Drug screening of Bisnaphthalimidopropyl derivatives on *Trypanosoma brucei*

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African trypanosomiasis (AT) is a parasitic vector-borne endemic disease in sub-Saharan Africa and approximately 60 million of people are currently at risk. It is transmitted by the tsetse fly bite. Despite the efforts made by the World Health Organization (WHO) this disease is a continuing problem in Africa. The causative agent is the extracellular protozoan parasite *Trypanosoma brucei* (*T. brucei*) which is sub-divided in three subspecies: *T. brucei brucei*, *T. brucei gambiense* and *T. brucei rhodesiense*. The two latter subspecies are responsible for Human African Trypanosomiasis (HAT) also known as sleeping sickness disease, which evolve to the slow onset chronic disease or to the fast onset acute disease, respectively [1, 2].

The current state of drug therapy is limited to five drugs. Suramin and pentamidine are applied against the early phase of the disease denominated as the hemo-lymphatic stage, and melarsoprol, efornithine and the combination therapy nifurtimox/eflornithine are used against the late meningo-encephalitic stage [3]. However, some drawbacks are associated with these compounds such as their general toxicity to human organism, expensive cost and difficulty in their administration [3-5]. Thus, there is an urgent need for new drugs that are effective against *T. brucei*, with lower cost, easy to administer, and safer to humans.

The anti-parasitic activity on *Leishmania infantum* (member of the Trypanosomatid family) of bisnaphthalimidopropyl (BNIP) derivative compounds has been described [6-10]. These are composed by two naphthalimidopropyl groups linked by a carbonated chain, varying in size and in the presence of amines, oxygens, cyclohexanes and/or benzene rings.

The present work had the objective to evaluate a small library of BNIP-derivative compounds as therapeutic drugs in the treatment of infections caused by *T. brucei*. At first, an in vitro optimization of two methods to assess parasite growth inhibition [resazurin-based assay and tetrazole 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay] was performed to defined the cell-based drug screening assay towards the bloodstream form *T. b. brucei* strain Lister 427. Mouse fibroblast L929 cells were used as a counter-screen for non-selective inhibitors determined by the MTT assay. The most potent compound against *T. brucei* was chosen to determine its reversibility and time kill action, in vivo efficacy on *T. brucei*-infected BALB/c mice, identification of its putative protein(s) target(s) and development of polyethylene glycol (PEG) – coated poly (lactic acid) (PLA) nanoparticles.

In this project, the current used drugs in therapy, pentamidine and suramin, exhibited IC_{50} (half maximal inhibitory concentration) values against *T. brucei* of ~4 nM and 70 nM,
respectively, in accordance with available literature [11]. From the small 15 BNIP library, 12 compounds exhibited an IC\textsubscript{50} in the nanomolar range and it was demonstrated that the presence of two cyclohexane or benzene rings in the linker chain hampers their anti- \textit{T. brucei} activity. Also it was verified a decrease in the anti-parasitic activity with the increment on the linker chain of the BNIP derivative compounds.

BNIPDabut was the most potent compound with ~3 nM activity, similar to the IC\textsubscript{50} of pentamidine and with a selectivity index for \textit{T. brucei} over fibroblasts of about 8000-fold higher. Also, it was characterized as an \textit{in vitro} trypanocidal compound dependent of concentration and time that is also able to reduce parasite quantity \textit{in vivo}. Moreover, it exhibits a specific interaction with some \textit{T. brucei} proteins, in particular with fructose-1,6-bisphosphate aldolase. In addition, BNIPDabut PEG-PLA nanoparticles were successfully produced and are effective \textit{in vitro} towards \textit{T. brucei} at the nanomolar range.

In conclusion, BNIP-derivative compounds showed promising toxic potential against \textit{T. brucei} pathogen at low concentrations which can be applied in the future for HAT treatment.

\textbf{Keywords:} African trypanosomiasis, \textit{Trypanosoma brucei}, drugs, bisnaphthalimidopropyl derivative compounds, BNIPDabut
Resumo

A Tripanossomíase Africana é uma doença parasitária endémica em África, transmitida por um vector, com aproximadamente 60 milhões de pessoas em risco. É transmitida pela picada da mosca tsé-tsé. Apesar dos esforços realizados pela Organização Mundial de Saúde, esta doença permanece um problema em África. O agente causador é um parasita protozoário extracelular denominado Trypanosoma brucei (*T. brucei*) que está classificado em 3 sub-espécies: *T. brucei brucei*, *T. brucei gambiense* e *T. brucei rhodesiense*. As duas últimas sub-espécies referidas são responsáveis pela Tripanossomíase Humana Africana também conhecida como doença do sono, que se desenvolve com um estabelecimento lento provocando uma forma crónica da doença ou com um estabelecimento rápido e induzindo a forma aguda da doença [1, 2].

Atualmente a terapia está limitada a cinco fármacos. Suramina e pentamidina são aplicadas na fase inicial da doença designada de estadío hemo-linfático, e o melarsoprol, a eflornitina e a terapia combinada entre nifurtimox/eflornitina são usados contra o estadío más tardío meningo-encefálico [3]. No entanto, alguns inconvenientes estão associados a estes medicamentos tais como a toxicidade geral provocada no organismo humano, o elevado custo e a dificuldade existente na sua administração [3-5]. Portanto, existe uma necessidade urgente de novos fármacos eficazes contra *T. brucei*, com reduzido custo, fácil administração, e mais seguros para os humanos.

A atividade anti-parasitária dos compostos derivados do bisnaftalimidopropilo (BNIP) está descrita em Leishmania infantum (membro da família dos tripanossomatídeos) [6-10]. Estes são constituídos por dois grupos naftalimidopropilo ligados por uma cadeia carbonada, que varia no tamanho e na presença de aminas, átomos de oxigénio, ciclohexanos e/ou anéis de benzeno.

O presente trabalho teve como objetivo avaliar um conjunto de compostos derivados do bisnaftalimidopropilo como fármacos terapêuticos no tratamento de infeções causadas por *T. brucei*. Primeiramente, foi realizada uma optimização in vitro de dois métodos para avaliar a inibição de crescimento parasitário [método baseado na redução de resazurina e método do brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT)] para definir o processo de screening de compostos na estirpe Lister 427 de *T. b. brucei*, forma tripomastigota sanguínea. Os fibroblastos de ratinho L929 foram utilizados para determinar os inibidores não selectivos de células mamíferas pelo método do MTT. O composto mais eficaz contra *T. brucei* foi selecionado para determinar o tipo de ação (reversível ou irreversível) e tempo de...
morte, eficácia *in vivo* em ratinhos BALB/c infectados com *T. brucei*, identificação da(s) proteína(s) alvo do composto mais promissor e desenvolvimento de nanopartículas poli(ácido láctico)-polietilenoglicol para o mesmo.

Neste projeto, os fármacos utilizados na terapia atual, pentamidina e suramina exibiram valores de concentração inibitória de 50% (IC$_{50}$) da população de *T. brucei* de aproximadamente 4 nM e 70 nM, respectivamente, e estão de acordo com a literatura [11]. Dos 15 compostos BNIP testados, 12 apresentaram atividade na ordem dos nanomolar e foi demonstrado que a presença de dois anéis de ciclohexano ou benzeno na cadeia de ligação diminui a sua atividade anti-*T. brucei*. Também foi verificada uma perda na atividade antiparasitária com o aumento da cadeia de ligação dos compostos derivados BNIP.

BNIPDabut foi o composto mais eficaz com atividade antiparasitária de aproximadamente 3 nM, semelhante ao IC$_{50}$ da pentamidina e com um índice de selectividade em *T. brucei* relativamente a fibroblastos cerca de 8000 vezes superior. Adicionalmente, foi caracterizado *in vitro* como um composto tripanocida dependente do tempo e da concentração que, também tem a capacidade de reduzir a quantidade de parasitas *in vivo*. Induz ainda uma interação específica com algumas proteínas de *T. brucei*, em particular com a frutose-1,6-bifosfato aldolase. Além disso, nanopartículas de BNIPDabut encapsulado em poli(ácido láctico)-polietilenoglicol foram produzidas com sucesso e foram eficazes *in vitro* contra *T. brucei* na ordem dos nanomolar.

Concluindo, os compostos derivados do bisnaftalimidopropilo mostraram um potencial tóxico promissor em concentrações reduzidas contra o patogénio *T. brucei*, que podem ser aplicados futuramente no tratamento da Tripanossomíase Humana Africana.

**Palavras-chave:** Tripanossomíase Africana, *Trypanosoma brucei*, fármacos, compostos derivados do bisnaftalimidopropilo, BNIPDabut
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ALD</td>
<td>Fructose-1,6-bisphosphate aldolase</td>
</tr>
<tr>
<td>AT</td>
<td>African Trypanosomiasis</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>a.u.</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>BNIP</td>
<td>Bisnaphthalimidopropyl</td>
</tr>
<tr>
<td>BNIPDabut</td>
<td>Bisnaphthalimidopropyl diaminobutane</td>
</tr>
<tr>
<td>BNIPDaCHM</td>
<td>Bisnaphthalimidopropyl diaminocyclohexanemethyl</td>
</tr>
<tr>
<td>BNIPDadec</td>
<td>Bisnaphthalimidopropyl diaminodecane</td>
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<tr>
<td>BNIPDahep</td>
<td>Bisnaphthalimidopropyl diaminooctane</td>
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<td>BNIPDahep</td>
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<td>BNIPDanon</td>
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<td>BNIPDaoct</td>
<td>Bisnaphthalimidopropyl diaminooctane</td>
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<td>BNIPDaoxoct</td>
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<tr>
<td>BNIPDapen</td>
<td>Bisnaphthalimidopropyl diaminopentane</td>
</tr>
<tr>
<td>BNIPDpta</td>
<td>Bisnaphthalimidopropyl dipropyltriamine</td>
</tr>
<tr>
<td>BNIPSpm</td>
<td>Bisnaphthalimidopropyl spermidine</td>
</tr>
<tr>
<td>BNIPSpm</td>
<td>Bisnaphthalimidopropyl spermine</td>
</tr>
<tr>
<td>BNIP(1,4)Dacyhex</td>
<td>Bisnaphthalimidopropyl – 1,4 - diaminocyclohexane</td>
</tr>
<tr>
<td>BNIP(4,4)Dapm</td>
<td>Bisnaphthalimidopropyl – 4,4 – diaminophenylmethyl</td>
</tr>
<tr>
<td>BSF</td>
<td>Bloodstream form</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>E.E.</td>
<td>Encapsulation Efficiency</td>
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FBS  Fetal Bovine Serum
HAT  Human African Trypanosomiasis
HDL  High-density lipoproteins
IC$_{50}$  Half maximal inhibitory concentration
i.m.  Intramuscular
i.p.  Intraperitoneal
i.v.  Intravenous
MTT  Tetrazole 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
MW  Molecular Weight
NPA  Naphthylphtalamic acid
NPs  Nanoparticles
OD  Optical density
ODC  Ornithine decarboxylase
PBS  Phosphate-buffered saline
PEG  Polyethylene glycol
PEG-PLA  Polyethylene glycol – coated poly(lactic acid)
PLA  Poly (lactic acid)
PVA  Poly (vinyl alcohol)
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Spd  Spermidine
Spm  Spermine
SRA  Serum resistance associated
T. brucei  Trypanosoma brucei
T. b. brucei  Trypanosoma brucei brucei
T. b. gambiense  Trypanosoma brucei gambiense
T. b. rhodesiense  Trypanosoma brucei rhodesiense
TEM  Transmission electron microscope
TLF-1  Trypanosome Lytic Factor-1
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Term</th>
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<tr>
<td>VSG</td>
<td>Variant surface glycoprotein</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction

1. Neglected tropical diseases – Human African trypanosomiasis

Mostly in tropical and subtropical developing countries human parasitic infections are a serious problem [12]. These parasitic diseases have no especial public and private attention in recent years being classified as neglected diseases. Currently the existing neglected diseases of parasitic origin are: tuberculosis, malaria, lymphatic filariasis, soil-transmitted helminthiasis, schistosomiasis, onchocerciasis, leishmaniasis, African trypanosomiasis, Chagas disease, ectoparasitic skin infections, parasitic zoonoses and others such as dengue, leprosy and Buruli ulcer [13]. Human African trypanosomiasis (HAT) also known as sleeping sickness is fatal if left untreated. It has resurfaced in the 80’s decade and reached epidemic proportions in many parts of the African continent. One century ago HAT was believed to be the main death cause in colonial territories. When its causative agents and vector were identified by David Bourne in 1903, systematic screening, treatment research and its application were established in western and central Africa. Thereafter, by the 1960s the rarity of cases lead to a loss of interest considering that the control of the disease was feasible [14]. The lack of health care, competent personnel, surveillance, new treatments and the emergence of parasite resistance to some of the currently used drugs, due to the feeling that HAT had been almost eradicated in the 60’s decade, contributed to this dramatic reappearance [15]. In the past years research of new drugs was limited by the low economic return for the manufacturers, subsequently only one combination therapy was discovered and applied. In 2001, HAT disease threatened over 60 million of people [16]. Since then, WHO and its partners made an effort to control the disease and, in 2009 and 2010 it was reported a drop below 10,000 HAT new cases for the first time in 50 years [14]. However, the few chemotherapy options available are difficult to administer, some with resistance and/or high toxicity problems associated [2]. The last collection of field data in 2010 considered that 55 million are at risk to contract HAT in 36 sub-Saharan Africa countries and the available estimates indicates that approximately 10,000 new cases per year still occurs (considering only the patients that are of WHO knowledge and are under treatment) [17].
2. African trypanosomiasis

2.1. Disease vector

African trypanosomiasis is caused by the extracellular protozoan parasites from *Trypanosoma brucei* (*T. brucei*) species, transmitted to their host by the bite of tsetse flies (genus *Glossina*) [18, 19]. These vectors are classified in three subgenera based on morphological differences in the structure of the genitalia: Morsitans, Palpalis and Fusca groups [20]. Most of HAT cases are transmitted particularly by *Glossina fuscipes* (Fig. 1) and *Glossina palpalis* [14, 21].

![Glossina fuscipes tsetse fly](image)

**Figure 1.** *Glossina fuscipes* tsetse fly [22].

2.1.1. *Trypanosoma brucei* taxonomy

Three subspecies of *T. brucei* are described: *T. brucei gambiense*, *T. brucei rhodesiense* and *T. brucei brucei*. HAT is caused by the subspecies *T. brucei gambiense* (slow onset chronic disease) and *T. brucei rhodesiense* (fast onset acute disease) (Fig. 2A, 2B) [1, 2]. These subspecies also infect wild and domesticated animals. *T. b. gambiense* infects humans as the main reservoir however for *T. b. rhodesiense* animals are thought to be the primary reservoir. Cattle are the major reservoir for human infection and contribute to the population malnutrition (Fig. 2C).

