



Nanocarriers for effective delivery of benznidazole and nifurtimox in the treatment of chagas disease: A review

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ABSTRACT

Neglected tropical diseases (NTDs) constitute a group of infectious diseases prevalent in countries with tropical and subtropical climate that affect the poorest individuals and produce high chronic disability associated with serious problems for the health system and socioeconomic development. Chagas disease or American trypanosomiasis is included on the NTDs list. However, even though this disease affects more than 10 million people, mostly in Latin America, causing the death of over 10,000 people every year, only two drugs are approved for its treatment, benznidazole and nifurtimox. These antiparasitic agents were developed almost half a century ago and present several biopharmaceutical disadvantages such as low aqueous solubility and permeability limiting their bioavailability. In addition, both therapeutic agents are available only as tablets and a liquid pediatric formulation is still lacking. Therefore, novel pharmaceutical strategies to optimize the pharmacotherapy of Chagas disease are urgently required. In this regard, nanotechnological approaches may be a crucial alternative for the delivery of both drugs ensuring an effective pharmacotherapy although the successful bench-to bedside translation remains a major challenge. The present work reviews in detail the formulation and *in-vitro/in-vivo* analysis of different nanoformulations of nifurtimox and benznidazole in order to enhance their solubility, dissolution, bioavailability and trypanocidal activity.

1. Introduction

Neglected tropical diseases (NTDs) are a group of infectious diseases produced by bacteria, virus and parasites that prevail in tropical and subtropical conditions in 149 countries affecting more than one billion people (one-seventh of the world's population) and putting nearly one billion at risk (WHO, 2015).

The term “neglected” is due to such diseases have been largely eliminated in the majority of the developing countries remaining only in the poorest regions of the less developed world, where the access to safe water and adequate health care system is lacking (Molyneux et al., 2005). To date, these NTDs place a substantial burden in those regions, increasing both the marginality and poverty of the population and cost developing economies billions of dollars every year. In addition, a remarkable growth of the population will take part in the less developed countries increasing, therefore, the spread of these infection diseases (Hotez, 2013).

NTDs reduce the physical and cognitive development of people and limit their opportunities for life and productivity at work, becoming a dangerous circle of poverty and disease. Even though NTDs produce massive but hidden and silent suffering, people most at risk do not have easy access to an effective treatment or prevention (Keenan et al., 2013).

In general, NTDs occur between individuals and families with a deficiency of proper sanitation and safe drinking water, inadequate housing, and crowded living conditions. These pathologies occur in communities living in areas of conflict, also (Hotez, 2017).

Most of them are very old and well-known diseases that have been affected larger populations for centuries. The scientific literature describes seventeen NTDs, many of them are chronic that may become progressively worse without treatment, however, the World Health Organization (WHO) recently added three other infectious disease to the commonly known classification (WHO, 2017).

Regardless the geographical regions, the consequences of NTDs are

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highly costly for societies and healthcare. The costs include intensive care required by patients of dengue hemorrhagic fever and rabies, surgical procedures and longer hospital stays in cases of Buruli ulcer, and rehabilitation required of those suffering from leprosy and lymphatic filariasis (Marchal et al., 2011).

Some of the NTDs are transmitted by vectors (vector-borne diseases) that can transmit infectious diseases between humans or from animals to humans. Commonly, the vectors are often found in tropical regions, where insects prevail, and access to drinking water and sanitation is not safe (Valenzuela and Aksoy, 2018). Many of them are bloodsucking insects, which ingest disease-producing microorganisms during a blood meal from an infected host (human or animal) and later inject it into a new host during their subsequent blood meal. These vector-borne diseases are disorders caused by pathogens, including parasites, in humans. Mosquitoes are the best-known vectors of diseases. Tick, flies, sand flies, fleas, triatomines and some freshwater snails are also vectors of several diseases (Fournet et al., 2018). To date, the world register every year more than 1 million deaths as a result of diseases transmitted by vectors (Eder et al., 2018), such as dengue, schistosomiasis, human African trypanosomiasis, leishmaniasis, Chagas disease, yellow fever, Japanese encephalitis and onchocerciasis. According to the literature, vector-borne diseases represent nearly 17% of the global burden of infectious diseases. Social, demographic and environmental factors are crucial for the spread of vector-borne pathogens (Eder et al., 2018).

