Nanotechnological approaches against Chagas disease

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Abstract

Over several thousand years, the flagellated Trypanosoma cruzi—causative agent of Chagas disease—developed a complex life cycle between the reduviidae vectors and its human hosts. Due to their silent and hidden location, the intracellular amastigotes are mainly responsible for the nearly 50,000 annual deaths caused by the chronic chagasic cardiomyopathy. Chagas disease is the most important parasitic disease in the Americas, though treatments have not evolved towards a more efficient pharmacotherapy that (i) eradicates the scarce amastigotes present at the indeterminate/chronic form and (ii) employs less toxic drugs than benznidazole or nifurtimox. Nano-drug delivery systems (nanoDDS) represent useful means to selectively deliver the drug to intracellular targets. However, preclinical research in Chagas must be extended in order to improve the chances of a clinical implementation. The stages involved in this process are (i) selection of the appropriate drug for a specific parasite, (ii) development of a drug-loaded nanoDDS structure that displays the adequate pharmacokinetics, biodistribution and intracellular transit and (iii) selection of the right parasite form to target and the right stage of the disease for the treatment to be started. In this review we will critically overview the few research works published in the last 20 years in the context of nanotechnology and Chagas diseases and highlight the gaps in knowledge towards the design of more efficient medicines to address this endemic.

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1. Introduction

Chagas disease is a major public health concern in Latin America (LA), affecting approximately 15–20 million people from Southern California to Argentina and Chile [1]; 2–3% of the Latin American population is infected, encompassing a burden of about 670,000 Disability Adjusted Life Years (DALYs) [2]. In LA, more DALYs are lost due to Chagas than meningitis, sexually transmitted diseases, hepatitis B and C, or malaria. In fact, only HIV, diarrhoeal diseases and
tuberculosis rank higher [3]. Also, the morbidity and mortality are more than one order of magnitude higher than those found for malaria, schistosomiasis or leishmaniasis [4]. Current estimates indicate 200,000 new cases every year and an annual mortality ~ 50,000 [3]. WHO estimated that early morbidity and mortality cause an annual economic loss in LA of over $6.5 billion [5]. However, after nearly 45 years of employing nifurtimox (NF) and benznidazole (BNZ) as first-choice drugs (Table 1)—both widely criticised because of their low efficacy and serious toxic side effects [6–10] leading to discontinuation of 20–30% treatments [11]—none of them could still be replaced [12]. Paradoxically, 100 years after the first report describing the morphology and the life cycle of the pathogen [13,14], neither a vaccine nor an effective treatment for the chronic cases is available [15]. A main reason is that Chagas belongs to a group of tropical infections especially endemic in low-income populations of developing regions of Africa, Asia, and the Americas [16], known as neglected diseases. The lack of interest of pharmaceutical companies and the absence of effective social policies from the endemic states [17] are responsible for the very limited evolution towards an improved pharmacotherapy. In this context, not-for-profit product–development partnerships (PDPs) such as the Drugs for Neglected Diseases initiative [18], amongst other organizations [19–21] have launched programs aimed to produce new drugs for infected patients. In general, these PDPs foster both the search of new drug candidates and the selection of those approved and commercially available to develop more efficient medicines. Regardless of the promissory technical background [22], neither profit nor non-profit organizations have addressed these challenges by means of nanotechnology.

The objective of this review is to extensively describe the state-of-the-art and to assess the impact of nanotechnology in the preclinical therapies against Chagas disease. Finally, new research avenues that could improve the therapeutic success will be discussed.

2. Pathogenesis of Chagas disease

The causative agent of Chagas disease is the flagellate protozoan Trypanosoma cruzi which is transmitted to the human by hematophagous reduviidae bugs; i.e., Triatoma infectans, Rhodnius prolixus, Triatoma dimidiata, Panstrongylus megistus and Triatoma sanguisuga [13,23]. Transmission is associated with the faeces of triatomiine bugs (>80%), blood transfusion or organ transplant (~15%) [24], congenital transmission (the parasite crosses placenta) (4%), ingestion of contaminated food (<1%) and laboratory accidents (<1%) [3]. The growing number of reported cases due to infected blood donors, most of them being Latin American immigrants [25], and the finding of autochthonous transmission makes Chagas a potentially emergent disease in the United States [26]. Also, immigration has brought the disease to other developed countries in Europe and Oceania [27–29]. Chagas disease is also an emerging opportunistic infection among immuno-compromised patients [30].

The biological cycle is comprised of three fundamental forms: (i) the infecting trypomastigotes (found in mammalian blood as disseminators of blood-borne infection and in the hindgut of triatomiine bugs), (ii) the epimastigotes (the proliferative form in the intestine of the bug), and (iii) the amastigotes (that multiply by means of binary fission inside mammalian host cells, producing their lysis, and releasing new trypomastigotes into the blood stream that can invade any nucleated cell to begin a new reproductive cycle) [31] (Fig. 1). There are three stages: (i) acute, (ii) indeterminate and (iii) chronic. However, some investigators consider only the existence of the acute and the chronic phases [3]. After the generally asymptomatic acute phase (characterized by detectable parasitemia of trypomastigotes) that lasts from a few weeks to several months, the infection is well controlled by the host immune response. Nonetheless, parasites continue to cycle in and out of the host cells and are only transiently in the blood stream, thus making the detection difficult; symptoms are not evident for years or even decades (indeterminate stage). Eventually, up to 40% of infected patients develop the chronic form of the disease, where intracellular amastigotes cause irreversible structural damage to the heart, the esophagus and the colon, with severe disorders of nerve conduction in these organs. Patients usually die from heart conditions [32,33].

3. New trypanocidal agents: new prescriptions for marketed drugs?

The different vital metabolic pathways and the drugs inhibiting these potential targets are presented in Table 2 [34]. It is remarkable that most of them are only effective in vitro [35–37], have shown toxicity in preclinical models [38], were not effective against the chronic form [39,40], or did not produce parasitological cure. On the other hand, they produce increased survivals [12,41,42]. Another drawback is the appearance of resistance [43].

