amastigotes differentiate into trypomastigotes, escaping from the cells and returning to the bloodstream. Thus, they make their way to target tissues such as the myocardium, skeletal muscle, visceral smooth muscle and central nervous system glia. Other important forms of transmission are blood transfusions and organ transplants (10% of cases) and transplacental transmission in Chagasic mothers[5]. The epidemiological importance of consumption of infected meat has not been dimensioned, even though oral transmission is possible[6,7].

The disease progresses in three phases. The acute phase, immediately after infection, with intense parasitemia, produces symptoms in only some patients (regional lymphadenopathies, bipalpebral, unilateral oedema or Romaña's sign and characteristic electrocardiographic alterations). In most cases, the acute infection does not present clinical manifestations and advances into a latent phase that can last months, or even years[8]. The chronic phase in 30% of infected patients is associated with megacolon, megaesophagus, denervation of the autonomous nervous system, cardiac arrhythmia, myocardial hypertrophy and progressive

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cardiac insufficiency and a very negative impact on the individual's working capacity[5]. In this phase, the disease can be incapacitating and directly or concurrently responsible for mortality.

It is therefore evident, that there exists a need to develop effective therapeutic strategies to fight this disease. Taking into consideration the limited effectiveness and collateral toxicity of currently available drugs for this disease, it is necessary to develop a multiple therapeutic approach, with additive or synergic effect to treat this disease.

Limitations for the use of conventional drugs: Chagas' disease must be treated at the moment it is diagnosed. Drugs currently in use for the treatment of Chagas' disease are Nifurtimox (4-[5-furfuriliden)amino]-3-mehtylthiomorpholyn-1,1-dioxide) and Benznidazole (N-benzyl-2-nitroimidazole-1-acetamide). Recommended dosage in the acute phase of disease is 8-10 mg/kg/day for 90 days in adults and 15 mg/kg/day in children for Nifurtimox and 5 mg/kg/day for 60-90 days for Benznidazole. Duration of therapy averages 60 days. Only in case of accidental infection, by a vector, blood transfusion, or laboratory contamination, treatment duration is 10 days.

Both nifurtimox and benznidazole are active against all parasite forms[9]. In spite of this, they can cause systemic toxicity and mild to severe side effects that include: anorexia, nausea, vomiting, vertigo, headache, amnesia, pruritus, fever, depression or excitation, convulsions, paresthesias, peripheral neuropathy and dermatitis[10]. In addition, they produce mutagenesis and DNA damage, reasons for which there are doubts on the benefits of their use[11,12]. Differences in susceptibility to these drugs have been observed between strains, which adds another element of complication to the pharmacological treatment of this disease, resulting in an important cause of treatment failure[13].

Mechanisms of action of nifurtimox and benznidazole: During the reduction process of both drugs, by action of nitroreductases, electrophylic metabolites and nitroradicals are produced[14]. As seen in Fig. 1, nitroradicals enter redox recycling with molecular oxygen, producing partial oxygen reduction, thus regenerating the drug[15] and favoring the appearance of the superoxide anion ($O_2^{\bullet -}$), H_2O_2[16] and the hydroxyl radical ($OH^{\bullet}$). These free radicals bind to macromolecules such as: lipids, proteins and DNA and can therefore produce mutagenesis and carcinogenesis[10,17,18].

Nifurtimox produces the nitro anion radical in *T. cruzi*[19-23] at concentrations that inhibit epimastigotes in culture (10-20 μM)[14,19,21].

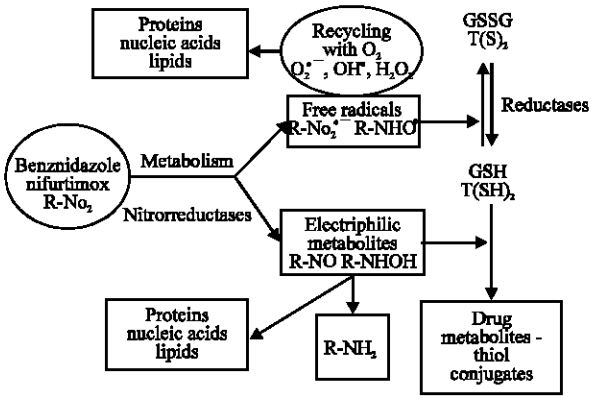

Fig. 1: Mode of action of Nifurtimox and Benznidazole. Glutathione (GSH) and trypanothione ($T(SH)_2$ neutralization effect of macromolecular damage

Benznidazole inhibits *T. cruzi* epimastigote growth at concentrations that do not induce production of $O_2^{\bullet -}$ or H_2O_2. On the contrary, Benznidazole's reduced metabolites, through covalent binding to macromolecules, are involved in its toxic and trypanocidal effects[17,18,21,24,25]. A variety of responses to Benznidazole and Nifurtimox have been observed in different *T. cruzi* strains[26]. The reason for this phenomenon is unknown, although an increase in concentration of detoxifying enzymes or modification of intracellular thiol content has been proposed[25,27].