In opposite to the previous subspecies, *T. b. brucei* only infect animals (Animal African trypanosomiasis), such as other trypanosome species also endemic in Africa (*T. congolense* and *T. evansi*). These subspecies don’t infect humans because they are susceptible to the human immunity system. In fact, the Human high-density lipoproteins (HDLs) in human innate immunity to infection by African trypanosomes includes an initially described as a minor subclass, Trypanosome Lytic Factor-1 (TLF-1) (contains apolipoprotein A-1, two primate specific proteins apolipoprotein L-1 and haptoglobin related protein) [23]. TLF-1 requires haptoglobin related protein bound hemoglobin to high selective cytotoxicity to the veterinary pathogen *T. b. brucei* but is not effective against the human sleeping sickness pathogens *T. b. gambiense* or *T. b. rhodesiense* [24]. *T. b. rhodesiense* has evolved the
serum resistance associated protein (SRA) that binds and confers resistance to TLF-1 while *T. b. gambiense* lacks the gene for SRA and is inherently resistant to TLF indicating that these parasites have diverse mechanisms of resistance to TLF-1 [25]. However, *T. b. brucei* is used as a model for human infections in laboratory studies as it shares many features with the human infective subspecies (*T. b. gambiense* and *T. b. rhodesiense*), such as antigenic variation.

2.1.2. Trypanosomes cell architecture

*T. brucei* pathogen is an unicellular eukaryotic parasite and is classified by its specific cellular organizations of the nucleus, kinetoplast (the single mass of mitochondrial DNA) and single flagellum position (Fig. 3A) [28]. The classical descriptions of *T. brucei* polymorphism in distinct stages of the life cycle are denominated as epimastigote and trypomastigote (includes slender and stumpy bloodstream forms, and also procyclic and metacyclic forms) (Fig. 3B) [29]. The life cycle will be further explored in this introduction at point 2.1.3.

The microtubule cytoskeleton defines the elongated cell shape and the single-copy organelles (flagellar pocket, flagellum, kinetoplast, mitochondrion and nucleus) are

---

Figure 2. Physical manifestations of African trypanosomiasis. (A) Early stage of HAT [2], (B) Late stage of HAT [26] and (C) Cattle trypanosomiasis [27].

Figure 3. Trypanosome cell architecture. (A) Simplified schematic representation of the major structural features location of *T. brucei*. (B) Position of the kinetoplast with respect to the nucleus and posterior end in trypomastigote bloodstream and procyclic forms, and in epimastigote form [29].
positioned between the posterior end and the center of the cell (Fig. 3A) [20]. The motility of the parasite is dependent of its single flagellum connected to a paraflagellar rod [19]. The mitochondrion is a single elongated structure that runs from the posterior to the anterior extremity of the cell. Energy production of procyclic trypanosomes is based in the mitochondria but in mammals, the bloodstream form trypomastigotes lack mitochondrial respiration and ATP generation exclusively depends on glycolysis, compartmentalized in glycosomes [21]. This is due to the lack of a mitochondrion viable citric acid cycle, since the bloodstream form of *T. brucei* has no lipid or carbohydrate reserves [22].

2.1.3. Life cycle and stages of infection

![Trypanosoma brucei life cycle](image)

**Figure 4. Trypanosoma brucei** life cycle [30].

As it has been described, trypanosome is transmitted by the tsetse fly to the mammalian host. After a blood meal on an infected host, the parasite establishes in the midgut of the tsetse fly. Then it migrates to the salivary glands and in the next blood meal it is prepared to infect a new mammalian host (Fig. 4, Fig. 5). When in the mammalian organism, the bloodstream form (BSF) trypomastigotes escapes immune recognition by switching the mono-allelic expression of one variant surface glycoprotein (VSG)-encoding gene to another (Fig. 5) [31]. This VSG coat is linked to the surface membrane by the glycosylphosphatidylinositol anchor. Moreover, VSGs can recombine to form novel mosaic VSGs, making the complexity of repertoire limitless [32]. Initially in the mammalian bloodstream, trypanosomes proliferate as slender forms in which the kinetoplast (mitochondrial genome of the parasite) is located at the posterior extremity of the cell and mitochondrial activity is relatively repressed, obtaining energy by glycolysis (Fig. 4, Fig. 5).
the slender form, trypanosomes achieve G1 phase of the cell cycle that corresponds to the major period of cell growth during its lifespan and obtain their mobility. As parasite quantity increases, these are replaced by division-arrested stumpy forms (Fig. 5) [29, 33]. The accumulation of parasite stumpy forms limits the trypanosome multiplication, maintaining the number of circulating parasites which prolongs host survival and subsequently a higher probability of disease transmission. Also the non-proliferative stumpy forms arrested in G1 phase of the cell cycle ensures that the morphological changes that occur upon transmission to the tsetse fly can be coordinated with re-entry in the cell cycle. Upon uptake by the tsetse fly in the mid gut bloodstream form trypanosomes replace the VSG coat with a less-dense coat composed by EP (form of procyclin rich in Glu-Pro repeats) and GPEET (form of procyclin rich in Glu-Pro-Glu-Thr repeats) procyclins being the trypanosomes denominated as procyclic forms (Fig. 4, Fig. 5) [34]. At this form, the kinetoplast is repositioned to sub-terminal location and mitochondrion-based respiratory system is activated, as a result the ATP production isn’t exclusively based on glycolysis. The parasite proliferates in tsetse midgut and when it establishes, trypanosomes arrest in division and migrate to the salivary gland, where they proliferate and attach to the gland wall by the flagellar membrane as epimastigote forms. Eventually, epimastigotes arrest multiplication originating non-proliferative metacyclic forms, in which a VSG coat is re-expressed and is kept into the salivary gland lumen being prepared for a new mammal infection (Fig. 4, Fig. 5).

Figure 5. Trypanosoma brucei polymorphism, a detailed representation in distinct environments [29].

BSF and procyclic forms of T. brucei are used in most studies due to the fact that these stages can be readily cultured in vitro.

In the absence of treatment, two African trypanosomiasis disease stages caused by the slender form of T. brucei can be distinguished (Fig. 6). Stage 1 known as the hemo-lymphatic stage that occurs in an early phase of the disease while the parasite is present in
blood and lymphatic system. The parasite resides extracellularly in the bloodstream and lymph nodes, then in organs, as slender forms [35, 36].

When trypanosomes cross the blood brain barrier and invades the cerebrospinal fluid, stage 2 is achieved. This phase is denominated also as meningo-encephalitic stage, and when pathogens are in central nervous system they are protected from the drugs toxic effect because most of the compounds are incapable of permeabilize the blood brain barrier [35, 36]. Consequently, the parasite establishes its infection and the mammalian host progresses to death.

Figure 6. A representation of the life cycle of *T. brucei* parasites showing the occurrence of the two disease stages in the mammalian organism caused by the slender form (2.1.) [36]. Legend: Replication via fission (*) and variable antigen coat (π).

2.2. Global distribution and impact

Sleeping sickness appears in sub-Saharan Africa areas (Fig. 7, Fig. 8). In Central and West Africa, infection with *T. brucei gambiense* gives rise to a chronic disease that mainly affects humans and accounts for greater than 90% of reported sleeping sickness cases [37]. Disease caused by *T. b. gambiense* is epidemic in Sudan, Uganda, Democratic Republic of Congo and Angola [20]. In East Africa, infection with *T. b. rhodesiense* generates acute disease in humans, and also circulates in livestock (cattle) reservoir inducing the nagana disease [38].

The occurrence of the disease is related to the distribution of the tsetse flies (*Glossina* sp.) that are present in Africa arable land for about 10 million square kilometers (Fig. 7) [39]. Tsetse populations are limited in the Sahara and Somali in the north, extending across the entire continent from Senegal in the west to southern Somalia in the east. Also they are denser in West and Central Africa, and are found more sporadically to the East and down to the borders of the Kalahari and Namibian Deserts in Southern Africa. The 3 groups of tsetse
flies are generally adapted to different habitats and ecozones, being the Morsitans and the Palpalis groups the main vectors of trypanosomes able to infect humans (T. b. gambiense and T. b. rhodesiense) (Fig. 7) [20]. Climate changes over the next century are expected to have a large impact on vector-borne diseases because temperature changes can alter vector development rates, shift their geographical distribution and alter transmission dynamics [40]. For this reason, African trypanosomiasis was identified as one of the 12 infectious diseases likely to spread due to climate change and 46 to 77 million additional people may be at risk of exposure by 2090. It was also predicted that epidemics can occur when mean temperatures are between 20.7°C and 26.1°C [40].

![Image](https://example.com/image.jpg)

**Figure 7.** Relation between HAT cases, distribution of T. brucei subspecies and tsetse flies location [41].

In the last available data of HAT new cases in Africa at 2009, 25 of the 36 countries at risk were evaluated (Fig. 8). Data processing is ongoing for the epidemic countries Angola and Democratic Republic of Congo. The number of new cases reported to WHO in 2009 has dropped to approximately 10,000. Only Central African Republic and Democratic Republic of Congo have reported more than 1,000 new cases, corresponding to 11% and 73% of the total cases reported, but it is important to notice that the data from Democratic Republic of Congo isn’t yet concluded [17]. One country, Chad, has reported more than 500 cases. Three countries considered epidemic (Angola, Sudan, and Uganda) have reported new cases in the range of 100 to 500. Once again, the data obtained from Angola isn’t completed. Finally, 19 countries listed as being HAT endemic reported no cases in 2009 however nine of
them have no regular surveillance activities. These countries deserve an assessment to clarify their epidemiological situation [17].

![Map of Africa with HAT cases reported.](Image)

Figure 8. Number of cases reported in 2009 for HAT [14].

During 2000 to 2010 period, 94 HAT cases were reported in disease non-endemic countries, mostly in Europe and North America. Portugal is included due to the diagnosis of 2 cases of sleeping sickness second stage caused by *T. b. gambiense* recently reported in 2009 and 2010 (Table I) [42]. One of the patients was diagnosed in Braga and the other in Lisbon.

Table I. Cases of Gambiense HAT diagnosed in non-endemic countries (2009 and 2010) (Adapted from [42]).

<table>
<thead>
<tr>
<th>Year</th>
<th>Place of diagnosis</th>
<th>Place of infection</th>
<th>Sex/age</th>
<th>Activity</th>
<th>Diagnosis</th>
<th>Stage</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>Sydney</td>
<td>Ulanga</td>
<td>F 24</td>
<td>Refugee</td>
<td>Brain biopsy</td>
<td>Second</td>
<td>Echinococcal</td>
</tr>
<tr>
<td></td>
<td>Cameroon</td>
<td>Croe</td>
<td>M 32</td>
<td>Export business</td>
<td>Blood smear/CSF</td>
<td>Second</td>
<td>Echinococcal</td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>Gabon</td>
<td>M 40</td>
<td>Export business</td>
<td>Blood smear</td>
<td>First</td>
<td>Pentamidine</td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>Kamina</td>
<td>M 55</td>
<td>Export business</td>
<td>Blood/CSF</td>
<td>Second</td>
<td>Echinococcal</td>
</tr>
<tr>
<td>2010</td>
<td>Lisbon</td>
<td>Angola</td>
<td>F 2</td>
<td>Migrant</td>
<td>Blood/CSF</td>
<td>Second</td>
<td>Echinococcal</td>
</tr>
<tr>
<td></td>
<td>Portugal</td>
<td>Angola</td>
<td>M 37</td>
<td>Migrant</td>
<td>Blood/CCT</td>
<td>Second</td>
<td>Echinococcal</td>
</tr>
<tr>
<td></td>
<td>USA</td>
<td>Cameroon</td>
<td>M 40</td>
<td>Migrant</td>
<td>Blood/CCT</td>
<td>Second</td>
<td>Echinococcal</td>
</tr>
<tr>
<td></td>
<td>USA</td>
<td>Cameroun</td>
<td>M 55</td>
<td>Migrant</td>
<td>Blood/CCT</td>
<td>Second</td>
<td>Echinococcal</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>DRC</td>
<td>M 25</td>
<td>Migrant</td>
<td>CSF</td>
<td>Second</td>
<td>Echinococcal</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Kinshasa</td>
<td>DRC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M = male; F = female; CSF = cerebrospinal fluid; CCT = capillary cerebrospinal fluid; BM = bone marrow.
2.3. HAT disease symptoms and current chemotherapies

HAT clinical symptoms depend on the subspecies infecting the patient (T. b. rhodesiense or T. b. gambiense) and also on the disease stage [2, 19]. T. b. rhodesiense causes a virulent quick fatal disease as it progresses rapidly to central nervous system (CNS), while T. b. gambiense is described with a slower time course before it reaches the CNS [2, 3].

As previously mentioned there are two stages of HAT disease, the early phase in which the parasite is in the bloodstream and lymph, and the late stage when the trypanosome invades the CNS. In the early stage disease the non-specific signs manifested are fever, headache, skin lesion, chancre, pruritus, weight loss and arthralgia (fever sometimes is accompanied by rigor and vomiting) [2, 3]. Cardiac, endocrine and gastrointestinal problems can also happen in this stage of HAT [2]. The characteristic symptoms in late stage disease are tremors, headache persistence, personality changes (such as anxiety, irritability, violence, delirium), motor weakness, walking difficulties, sensory disorders, visual impairments and sleep disorders that deteriorate into coma [2, 3, 19]. Patients with HAT second stage usually die within weeks to months.

Disease diagnosis relies on the microscopic identification of trypanosome subspecies and life cycle forms in the blood, lymph or cerebrospinal fluid. A card agglutination test is used to screen for possible T. b. gambiense infections with conformation relying on microscopic identification [3]. A simple serological diagnostic test for T. b. rhodesiense infection is still lacking, since its proliferation in the mammalian organism occurs in a lower rate than T. b. gambiense [43]. Polymerase chain reaction (PCR) techniques are an available option to increase detection sensitivity. In addition, increased leukocytes cells and IgM levels also suggest the parasite infection.

The current state of drug therapy is resumed in Table II. Briefly, only five options are available for HAT treatment. Suramin and pentamidine are applied against the first stage of the disease because they’re unable to cross the blood brain barrier [4]. Melarsoprol, eflornithine and the combination therapy nifurtimox/eflornithine are used against the second stage of the disease. Drawbacks are associated to these currently used compounds such as their general toxicity to humans organism (some examples are presented in Table II); temporary side effects such as headache, vomiting or tremors common to all drugs; unknown mechanism of action except for eflornithine; some present drug resistance in the field; expensive cost and all require long treatments regimen as described in Table II [3-5]. For the appliance of these drugs trained personnel is required creating the problems of the little existence of infrastructures for treatment and limited access to patients in Africa [14]. There is an urgent need for new drugs that are effective against T. brucei (preferably against both
subspecies and stages of the infection); with lower cost; easy to administer, being the oral via of administration more desirable; and safer in terms of not causing permanent or temporary toxic effects.