Urbina indicated theazole posaconazole (POS) and the currently marketed bisphosphonates (BP) (e.g., pamidronate, ibandronate) as the most promising candidates about to enter clinical trials [44]. In particular, POS induces parasitological cure in acute and chronic

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**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mode of action</th>
<th>Dose</th>
<th>Cure rates</th>
<th>Associated problems</th>
<th>Manufacture problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifurtimox 1-methyl-4-</td>
<td>Reduction of the nitro group to</td>
<td>8–10 mg/kg/day 90 days</td>
<td>Significant activity in the acute (up to 80% of parasitological cures in</td>
<td>1. Low effectiveness in the chronic phase of the disease (~20%), that is the most frequent clinical presentation in LA [9]</td>
<td>NF has been only intermittently produced since 1997 by Bayer</td>
</tr>
<tr>
<td>[(5-nitrofururylidene)</td>
<td>unstable nitroanion radicals, which in turn react to produce highly</td>
<td></td>
<td>treated patients, defined as a negative result for all parasitological and</td>
<td>2. Regional variations in efficacy due to naturally resistant</td>
<td></td>
</tr>
<tr>
<td>aminothiomorpholine-1,</td>
<td>toxic reduced oxygen metabolites (i.e. superoxide anion, hydrogen peroxide).</td>
<td></td>
<td>serological tests) and early chronic phases (up to 60% cures) [7,8,10]</td>
<td>T.cruzi strains</td>
<td></td>
</tr>
<tr>
<td>1-dioxide</td>
<td>T. cruzi is deficient in detoxification mechanisms for oxygen metabolites,</td>
<td></td>
<td></td>
<td>3. High rate of patient noncompliance due to drug side effects</td>
<td></td>
</tr>
<tr>
<td></td>
<td>particularly hydrogen peroxide, and is more sensitive to oxidative stress</td>
<td></td>
<td></td>
<td>4. Long period of treatment (30–60 days), and dose-dependent toxicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>than mammalian cells</td>
<td></td>
<td></td>
<td>5. Need for monitoring under specialized medical supervision</td>
<td></td>
</tr>
<tr>
<td>Benznidazole N-benzyl-2</td>
<td>Covalent modification of macromolecules by nitroreduction intermediates</td>
<td>5 mg/kg/day 30–60 days</td>
<td>Reactivated chronic disease, such as in immuno-compromised patients,</td>
<td>BNZ, Roche has transferred rights and technology for BNZ to</td>
<td></td>
</tr>
<tr>
<td>-nitroimidazolylacetamide</td>
<td></td>
<td>treatment can last 5 months or longer</td>
<td>treatment can last 5 months or longer</td>
<td>Brazilian government in 2007, making Pharmaceutical Laboratory of Pernambuco the drug sole manufacturer in world (with help of DNDI)</td>
<td></td>
</tr>
</tbody>
</table>
preclinical models of mice infected with BNZ-resistant strains (see below).

Bisphosphonates (BP) are biological analogues of naturally occurring pyrophosphates (P–O–P) where the oxygen atom is substituted by a carbon one (P–C–P). Amino BP (aBP) are novel and more potent nitrogen-free BP [45]. T. cruzi contains massive amounts of pyrophosphate and polyphosphates stored in an acidic organelle named the acidocalcisome [46,47]. aBP inhibits parasite growth upon inhibition of protein prenylation (at the level of FPPS) [48]. In a pioneering study, Urbina et al. showed that seven daily doses of pamidronate disodium (IV, 10 mg/kg/day) on NMRI albino mice infected with the Y strain led to a reduction of parasitemia, hypothesizing that the presence of the calcium- and pyrophosphate-rich acidocalcisomes should be responsible for the selective target of aBP to the parasites in vivo [49]. In 2004, Garzoni et al. evaluated seven daily doses of risedronate (50-fold higher anti-osteoclastic activity but with a lower anti-amastigote activity than pamidronate) on mice infected with the Y strain. A pronounced decrease in the parasitemia (>90%) and an increased survival of treated animals was found [50]. Higher doses (up to 10 mg/kg/day) led to further reductions in parasitemia and less mortality. Bouzahzah et al. explored the effect of lower doses of risedronate (SC, 25–40 μg/kg, 3 times a week) on CD 1 mice infected with the Brazil strain over 40 days. Parasitemia levels decreased though the myocardial pathology and the right ventricular dilation remained unchanged and the disease was not eradicated. When administered over 25 days (SC, 50–100 μg/kg, 3 times a week) on C57 black C/mice infected with the Tulahuen strain, no change in mortality was apparent [51]. Based on the Food and Drug Administration approval and the current commercialization of aBP, these preclinical studies sufficiently supported the use of aBP in Chagas disease. However, recently, serious acute complications, such as ocular or systemic inflammatory reactions, electrolyte imbalance, nephrotic syndrome and renal failure were associated to IV administration of aBP. In 2003, BP-associated osteonecrosis of the jaws (BRONJ) and avascular necrosis of the hip (ANH) were described as the first long-term adverse effects associated to IV aBP pharmacotherapy. Moreover, they appear several months or years after therapy discontinuation. In view of the hundreds of cases reported worldwide [52–54], BRONJ and ANH are regarded as potentially important, long-term skeletal complications of IV aBP [45,55]. Thus, the management demands the intervention of a solid health system, not always available in constrained-setting populations. Finally, the trypanocidal activity of aBP has only been evaluated on the acute stage of infected mice after the parenteral administration of several doses. The difficult oral absorption together with the relatively high IC50 for amastigotes [56] suggests that high IV doses should be administered in humans to achieve therapeutic concentrations. Having expressed this, the absence of successful preclinical results at lower aBP doses, such as this resultant from oral intake, makes uncertain the true cost/benefit of IV aBP for Chagas patients.

In this complex scenario, the main challenges in the pharmacotherapy of Chagas are (i) the reduction of the frequency of administration to improve compliance and adherence, (ii) the use of lower doses to prevent adverse effects and (iii) a more precise and accurate diagnosis.