Thiol metabolism and defense against free radicals: The defense mechanisms used by mammalian cells to eliminated free radicals are multiple and diverse[28-30]. They possess enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and reductive compounds such as reduced glutathione (GSH), α-tocopherol, ascorbic acid and beta-carotene. In addition, it has been proposed that metalothioneins, due to their high-SH group content, can participate in the metabolism of free radicals and electrophylic agents[31-33]. On the other hand, the parasites' defense mechanisms against oxidative stress are deficient[34,35]. Selenium dependent glutathione peroxidase and catalase activity have not been detected[35,36] and superoxide dismutase activity is very low[16,37]. In addition, the existence of beta-carotene and α-tocopherol has not been published either. Not withstanding, the presence of ascorbate reductase and dehydroascorbate reductase activity has been detected in *T. cruzi*, suggesting the existence of an ascorbic acid redox cycle, both in epimastigotes and trypomastigotes[38].

Due the fact that trypanosomatids are deficient in enzymes that protect them from oxidative stress, the main mechanisms that *T. cruzi* has to protect itself from free

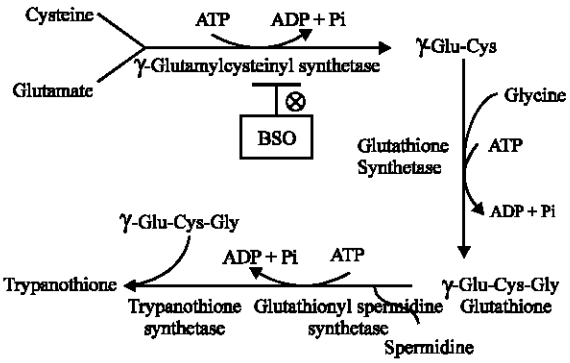

Fig. 2: Glutathione and trypanothione synthesis. Inhibition by buthionine sulfoximine (BSO)

radicals are reduced GSH and a GSH-spermidine conjugate named trypanothione. This compound is characteristic of all trypanosomatids and is indispensable for GSH reduction[34,35].

Glutathione (γL-glutamyl-L-cysteinyl-glycine) is the low molecular weight thiol that is found in highest concentration in mammalian cells. It protects against toxicity from highly electrophylic compounds or from their metabolites and against free radicals[39]. It also participates in detoxification processes of xenobiotics of electrophylic character, via glutathione-S-transferases[40-42]. In addition, it is important in protecting DNA[43] and lipid membranes[44,45].

Glutathione reductase irreversibly reduces oxidized glutathione in mammals, maintaining a very high GSH:GSSG ratio. In *T. cruzi*, the enzyme glutathione reductase has not been found[46], but reduction of GSSG by means of trypanothione has been demonstrated[47].

Glutathione is synthesized by the successive action of γ-glutamylcysteinyl synthetase (GGCS) and glutathione synthetase (Fig. 2). Both require ATP. Reduced glutathione inhibits GGCS, by non allosteric feedback. Glutathione is degraded by γ-glutamyl transpeptidase, γ-glutamyl cyclotransferase and 5-oxo-prolinase and by a peptidase, thus conforming the γ-glutamyl cycle[48].

GGCS can be inhibited by a group of chemical analogs[49], one of them being L-buthionine (SR) sulphoximine (BSO) (Fig. 2).

Trypanothione (N1,N8-bis(glutathionyl) spermidine, $T(SH)_2$) is a low molecular weight thiol specific of trypanosomatids. It is synthesized by the conjugation of two molecules of reduced glutathione and spermidine, in an ATP dependent reaction (Fig. 2) and catalyzed by glutathionyl-spermidine synthetase and trypanothione synthetase[47,50,51]. Trypanothione needs two electrons in order to be reduced and at physiologic pH has a +1 charge and is slightly more electronegative than GSH[50]. These characteristics results in its important reductive power. There are studies that showing that $T(SH)_2$ is a better radioprotector of DNA than GSH or spermidine, presumably because T(SH)2's spermidine allows a greater local concentration of SH groups in the proximity of DNA, due to its positive charge[52].

$T(SH)_2$ in trypanosomatids reduces glutathione and also acts as a intracellular protector against endogenous and exogenous oxidative agents[50,53]. Other functions of $T(SH)_2$ include ascorbate homeostasis[54], reduction of hydroperoxides[55,56], desoxyribonucleotide synthesis[57] and conjugation with metals and drugs (Fig. 1)[25].

In conclusion, trypanosomatids have a more deficient defense system than mammals against oxidative stress and their principal defense is through GSH and $T(SH)_2$.