In literature, a variety of new natural or synthesized compounds have been reported to be toxic against *T. brucei* parasites. Among them two compounds, a nitroimidazole drug denominated fexinidazole and a benzoxaborole substance designated SCYX-7158 (Fig. 9), stand out due to their great results being able to enter phase I clinical trials in 2009 and 2011, respectively [44-46]. In fact, preclinical pharmacological and safety studies showed that fexinidazole and SCYX-7158 are safe effective oral drugs with a promising future [44-46].

![Figure 9](image-url). Chemical structure of fexinidazole and SCYX-7158 [44, 46].
<table>
<thead>
<tr>
<th>Drug and class</th>
<th>Chemical structure</th>
<th>Year</th>
<th>Use</th>
<th>Mechanisms of action</th>
<th>Resistance in the field</th>
<th>Limitations</th>
<th>Dosage scheme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suramin</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>1916</td>
<td><em>T. b. rhodesiense</em> early stage; but is also effective against <em>T. b. gambiense</em> stage 1</td>
<td>Unknown; Concentrated in the parasite via receptor-mediated endocytosis</td>
<td>Not occurred</td>
<td>Toxicity (renal toxicity and neurological complications); incapable of crossing blood brain barrier</td>
<td>Intravenous (IV) injection; 1g given on days 1, 3, 7, 14 and 21</td>
</tr>
<tr>
<td>Pentamidine</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>1937</td>
<td><em>T. b. gambiense</em> early stage</td>
<td>Unknown; Accumulated in cells by transport through the adenine/adenosine P2 transporter</td>
<td>Occurred but does not develop readily</td>
<td>Toxicity (life threatening hypoglycemia); Incapable of crossing blood brain barrier</td>
<td>Intramuscular (IM) injection; 4 mg/kg/day for 7 days</td>
</tr>
<tr>
<td>Melarsoprol</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>1946</td>
<td><em>T. b. rhodesiense</em> late stage; <em>T. b. gambiense</em> late stage</td>
<td>Unknown; Transported through the adenine/adenosine P2 transporter</td>
<td>Occurred; Field isolates that are tenfold less sensitive to the drug have been identified</td>
<td>Severe toxicity (cause reactive encephalopathy)</td>
<td><em>T. b. rhodesiense</em>: 3 series of 3 daily IV doses with a 7 day rest period in between: 1.8, 2.7 and 3.6 mg/kg on days 1, 2, and 3 respectively with subsequent series at 3.6 mg/kg daily; <em>T. b. gambiense</em>: 2.2 mg/kg/day IV for 10 days</td>
</tr>
<tr>
<td>Eflornithine</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>1977</td>
<td><em>T. b. gambiense</em> late stage</td>
<td>Mechanism-based inhibitor of the ornithine decarboxylase enzyme which catalyzes polyamine biosynthesis</td>
<td>Not occurred</td>
<td>Not effective against <em>T. b. rhodesiense</em>; prolonged iv administrations; side effects: seizures, fever, infections, neutropenia, hypertension and diarrhea</td>
<td>400 mg/kg/day in divided doses IV every 6 h for 14 days</td>
</tr>
<tr>
<td>Nifurtimox/ Eflornithine combination therapy (NECT)</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>1990</td>
<td><em>T. b. gambiense</em> late stage</td>
<td>Nifurtimox is activated by a NADH-dependent mitochondrial nitroreductase leading to the generation of intracellular free radicals</td>
<td>Not occurred</td>
<td>Not effective against <em>T. b. rhodesiense</em>; side effects: nausea, vomiting and tremors</td>
<td>Eflornithine 400 mg/kg/day IV in divided doses every 12 h for 7 days and nifurtimox 15 mg/kg/day orally every 8 h for 10 days</td>
</tr>
</tbody>
</table>
3. Bisnaphthalimidopropyl (BNIP) derivative compounds

In the cancer context, development of new compounds based on naturally occurring DNA grove-binding agents expanded in research [47]. Naphthalimides (originated in the 1970s) and bis-naphthalimido compounds (developed in the early 1990s) are well-known cytotoxic DNA intercalating agents by the naphthalimido planar aromatic ring interfering with DNA-protein interactions and were shown to have potential anticancer activity, especially the latter compounds [48-51]. However, bis-naphthalimides, composed by two naphthalimide rings covalently attached at the end of a linker chain containing two nitrogen atoms, were shown to be highly insoluble in aqueous solutions making their potential development into therapeutic agents difficult [50-52]. The majority of the research traditionally focused on the modification of the naphthalimido rings to enhance anticancer activities and compound aqueous solubility. However, in the year 2000 was reported the synthesis of bisnaphthalimidopropyl (BNIP)-derivative compounds that were based in the theory that by increasing the linker chain between two naphthalimido rings connected to three carbon propyl groups would enhance compound aqueous solubility and exert potential toxicity to human breast cancer cells [48]. Indeed, Lin and Pavlov (2000) [48] have verified that the newly synthesized BNIP compounds - BNIPPutrescine, BNIPSpermidine and BNIPSpermine (based in the structure of the naturally occurring polyamines: spermidine, spermine and their precursor putrescine) increased solubility and induced promising toxic effects in cancer cells (Fig. 10) [8, 48, 53].

Figure 10. Polyamine derivative compounds chemical structures. (A) BNIPPutrescine, (B) BNIPSpermidine, (C) BNIPSpermine [8].
Since the first BNIP-polyamine drugs, the synthesis of the next generation of bis-naphthalimidopropyls were based on the modification of the linker chain maintaining the primary goal to create potential anti-proliferative compounds and increase their solubility (Fig. 11) [6, 8, 9, 48, 49, 53-56]. The compounds were prepared with variations in the number of carbons and insertion of heteroatoms (nitrogen and/or oxygen), cyclohexane ring(s) and/or benzene ring(s).

![Figure 11. Basis structure of BNIP-derivative compounds. Two naphthalimido rings linked to propyl chains and the point of diversity are represented (Adapted from [6]).](image)

The use of terminally alkylated polyamine analogues has been shown to induce inhibitory effects on distinct cancer cell lines and *Leishmania infantum* parasites growth [6, 8, 9, 57-59]. Like cancer cells and *Leishmania* parasites, *T. brucei* also has the feature of rapid cell proliferation. Into cells, BNIP-derivatives are able to mimic the natural polyamines in their self-regulatory function, but incapable of acting as substitutes for polyamines in their cell growth regulatory role. The natural polyamines, spermidine and spermine, and their precursor diamine putrescine, have an essential role in cell proliferation and differentiation, and are present in most eukaryotic cells [60]. In *T. brucei* parasites, only putrescine and spermidine are present and are synthesized by ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase and spermidine synthase (Fig. 12) [61]. The polyamine biosynthetic enzymes have been shown to be essential in *T. brucei* based on genetic knockdown studies and on the evaluation of small-molecule inhibitors specific to a number of pathway enzymes [62].
Parasites and mammalian cells have distinct polyamine metabolisms. Subsequently, interfering with the parasite specific polyamine metabolism pathway will have more severe consequences for the parasite than for its host [8, 62]. Furthermore, targeting parasitic protozoan polyamines and their associated enzymes will lead to an alteration of natural defense mechanisms of the parasite. In fact, the inhibition of ODC is an actual mechanism of action used in the field against T. b. gambiense HAT late stage by the administration of efornithine described in section 2.3 (Table II). Thus, the interference with the usual functions of polyamine has been one strategy in research against this parasite [63].

In addition, some relevant findings about BNIP-compounds include: the inherent fluorescence properties of BNIP compounds has shown their preferential location in the nucleus; in Leishmania promastigotes the cell death by BNIP-derivative compounds occurs by apoptosis; and it was verified that these drugs induced alterations in Leishmania mitochondrial membrane [6-8, 53]. Moreover, one BNIP-derivative was shown to be highly effective in reducing parasite load in Leishmania infected BALB/c mice [9].
4. Drug delivery systems

4.1. HAT and nanoparticles as drug delivery systems

Due to nanoparticles small size and large modifiable surface, they have unique advantages compared with other drug carriers [64]. Nanobiotechnology and nanomedicine development for neglected diseases is a novel strategy in finding new therapies or improve currently used therapies. By designing delivery devices containing the active molecule, drug therapeutic efficacy and pharmacokinetic characteristics are improved, and its doses and toxicity can be diminished [36, 65, 66]. Also targeted delivery systems can reduce drug-resistant infections [67, 68]. The drawbacks of trypanocidal chemotherapies (described in section 2.3) justify the use of nanoparticles (NPs) delivery systems that optimize drug absorption, distribution, metabolism and elimination. Furthermore, nanotechnology appears to be more cost-effective and a short period of time is required for its development than of creating new antiparasitic molecules [36].

Nanoparticle is a general name to describe nanocapsules and nanospheres [69]. A nanocapsule corresponds to a solid wall enveloping a liquid core (e.g., liposomes, lipid nanocapsules, etc.), whereas a nanosphere consists of a full or porous matrix (e.g., micelles, nanoparticles of maltodextrine, etc.). Among the existing NPs, polymeric nanoparticles technology showed advantages over liposomes due to their higher stability during storage or in biological fluids. These polymeric NPs are especially used to encapsulate lipophilic drugs, but it also is able to encapsulate hydrophilic compounds [13]. It depends on the drug loading method that can be performed by adsorption, entrapment or covalent attachment in the matrix, and binding can occur via hydrogen bonds, or electrostatic or hydrophobic bonds [36]. In addition, NPs are characterized by their composition, size, shape, and inner and surface physicochemical properties. The main properties under consideration are the drug loading efficiency, dispersion stability, release kinetics, and in vitro and in vivo pharmaceutical behaviors [36]. Moreover, it has been shown that NPs can cross the blood brain barrier, which is an important characteristic for the compound to target the second stage of HAT that occurs in CNS.

Some polymeric NPs systems already developed with the intention to act against HAT are: melarsoprol nanosuspensions with poloxamer 188 or 407 and mannitol, cationic starch nanoparticles with a lipid core in which diminazene was loaded, and PEGylated (polyethylene glycol-linked) solid NP system named Bdellosomes with daunomycin [70-72]. The first example, melarsoprol nanosuspensions with poloxamer 188 or 407 and mannitol, were used in non-infected mice with a size around 350 nm. The distribution in mice showed clear reticuloendothelial system targeting with five- to nine fold higher liver concentrations
than the free drug injection and due to their excessive size it was impossible for these NPs to cross blood brain barrier and target the brain. Thus, these formulations were not appropriate for testing on infected animals because they can’t act on the second stage of HAT disease as the authors intended [70]. Second, a colloidal formulation based on a porous cationic nanoparticle with an oily core containing diminazene presented a size between 250 and 450 nm and the surface charge was ~ -34 mV. Improved efficacy was observed on *T. b. brucei in vitro* where NPs killed parasites more rapidly, but no *in vivo* testings were conducted [71].

Another example, the solid NP system named Bdellosomes varied in size from 40 to 100 nm. This drug delivery particle was made of polyvinyl alcohol grafted with polylactide side chains terminated by amino groups, loaded with daunomycin and, coated with polyethylene glycol-3400 to evade immune reactions and linked to target-specific ligands, such as transferrin or anti-transferrin receptor-antibody fragments. *In vivo* distribution studies showed prolonged circulation time of the PEGylated particles. When the daunomycin loaded particles were linked to transferrin or transferrin receptor-antibody fragments, a significant cytotoxic effect against the bloodstream forms of *T. brucei*, could be observed in comparison with the particles that weren’t linked to the cell specific ligand [72].

Polymeric NPs coated with PEG (polyethylene glycol) have innumerable advantages further described in point 4.2.

4.2. **Characteristics and applications of polyethylene glycol – coated poly(lactic acid) nanoparticles**

Poly(lactic acid) (PLA) is a synthetic biodegradable polymer with good security, low immunity, and good mechanical strength [64]. Due to the previous characteristics, PLA has been approved by the US Food and Drug Administration for application in tissue engineering, medical materials and drug carriers [10, 73]. However, PLA applications are limited by its excessively long degradation time, weak hydrophilicity, and low drug loading of polar drugs [74]. To overcome these drawbacks, polyethylene glycol (PEG) can be conjugated to PLA (Fig. 13).
Many advantages are associated to PEG, such as biocompatibility, non-combination with proteins, increment in hydrophilicity, flexibility, and in particular anti-phagocytosis against macrophages and resistance to immunological recognition [75, 76]. Through copolymerization with PEG, PLA can be improved in hydrophilicity, degradation rate, and crystallization, showing great potential for development in drug delivery [64, 74]. Thus, the copolymerization of PLA and PEG can increase the drug loading, reduce the burst effect, and prolong the in vivo residence time of drugs and avoid them being engulfed by macrophages. These interesting properties provide new therapeutic opportunities and open viable possibilities to improve BNIP-derivative compounds therapies, especially since it is well-known that these compounds present low aqueous solubility [10] and the prolonging time in circulation is an enormous advantage since trypanosomes proliferate in blood circulation.

In addition it is noteworthy to mention that the, encapsulation of BNIP-derivatives in PEG-PLA nanoparticles was already successfully achieved in a previous work performed in our laboratory [10].
Aims

New alternative treatments are urgent to HAT therapy. In this context, the primary objective of this project was to evaluate a 15 BNIP-derivative compounds library activity against *T. brucei* parasite and to select the most active compound to study its mechanism of action and improve its chemotherapeutic features by incorporation on polymeric nanoparticles. The specific aims of this thesis were:

1. To conduct an *in vitro* optimization of two methods to evaluate trypanosome growth inhibition in 96-well plates, a resazurin-based assay and the tetrazole 3-(4,5-Dimethylthiazol-2-y1)-2,5- diphenyltetrazolium bromide (MTT) assay.

2. To perform a cell-based drug screening of the 15 BNIP-derivatives library, using the parasite growth inhibition assay selected previously against the bloodstream form *T. b. brucei* strain 427 (a clinically relevant subspecies) and, simultaneously assess their toxicity on mammalian cells (mouse fibroblast L929 cells) by the MTT assay.

3. To characterize and improve the properties of the most potent compound against *T. brucei* (BNIPDabut) through:
   3.1. Reversibility and time-kill tests;
   3.2. *In vivo* efficacy in BALB/c mice;
   3.3. Identification of its protein(s) target(s);
   3.4. Development and characterization of PEG-PLA nanoparticles loaded with BNIPDabut, and their *in vitro* evaluation.
1. Chemical reagents

Poly (vinyl alcohol) (PVA) [87-89 % hydrolysed, molecular weight (MW) 13 000 – 23 000 Da], Polyethylene glycol – coated poly(lactic acid) (PEG-PLA, Sigma-Aldrich), Acetone (CH₃COCH₃, MW 58.07 g/mol, Merck), Isopropanol (2-Propanol, MW 60.1 g/mol, Merck), Resazurin sodium salt (MW 251.17 g/mol, Sigma-Aldrich), Thiazolyl Blue Tetrazolium [tetrazole 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, MTT, MW 414.32 g/mol, Sigma-Aldrich], Dimethylsulfoxide (DMSO; MW 78.13 g/mol, Sigma-Aldrich), N,N-Dimethylformamide (DMF, MW 73.09 g/mol, Sigma-Aldrich).