Even when the physical definition of Nanotechnology [57] limits the upper size of the nano-objects to the 100 nm, in a biological context the new properties of nano-objects remain at least up to a size of 200–300 nm. Their high area/volume ratio is responsible for their ability to surpass anatomical and phenomenological barriers such as the gastrointestinal tract, skin and blood–brain barrier [58]. Nanomedicine is “the employment of nano-objects to execute actions in Medicine” [59]. The last two decades have witnessed the first nanoDDS to enter clinical trial and, in some cases, routine clinical use (reviewed in [60]). They include antibody conjugates (e.g. Mylotarg R®, Tositumomab® or Zevalin®) [61–63], liposomes (e.g. DaunoXome™ or Doxil®/Caelyx®) [64], the first anticancer nanoparticle, Abraxane® [65] and polymer therapeutics. The latter include polymeric drugs, polymer–protein and polymer–drug conjugates [66,67]. In the following sections, the different strategies aiming to improve the pharmacotherapy of Chagas disease will be discussed.

4. NanoDDS-based trypanocidal therapy: higher accessibility + improved selectivity + delivery of active principle to intracellular targets

The main challenge of Chagas disease pharmacotherapy is to reach the disseminated intracellular parasites. In viral, intracellular bacterial and protozoan infections, there are present physical barriers that
<table>
<thead>
<tr>
<th>Drug</th>
<th>Via and dose</th>
<th>Main results</th>
<th>Problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioridazina</td>
<td>Oral 80 mg/kg day for 3 days</td>
<td>Reduce parasitemia, increase survival and prevent cardiac damage in murine acute model [41,42]</td>
<td>No parasitological cures were obtained and the selectivity of the drug action against the parasite has not been demonstrated</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>Oral 6 mg/kg day for 120 days</td>
<td>Markedly reduced the number of positive xenodiagnostic tests and was able to regress (50% of the cases) or prevent (97.8% of the cases) ECG abnormalities [138]</td>
<td>Treated patients maintained positive serology after a 9 year follow-up, indicating that no parasitological cures were achieved according to accepted standards [12]</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>Oral 20 mg/kg day, into two daily doses</td>
<td>Parasitological cure both acute and chronic murine models. Eradicate nitrofuran and nitroimidazole-resistant T. cruzi strains from infected mice, even if the hosts were immunosuppressed [139]</td>
<td></td>
</tr>
<tr>
<td>D0870</td>
<td>Oral N10 mg/kg/ day 20 doses</td>
<td>Parasitological cured both acute and chronic murine models. Eradicate nitrofuran and nitroimidazole-resistant T. cruzi strains from infected mice, even if the hosts were immunosuppressed [140,94]</td>
<td>Discontinued Zeneca Pharmaceuticals</td>
</tr>
<tr>
<td>UR-9825</td>
<td>Oral N10 mg/kg/ day 43 doses</td>
<td>Cured in a canine model with established infections of the virulent Y strain with very low toxicity [141]</td>
<td>Drug resistance was encountered with the Berenice-78 strain [43]</td>
</tr>
<tr>
<td>TAK-187</td>
<td>Oral 20 mg/kg/day 20 doses</td>
<td>Parasitological cured both acute and chronic murine models, even when the infecting strain is nitrofuran and nitroimidazole-resistant [142]. Superior to BNZ in preventing cardiac damage in murine model [143]</td>
<td></td>
</tr>
<tr>
<td>Ravuconazole</td>
<td>Oral 10 mg/kg/day, twice a day</td>
<td>Very active in vitro and in vivo against both acute and chronic murine models; Eradicate nitrofuran and nitroimidazole-resistant T. cruzi strains from infected mice, even if the hosts were immunosuppressed [139]</td>
<td></td>
</tr>
<tr>
<td>E7700 and ER 11884</td>
<td>Oral 50 mg/kg/day</td>
<td>Active in vitro and in vivo against extracellular epimastigotes and intracellular amastigotes; Significant selective toxicity against T. cruzi with minimal toxicity against mammalian cells [17]</td>
<td></td>
</tr>
<tr>
<td>Squalene synthase</td>
<td>E5700 and ER-119884 Oral 50 mg/kg/day</td>
<td>Active in vitro against extracellular epimastigotes and intracellular amastigotes; Significant selective toxicity against T. cruzi with minimal toxicity against mammalian cells [17]</td>
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</table>

E7700 provides full protection against death and parasitism in murine model [17].
<table>
<thead>
<tr>
<th>General target</th>
<th>Particular target</th>
<th>Drug</th>
<th>Via and dose</th>
<th>Main results</th>
<th>Problems</th>
<th>Other uses and companies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidosqualene cyclase (OSC)</td>
<td>OSC inhibitors</td>
<td>Potent and selective in vitro antiparasitic activities [35]</td>
<td>No evidence of in vivo activity</td>
<td></td>
<td></td>
<td>antiarrhythmic drug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In vitro and in vivo activity by disruption of Ca^{2+} homeostasis and blockade of de novo ergosterol biosynthesis at the level of OSC [144,145]</td>
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<tr>
<td></td>
<td>amiodarone</td>
<td></td>
<td>Hepatotoxicity and problems with the manufacture process in 2005 [38]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine Protease Inhibitors</td>
<td>Cruzipain</td>
<td>K-777</td>
<td>Reduce the parasitemia levels and prolong survival in acute and chronic murine models, with minimal toxicity [146]</td>
<td>No in vivo data were provided</td>
<td></td>
<td>Medivir UK Ltd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>substituted amides and 2-acycloamino-bycyclic ketone derivatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrophosphate Metabolism Inhibitors</td>
<td>Farnesylpyrophosphate synthase (FPPS)</td>
<td>Risedronate</td>
<td>In vitro and in vivo in acute model induced &gt;90% reductions in parasitaemia and increased survival. Higher doses (up to 10 mg/kg per day) led to further reductions in parasitaemia and mortality. No relapse of parasitaemia. Almost complete disappearance of amastigote nests in the hearts of treated animals [50,147].</td>
<td>No parasitological cures were observed in infected animals that received the bisphosphonate, probably due to the short treatment period [50]</td>
<td></td>
<td>Actonel, P&amp;G, marketed for the treatment of osteoporosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pamidronate</td>
<td></td>
<td>No cures were reported in a acute murine model [50]</td>
<td></td>
<td>Aredia, Novartis treatment of hypercalcemia associated with some types of cancer.</td>
</tr>
<tr>
<td>Inhibitors of purine salvage</td>
<td>Hypoxanthine-guanine phosphoribosyl transferase</td>
<td>Allopurinol</td>
<td>Activity both in vitro and in vivo [148]</td>
<td>Marked differences in susceptibilities among different T. cruzi strains [146] Conflicting reports of the therapeutic efficacy in humans. An early report from Brazil indicated its ineffectiveness in acute Chagas disease patients [150], a finding confirmed by a multicentric study in chronic patients launched in 1992 in Argentina, Brazil and Bolivia, which was stopped as it was unable to control parasitemia in treated patients [151]. In contrast, Apt et al. [152] found that allopurinol induced disappearance of positive xenodiagnosis tests in a high percentage of chronic patients in Chile and was able to reverse (in 49% of the cases) or prevent (75% of the cases) the development of ECG abnormalities after a 9 year follow-up [40]</td>
<td></td>
<td>Roche, treatment of osteoporosis</td>
</tr>
</tbody>
</table>