In the American continent, Chagas disease is a major NTD caused by infection with the protozoa, *Trypanosoma cruzi* (*T. cruzi*), transmitted, mainly, by the “kissing bugs” insects, through the bite wound or mucous membranes (Pérez-Molina and Molina, 2018). It is the most important public health issue and one of the leading causes of morbidity, long-term disability and mortality in such geographical region, with estimates of nearly 80 million people at risk of infection, and more than 10 million infected in 21 endemic countries (Ventura-García et al., 2013).

2. Chagas disease

Chagas disease, also known as American trypanosomiasis, it was discovered by the Brazilian Carlos Chagas, who investigated the infection produced by the protozoan *T. cruzi*, more than a century ago. He was able to describe the entire life cycle of *T. cruzi* as well as the corresponding vectors and the final hosts (animals and human) (Aufderheide et al., 2004). It has been reported that, annually, the global burden of Chagas disease is around \$627 million in terms of health costs and more than 800,000 disability-adjusted life-years (DALYs) (Lee et al., 2013).

In regions where Chagas disease is endemic, the main form of transmission is through vectors (triatomines). These blood sucking insects are infected after biting an infected animal or human. Once they bite and ingest the blood of a new host, they defecate close to the bite. The host may become infected if parasites of *T. cruzi* present in insect feces get into the body through mucous membranes or cuts on the skin or when the person rubs the area of the bite in an instinctive reaction (Yamagata and Nakagawa, 2006). Additionally to vector transmission, there are other routes of Chagas disease transmission including blood transfusions, organ transplants, transmission from mother to child during pregnancy and the intake of contaminated food (Angheben

et al., 2015). It is not transmitted by direct contact with infected people (WHO, 2010; Howard et al., 2014). Lately, a remarkable advance in the control of both the vector and blood supply sources for *T. cruzi* infection has been noticed in many countries, by means of governmental control programmes (Antinori et al., 2017).

It is worth of mentioning that every year more than 15,000 babies are born infected by *T. cruzi*, worldwide. However, less than 20%, will be early diagnosed and treated. According to the experts, the congenital transmission (mother to child) is the route that produces greater number of new acute cases and, therefore, such way of transmission is the key to controlling the disease, both in endemic and non-endemic regions (Costales et al., 2015).

In this regard, more than 1 million women in fertile age are infected with *T. cruzi*, which poses a high risk of new infections due to an infected woman may transmit the parasite to the child during pregnancy or childbirth (it is calculated a risk between 3% and 5%) (Dumontel et al., 2019).

Parasitological treatment has success rates in acute and congenital disease, reactivations and indeterminate chronic phase in young patients (< 18 years) (Reithinger et al., 2009). However, its efficacy in the chronic stage in adults is still subject of intense controversy though it has increased in the past decade due to recommendations to reduce cardiac symptoms (García et al., 2005). According to a study conducted in different health centers of Argentina the risk of the occurrence of congenital transmission in treated mothers was 25 times lower than those untreated before pregnancy (Fabbro et al., 2014).

3. Chagas disease chemotherapy

According previous reports, the discovery of new chemical entities (NEC) for the treatment of global diseases including cancer, Parkinson disease and other neurodegenerative pathologies, diabetes and cardiovascular disorders has been remarkable increased in the last decades in both the academy and pharmaceutical industry (Dixon et al., 2010). However, a complete opposite situation has occurred in the case of the NTDs, mainly due to the lack of economic interest by the pharmaceutical companies and the almost complete absence of economic support to the academic research groups (Hotez et al., 2009). Thus, even though few medicines were discovered, lately, for the treatment of some NTDs, unfortunately, none of them was for the treatment of Chagas' disease (Rassi et al., 2010; Weng et al., 2018). On the other hand, although a remarkable advance in the study of *T. cruzi* genome was achieved, a vaccine is still lacking for the successful treatment of Chagas disease (Villanueva-Lizama et al., 2018).