2. Parasite and cell cultures

2.1. Trypanosome culture

The bloodstream-form (BSF) *T. brucei brucei* strain 427 was grown in HMI-9 medium – 17.66 mg/mL IMDM (Gibco), 3.020 mg/mL sodium bicarbonate (Sigma-Aldrich), 0.136 mg/mL hypoxanthine (Sigma-Aldrich), 0.039 mg/mL thymidine (Sigma-Aldrich) and 0.028 mg/mL bathocuproine sulfonic acid (Sigma-Aldrich) - supplemented with 10% heat-inactivated fetal bovine serum (FBS) (BioWhittaker), 1.5 mM L-cysteine hydrochloride monohydrate (Merck), 0.2 mM β-mercaptoethanol (Sigma-Aldrich), 100 U/ml penicillin and 100 U/ml streptomycin (BioWhittaker) at 37ºC under a humidified 5% CO₂ atmosphere [77]. Cultures were grown in T25 or T75 vented cap culture flasks (Sarstedt) and sub-cultured every 2-3 days by 100 to 1000-fold dilution, respectively. Parasites were counted directly using a Neubauer chamber (Marienfeld) and diluted appropriately in complete HMI-9 medium.

2.2. Mammalian cell culture

Murine fibroblasts L929 cell line were cultured in RPMI 1640 medium (BioWhittaker), supplemented with 10% heat-inactivated FBS (BioWhittaker), 2 mM L-glutamine (BioWhittaker), 100 U/ml penicillin and 100 U/ml streptomycin (BioWhittaker). Cells were maintained in T75 vented cap culture flasks (Sarstedt), at 37ºC in an atmosphere containing 5% CO₂, by sub-passage at each 2-3 days. These cells were counted in a Neubauer chamber (Marienfeld), in the presence of trypan blue to exclude non-viable cells.
3. Testing compounds

Bisnaphthalimidopropyl derivative compounds were kindly provided by Professor Paul Kong Thoo Lin from the Robert Gordon University, Scotland. Pentamidine isethionate salt, suramin sodium salt, spermidine and spermine were purchased from Sigma-Aldrich (Table III).

All compounds were prepared at 10 or 20 mM in 50% or 100% dimethylsulfoxide (DMSO), except for suramin, spermidine and spermine that were dissolved in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2.0 mM KH₂PO₄) at pH 7.4 and were sterilized by filtration in a sterile syringe filter 0.2 µm (Frilabo). For in vitro studies the compounds were diluted in culture medium prior to assay and for in vivo experiments they were dissolved with PBS pH 7.4 and filtered in a sterile syringe filter 0.2 µm (Frilabo).

Table III – Structure and molecular weight (MW) of the compounds studied

<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Chemical structure</th>
<th>MW (g.mol⁻¹)</th>
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<tbody>
<tr>
<td>Currently used drugs</td>
<td>Pentamidine</td>
<td><img src="image" alt="Chemical structure" /></td>
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<td>Suramin</td>
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<td>Natural poliamines</td>
<td>Spermidine</td>
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<td>Lead compounds</td>
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<td>Structure</td>
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<tr>
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<tr>
<td>NPA</td>
<td><img src="image" alt="NPA" /></td>
<td>290.78</td>
<td></td>
</tr>
</tbody>
</table>

4. **Evaluation of trypanosomes growth**

The trypanosome fold-increase *in vitro* is an important characteristic to determine the starting density and the period of time that a culture can be maintained in continuous growth.
For that purpose, parasites counted in a Neubauer chamber (Marienfeld) after 1, 2 and 3 days of incubation at 37°C, 5% CO₂ were considered in this evaluation.

5. Optimization of a 96-well plate assay for *T. brucei* drug screening

The *T. brucei* BSF was used to optimize an *in vitro* 96-well plate assay for drug screening. The two viability assays chosen were the resazurin-based cell viability and the MTT assay.

5.1. Linearity of the resazurin-based cell viability assay

The conversion of resazurin by viable parasites results in resofurin with a fluorescent signal that was determined after 0, 2, 3, 4 and 5h of incubation at 37°C as well as at five different cell densities ranging from 1.25 to 20x10⁵ trypanosomes/well. These were in a total volume of 200 µL culture medium and resazurin (Sigma-Aldrich) was added immediately at a 45.5 µM concentration per well [11]. Fluorescence intensity was quantified in a black 96 well-plate with clear bottom (Sarsted) at the periods previous described by measuring the fluorescence intensity at 590 nm, after excitation at 528 nm, in a microplate reader (Sinergy 2, BioTek).

5.2. Linearity of the MTT viability assay

The substrate MTT is reduced to purple formazan in the mitochondria of living cells. The absorbance was measured for 1.25 – 20x10⁵ trypanosomes per well after 0, 2, 3, 4 and 5 hours of incubation, with 0.05 or 0.5 mg/mL of MTT concentration. At those times the 96 well U-bottom plates were centrifuged at 2000 x g for 5 minutes, the supernatant was removed and 200 µL of isopropanol were added in each well to dissolve the formazan crystals. This solution was quantified by measuring absorbance at 660 and 570 nm in a microplate reader (Synergy 2, Biotek) using flat–bottom 96 well plates.

6. Evaluation of dimethylsulfoxide (DMSO) tolerance

DMSO was diluted in culture medium prior the assay to 0.1, 0.25, 0.5, 0.75, 1 and 5%. These percentages were tested in a final volume of 200 µL into a sterile flat-bottom 96 well plate in the presence of 10⁵ and 5x10⁵ trypanosomes per well and, after 72h of incubation at 37°C and 5% CO₂, the optimized resazurin-based viability assay was performed.
To measure the parasite viability in the presence of DMSO with the MTT assay, the initial densities tested were $10^2$ and $2 \times 10^2$ per well. The optimized MTT assay was applied after 72h of incubation in a humidified atmosphere at 37°C.

In both assays the parasites were counted at the ending point (3rd day of experiment) to determine their quantity to initiate the assay.

7. Screening of a small-library of BNIP derivative compounds

For all assays in sterile 96-well plates (Sarsted), two controls were maintained: column 1 corresponded to the negative control condition in which parasites or cells were only in their respective medium and column 2 presented the positive control condition where 100 µL of DMSO in the same quantity as the major compound concentration tested was added.

7.1. Toxicity against Trypanosoma brucei

The resazurin-based assay optimized previously was applied to determine the half maximal inhibitory concentration ($IC_{50}$) of a series of BNIP derivative compounds towards *T. brucei*. As reference drugs pentamidine and suramin were used in a concentration range of 1.3 to 100 nM.

In a sterile flat bottom 96-well plate, 100 µL of $2 \times 10^3$ parasites were added in each well. The compounds were tested in a serial dilution assay with complete HMI-9 in a final volume of 100 µL. The plate was incubated at 37°C and 5% CO$_2$ for 72h and the trypanosome viability was measured by the optimised resazurin-based assay (Fig. 14).

The $IC_{50}$ value was determined by linear regression analysis.

![Figure 14](image.png)

**Figure 14.** Example of a resazurin-based assay after 4h incubation at 37°C, in a 96 well-plate assay set.
7.2. Toxicity against mouse fibroblast cells

Mouse fibroblast L929 cells were used as a counter-screen for non-selective inhibitors determined by the MTT assay. This viability method for mammalian cells was already implemented in the research group [78].

In a sterile flat bottom 96-well plate, 10^5 cells/well were cultured and incubated at 37°C, 5%CO₂ during 12-16 hours to allow cell adherence. After this time, the supernatant was aspirated and a serial of diluted compounds were added in each well. The plate was kept in a humidified incubator (37°C and 5% CO₂) for 3 days. Afterward, the supernatant was removed and the well was supplemented with 200 µL containing 0.5 mg/mL MTT reagent. After 4 hours of incubation at 37°C the medium was removed and 200 µL of isopropanol was added to dissolve the formazan crystals formed by viable cells (Fig. 15). Optical density (OD) was read at 660 and 570 nm in a microplate reader (Synergy 2, Biotek) and the percentage of cell viability was obtained by the equation:

\[
\% \text{ of cell viability} = \frac{(OD_{570} - OD_{660}) \text{ treated cells}}{(OD_{570} - OD_{660}) \text{ untreated cells}} \times 100
\]

The IC₅₀ value was determined by linear regression analysis.

8. Time-kill assay

The assessment of in vitro compound-mediated killing of trypanosomes over time was based on the resazurin assay. Trypanosomes were seeded in 96-well U-bottom plates at the density 2x10^5 parasites/well in 100 µL of medium and incubated with 100 µL of serially diluted compound. To wash the compound at the selected time (1, 8, 16, 24 or 48 hours) the plate was spun at 2450 xg for 10 minutes (Centrifuge 5810 R – Eppendorf) to pellet the
parasites, then the supernatant was removed and 200 µL of warmed drug-free HMI-9 medium plus 20 µL of resazurin (500 µM) were added. The fluorescence measurement (590 and 528 nm) was done at the optimized time (4 hours of incubation with resazurin). The IC$_{50}$ value was determined by linear regression analysis.

9. **Examination of reversibility of trypanocidal effects**

To establish the time and concentration required for the most potent compound to cause persistent or irreversible effects, *T. b. brucei* parasites were assessed for their ability to recover from the conditions of exposure to the compound tested.

In 96 well plates with U-bottom, parasites were cultured $5 \times 10^4$/well in a total volume of 200 µL including the diluted compound. After 1, 8, 16, 24, 48 or 72 hours of incubation time the plate was spun at 2450 xg for 10 minutes and the supernatant was aspirated to remove the compound. To remove residual compound the wash was applied two more times with sterile PBS pH 7.4 and a final one with HMI-9 medium. After the last centrifugation, the supernatant was removed and the parasites were resuspended in warmed culture medium. Into a flat bottom 96-well plate, 40 µL of this suspension was added to 160 µL of HMI-9 medium in quadruplicate and incubated for 3 days. The parasite viability was assessed by the resazurin-based assay. The IC$_{50}$ value was determined by linear regression analysis.

10. **Evaluation of the in vivo efficacy of the most potent compound in vitro**

The most promising BNIP compound was studied *in vivo* to determine its efficacy and safety by using 6-8 weeks old male BALB/c mice (from Instituto Biologia Molecular e Celular animal facilities) weighting between 20 and 25g at the beginning of the experiment. Mice were kept with food and water *ad libitum* in sterile cabinets. Animal care and procedures were in accordance with institutional guidelines. All conducted experiments were done in accordance with the IBMC:INEB Animal Ethics Committee and the Portuguese Veterinary Director General guidelines. Each mouse was inoculated intraperitoneally (i.p.) with a PBS solution (100 µL) containing $10^4$ *T. b. brucei* BSF (Lister 427). The infection was allowed to progress 24 hours and the treatment was given by intravenous (i.v) injection, except for pentamidine (i.p. injection) (*Table IV*). For controls were used *T. brucei*-infected mice without any treatment administration (developing the disease), and infected mice inoculated intraperitoneally with pentamidine at 5 mg/kg of weight administered for 4-consecutive days (did not develop the disease for 30 days) (*Table IV*).

The animals were weighted and observed for the presence of clinical and adverse changes during the course of the experiment. Parasitemia was monitored two to three times a week with tail vein blood by microscopic counting in a Neubauer chamber (Marienfeld).
Aparasitemic mice for 30 days after the treatment period were considered cured. Mice that exceeded $10^8$ parasites/mL were humanely euthanized.

Table IV. Doses, schemes of administration and number of mice used for in vivo experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total dose (mg/kg)</th>
<th>Scheme of administration$^a$</th>
<th>Dosing route</th>
<th>Number of mice</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pentamidine</td>
<td>20</td>
<td>4x5</td>
<td>i.p.</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1x1</td>
<td>i.v.</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1x10</td>
<td>i.v.</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>BNIPDabut</td>
<td>40</td>
<td>4x10</td>
<td>i.v.</td>
<td>6</td>
<td>Doses given daily</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4x10</td>
<td>i.v.</td>
<td>3</td>
<td>Doses given in alternate days</td>
</tr>
</tbody>
</table>

$^a$ Number of doses x Dose concentration (mg/Kg); i.p. intraperitoneal; i.v. intravenous

11. Identification of protein targets of the most potent compound in vitro

11.1. Preparation of protein lysates from T. brucei

Cultures of T. brucei BSF were centrifuged (2,450 xg, 10 minutes) (Centrifuge 5810 R – Eppendorf), the supernatant was removed and centrifuged once more in sterile PBS pH 7.4. Then, the pellet was resuspended in an appropriate volume of PBS considering that $10^9$ parasites are kept in 1 mL PBS. The protein extraction was prepared in the presence of PBS pH=7.4 or RIPA pH=8 (50 mM Tris, 150 mM NaCl, 0.1% SDS, 2.0 mM EDTA - with protease inhibitor 1:7) lysis buffer by ten freeze-thaw cycles. At the end of the cycles, the parasites were centrifuged 21,100 xg for 10 minutes at 4ºC and the supernatant was recovered (contains the proteins). Protein quantification was done by the Bradford method (Bio-Rad Dc protein assay kit). Cell lysates were stored at -20ºC.

11.2. Compound immobilization, extract binding to the immobilized compound and recovery

For compound-immobilized or control blocked sepharose beads 0.5 g of resin – epoxy activated Sepharose 6β (Sigma-Aldrich) – was washed with distilled water (approximately 2 mL) in a 15 mL tube. After the water was removed, the moist beads were suspended in DMF/water 1:1 and washed 3 times. Then a solution of compound (4 mg) or DMF/water (control beads) was added to the suspension and the pH was adjusted to 11 (with aqueous NaOH – 0.1M). The reaction was shaken for 16h at 40ºC before a solution of ethanolamine (0.14 mL, 2.4 mmol) was added and allowed to shake for additional 24 h at the
same conditions. The product was washed at least 3 times with alternating pH – acetate buffer pH 4 and Tris-HCl pH 8. The remaining solid was suspended in PBS buffer pH 7.4 (2x volume of solid). Sepharose beads can be stored at 4ºC as 50% slurry in PBS buffer pH 7.4 containing 0.2% sodium azide.

To perform the extract binding to the compound immobilized resin 100 µL of BNIP-immobilized epoxy activated sepharose 6β was incubated with 1 mg of total protein from the parasite extract, overnight at 4ºC with orbital shaking. To elute the unbound proteins the beads were washed twice with the same buffer used to prepare the extract, then washed two times with two buffers with increased ionic strength (0.1 M NaCl and 1.0 M NaCl) and finally a competitive elution was performed with a buffer containing 1.0 M NaCl and 10% the amount of BNIP immobilized on the agarose. All these washing steps were performed by centrifugations at 10,000 xg for 10 minutes (4ºC) and the supernatant was recovered (which contains the eluted proteins).