impair the access of sufficiently high drug amounts [68,69]. In Chagas disease, the plasma membrane and the complex microenvironment of the host cells spoil the selective and massive delivery of trypanocidal agents to the amastigotes nests [70–72]. On the other hand, selectivity is a main goal if therapeutic doses are also toxic. Due to the disseminated nature of the disease (T. cruzi) parasites are distributed in mucous membranes, cardiac, skeletal and smooth muscle, and glial cells, the ideal anti-Chagas drug should display a high volume of distribution (Vd) and a long half-life time; this drug would be effective during both the indeterminate and chronic phase as well as in the acute phase [3]. BNZ and NF show high Vd, though the impaired access to the intracellular targets results in high plasma concentrations and toxicity (Table 2). The ability to modify the surface to target cells and tissues and the uptake by pin or phagocytic mechanisms confers nanoDDS the ability to overcome intracellular barriers and to massively deliver trypanocidal drugs into an extremely small volume, reducing their concentration in blood circulation and non-targeted tissues.

Even though nanoDDS can easily cross any anatomical barriers, those made of biodegradable polymers are degraded when administered by the oral route [73,58]. Only the M cells of the Peyer’s patches at the small intestine can uptake particulate material from the intestinal lumen and send it by transcytosis across the basolateral pocket to the inner dome of the patch. Such pathway however, is not suitable for the intake of an important amount of nanoDDS, since the M uptake is a natural via of entrance for microorganisms [74]. The transcytosed particles are delivered to antigen-presenting cells that translocate to the regional lymph node to unravel a protecting immune response. There are not clear data on the uptake of particulate material by enterocytes, nor on their capacity to perform transcytosis and further delivery to the blood [72]. The paracellular route is followed by certain generations of dendrimers [75–77] but it is uncertain whether the dendrimer structure is maintained upon crossing. Biostable nanoDDS are associated to toxic effects that could hurdle the approval by regulatory authorities [78]. Also, they have been disregarded on public statements by pharmaceutical companies recently interested in the development and commercialization of nanoDDS. Thus, the only way to directly access the blood stream is using injectables [79]. Then, the nanoDDS structure will govern the specific cellular uptake mechanism and the intracellular pathway [80,81].

The design of effective trypanocidal-loaded nanoDDS against different T. cruzi forms needs to take in consideration that only mammalian host cells and epimastigotes present uptake mechanisms [82]; neither amastigotes nor trypomastigotes can take up nanoDDS. In addition, it is worth reminding that both the asymptomatic indeterminate and the chronic stages are caused by the resident amastigotes. Therefore, the circulating and accessible trypanomastigotes characteristic of the acute stage and the intracellular amastigotes (indirectly and restrictedly accessible for nanoDDS) are two potential targets (Fig. 2).

4.1. Stearylamine liposomes

The first study employing nanoDDS as antichagasic agents was published in 1987 and comprised the incorporation of stearylamine (SA) in phosphatidylcholine liposomes [83]. Epimastigotes, trypomastigotes and amastigotes (harvested from culture supernatant of T. cruzi Tulahuen strain-infected HeLa cells) incubated with SA-liposomes (15% mol SA, 7.5 mM total lipids) at 28 and 37 °C, respectively, were rapidly killed. The time required to kill 50% of all the parasite forms was inversely proportional to the surface negative charge of each parasite stage [84–86]. In addition, the trypomastigote death rate was higher as the liposomal concentration (15 mol% SA 10, 20, 50 and 1000 μM)
and %SA (up to 25% SA) increased. Overall results indicated that a key factor governing the susceptibility of the parasites for SA-liposomes was the surface negative charge, the trypomastigotes being about 8- and 26-fold more susceptible than amastigotes and epimastigotes, respectively. Finally, when the hemocompatibility of 100 μM, 15% SA-liposomes was tested on 10% human erythrocytes, less than 1% haemolysis was found after 4 h. Circulating trypomastigotes are suitable targets during the short window time of the acute stage in humans (5–20 days), though clinical detection (e.g., Roman’s sign) is possible in only 1–2% of the cases [3]. The short half-life of SA-liposomes in blood is a main limitation against trypomastigotes. Moreover, to reach the intracellular amastigotes in vivo, SA-liposomes should target the inflammation zones and escape the endolysosomal pathway after the uptake towards the cytoplasm. SA-liposomes lack this property.