Numerous chemical compounds exist, of natural and synthetic origin, that have been evaluated as potential trypanocidal agents[58,59], the main ones are: Allopurinol and its analogs[60], Ketoconazole and Itraconazole among other antimycotics[61-63], numerous quinine derivatives[64], Nitroheterocyclic derivatives such as megazole[27], antioxidants[35] and drugs in clinical use such as Phenothiacins[65] and oxazol(thiazol)piridine derivatives[66]. However, for various reasons including lack of susceptibility, resistance, insolubility, toxicity, or poor clinical response, these drugs have not had better results than Nifurtimox and Benznidazole. The logic of testing a combination of BSO with Nifurtimox and Benznidazole, in order to boost therapeutic effects and lower toxicity of these drugs, is based on the previous considerations.

This hypothesis is based on the following: a) *T. cruzi* has levels of free and conjugated GSH that are much lower than those of the mammalian host[25,67-69]. Electrophylic metabolites of Nifurtimox and Benznidazole conjugate with GSH and $T(SH)_2$, lowering the intracellular concentration of these thiols[25,69], thus aggravating the parasite's already precarious defense against oxidative stress and electrophylic metabolites (Fig. 1). b) In mammals, GSH synthesis can be inhibited in up to 80-90% without evidence of toxicity, since they possess other defense mechanisms against oxidative stress and electrophylic agents. Thus oral adninistration of 20 mmol BSO kg^{-1} in mice inhibits GSH synthesis in all examined tissues, except the brain[70], without producing toxic effects[71]. c) GGCS has been isolated in species like *E. coli*[72], protozoans such as *T. brucei*[73] and Leishmania[74], nematodes[75], rats[76] and humans[77]. The Ki of BSO for this enzyme can vary greatly depending on the species. For example, in *E. coli* the magnitude of inhibition of GGCS by BSO and other analogs is low compared to

the inhibition in rats or humans[78], which suggests that the selective inhibition of GSH synthesis is possible and that this selectivity has a potential therapeutic application. In fact, a similar approach has been tried in trypanosomatid protozoans, in which the dependence on GSH and $T(SH)_2$ is essential for survival. Mice infected with *T. brucei* have been cured with a single dose of 2-4 mmol kg^{-1} BSO[79]. Apparently, the same strategy has also worked in Leishmaniasis *in vitro*[80]. All of the above indicates that there are structural differences in GGCS that could be exploited in Chagas' disease drug therapy. d) Resistance to some anti-neoplasic agents is partly related to GSH levels since this compound can protect against oxidative stress induced by these agents on its own by: I) protecting against drug induced oxidative stress, ii) conjugating with the drug[81] , or iii) participating in DNA repair processes[43]. The fact that GGCS activity and GSH turnover in tumor cells is lower than in normal cells has also been demonstrated[82,83]. For this reason, selective inhibition of GSH synthesis with BSO is possible. In addition, this strategy has been demonstrated to be effective in reverting resistance processes[82,84] and in potentiating the effect of antineoplasic agents such as doxorubicin[85], melphalan[86,87] and cyclophosphamide derivatives[88] in preclinical studies and in human phase I and II clinical studies in the case of melphalan[84,89-91]. In these experiments, blood levels of 0.5 to 1 mM of BSO were obtained and up to 80% inhibition of white blood cell GSH content and 50% inhibition of GGCS. It has also been demonstrated that tumors can be sensitized to treatment with radiation by lowering GSH levels[92]. Decrease in glutathione content in humans might pose a therapeutic problem, but apparently this strategy does not have toxic consequences according to studies in mice and clinical studies[71,84].

Finally, in our laboratory we have proven that in *T. cruzi* epimastigote cultures inhibition of GSH by approximately 50%, using BSO, increases Nifurtimox and Benznidazole parasite toxicity[68]. Nevertheless, there are no references in the literature where BSO has been used to boost other anti-parasitic drugs, except experiments carried out in our laboratories and the use of BSO to modulate GSH levels in cancer therapy.

The fact that different *T. cruzi* strains have different susceptibility profiles to Nifurtimox and Benznidazole[13], partly explained by differences in glutathione and trypanothione content[25,69], supports the use of BSO in combination with drugs of similar profiles of action as those in clinical use in order to improve efficiency in Chagas' disease treatment.

In recent studies in our laboratory[93] we show that L-buthionine (S,R)-sulfoximine (BSO) increased the toxicity of nifurtimox and benznidazole toward the epimastigote, trypomastigote and amastigote forms of *Trypanosoma cruzi*. In Vero cells infected with amastigotes, 25 μM BSO was able to potentiate the effect of nifurtimox and benznidazole as measured by the percentage of infected Vero cells multiplied by the average number of intracellular amastigotes (endocytic index). At 0.5 μM nifurtimox, the proportion of Vero cells infected decreased from 27 to 20% and the endocytic index decreased from 2500 to 980 when 25 μM-BSO was added. Similar results were obtained with benznidazole and BSO-benznidazole treated cells.

This study indicates that potentiation of nifurtimox or benznidazole by BSO could decrease the clinical dose of both drugs and diminish the side effects or the length of therapy.

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