11.3. **SDS polyacrylamide gel electrophoresis (SDS-PAGE)**

The separation of proteins according to their size from the total lysates and the various eluted samples occurred in 10% polyacrylamide gel. The gel electrophoresis was carried out in Mini-Protean Tetra Cell apparatus (Bio-Rad) at 200 volts [79].

11.4. **Silver staining and sample preparation for mass spectrometry**

Protein detection was done by silver staining following a procedure compatible with mass-spectrometry described by Gromova and Celis [80]. The specific protein bands binding to the BNIP compound were excised, and then were sent to Peptide Mass Fingerprint Analysis service at the IPATIMUP institute.

11.5. **Protein identification by database search**

In IPATIMUP proteomics service, the gel bands samples were digested with trypsin and then a search of proteins was obtained by peptide mass spectrum analysis (Peptide Mass Fingerprint Analysis). Protein(s) identification was searched in TriTryp database of *T. b. brucei* (http://tritrypdb.org) by protein sequences (in our laboratory).
12. Polymeric nanoparticles

12.1. Preparation of unloaded and loaded polyethylene glycol (PEG) – coated poly(lactic acid) (PLA) nanoparticles

Unloaded and loaded with BNIPDabut PEG-PLA nanoparticles (Sigma-Aldrich) were prepared by the nanoprecipitation method [81].

A mass of ± 20 mg PEG-PLA was weighed (Analytical Balance Kern ABS) in a glass tube and dissolved in a defined volume of acetone, in order to give a proportion of ≈ 7 mg of polymer/mL of acetone. In the case of loaded nanoparticles, a volume of stock solution at 10 mg/mL of BNIP compound was added to the glass tube in order to achieve the pretended drug loading (w/w):

\[
\text{% Drug loading (w/w) } = \frac{\text{Mass of BNIP compound used in nanoparticle preparation}}{\text{Mass of polymer used in nanoparticle preparation}} \times 100
\]

The content of the glass tube was injected using a 19G syringe in 20 mL of an aqueous solution of PVA 1% (w/v). The suspension was left under magnetic stirring for 10 minutes and then in the dark over a lab rotator for a period of 5 hours or overnight to promote the organic solvent evaporation.

Nanoparticles were then recovered and washed by centrifugation. To remove larger particle aggregates, an initial centrifugation was performed at 5,000 xg for 5 minutes at 20ºC (Sigma 3K18 Laboratory Centrifuges). The resulting supernatant was recovered and centrifuged at 20,000 xg for 20 minutes at 20ºC and the supernatant (S1) was stored at 4º. The nanoparticle sediment was washed two times in distilled water by centrifugations at 20,000 xg for 20 min at 20ºC, both supernatants (L1 and L2) were recovered and the pellet was resuspended in 1 mL of PBS buffer at pH 7.4 previously to storage at 4ºC. The supernatants obtained from the three washings S1, L1 and L2 were also stored at 4ºC for drug encapsulation quantification.

12.2. Nanoparticle characterization

12.2.1. Size, polydispersity index and zeta potential

These determinations were performed by dynamic light scattering methods using a Zetasizer Nano ZS (Malvern Instruments), with a detection angle of 173º. Measurements were made in triplicate at 25ºC. Samples for dynamic light scattering analysis were prepared as a 10-fold dilution of the nanoparticle suspension in distilled water.
The morphology of PEG-PLA nanoparticles was also observed in a transmission electron microscope (TEM). A volume of 10 µL of diluted aqueous nanoparticle dispersions was placed on carbon-coated copper grids. After 1 minute, they were stained with a solution of uranyl acetate (3% in water) for 30 seconds. Samples were dried and viewed in a TEM (Jeol JEM-1400, Tokyo, Japan) at the accelerating voltage of 60kV.

12.2.2. Encapsulation efficiency

The encapsulation efficiency of BNIPDabut in PEG-PLA nanoparticles was determined indirectly by quantification of the non-encapsulated compound. BNIPDabut compound have intrinsic fluorescent properties exhibiting two peaks in the emission spectra, at 437 and 576 nm, after excitation at 350 nm. Non-encapsulated drug, present in the supernatants obtained after recovery of the nanoparticles (S1, L1 and L2), was quantified by measuring the fluorescence intensity at 460 nm, after excitation at 360 nm, in a spectrofluorimeter (Perkin Elmer L550B). Linear calibration curves were elaborated to determine the compound quantity in S1, L1 and L2, in distilled water. Encapsulation efficiency (E.E.) was determined as:

\[
E.E.(\%) = \frac{(C_{\text{BNIP initial solution}} \times V_{\text{initial solution}}) - (C_{\text{BNIP(S1+L1+L2)}} \times V_{(S1+L1+L2)})}{(C_{\text{BNIP initial solution}} \times V_{\text{initial solution}})} \times 100
\]


12.2.3. Toxicity against *Trypanosoma brucei*

Previous to the biological evaluation the nanoparticles were sterilized by ultraviolet exposure for 20 minutes. The polymeric nanoparticles PEG-PLA with and without compound were tested following the *in vitro* procedure described in sections 5.1 and 5.2. in a concentration range of 1000, 500, 250, 125, 62.5, 31.3, 15.6 nM considering the quantity of BNIPDabut incorporated in the NPs.
Results

1. Trypanosome culture growth

The trypanosome-fold growth was determined after 24, 48 and 72h of incubation at 37°C, 5% CO₂ to define the growth conditions necessary to have the BSF trypanosome culture in the log-phase, reached in vitro at 2 to 3x10⁶ parasites/mL [2]. Two independent cultures (Fig. 16) showed similar proliferations during time and both indicate that in a period of 24 hours the culture presented 10 to 15-fold increase.

![Figure 16](image)

Figure 16. Two independent cultures showing the number of trypanosomes grown overtime – 24, 48 and 72 hours. White bars – Culture A; Grey bars – Culture B. The number of parasites was estimated from 4 to 10 independent counts.

2. Linearity of the cell viability assays (resazurin and MTT)

Resazurin and MTT are two reagents used commonly in vitro to determine cell viability and we intended to implement one of these for routine drug screening with T.brucei in the laboratory [11, 82, 83].

To perform the resazurin-based assay, resazurin is added to the wells and is metabolically reduced by viable cells to resofurin, a product that emits fluorescence which can be measured in a microplate reader apparatus. The reduction of resazurin in bloodstream forms occurs by the alternative oxidase pathway [84]. T. brucei BSF were incubated at 37°C at different densities in the presence of 45.5 µM of resazurin and the fluorescence was determined at defined intervals of time. For each density, fluorescence increased linearly with time presenting linear correlation coefficients (r²) higher than 0.99 (Fig. 17A). Fluorescence was also proportional to cell density at all periods of time tested with an r² > 0.98, except for time 0 (r² = 0.002) (Fig. 17B). Therefore, the fluorescence signal was linear for all trypanosome densities tested (1.25 – 20x10⁵ trypanosomes/ well) between 2 to 4 hours of incubation.
Figure 17. Linearity of the resazurin-based assay. (A) Fluorescence intensities in arbitrary units (a.u.) as a function of incubation time (0, 2, 3, 4 and 5h) for parasite densities ranging from 1.25 to 20x10^5 trypanosomes/ well. (B) Fluorescence signal plotted as a function of parasite densities (1.25 – 20x10^5 trypanosomes/ well) at various time points (2, 3, 4 and 5h). Values are the mean and standard deviation of three independent experiments (n=3) conducted in quadruplicate.

In the mitochondria of living parasites the substrate MTT is reduced to purple formazan which absorbance can be measured, after being dissolved [83]. Concerning the two MTT concentrations used in the wells, it was verified that the lowest concentration used (0.05 mg/mL) produced inconstant data which hamper its application to yield reproducible results. Thus, the MTT assay optimization was proceeded with 0.5 mg/mL per well. The absorbance was measured for 1.25 – 20x10^5 trypanosomes per well after 0, 2, 3, 4 and 5 hours of incubation at 37ºC with the substrate. Absorbance increased linearly with time between 2.5 and 10x10^5 trypanosomes/well (r^2 > 0.94) (Fig. 18A). For each time point absorbance was proportional to all cell densities at equal or above 2 hours points with higher correlation coefficient at 4 and 5 hours of incubation (r^2 > 0.99) (Fig. 18B). Thus, absorbance is proportional to cell density in a range of 2.5 – 10x10^5 parasites/well within 3 to 5 hours incubation.

Figure 18. Linearity of MTT assay. (A) Absorbance signal as a function of incubation time (0, 2, 3, 4 and 5h) at 2.5 – 10x10^5 trypanosomes/well densities. (B) Absorbance values plotted as a function of parasite densities (1.25 – 20x10^5 trypanosomes/well) at various time points (2, 3, 4 and 5h). Values are the mean and standard deviation of three independent experiments (n=3) conducted in quadruplicate.
Comparing the two viability assays evaluated it is possible to see that the MTT assay is more restrictive as it is less reproducible (linear only at 2.5 to 10x10^5 parasites/well) and is more time consuming (involves more steps, centrifugations and crystals solubilization).

3. DMSO tolerance of *T. b. brucei* culture

Most of the test compounds were prepared at 10 or 20 mM in 50 - 100% DMSO, which is the best solvent for BNIP derivative compounds, but this solvent may be toxic to the parasites. Therefore, trypanosome tolerance was defined at 0.1, 0.25, 0.5, 0.75, 1 and 5% DMSO concentrations.

The results obtained with the optimized resazurin-based assay and MTT assay revealed similar values for the 50% inhibitory concentration (IC_{50}) on the parasites of approximately 1% and is also demonstrated that 0.1% is well tolerated by trypanosomes in which viable parasites are around 80 to 90% (Fig. 19).

**Figure 19.** DMSO tolerance determined by resazurin-based (A) and MTT (B) assays. Each experiment was conducted in quadruplicate. Values are the mean and standard deviation of three (n=3) and two (n=2) independent experiments, respectively.

Taking into consideration the results obtained for the *T. brucei* growth on both viability assays, it was possible to determine the most suitable inoculum density for a 96-well plate in order to obtain optimal parasite growth over a 72-hour period. For the resazurin-based assay the parasite density at 72h should not exceed ~2x10^6 cells/mL, which can be achieved by initiating the assay with 10^3 parasites per well, instead of 5x10^3 parasites (data not shown). While for the MTT assay the initial inoculum must be 10^2 trypanosomes rather than 2x10^5 parasites per well, to don't exceed the linearity limit of 1x10^6 cells/well (data not shown). Therefore to perform the MTT assay a smaller inoculum is required.

As a consequence of the results obtained in section 2 and 3, the resazurin-based procedure was chosen to perform the drug-screening assays.
4. Toxicity of standard drugs

Standard drugs used in sleeping sickness therapy (pentamidine and suramin) were tested as control to validate the previous selected and optimized resazurin-based cell viability assay. Suramin (IC$_{50}$ 76.71 ± 2.22 nM) was 22.9-fold least effective than pentamidine (IC$_{50}$ 3.35 ± 0.15 nM) in reducing T. brucei growth. Pentamidine is toxic only in a concentration approximately to 70 µM on mouse L929 fibroblasts, resulting in a high selectivity index. In the case of suramin, no toxic effect was observed by the highest concentration tested (1mM) (Table V).

Table V. Effect of currently used drugs in T. brucei and fibroblasts$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>T. brucei IC$_{50}$ (nM)</th>
<th>L929 fibroblasts IC$_{50}$ (nM)</th>
<th>Selectivity index$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentamidine isethionate</td>
<td>3.35 ± 0.15</td>
<td>70 810 ± 2380</td>
<td>21 137.31</td>
</tr>
<tr>
<td>Suramin sodium salt</td>
<td>76.71 ± 2.22</td>
<td>&gt; 1 000 000</td>
<td>&gt; 13 036.11</td>
</tr>
</tbody>
</table>

$^a$Values are presented as mean±standard deviation of at least 3 independent experiments conducted in quadruplicate; $^b$Selectivity index = IC$_{50}$ (mammalian cells)/IC$_{50}$ (T. brucei).

5. Screening of BNIP-derivative compounds library

A library of 15 BNIP-derivative compounds, including the lead compounds (BNIPSpermidine, BNIPSpd and BNIPSpermine, BNIPSpm) was screened against T. brucei BSF. Both induced submicromolar toxicity and exhibited a higher potency than their precursors (spermidine and spermine) in bloodstream form parasites (Table VI). BNIPSpd is more toxic than BNIPSpm. A counter-screen using mouse fibroblasts of the BNIPSpd and BNIPSpm revealed a more effective toxic activity on the parasite than in the fibroblast, as can be inferred from the selectivity index values (Table VI).

Table VI. Comparison between BNIPSpermidine and BNIPSpermine toxicity on T. brucei parasites and L929 fibroblasts with their respective precursors$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>T. brucei IC$_{50}$ (nM)</th>
<th>L929 fibroblasts IC$_{50}$ (nM)</th>
<th>Selectivity index$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNIPSpd</td>
<td>84.91 ± 7.91</td>
<td>137 065 ± 5 365</td>
<td>1614.24</td>
</tr>
<tr>
<td>BNIPSpm</td>
<td>824.06 ± 56.60</td>
<td>258 414 ± 23 343</td>
<td>313.58</td>
</tr>
<tr>
<td>Spermidine</td>
<td>&gt; 4000</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Spermine</td>
<td>&gt; 20 000</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^a$Values are presented as mean±standard deviation of at least 3 independent experiments conducted in quadruplicate; $^b$Selectivity index = IC$_{50}$ (mammalian cells)/IC$_{50}$ (T. brucei); n.d. not determined
Only the hits with IC\textsubscript{50} lower than 1 \textmu M in \textit{T. brucei} BSF were tested in mouse fibroblasts cells (L929) to determine their cytotoxicity in mammalian cells and define safe concentrations to apply in animal organisms (\textit{Table VII}). BNIPDabut was the most potent compound against \textit{T. brucei}, followed by its modified analogue BNIP(1,4)Dacyhex and both presented the highest fold-specificity values for \textit{T. brucei} over fibroblasts. The introduction of oxygen atoms in the linker chain of BNIPDaoct (IC\textsubscript{50}=78.88 nM) increased its anti-parasitic activity – BNIPDaoxoct, IC\textsubscript{50}=18.05 nM. In opposite, the introduction of one more nitrogen atom appears to decrease the toxicity of the compound, showed by BNIPDpta in comparison to BNIPDahep (IC\textsubscript{50} increased 3.12-fold). The results also show that occurs a decrease of anti-\textit{T. brucei} activity as longer is the linker chain of BNIPDi-aminoalkyl compounds. Only BNIPDadec has a selectivity index lower than 100-fold when compared to the other BNIP compounds with submicromolar toxicity in BSF parasites.