4.2. Polyalkylcyanoacrylate (PACA) nanospheres

4.2.1. NF-loaded nanospheres (NF–NS)

Only a decade later, a new study on the nanoencapsulation of a trypanocidal drug was published. Gonzalez-Martín et al. prepared NF-loaded polyalkylcyanoacrylate nanoparticles (NF–NS) by the emulsion polymerization technique [87]. The encapsulation efficiency was 33.4% (8.2 ± 2.1 μg NF/mg NF–NS) and the size was ~200 nm. The trypanocidal activity was evaluated against epimastigotes isolated from a North Chilean patient suffering the chronic stage of the disease. At low concentrations (<2 μg/mL NF corresponding to ~0.002% NS), a 100% anti-epimastigote activity was found, whereas the drug-free nanocarriers (NS) and free NF showed ~50% activity. Higher NF–NS concentrations (5 μg/mL NF) showed the same activity of NS and NF (~90%). Similar results were observed in metacyclic form-infected Vero cells; at low and intermediate NF concentrations (1 and 10 μg/mL), NF–NS showed ~92% activity, whereas NS and NF had 66% and 24.8% activity, respectively. Again, higher NF–NS concentration showed the same anti-amastigote activity of NS and NF. It remains unclear if the anti-epimastigote activity could be ascribed to a superficial interaction or to the NS–NF uptake by the parasites. Epimastigotes though capable of endocytosis, are not the parasite form found in human hosts. Thus, activities other than superficial interaction against trypomastigotes or lacking the ability to deliver the drug to intracellular amastigotes would probably be poorly successful. On the other hand, 65.4% NF was released from NF–NS upon 6 h incubation at pH 7.4. Since the in vitro assays were conducted over 72 h (enough time for the total release of NF from the carrier), the trypanocidal activity was probably a contribution of the intrinsic NS toxicity and the activity of the released NF. The study with carrier), the trypanocidal activity was probably a contribution of the NS and NF (~90%). Similar results were observed in metacyclic form-infected Vero cells; at low and intermediate NF concentrations (1 and 10 μg/mL), NF–NS showed ~92% activity, whereas NS and NF had 66% and 24.8% activity, respectively. Again, higher NF–NS concentration showed the same anti-amastigote activity of NS and NF. It remains unclear if the anti-epimastigote activity could be ascribed to a superficial interaction or to the NS–NF uptake by the parasites. Epimastigotes though capable of endocytosis, are not the parasite form found in human hosts. Thus, activities other than superficial interaction against trypomastigotes or lacking the ability to deliver the drug to intracellular amastigotes would probably be poorly successful. On the other hand, 65.4% NF was released from NF–NS upon 6 h incubation at pH 7.4. Since the in vitro assays were conducted over 72 h (enough time for the total release of NF from the carrier), the trypanocidal activity was probably a contribution of the intrinsic NS toxicity and the activity of the released NF. The study with

4.2.2. Allopurinol-loaded nanospheres (ALL–NS)

High allopurinol (ALL) doses (300 and 600 mg/day [19–21], respectively) are required to decrease parasitemia levels between 45 and 85%. Aiming to reduce the effective therapeutic dose, the use of ALL–NS (187 nm, 62.8 μg ALL/mg NS) was proposed in 2000 [89]. The in vitro pan-trypanocidal effect of ALL–NS was determined on epimastigotes isolated from a chronic Chagas patient. Upon 72 h incubation, 16.7 μg/mL ALL (corresponding to 265 μg/mL NS) killed 91.5% of the parasites. However, drug-free NS and free ALL presented 87.2 and 45.9% activity, respectively. The work disregarded the intrinsic cytotoxicity of NS on Vero cells (>25% upon 24 h incubation). Also, the anti-epimastigote activity was determined after 72 h, regardless the fact that 7.4% ALL was released from NS after 6 h. The high intrinsic toxicity of PACA on both parasites and cells together with the fast drug release raises serious doubts about the feasibility of employing PACA NS as carriers for trypanocidal agents. Besides, due to their fast degrada
dation in vivo PACA NS are not suitable as sustained release depots [90,91]. Moreover, NS are aimed for IV administration and from blood circulation they should be uptaken by clathrin-mediated endocytosis to follow a lysosomotropic pathway. In this framework, the success against in vivo trypomastigotes is doubtful; trypomastigotes lack endocytotic capacity and are only present during the short and often asymptomatic period of parasitemia.

4.3. Poly(ethyleneglycol)–co-poly(lactic acid) nanoparticles

To minimize opsonization and RES uptake and passively target the diseased tissues and act as a depot intracranial (IT), ketokonazole (KET) and the fourth generation bis-triazole D0870 [92] were separately loaded in poly(ethyleneglycol)–co-poly(lactide) nanospheres (IT–TET, D0870–PLA-PEG5000 NS) prepared by the simple emulsification method and displaying monodisperse sizes between 100 and 200 nm [93]. Swiss albino female mice were ip infected with two trypanosome strains: (i) a nitroimidazoles/nitrofurans-susceptible and (ii) a partially resistant Y strain. IT, KET, D0870 and BNZ were daily administered by the oral route (5 mg/kg/day 4 days post infection) and continued for 30 days, whereas IT–TET, and D0870–PLA-PEG5000 NS were IV administered (1.5–3 mg/kg/day) over the same time period. PLA-PEG5000 NS did not show any deleterious effect against trypanosome. Thirty IV D0870–PLA-PEG5000 (3 mg/kg) performed similarly to free D0870 (30 oral doses, 5 mg/kg) on the susceptible strain, the cure percentages being 90 and 80%, respectively. In the case of the virulent Y strain, D0870–PLA-PEG5000 NS (60% cure) were more effective than IT–TET–PLA-PEG5000 NS (0% cure) and 30 daily oral doses of free BNZ (100 mg/kg) that led to 47% cure. However, 20 oral doses of D0870 (5 mg/kg/day) in the same animal model produced 4/4 animals/survivors [94].