\textbf{Table VII.} BNIP derivative compounds with submicromolar toxicity against \textit{T. brucei} BSF and mouse fibroblasts ranked by their potency on the parasite\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (nM)</th>
<th>Selectivity index\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{T. brucei}</td>
<td>L929 fibroblasts</td>
</tr>
<tr>
<td>BNIPDabut</td>
<td>2.37 ± 0.15</td>
<td>19 860 ± 1 260</td>
</tr>
<tr>
<td>BNIP(1,4)Dacyhex</td>
<td>4.41 ± 0.02</td>
<td>75 994 ± 6 463</td>
</tr>
<tr>
<td>BNIPDapen</td>
<td>7.19 ± 0.53</td>
<td>17 930 ± 2 40</td>
</tr>
<tr>
<td>BNIPDaoxoct</td>
<td>18.05 ± 0.81</td>
<td>100 039 ± 8 740</td>
</tr>
<tr>
<td>BNIPDaheax</td>
<td>23.72 ± 1.96</td>
<td>74 582 ± 3 498</td>
</tr>
<tr>
<td>BNIPDahep</td>
<td>37.52 ± 2.97</td>
<td>18 550 ± 4 60</td>
</tr>
<tr>
<td>BNIPDaoct</td>
<td>78.88 ± 4.01</td>
<td>22 139 ± 1 409</td>
</tr>
<tr>
<td>BNIPDanon</td>
<td>113.26 ± 1.66</td>
<td>396 983 ± 7 727</td>
</tr>
<tr>
<td>BNIPDpta</td>
<td>117.31 ± 5.19</td>
<td>89 229 ± 8 411</td>
</tr>
<tr>
<td>BNIPDadec</td>
<td>178.78 ± 7.45</td>
<td>6 866 ± 320</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are presented as mean±standard deviation of at least 3 independent experiments conducted in quadruplicate; \textsuperscript{b}Selectivity index = IC\textsubscript{50} (mammalian cells)/IC\textsubscript{50} (\textit{T. brucei}).

The remaining compounds exhibit an IC\textsubscript{50} against \textit{T. brucei} higher than 1 \textmu M or had no effect in the growth of the parasites in study – BNIP(4,4)Dapm (\textit{Table VIII}). These compounds have more rigid structures in the linker chain and NPA only presents one naphthalimido ring.

\textbf{Table VIII.} BNIP and mononaphthalimide compounds with IC\textsubscript{50} in \textit{T. brucei} higher than 1 \textmu M.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} \textit{T. brucei} (\textmu M)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNIPDaCHM</td>
<td>4.49 ± 0.28</td>
</tr>
<tr>
<td>BNIP(4,4)Dapm</td>
<td>N/E</td>
</tr>
<tr>
<td>NPA</td>
<td>4.53 ± 0.26</td>
</tr>
</tbody>
</table>

N/E – no effect; \textsuperscript{a}Values are presented as mean±standard deviation of at least 3 independent experiments conducted in quadruplicate.
The BNIPDabut was the most promising compound from the in vitro drug screening tests since it induced the lower IC\textsubscript{50} against \textit{T. brucei}, and was selected to further in vitro characterization to ascertain its mechanisms of action and in vivo efficacy evaluation.

6. \textbf{Time-kill and reversibility of BNIPDabut compound on \textit{T. brucei} BSF}

Parasite survival to different BNIPDabut concentrations for intervals in a total 48h period of time was measured (\textit{Fig. 20}). This graph shows a decreasing tendency of parasite viability over time in the presence of all BNIPDabut concentrations tested. For 125 to 500 nM concentrations, a similar effect is verified during time, inhibiting 50% of parasite growth approximately at 30 hours of exposure. Lower concentrations require more time to induce a toxic effect, for example 1.95 nM and 3.91 nM concentrations of BNIPDabut have no effect on the parasites until 15 hours of contact. In an overall view of the time-kill results, it is clear that the compound effect is time and concentration dependent requiring a prolonged period of time to induce a pronounced toxic effect on \textit{T. b. brucei} (\textit{Fig. 20}).

\textbf{Figure 20.} BNIPDabut dose and time to kill experiment. Trypanosome viability measured after 1, 8, 16, 24 and 48 hours of exposure to compound in distinct concentrations. Values are presented as mean±standard deviation of at least 2 independent experiments conducted in quadruplicate.

Reversibility of trypanocidal effects caused by BNIPDabut was assessed after compound washout at specified times and 3 days later parasite viability was measured. Compound concentrations in a range of 1.95 to 62.5 nM are not enough to cause a severe toxic effect in \textit{T. brucei} culture at the times tested (1, 8, 16, 24 and 48 hours) (\textit{Fig. 21}). Concentrations above 125 nM are required to induce a cytotoxic result and produce an irreversible effect on parasite survival. At least a 50-fold increase of the IC\textsubscript{50} in vitro of this compound (IC\textsubscript{50}=2.37 nM, described in point 5 – \textbf{Table VII}) is necessary to create a persistent and irreversible effect which is time dependent. Thus, the irreversibility produced by BNIPDabut is concentration and time dependent.
Figure 21. BNIPDabut reversibility effect on *T. brucei* BSF. After compound removal at 1, 8, 16, 24 and 48 hours of exposure, trypanosome viability was measured after 72h incubation in free-medium. Values are presented as mean±standard deviation of at least 2 independent experiments conducted in quadruplicate.

7. *In vivo* treatment of *T. b. brucei* infections with BNIPDabut

The survival of BALB/c mice infected with BSF *T. b. brucei* was studied without administration of any treatment (control mice). In those conditions, parasites reached $10^8$ trypanosomes/mL in approximately 7 days post-infection and mice were euthanized accordingly with the established humane end-point (Table IX).

A currently used drug (pentamidine) was administered for 4-consecutive days at 5 mg/kg intraperitoneally to *T. brucei* infected mice as a positive control [85]. All the mice remained for more than 30 days without parasites and were considered cured.

To test BNIPDabut effectiveness *in vivo*, five distinct schemes of treatment were defined (Table IX). A single intravenous administration of 1 mg/kg led to a slight diminution of parasite proliferation (parasite count was maintained in $10^5$ parasites/mL range from day 4 to day 6 – data not shown) and mice were euthanized 9 days post-infection, 2 more days than the untreated mice. The infection progressed rapidly with one dose of 10 mg/kg, and the animals were euthanised 6 days after the infection (Table IX). This group of mice at day 4 already had $10^7$ trypanosomes/mL in their blood.

A single dose of BNIPDabut was able to reduce the *T. brucei* proliferation, but unable to eliminate completely the parasite, then a multiple-dosage scheme was set.

Mice that received treatment at 3.33 mg/kg for 3 consecutive days, relapsed at 10th day after infection showing that this treatment caused a decrease of parasite growth and consequently on progression of the infection. In this group (3 mice), it was verified in one mouse a decrease of trypanosome density between day 6 and 7 from $10^7$ parasites/mL to $10^6$ parasites/mL (data not shown).
The results obtained in mice that were treated with 10 mg/kg in each day during four consecutive days (a total of 40 mg/kg BNIPDabut were administrated) show a mean survival of 15 days (Table IX), but it was noticed a variability in the progression of parasites in each mouse. Among the 6 BALB/c mice, 2 of them were euthanised at 10th day, another 2 at 15th day and the last ones at day 20. Regardless of this fact, it was more effective in comparison with previous dosages.

In another scheme, the same dosage of 40 mg/kg of BNIPDabut was applied but in every odd day: 1, 3, 5 and 7 post-infection (alternate days). Mice were euthanised in an average of 18.3 days (Table IX). An interesting parasite regression was detected in one mouse (out of 3 mice) from day 6 (the first day that parasites were viewed in this rodent at 2.75x10^4 parasites/mL) to day 8 in which no parasites were observed in parasitemia and they only reappear at day 17. In the other two animals, parasites started to be detected only after 10 days post-infection (data not shown). With this treatment the survival days were similar to two mice from the previous group, but the capacity to delay parasite proliferation until more than ten days is an important improvement when compared with the previous scheme. So the significant delay in mortality in mice is correlated directly with the delay in parasitemia. In this case mortality was delayed to more than twice the time of control mice.

### Table IX. Anti-trypanosomal activity in BALB/c mice.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total dose (mg/kg)</th>
<th>Scheme of administration</th>
<th>Dosing route</th>
<th>Cured/Infected</th>
<th>Survival days (mean)</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0/4</td>
<td>7.25</td>
<td></td>
</tr>
<tr>
<td>Pentamidine</td>
<td>1</td>
<td>4x5</td>
<td>i.p.</td>
<td>3/3</td>
<td>&gt;30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1x10</td>
<td>i.v.</td>
<td>0/3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>BNIPDabut</td>
<td>10</td>
<td>3x3.33</td>
<td>i.v.</td>
<td>0/3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4x10</td>
<td>i.v.</td>
<td>0/6</td>
<td>15 Doses given daily</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4x10</td>
<td>i.v.</td>
<td>0/3</td>
<td>18.3 Doses given in alternate days</td>
<td></td>
</tr>
</tbody>
</table>

*Number of doses x Dose concentration (mg/Kg); i.p. intraperitoneal; i.v. intravenous|

8. **BNIPDabut protein targets**

To identify potential protein targets for BNIPDabut, *T. brucei* total protein extracts were incubated with BNIPDabut-immobilized on epoxy-activated sepharose beads (as control, resin without immobilized-compound was used). Visualization by silver staining of a SDS-PAGE gel containing the resin without compound, the BNIPDabut-immobilized resin and the eluted proteins obtained from the washing steps is illustrated on **figure 22A**. Comparison between the resin without compound and the BNIPDabut-immobilized resin (**Fig. 22A**, 37)
columns 1 and 2, respectively) show some proteins that interact nonspecifically with BNIPDabut, because they are also linked to the resin without compound. From the column with the extract incubated with the BNIPDabut-immobilized resin, it is possible to see that the compound has a more marked specificity with proteins that present a MW lower than 90 kDa (Fig. 22A, column 2). It is verified that most of the proteins that aren't bound to the BNIPDabut-immobilized resin are efficiently removed with the washing procedure, especially in the first two washes (Fig. 22A, columns 7 and 8).

For comparison, a similar analysis of a less potent BNIP-derivative compound on T. brucei was performed with BNIPDaoct (IC$_{50}$=78.88 nM for T. brucei BSF) (Fig. 22B). Likewise in the previous gel, some proteins also bind nonspecifically to BNIPDaoct-immobilized resin (Fig. 22B, lane 8 and 9). Most of the proteins that aren't linked by BNIPDaoct-immobilized resin are efficiently removed with the washing procedure. In addition, this compound neatly has a strong specificity for protein(s) around 36.5 kDa order (Fig. 22B, columns 8 and 9 respectively). These proteins must be abundant since they were also eluted using ionic strength (Fig. 22B, columns 5, 6) and competitive elution (Fig. 22B, columns 7).

![Figure 22](image)

**Figure 22.** Silver stained SDS-page gels loaded with T. brucei lysates (obtained with RIPA) onto compound-immobilized or control sepharose beads and their respective eluted proteins. (A) *T. brucei* total proteins extract incubated with BNIPDabut-immobilized resin. Protein samples are: (1) Control resin, (2) BNIPDabut-immobilized resin, (3) Competitive eluted proteins, (4,5) Eluted proteins using ionic strength, (6 - 8) Eluted unbound proteins, (9) MW marker. (B) *T. brucei* total proteins extracts incubated with BNIPDaoct-immobilized resin. Protein samples are: (1) MW marker, (2 - 4) Eluted unbound proteins, (5,6) Eluted proteins using ionic strength, (7) Competitive eluted proteins, (8) BNIPDaoct-immobilized resin, (9) Control resin.
For a more detailed analysis of the BNIPDabut targeted proteins, a SDS-PAGE gel with silver staining was performed with the resins resulting from the *T. brucei* protein extract interaction with the BNIPDabut-immobilized resins. PBS or RIPA buffers were used to prepare *T. brucei* protein total extracts (Materials and methods, section 10.1) and each protein extract was incubated with BNIPDabut-immobilized resins. RIPA buffer is particularly useful to obtain nuclear proteins by the induction of nuclear membrane disruption. Moreover, in its constitution the ionic detergents sodium dodecyl sulfate and sodium deoxycholate are present, which can change proteins native conformation and denature them [86]. Thus, the extract preparation without detergent (PBS) or with detergents (RIPA) was performed to assure that parasite protein extract would represent most of the proteins that could interact with the BNIPDabut. Observation of figure 23 reveals that more protein bands are found resulting from the interaction of the total protein extract obtained with RIPA buffer (which contains detergent) (lane 3 in comparison to lane 4). Specificity of the protein bands can be determined by excluding the ones present in the resin without compound (Fig 23, lane 2) and comparing with the bands present in the BNIPDabut-immobilized resins (Fig. 23, 3 and 4). A strong band is observed around 36.5 kDa weight (Fig. 23, lane 4, box 3). Three bands were selected (Fig. 23, boxes 1 to 3) to be sent to Peptide Mass Fingerprint analysis, based on their specificity for BNIPDabut. This compound seems to be targeting some proteins that are not yet discovered and described for *T. b. brucei* BSF Lister 427 (Conserved hypothetical proteins) (Fig. 23; Table X). Only one protein, Fructose-1,6-Bisphosphate Aldolase (ALD), was identified with high protein score (confidence interval > 99.6 %) (Fig. 23, box 2; Table X).

![Figure 23](image.png)

**Figure 23.** Silver stained SDS-PAGE showing the interactions between the *T. brucei* total protein extracts with the BNIPDabut-immobilized resin. Columns represent: (1) MW marker, (2) Resin without immobilized compound, (3) BNIPDabut-immobilized resin with *T. brucei* total protein extract obtained with PBS, (4) BNIPDabut immobilized resin incubated with *T. brucei* total protein extract obtained with RIPA. The boxes 1 to 3 represent the specific protein bands binding to the BNIPDabut compound excised and sent to Peptide Mass Fingerprint analysis, and in the right of the gel is presented the putative proteins identified.
Table X. BNIPDabut protein targets identified from *T. brucei* by Peptide Mass Fingerprint analysis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-bisphosphate aldolase, glycosomal (ALD)</td>
<td>Tb427.10.5620</td>
<td>41.1</td>
</tr>
<tr>
<td>Conserved hypothetical protein</td>
<td>Tb427tmp.01.5420</td>
<td>29.7</td>
</tr>
<tr>
<td>Conserved hypothetical protein</td>
<td>Tb427.04.4640</td>
<td>21.8</td>
</tr>
<tr>
<td>Conserved hypothetical protein TCIL3000_4_3990</td>
<td>Tb427.08.1750</td>
<td>24.0</td>
</tr>
</tbody>
</table>

* Proteins ordered by its appearance in the right of the gel in figure 23.

b Accession numbers and MW obtained in TriTrypDB [http://tritrypdb.org/tritrypdb/](http://tritrypdb.org/tritrypdb/).