The activity of KET and IT against intracellular T. cruzi amastigotes is comparable to that of the new triazoles, but they are unable to eradicate the parasite from infected patients or experimental animals [95–97], even if the infecting strain is susceptible to nitrofurans and nitroimidazoles. This behavior was ascribed to the relatively short half-lives (e.g., 6–9 h for KET in humans [98]). Encapsulation in PLA-PEG5000 NS could protect them from degradation. However, KET and IT did not show activity against the Y strain in vivo probably due to a fast release form the nanocarrier; the authors however did not test the structural stability of any of the drug in PLA-PEG5000 NS. On the other hand, D0870 has a remarkable trypanocidal activity in vivo [99,100]. Theoretically, D0780 fulfills the pharmacokinetic specifications of an ideal trypanocidal agent [38]. D0870 induced parasitological cure in both acute and chronic in murine models of Chagas disease and also cured the acute infection caused by NF- and BNZ-resistant strains [94].
In an acute model, 20 oral doses (≥ 10 mg/kg) resulted in 80–100% protection against death for up to 110 days in seven different strains. It was also effective (20 mg/kg) in immunosuppressed animals infected with both susceptible and highly resistant strains, but the rate of cure decreased with intermediate resistant strains. When administered in a chronic model (20 mg/kg/day), the survival was similar to that of oral BNZ (100 mg/kg/day) [94]. Its activity was similar to that of POS when orally administered (20 mg/kg/day) to chronic models, inducing 50–60% parasitological cure, independently of the strain.

In the framework of a strategic design, nanoDDS systems should display improved performances, if possible far beyond those already available with the conventional technology. The development of D0870 by Astra Zeneca was finished in 1995 due to a cardiac adverse event in one HIV-positive patient and the propensity to QT prolongation at modest serum concentrations [101]. Depot formulations (e.g., PLA-PEG<sub>5000</sub> NS) are limited to the release of free D0870 and the intrinsic toxicity of the drug, if not avoided, could be reduced. On the other hand, D0870 and POS had comparable activity on the chronic stage. Thus, an interesting research avenue could have been the evaluation of POS-PLA-PEG<sub>5000</sub> NS on a chronic model infected with NF- and BNZ-resistant strains. However, POS is reasonably effective upon a relatively high number of oral doses. Thus, the only reason that justifies the development of an injectable depot is envisioning that D0870/POS-PLA-PEG<sub>5000</sub> NS could reduce the therapeutic dose to a single administration, in the absence of toxicity. This work clearly showed that it was not possible to achieve such goals.

4.4. Lipid formulations of amphotericin B

In 1999, the activities of Fungizone™ and of other lipid formulations of the macroalide antimycotic Amphotericine B (AMB) (Amphoticil™, Abelcet® and Ambisome®) both in vitro and against an acute murine model infected with the Y and the Thulahuuen strains were tested for the first time [102].

When tested against trypomastigotes of the Y strain upon 24 h incubation, Fungizone showed the highest activity; MIC values were between 0.3 and 0.1 μg/mL at 37 and 4 °C, respectively, followed by Amphocil with a MIC between 1 and 0.5 μg/mL. Abelcet and Ambisome did not show activity up to a 30 μg/mL concentration and 24 h incubation; Abelcet was active upon 48 h (MIC 0.3 and 10 μg/mL, at 37 and 4 °C), but Ambisome and free BNZ were not.

When tested against amastigotes infecting murine peritoneal macrophages, Fungizone and Amphocil showed the highest activities. IC<sub>50</sub> were between 0.027 and 0.028 μg/mL, followed by Ambisome, Abelcet and BNZ, with IC<sub>50</sub> of 0.19, 1.2 and 1.43 μg/mL, respectively. On amastigote-infected Vero cells, Fungizone, Amphocil, Ambisome, Abelcet and BNZ IC<sub>50</sub> increased to 2, 4.1, 3.6, 2.3 and 4.2 μg/mL, respectively. Microscopic examination of macrophages showed that Ambisome caused no cell damage at 3 μg/mL, whereas at the same AMB concentration, the remaining lipid formulations obliterated the cells. The effect of multiple and single doses on Balb/c mice infected with Y strain was also evaluated. When 6 IV Fungizone (0.5 mg AMB/kg, maximal tolerated dose) doses were administered as bolus on alternate days 4 out of 5 animals survived. In contrast, Ambisome (12.5 mg AMB/kg) resulted in 100% survival at day 60 post infection. Control mice died at day 18. The fact that parasites were found in blood 3 weeks post-treatment indicated that Ambisome does not lead to the total eradication of the parasite. Multiple Ambisome doses, though at lower concentration (6.25 mg AMB/kg), administered to Tulahuen strain-infected animals were sufficient to induce survival of all the infected mice until day 60. This multiple doses regimen did not show a correlation between survival and dose level. A single dose of Ambisome (25 mg AMB/kg) induced the survival of 5/5 mice, whereas 5 consecutive dosis of BNZ at 45 mg/kg induced the survival of 3/5 at the end of 60 days (lower doses failed). Abelcet and Amphocil at 25 mg AMB/kg induced 3/5 survivals, whereas the controls died at day 11. In vitro infections caused by lower parasite inoculums, Ambisome (25 and 5 mg AMB/kg) induced 5/5 survivals at day 60, whereas 0.2 and 1 mg/kg induced 1/5 and 2/5 survivals, respectively. Finally, Abelcet and Amphocil 25 mg AMB/kg induced 3/5 survivals. In general terms, in vivo Ambisome was more effective and less toxic than other lipid AMB formulations, despite of a delayed elimination of blood parasites when compared to BNZ (3 vs. 1 week). On the other hand, the activities on trypomastigotes and intracellular amastigote-infected macrophages and Vero cells were Fungizone-Amphocil (both mixed micelles of AMB and surfactant)-Ambisome (AMB intercalated with phospholipid and sterol in a unilamellar liposome)-Abelcet (AMB intercalated with sheets of phospholipid). The stronger activity on infected macrophages was inversely related to the size of the lipid particles. Finally, non-phagocytic Vero cells should only uptake the AMB released from Fungizone and Amphocil and trypanomastigotes should only be sensitive to the AMB released from any formulation.

This work is a clear example of nanotechnology as a tool to improve the performance of an already approved drug. AMB as Fungizone cannot be used in vivo as anti-T. cruzi agent due to its high toxicity [103], while the same active agent loaded in a lipidic nanocarrier (Ambisome) is less toxic and a good therapeutic alternative [104].