9. Physicochemical characteristics of PEG-PLA nanoparticles

The physicochemical characteristics of the PEG-PLA nanoparticles obtained by the nanoprecipitation method were determined (Table XI, Fig. 24). BNIPDabut compound was successfully encapsulated into PEG-PLA NPs with a diameter size of 174.97 ± 9.27 nm and 33% of encapsulation efficiency (E.E.). Also present a lower polydispersity index (PDI=0.2), representing a monodisperse population (Table XI, Fig. 24) [87]. The incorporation of the BNIP-derivative compound influenced the surface charge of the nanoparticle (-27.4 ± 10.6 mV) when compared with empty PEG-PLA nanoformulations (-21.2 ± 3.3 mV) (Table XI). The physicochemical characteristics of the produced empty nanoparticles were very distinct from the BNIPDabut-loaded PEG-PLA nanoparticles, with an elevated PDI (0.68 ± 0.14), high size (895.6 ± 67.03 nm) and a heterogeneous population (Table XI, Fig. 24).

Table XI. Size (diameter), polydispersity index (PDI), zeta-potential and E.E. of PEG-PLA nanoparticles, empty and loaded with BNIPDabut.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Compound</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>ζ-potential (mV)</th>
<th>E. E. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PLA</td>
<td></td>
<td>895.60 ± 67.03</td>
<td>0.68 ± 0.14</td>
<td>-21.2 ± 3.3</td>
<td>N/A</td>
</tr>
<tr>
<td>BNIPDabut</td>
<td></td>
<td>174.97 ± 9.27</td>
<td>0.20 ± 0.03</td>
<td>-27.4 ± 10.6</td>
<td>33.01 ± 3.03</td>
</tr>
</tbody>
</table>

N/A – not applicable; *Values are presented as mean±standard deviation of at least 2 independent experiments.

Figure 24. Size (radius) distributions for BNIPDabut PEG-PLA (---) and PEG-PLA (---) nanoparticles. Values presented are from 1 representative experiment.
The morphology of PEG-PLA nanoparticles, either unloaded or loaded with BNIPDabut, was analyzed by TEM (Fig. 25). Empty PEG-PLA nanoparticles micrographs show the presence of smooth surface structures agglomerates (Fig. 25A, 25B). In PEG-PLA nanoparticles the population is well dispersed and a spongy surface is observed (Fig. 25C, 25D).

Figure 25. Transmission electron micrographs (TEM) of the developed PEG-PLA nanoparticles. (A,B) Empty PEG-PLA; (C,D) – BNIPDabut PEG-PLA. Images presented are from 1 representative experiment.

10. Anti-trypanosomal activity of empty and loaded with BNIPDabut nanoformulations

To ensure that toxicity induced by loaded nanoparticles was due to the compound incorporated, empty nanoparticles were tested in the same concentration range defined for loaded nanoformulations (Fig. 26, Table XII). In figure 26, is demonstrated the difference of effect on trypanosome viability between empty and loaded nanoformulations by the following concentrations: 1000, 500, 250, 125, 62.5, 31.25 and 15.6 nM in BNIPDabut. Empty PEG-PLA nanoparticles didn’t cause any toxic effect on trypanosomes (Fig. 26). Concerning the result with BNIPDabut PEG-PLA nanoparticles, a substantial reduction of parasite percentage occurs with 125 nM of polymer concentration (Fig 26). The BNIPDabut-PEG-PLA nanoparticles IC_{50} against T. brucei achieved in a 72 hours period was 99.28 ± 5.86 nM, a value that is 41.9-fold higher than the free-compound IC_{50} (Table XII).
Figure 26. Growth inhibition of unloaded (○) and loaded with BNIPDabut (●) PEG-PLA nanoparticles. Values are presented as mean±standard deviation of 2 different nanoparticle preparations, each with 3 concordant independent assays.

Table XII. Activity of free BNIPDabut compound and nanoformulations (PEG-PLA nanoparticles empty and loaded) against *T. brucei*.  

<table>
<thead>
<tr>
<th>Formulation</th>
<th>IC$_{50}$ T. brucei (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free BNIPDabut*</td>
<td>2.37 ± 0.15</td>
</tr>
<tr>
<td>PEG-PLA$^a$</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>BNIPDabut PEG-PLA$^a$</td>
<td>99.28 ± 5.86</td>
</tr>
</tbody>
</table>

$^a$Values are presented as mean±standard deviation of 2 different nanoparticle preparations, each with 3 concordant independent assays.

* Value from table VI.
Discussion

1. Trypanosome culture growth

The knowledge of trypanosome-fold during time indicates that a starting culture of $10^3$, $10^4$ or $10^5$ trypanosomes per milliliter will reach log-phase (1-3x$10^6$ parasites/mL) after 72, 48 or 24 hours, respectively. The 10 to 15 trypanosome-fold increase in growth estimated corresponds to a doubling time of 4.8 hours (Fig. 16), similar to the 6.8 hours of doubling time in logarithmic phase already described for the same subspecies [82]. This growth assay was essential to determine the starting inoculum of *T. brucei* BSF for maintenance and all the assays described on this thesis.

2. Linearity of the cell viability assays (resazurin and MTT)

The selection of one viability assay in early drug discovery is the first step to accurately identify potential cytotoxic compounds. Here we report two different viability assays (resazurin-based and MTT assays) successfully optimized for *T. b. brucei* BSF Lister 427 in 96 well plates for drug screening research. However, one assay is more efficient than the other, a parameter that is explored hereafter.

In our results it was establish that for resazurin-based assay the measurement of trypanosome viability can be evaluated for all densities (1.25 – 20x$10^5$ trypanosomes/well) with 45.5 µM of substrate (resazurin) into a time period of incubation around 2 to 4 hours at 37ºC, 5% CO₂ (Fig. 17). Jones and coworkers [11] obtained the similar assay conditions, using *T. b. brucei* Lister 427 strain, up to 10⁶ trypanosomes/well, the maximum cell density applied in their work. In addition, this viability assay has been extensively reported by a number of authors for whole cell *T. brucei* species in 96-well drug screening format [11, 45, 88-90].

On the other hand, the absorbance measurement in MTT assay was linear after a period of 3 to 5 hours of incubation with the substrate and the parasite density in the well must be within a range of 2.5 – 10x$10^5$ parasites/well (Fig. 18). In the work of Ellis and coworkers [91] it was described that this method could detect as low as $10^4$ parasites of *T. b. rhodesiense* strain, which is not observed in our results with *T. b. brucei* strain which show that absorbance is not proportional to time for the lower density tested: 1.25x$10^5$ parasites per well (Fig. 18).
In T. brucei drug screening literature, reports describing MTT assay as the chosen method are fewer than the resazurin-based assay, commercially designated Alamar blue. In fact, our work demonstrates that the MTT viability assay is more restrictive, less reproducible and more time consuming than the resazurin-based assay. The later has many benefits reported previously such as sensitivity, simplicity, lack of radioactive materials, economy and is non-toxic to trypanosomes [82, 90, 92].

3. DMSO tolerance of T. b. brucei culture

DMSO is the best solvent for BNIP-derivative compounds although it is well-known that this solvent solely induces toxic effects to cells [93]. Consequently, in all drug discovery assays that use DMSO as a solvent it is crucial to establish the tolerance of the organism in study.

In our experiment, the tolerance to DMSO determined by the resazurin-based and the MTT assays produced similar IC50 values (~ 0.9 %) but in the first one is shown a decreasing tendency dependent on the increase of DMSO quantity (Fig. 19). In MTT assay this tendency is not observed in the range of 0.25 to 0.75 % of the trypanosome viability that show a really similar level of trypanosome toxicity.

Since resazurin-based methodology has already been described in this work as more efficient and was the chosen method to perform drug screening experiments, the selection of the maximum DMSO percentage to use in the wells was based on the values of DMSO toxicity evaluated by the resazurin-based assay. The sensitivity to DMSO obtained with resazurin-based parasite viability assay demonstrated an IC50 value of 0.98 ± 0.2 % approximately to a value that has been described previously (0.8%) [11]. The selected value for maximum DMSO percentage in the wells considered acceptable was 0.1% since this concentration does not induce a notorious impact in parasite survival (Fig. 19A). This percentage is safer to use comparing to the 0.25 or 0.5% DMSO applied in the previous works for the same T. brucei strain used in this study [11, 94].

4. Toxicity of standard drugs

Only four drugs (pentamidine, suramin, melarsoprol and eflornithine) and a combination therapy (nifurtimox-eflornithine) are registered for HAT treatment. Pentamidine and suramin are effective against the early stage of the disease, the hemo-lymphatic phase, and were used in a number of laboratory works with T. brucei strains as control for in vitro and/or in vivo assays [11, 45, 90, 95, 96]. The IC50 values described here for pentamidine and suramin (3.35 and 76.71 nM, respectively) are in the same range as described by others authors in a
variety of *T. brucei* subspecies [11, 45, 90, 95, 96]. Therefore the optimization of the resazurin-based assay can be considered validated.

The toxicity of these compounds was also evaluated in mouse fibroblast cells (L929). Pentamidine exhibited a toxic effect in these mammalian cells at 70 µM concentration (Table V). Nevertheless, the selectivity index is high (21 137) which undoubtedly shows that this drug has specificity towards *T. brucei*. On the other hand, no toxic effect was induced by suramin up to 1 mM.

Pentamidine toxicity was also determined by the optimized MTT assay (data not shown) and it was obtained an IC$_{50}$ value of 5.4±0.67 nM (n= 3), identical to the value obtained with the resazurin-based assay, although the MTT assay was less reproducible. Nevertheless the optimized conditions for this assay were validated by this result.

5. Screening of BNIP-derivative compounds library

BNIP-derivative compounds are composed by two naphtalimidopropyl groups linked by a carbonated chain, varying in size, presence of heteroatoms (oxygen or amine) or cyclohexane/benzene rings (Table III). In cancer cells, their mode of action consists to intercalate in DNA and restrain proliferation capacity [49, 56, 97-99]. Previously they have been explored as potential therapeutic agents for cancer and leishmaniasis [6, 8, 48, 49, 53, 55, 56]. This study evaluated their application in the treatment of infections caused by *T. brucei*. Although *T. b. brucei* subspecies used in our experiments are non-pathogenic to humans, it is a clinically relevant species and a suitable model for human African disease to test advanced compounds [61].

BNIP derivatives such as BNIPSpermidine (BNIPSpd) and BNIPSpermine (BNIPSpm) were the first to be synthesized consisting in the addition of one naphtalimido ring linked to a propyl chain in each extremity of the polyamines spermidine and spermine [48]. Clearly the presence of naphtalimidopropyl rings increased the cytotoxic effect of these polyamines demonstrated by their activity in nanomolar order against trypanosomes, and comparing to their effect in fibroblasts these compounds show a strong tendency to cause more toxic effects on *T. brucei* (Table VI). This justified by the different polyamine metabolisms between parasites and mammalian cells, and interfering with the parasite polyamine pathway causes more severe consequences than for its host given the fact that polyamines are essential for parasite growth and proliferation [8]. The polyamines that trypanosomes are able to synthetize are spermidine and putrescine [49, 61]. Thus BNIPSpd probably was recognized by the parasite, having a facilitated entry into trypanosomes. This justifies the higher anti-*T. brucei* activity verified for BNIPSpd (IC$_{50}$ = 85 nM) than BNIPSpm (IC$_{50}$ = 824
Also the higher toxicity of spermidine, comparing to spermine, is probably due to the fact that it interferes with the activity of the natural spermidine produced by the *T. brucei* parasite required for growth mechanism.

From the library of BNIP-derivative compounds assessed in this project, 10 drugs exhibited an anti-trypanosomal IC\textsubscript{50} lower than 200 nM. Here we report the lower IC\textsubscript{50} values ever seen, induced by these drugs (Table VII). The low concentration (in nanomolar range) necessary to induce a wide decrease in *T. b. brucei* population confirms the therapeutic potential of this class of compounds. All drugs with submicromolar toxicity against *T. brucei* only induce negative effects in fibroblasts in the micromolar range (Table VII). This level of cytotoxicity suggests a low selectivity towards fibroblast cells and provides an initial indication of differences between trypanosomes and mammalian target proteins.

Interestingly, a tendency of reduction in anti-trypanosomal potency occurs as longer is the linker chain of BNIPDi-aminoalkyl compounds (Table III; Table VII). Exactly the opposite tendency was described for the majority of BNIP-derivative compounds in cancer and *Leishmania* cells (longer the linker chain, higher the toxic effect of the drug) [6, 55].

It was described that by increasing the length of the alkyl central chain helps to increase aqueous solubility, since the two naphtalimido rings do not tend to be on top of each other by π-π interactions between the aromatic rings and hence favor aqueous solubility [6]. This is in accordance to what was noticed in the preparation of compounds with a decreased length in the linker chain that tends to precipitate when the solvent is added.

The most potent *T. brucei* inhibitor is BNIP diaminobutane (BNIPDabut), which has an alkyl linker chain containing four methylene groups and two amine groups, with the lower IC\textsubscript{50} ever shown caused by the activity of BNIP-derivative compounds – IC\textsubscript{50} = 2.37 ± 0.15 nM (Table III; Table VII). BNIPDabut is also known by BNIPPutrescine (Fig. 10) that is based on the polyamine putrescine also synthesized by *T. brucei* parasites [48, 61]. This suggests that this compound might interfere with polyamine synthesis and consequently in parasite proliferation. Recently its structure was modified to confer rigidity to the structure resulting in the analogue, BNIP(1,4)Dacyhex, which retains high toxicity towards the parasite (IC\textsubscript{50} = 4.41 ± 0.02 nM), but with a statistically high significant decrease on the mammalian cells toxicity. However in cancer cells this analogue is more active than BNIPDabut considering that by introducing ring systems in the linker chain it is expected to reduce its flexibility and hence should in principle enhance binding to the target (P.K.T. Lin, personal communication). Both compounds had nanomolar activity similar to the IC\textsubscript{50} of pentamidine (3.35 ± 0.15 nM) and the highest values in selectivity for *T. brucei* over fibroblasts.

Introduction of heteroatoms (oxygen or nitrogen) results in a significant increase in their solubility in aqueous medium [49]. In our work an increase of the number of nitrogens
(comparison between BNIPDahep and BNIPDpta) decreased its toxic effect and an introduction of two oxygen atoms increased the compound activity in BNIPDaopt case (Table III; Table VII). However, for example in cancer cells by introducing oxygen atoms in BNIPSpm and BNIPSpd linker chain tends to decrease the cytotoxicity activity, and the removal of a nitrogen atom does not appear to substantially affect the cytotoxic properties of these compounds [6, 49, 53]. Since in T. brucei the presence of oxygen atoms seems to increase the compound toxicity and pentamidine presents two of these heteroatoms in its structure, in future work it would be interesting to test more BNIP-derivative compounds with oxygen incorporated.