A crucial difference between the use of Ambisome against leishmaniasis (a parasite that colonizes the phago-lysosomal system in cells of the RES) and against Chagas is that in the latter, even if properly biodistributed, the intracellular pathway followed by AMB could not be appropriate. It is possible that once inside the target cell, the hydrophobic nature of the AMB impairs its diffusion from the endo/phago-lysosomal confinement. The importance of this fact remains invisible for nanodDS directed against Leishmania parasites, because the endo/phago-lysosomal system is the natural pathway followed by any nanoparticulate agent entering by clathrin-dependent endocytosis. In Chagas disease however, the right intracellular traffic followed by the nanoDDS is a key for therapeutic success.

4.5. Conventional and pH-sensitive liposomes loaded with nitroimidazoles

Aiming to increase the efficacy of BNZ as trypanocidal agent by means of the modification of its pharmacokinetics and biodistribution, our research group developed a multilamellar lipidosomal formulation of BNZ (hydrogenated phosphatidylcholine from soybean (HSCP); Cholesterol (Chol);distearoyl-phosphatidylglycerol (DSPG) (molar ratio 2:2:1) at 0.7% w/w BNZ/liposomal lipid (MLV, multilamellar vesicles) [105]. When IV administered in rats as bolus (0.2 mg BNZ/kg), a 3-fold higher BNZ accumulation in the liver than the free drug was found. However, liposomal BNZ (IV, 0.4 mg/kg, twice-a-week from day 5 to day 22 post infection) did not decrease parasitemia levels in mice infected with the RA strain. These results indicated that the relationship between the increased selectivity of the nanoDDS for a given tissue and the therapeutic effect is not always straightforward.

We determined that both the aqueous and the hydrophobic components of liposomal BNZ remained entrapped within lysosomes after phagocytosis. Thus, factors such as the intracellular pathway followed by the BNZ upon MLV uptake should be strongly responsible for this low trypanocidal activity [106].

pH-sensitive liposomes experience a phase transition from bilayer to inverted hexagonal phase II that is triggered by a pH decrease (Fig. 3). Due to its hydrophobic nature, BNZ loaded in pH-sensitive matrices remains partitioned in the lipidic phase, instead of being released to the aqueous medium. Based on these preliminary results, etanidazole (ETZ)-pH-sensitive liposomes made of dioleoyl-phosphatidylethanolamine:cholesteryl hemisuccinate (DOPE:CHEMS, 6:4, mol:mol), (~400 nm) were prepared [107]. ETZ is hydrophilic and dissolves in the inner aqueous phase. This formulation ensured a fast and massive delivery of the ETZ to the cytosol of murine J774 macrophages.
ETZ-loaded nanoDDS were phagocytosed by both uninfected and *T. cruzi*-infected macrophages. Also, a 200 μg/mL ETZ dose showed 72% anti-amastigote activity in J774 cells after 2 h. Contrary to this, the same dose of free ETZ rendered 0% activity. IV administration of ETZ nanoDDS (0.56 mg ETZ/kg, starting 5 day post infection, three days-a-week over 3 weeks) resulted in a significant decrease in parasitemia (days 12, 19, 21 and 23 p < 0.05) of Balb/c mice infected with 50 trypomastigotes of the RA strain. Administration of a 180-fold higher dose of free ETZ failed to reduce the number trypomastigotes in blood. Previous *in vitro* determinations of free ETZ trypanocidal activity showed that on RA trypomastigotes, the LD<sub>50</sub> was 18 μM (8.2-fold less active than BNZ). In the case of amastigote-infected Vero cells, IC<sub>50</sub> for ETZ was 40 μM (23.5-fold less active than BNZ). Finally, on amastigotes infecting J774 cells, 15-fold lower activity than BNZ was found [108]. According to the classical interpretation, these *in vitro* data should rule out the ETZ as trypanocidal agent. However, we showed that this poor performance could be rescued without modifying its chemical structure just by encapsulating the drug in pH-sensitive liposomes. The example of ETZ, that *in vivo* proved to be less toxic than BNZ, could be extensive to other trypanocidal drugs [107]. Mice infected with 10<sup>2</sup> trypomastigotes (Tulahuen strain) and treated with liposomal ETZ at 3.2 mg ETZ/mouse led to the complete elimination of parasitemia (p < 0.05). The administration of an equivalent dose of empty nanoDDS (293 μg liposomal lipid/mouse) or free ETZ at 3.2 mg ETZ/mouse had no effect on parasitemia (unpublished results). The ETZ nanoDDS therapy however, remains to be tested against higher infecting inoculums from different *T. cruzi* strains, both in the acute and the indeterminate and chronic stages of the disease.

5. NanoDDS: targeting to the heart in Chagas disease?

Chagas cardiomyopathy is essentially a myocarditis and the inflammatory process, although more conspicuous in the acute phase, is clinically silent but gradual in patients with the indeterminate and chronic phases of the disease. Inflammation becomes mild and focal and undergoes cyclic changes leading to complete resolution. However, the process is maintained due to the disappearance of old focal lesions and the resurgence of new ones. This equilibrium allows a prolonged host survival in the absence of symptoms or signs. Chronic chagasic cardiomyopathy (CCCM) is a dilated cardiomyopathy [109] accompanied by acute and chronic inflammation, fibrosis, and vasculitis. The mechanism by which this persistent inflammatory reaction occurs is not clear and it is found only in about 25% of *T. cruzi*-infected individuals. Several reports have recently demonstrated the role of autoimmune responses in experimental models of chronic chagasic myocarditis [110]. However, although the intensities of tissue parasitism and inflammation do not have a direct correlation, parasite persistence is probably required for disease development and maintenance [111].

Only a few articles reported on the delivery of nanoparticulate material to the myocardium. Since the success of nanoDDS-based strategies depends on the targeting ability taking place when nanoDDS leak from circulation to a defined micro or macrosite in the body, the targeted gene delivery has to be achieved employing highly invasive techniques [112–114]. Still under development, a non-invasive technology is the ultrasound-targeted micro-bubble destruction (UTMD) [115]. The UTMD consists of the destruction of gas-filled...
microbubbles with an albumin or phospholipid shell IV administered, by means of the application of local ultrasound. Micro-bubble destruction causes the rupture of the endothelial capillary wall, a phenomenon leading to increased local leakage of nanoparticulate material up to 100 nm diameter [116], namely naked plasmid DNA or viral vectors [117,118]. However, in this technique the heart is exposed to high US peak pressure and high concentration of microbubbles for a prolonged period of time and to the induction of significant bio-effects such as transient tissue damage, microvascular rupture, premature ventricular contraction and left ventricle dysfunction. Thus, the benefits of delivering trypanocidal drugs in the case of patients suffering Chagas disease must be carefully evaluated.