The presence of more rigid structures decreases compound toxicity represented by BNIPDaCHM and NPA with an IC$_{50}$ in T. b. brucei over 1 µM (Table III; Table VIII). The lower cytotoxicity for NPA is in accordance with literature since this drug is a mononaphthalimido compound and previously it has been described that bisnaphthalimides have higher activity than the mononaphthalimides [52]. BNIP(4,4)Dapm has two benzene rings in the linker chain and is non-toxic to T. brucei (Table III; Table VIII). These observations indicate that it is not worthy to apply BNIP-derivative compounds with only one naphtalimido ring in chain extremity and compounds with more than one cyclohexane or benzene rings in the discovery of new BNIP-derivative potential drugs for HAT disease. The two naphthalamido-chromophore units linked by alkyl chains containing at least one amino group has previously been shown to be essential for cytotoxicity in the naphthalimide series [50]. Also it is noteworthy to mention that only one cyclohexane ring in the linker chain doesn’t cause a substantial decrease in compound toxicity, showed by BNIPDabut and its analogue (Table III; Table VII).

Even though in vitro assays can’t assuredly predict in vivo toxicity they provide a useful tool to compare and rank new compounds. BNIPDabut was the most promising compound among the BNIP-derivative drugs and was selected to further explore and discover its mode of action.

6. Time-kill and reversibility of BNIPDabut compound

The first topic to understand BNIPDabut activity was to determine the time that this compound needs to induce a severe toxic effect in parasites. In a total period of 48 hours, distinct concentrations caused a percentage of trypanosome inhibition in different degrees but all have in common a tendency to decrease parasite viability during the exposure time to the compound. Thus, BNIPDabut time-kill study yielded a concentration and time dependent toxic effect on viability of T. brucei (Fig. 20). Unfortunately, a total kill (100% death) of
parasites was not observed in this time period in all concentrations tested (from 1.95 to 500 nM), as it has been described by others authors with different compounds [44, 100]. In Mercer and collaborators work they applied a 320-fold difference between the IC<sub>50</sub> and the maximum concentration used in their time-kill assay (0.07 µM to 22.4 µM) [100]. In our study the time-kill assay was performed starting with a maximum concentration (500 nM), 211 times higher than the IC<sub>50</sub> (2.37 nM), which is a lower fold than the previous work described (Table VII, Fig. 20). This example suggests that with a higher concentration and a prolonged time of exposure it would be possible to observe total parasite death, without forgetting the fibroblasts IC<sub>50</sub> (19.9 µM). This observation could be applied in future experiments.

Until this point of the project, the viability assay does not indicate the type of compound anti-trypanosomal effect. Indeed, the fact of an existing decrease in parasite viability is not directly correlated to trypanosome death. Drugs could be inducing a cytocidal or a cytostatic activity on trypanosomes growth. In order to assess this parameter the reversibility of BNIPDabut was evaluated by allowing parasite growth to occur during 72 hours in free-compound medium after compound removal at 1, 8, 16, 24 and 48 hours to determine if trypanosomes could recover or not from the toxic effects induced in the exposure period (Fig. 21). The incapability to recuperate from transient exposure to BNIPDabut obtained with concentrations equal or above 125 nM at an exposure time more than 24 hours indicates that this compound caused irreversible effects on the potential target(s) within the parasites. Subsequently this compound is definitely trypanocidal in high concentrations. However, to induce severe toxic effects BNIPDabut requires a prolonged time of contact with parasites (Fig. 21). Like our BNIP-derivative compound, in literature some other new drugs have been described as originators of persistent and irreversible effects in <i>T. brucei</i> [44, 45, 100].

In addition, <i>in vitro</i> BNIPDabut is a trypanocidal compound with a mechanism of action dependent on time and concentration.

7. <i>In vivo</i> treatment of <i>T. b. brucei</i> infections with BNIPDabut

Encouraged by the potency and cytocidal profile of BNIPDabut, <i>in vivo</i> tests were performed to determine the potential of this compound as treatment to animals infected with <i>T. brucei</i>. Here is reported the first study that applied BNIPDabut <i>in vivo</i>. The efficacy to maintain trypanosome growth in blood circulation was observed with a reduced single dosage of 1 mg/kg. However, a multiple dosage scheme showed the best results with increasing time duration of mice relatively to non-treated animals (Table IX). Among all dosages the administration of a total 40 mg/kg in four alternate days was the most reproducible and effective in reducing parasite load in the first 10 to 18 days post-infection. In
one BALB/c mice of this group, parasites were detected at day 6 and after that they only resurfaced at day 17 of infection. Although no pharmacokinetic data is currently available, this occurrence strongly suggests that the compound had a trypanocidal effect during the time it was in circulation. In future work it is important to explore the pharmacokinetic of this compound because it includes the following parameters which are crucial to have a full understanding of BNIPDabut 'behavior’ in living organisms: mechanisms of absorption, distribution, rate at which a drug action begins, duration of the effect, chemical changes in the body and excretion of the drug [101].

8. BNIPDabut protein targets

In the preparation of total protein extracts two buffers were used, containing (RIPA) or not detergents (PBS). As expected, the presence of detergents yielded more proteins as can be observed in the gel (Fig. 23, column 3 and 4). This fact occurred because with detergent the extraction is done by physical and chemical processes and without detergent the cell lysis only occurs by physical pressure, which is applied by the freeze-thaw cycles [86].

BNIPDabut potential protein targets in *T. b. brucei* were identified. In a preliminary analysis in a SDS-PAGE gel stained by silver coloration, proteins specific for BNIPDabut were first visualized. Among the bands sent to proteomics analysis only one protein in band 2 (slightly above 36.5 kDa) was identified with high confidence interval percentage, which is Fructose-1,6-Bisphosphate Aldolase (ALD) (Fig. 23, column 4). This protein is involved in the glycolysis pathway, which is the only ATP-generating metabolism of *T. brucei* BSF [102]. During infection of the human host, the mitochondrial activity is restricted and ATP production is exclusively dependent of glycolysis in parasite glycosomes by using the host sugar [103]. ALD is the fourth enzyme in the pathway which effects reversible aldol cleavage of fructose-1,6-bisphosphate to dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate (Fig. 27). It has already been genetically validated as a drug target in *T. brucei* BSF [104]. In the previous study cited, the authors found that a partial depletion of ALD is sufficient to rapidly kill the parasites. Actually, in the last years one approach of new drug discovery against HAT disease is to find specific inhibitors of this glycolytic enzyme [105-107]. Thus, finding that the BNIPDabut compound seems to interact with this promising enzyme is a valuable discovery in therapeutic drugs for HAT disease. Moreover, specific protein targets for BNIPDaoct (Fig. 22B) also show a marked band in the 36.5 kDa order, which might contain ALD enzyme. This could be explored in the future and if BNIPDaoct also targets ALD, more BNIP derivative compounds can be searched for this inhibition.
Fig. 27. Glycolysis pathway in *T. brucei* BSF glycosomes. ALD: aldolase; DHAP: dihydroxyacetone phosphate; 1,3BPGA: 1,3-bisphosphoglycerate; ENO: enolase; F-6-P: fructose-6-phosphate; FBP: fructose-1,6-bisphosphate; G-3-P: glyceraldehyde-3-phosphate; G-6-P: glucose-6-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Glc: glucose; Gly-3-p: glycerol-3-phosphate; GPDH: glycerol 3-phosphate dehydrogenase; Mito: mitochondrial enzymes; PEP: phosphoenolpyruvate; 2-PGA: 2-phosphoglycerate; 3-PGA: 3-phosphoglycerate; PGI: glucose-6-phosphate isomerase; PGM: phosphoglycerate mutase; PFK: phosphofructokinase; PGK: phosphoglycerate kinase; PK: pyruvate kinase; PYR: pyruvate; TbHK: *T. brucei* hexokinase 1 and/or 2; TPI: triose-phosphate isomerase (Adapted from [103]).

9. Production of PEG-PLA nanoparticles - physicochemical characterization

The development of a nanotechnology-based drug delivery system would improve the BNIP polyamine derivative compounds biopharmaceutical properties to further application in the treatment of parasitic infections. The formulation of BNIPDabut encapsulated into PEG-PLA nanoparticles will, in theory, improve their *in vivo* circulation and consequently meet human African trypanosomiasis therapeutic need.

BNIPDabut exhibited potential effects *in vitro* and *in vivo*. However, this compound has some drawbacks such as low aqueous solubility and some toxic effects [9]. In recent years, polyethylene glycol-polylactic acid (PEG-PLA) block copolymer nanoparticles have been in focus due to the several benefits associated such as, enhance the drug loading of hydrophobic substances, reduce burst effect, avoid being engulfed by phagocytes, increase the blood circulation time of drugs and improve bioavailability [64]. The PEG hydrophilic layer solely can increase the solubility of insoluble drugs, prevent the protein absorbed on the nanoparticle surface and make nanoparticles unrecognizable by the reticuloendothelial system as foreign bodies allowing a long period of circulation. This improves BNIPDabut effect since it was already seen that is required a long time of exposure to the compound to
induce a severe trypanocidal action (Fig. 20, Fig. 21, Table IX). Moreover, after linking modifiers of special biological activity in the PEG–PLA chain end, such as peptide, amino acid or proteins, drugs in PEG–PLA nanoparticles can enter the brain through the blood–brain barrier. Indeed to achieve nanoparticles capable of entering brain would be an incredible development because HAT second stage occurs in central nervous system [108].

In our laboratory, a successful PEG-PLA nanoparticles preparation with BNIP-derivative compounds (BNIPDadec and BNIPDaoct) by the nanoprecipitation method was previously achieved [10]. Encouraged by this fact, BNIPDabut PEG-PLA nanoparticles were also manufactured by nanoprecipitation which has the advantages of not requiring any emulsification step or special laboratory equipment.

Our results show that hydrophobic BNIPDabut compound was successfully incorporated on the polymeric matrix, demonstrated by the physicochemical characteristics (Table XI, Fig. 24, Fig. 25). The diameter size measured in these nanoformulations was around 175 nm and, therefore presents acceptable size for parenteral administrations (ideally between 100 to 300 nm), since it won’t obstruct tiny vessels or pores existing in living organisms [87]. Polydispersity indices (PDI) values were approximately 0.2, which is acceptable since it has been described that a high PDI is considered between 0.2 and 0.5 and may result in particle overlapping and hamper accurate size-dependent interpretation of the in vivo results [109, 110]. Zeta potential gives an indication of the charge on particle surface and a marked negative potential was observed in the synthesized nanoparticles incorporated with compound [81]. This potential is much more negative than the previous reported with compounds in the same class [10]. Therefore, the differences in the linker chain length of BNIPDabut to BNIPDaoct or BNIPDadec compounds have a strong influence in this characteristic. The encapsulation efficiency indicates the percentage of the drug that was encapsulated in the nanoparticle. Only 33% of BNIP-encapsulation was achieved. This parameter could be improved because it isn’t in accordance with the literature that indicates high encapsulation efficiencies for hydrophobic compounds in nanoformulations prepared by nanoprecipitation procedure [10, 81]. The spongy surface morphology in PEG-PLA nanoformulations has already been described and could be due to the organization of the PEG and PLA blocks at the organic solvent water interface, during solvent evaporation [74].

The characteristics of unloaded polymeric nanoparticles weren’t optimized due to lack of time. The high diameter size and PDI is justified by the formation of aggregates visualized in nanoparticles micrographs (Fig. 25A, 25B).
10. Anti-trypanosomal activity of empty and loaded with BNIPDabut nanoformulations

Polymeric nanoparticles have already been tested as a drug vehicle against *T. brucei* showing good results, however our report is the first to use PEG-PLA nanoparticles against these parasites [36].

A decrease in toxicity against *T. brucei* of BNIPDabut occurred when it was encapsulated in PEG-PLA nanoparticles \( \text{IC}_{50} = 99.28 \pm 5.86 \text{ nM} \) compared to the free compound \( \text{IC}_{50} = 2.37 \pm 0.15 \text{ nM} \) (Table VII, Fig. 26, Table XII). The same fact was verified for BNIPDadec in *Leishmania infantum* amastigotes [10]. This suggests a controlled drug release by the nanoformulation that subsequently induces a slow liberation of the BNIP-derivatives compounds. In this work, it may be considered an advantage, since the continuing exposure of the compound to trypanosomes is crucial to induce all its toxic potential. *In vitro* drug release assays will be done in future, to clarify the quantity of compound released during time. As expected, empty PEG-PLA nanoparticles did not induce any toxic effect to trypanosomes growth [111].
Concluding remarks and Future perspectives

In this work, a BNIP-derivative compounds library was evaluated against *T. brucei* parasite, towards the identification of new alternative drugs, and a compound emerged as a promising candidate. The successful optimization of two cell viability assays (resazurin-based and MTT methods) was achieved, enabling the screening of a series of BNIP-derivative compounds that exhibit parasitical activity against *T. brucei*. BNIPDiaminobutane was identified as the most potent and promising compound to apply against African trypanosomiasis infection.

In general, it was demonstrated that as longer is the linker chain between the two naphthalimidopropyl groups, a loss in the anti-*T. brucei* activity *in vitro* occurs. Moreover, addition of rigid structures to BNIP compounds induces a decrease of their toxic effect.

A detailed analysis of BNIPDabut activity, showed that it is an *in vitro* trypanocidal compound that required a high concentration and a long period of exposure time to kill the trypanosomes. Since it has high fold selectivity to *T. brucei* towards mammalian cells (8000-fold higher) it is an attractive compound to be applied in living mammalian organisms. In animal models infected with African trypanosomiasis, BNIPDabut has shown a great efficacy in reducing parasites quantity when given by intravenous parental route in four alternate days at 10 mg/kg doses. Indeed, mice treated with the referred dosage scheme were able to survive more than the double time of untreated mice.

An important fact verified was the specific interaction between BNIPDabut and some *T. brucei* proteins, in particular the fructose-1,6-bisphosphate aldolase, which is involved in parasite ATP production exclusively by the glycolysis metabolism in *T. brucei* BSF. Finally, in order to improve BNIPDabut efficacy, the encapsulation of this compound in PEG-PLA nanoformulations was effectively developed. The fact of the nanoparticles originate a controlled drug release is an advantage, since the continuing exposure of the compound to trypanosomes is crucial to induce all its toxic potential.

In conclusion, BNIP-derivative compounds showed promising toxic potential against *T. brucei* pathogen at low concentrations which can be applied in the future for HAT treatment.

Relatively to future perspectives associated to this work, some possible items to be explored are:

- Evaluation of BNIPDabut at higher concentrations in time-kill and reversibility tests;
• Performance of BNIPDabut pharmacokinetic studies (mechanisms of absorption, distribution, rate at which a drug action begins, duration of the effect, chemical changes in the body and excretion of the drug);
• Analysis of the nanoparticle in vitro release of BNIPDabut;
• Test a new scheme dosage of free BNIPDabut and loaded PEG-PLA nanoparticles in vivo;
• Determine if the compound causes trypanosomes apoptosis/ mechanism of death;
• Perform a kinetic assay of fructose-1,6-bisphosphate aldolase to determine if BNIPDabut inhibits this enzyme.
References


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