Another alternative to target the myocardium could be passive targeting. It relies on the local increase of vascular permeability. Unfortunately, in the Chagas-infected heart, amastigote nests (which are very difficult to be found due to their scarce number) are poorly correlated to extensive vasculature damage or cardiomyocyte death [119,120]. The vascular damage is initially caused by the invasion of the endothelial cells by the parasite and the over-expression of cell adhesion molecules that leads to platelet aggregation and thrombus formation. The destruction of vascular endothelium causes hyperplasia of the intima smooth layer and thickening of its basement membrane (BM) [121]. These changes result in micro spasms, impaired irrigation and tissue hypoxia [122,123], and have been postulated to be causative mechanisms of CCMC [124,125].

Upon the silent transition from indeterminate to chronic phase, the tissue damage is more extensive and the destruction of vital structures required for conduction leads to irreversible cardiac dysfunction. Paradoxically, although the indeterminate stage should be ideal for eliciting a nanoDDS therapy, the vascular damage and the more pronounced extravasation is associated to the chronic stage [126]. Until now, there are no data on the feasibility of employing the vascular damage associated to the indeterminate or chronic stages to favour the extravasation of nanoDDS to cardiomyocytes. Available data on the flow of injected 99mTc-labelled microspheres in the left ventricle show the existence of perfusion defects [127].

In sum, to passively target infected cardiomyocytes or, at least, their neighbourhoods, nanoDDS must first extravasate. The loss of filtering/sieving function of the basal membrane could enable the access of nanoDDS to the sarcolemma. Then, cardiomyocytes should recognize and uptake the nanoDDS by means of clathrin or caveolin-dependent endocytosis and follow an appropriate pathway towards cytoplasmic amastigote nests. A potential drawback associated with the use of the clathrin route has aroused from recent in vitro studies, where T. cruzi-infected cardiomyocytes were employed to show that the parasite induces a decrease of the clathrin-dependent endocytic activity [128]. On the other hand, cardiomyocytes can also perform caveolin (3)-dependent endocytosis [129], a pathway that does not end up in lysosomes and allows the escape to the cytoplasm upon endoplasmic reticulum transposition. A recent study showed the delivery to caveolea of rat lung endothelium with aminopeptidase P antibody specifically targeted nanoparticles [130]. The caveolea then operated as a pump, transporting the antibody-conjugated nanoparticles from the blood across the endothelium into the lung tissue. Approximately 80% injected dose/g of tissue was uptaken within 30 min, with minimal uptake by other tissues. This new approach opens a new window for improving the tissue targeting of nanoparticles to the heart.

6. Challenges in nanomedicine and Chagas in developing countries

The restriction of the Chagas disease to LA, together with a poor know-how of the new possibilities offered by nanoDDS-based therapies could explain the limited number of local scientists addressing the challenges of this complex disease by means of nano-technology. The challenges and possibilities could be summarized as follow:

(i) Evaluation of passive targeting during the acute stage; important inflammation plus increased vascular permeability mostly in RES organs colonized by amastigotes, allows for passive targeting of IV injected nanoDDS.
(ii) Existence of cell uptake mechanisms both phagocytic and non-phagocytic employing clathrin-caveolin or non-clathrin and non-caveolin mediated mechanisms.
(iii) Availability of pH-sensitive nanoDDS to ensure cytoplasmic delivery to amastigote nests; pH-sensitive liposomes are now being designed to deliver macromolecular therapeutics into the cytosol [131]. Also, second-generation polymeric carriers that are pH-sensitive in the main chain (PEG-polyacets [132]) can be advantageous as they undergo degradation at the lower pH of the endosomes and lysosomes.
(iv) Availability of nanoDDS with enormous superficial activity capable of establishing strong interactions with cell membranes. For instance, cationic dendrimers could be used in replacement of the toxic SA-liposomes in order to defeat circulating trypomastigotes (even though this should not be the ideal therapeutic target) [133]
(v) The passive targeting of nanoDDS to the heart at the indeterminate/chronic stages is a key pending issue.

Preclinical nanoDDS-based therapeutic strategies must be faced after the full characterization of the drug-loaded nanoDDS structure (e.g., size and ζ potential) and its structural stability. Also, a suitable selection of the active agent must be done. Scalable and validated methods should be used to prepare the nanoDDS [134]. Cell culture assays are suitable tools to predict cytotoxicity and intracellular traffic of nanoDDS [135]. Toxicity studies both in mono and multiple doses will be relevant in the search for immune reactions otherwise invisible in vitro or as single bolus (case reference: Doxil® [136]). Finally, any preclinical development must be unavoidably checked for its in vivo efficacy, since only in vivo the anatomo and phenomenological barriers interposed to the AP will become evident [137]. In the future, more friendly administration routes must be found to improve patient compliance.

Even though considered as a nanoDDS, it is unlikely that lipid cochleates loaded with AMB (Bioral® AMB) could be absorbed without being destroyed along the gastrointestinal transit. Only an increased bioavailability of AMB could be achieved. This should not prevent the systemic toxicity of AMB, though at least would discard the use of injectables. The first research collaboration and licensing agreement between the DNDi and BioDelivery Sciences (www.biodeliverysciences.com Raleigh, North Carolina) for the development of a clinical program to assess the efficacy and safety of Bioral® AMB for the treatment of neglected diseases like leishmaniasis and Chagas disease is highly auspicious.

Finally, the development of suitable areas of nanotechnology is strategic for each nation and is directly linked to state policies. Until now, at least in Argentina, a plan to develop and consolidate a nanomedical platform on nanoDDS is absent and the use of nanotechnology to defeat infectious diseases is not considered a priority. Hopefully this review will encourage both the endemic states and the academia to address the challenge of using nanotechnology against Chagas, the hidden disease.

References