Unfortunately, the investment needed to develop a new medicine is a critical step due to nearly US \$1400 million is the cost for approving a new biological active molecule (DiMasi et al., 2016). In the particular case of NTDs, including Chagas disease, the lack of interest showed by the pharmaceutical companies is due to, mainly, the majority of the infected population lives in rural and poor urban areas. Thus, it is almost impossible for them to afford the costs of the novel medicines confirming that such economic market is not attractive, in terms of business, for the corresponding companies (Frew et al., 2009).

To date, there are only two approved drugs for the treatment of Chagas disease: benznidazole (BNZ) and nifurtimox (NFX) (Fig. 1). Both of them discovered more than 45 years ago, are included in the WHO

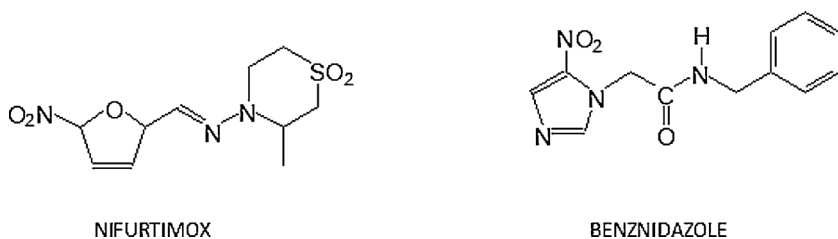


Fig. 1. Nifurtimox and Benznidazole. Unique treatment available for Chagas disease.

Model List of Essential Medicines (WHO, 2015)

Unfortunately, these medicines are not always available in many regions of Latin America (Salomon, 2012). Even though The London Declaration on Neglected Tropical Diseases announced the elimination or control of ten neglected diseases, including Chagas disease, by 2020, current estimations indicate that less than 1% of infected population with Chagas is treated (Bartsch et al., 2018).

BNZ [2-Nitro-N-(phenylmethyl)-1H-imidazole-1-acetamide] is the antichagasic drug of choice due to its biological effects and acceptable biopharmaceutical performance. The recommended dose for adults is 5–7 mg/kg orally while the corresponding dose for children up to 12 years old is 5–10 mg/kg orally, both in two divided doses administered daily for 30–60 days (Viotti et al., 2009). It was launched in 1971 by Roche and available as Rochagan® in Brazil and Radanil® in Argentina. In 2003, the license and rights of benznidazole were transferred to a Brazilian public pharmaceutical laboratory of the Pernambuco State (LAFEPE) (Alpern et al., 2017). Then, Drugs for Neglected Diseases Initiative (DNDi) and LAFEPE formulated a pediatric formulation for children weighing < 20 kg (12.5 mg BNZ per tablet). Registered in Brazil (Brazilian Health Surveillance Agency, 106 ANVISA), in 2013 was added to the WHO Model List of Essential Medicines for Children (Seremeta et al., 2019; Sales Junior et al., 2017). On the other hand, a public and private Argentinean consortium constituted by the Ministry of Health, Maprimed and Elea, both pharmaceutical companies, and Mundo Sano Foundation, started in 2012 the synthesis and production of generic version of BNZ and the development of the respective 100, 50 and 12.5 mg scored tablets. Currently, this consortium is the unique source of BNZ worldwide. In 2017, the U.S. Food and Drug Administration (FDA) approved BNZ for the treatment of children (2–12 years old) with Chagas disease, being the first drug registered by the institution for the treatment of Chagas disease (FDA, 2017).

NFX [5-nitrofurán (3-methyl-4-(5-nitrofurfurylideneamino) tetrahydro-4H-1,4-tiazine-1,1-dioxide)] it was registered and manufactured by Bayer Health Care (Lampit®). Since 1967, it is commercially available as tablets prescribed at dosages of 8–10 mg/kg/day for 90–120 days (Chirac and Torreale, 2006). Even though NFX is prescribed in all the phases of the disease, the higher efficacy of NFX is during the acute phase of the infection in children and patients recently infected (Rodrigues Coura and de Castro, 2002). NFX production was discontinued in 1997 and it was restarted in 2000 after the requirements of WHO for the treatment of both the African trypanosomiasis and Chagas disease (Jannin and Villa, 2007; Shegokar, 2013). In 2013, a clinical trial (phase I) with infected chronic patients was carried out to analyze the biopharmaceutical properties of a new 30 mg tablets of NFX compared with the commercially available 120 mg tablets. The results showed that both solid dosage forms were bioequivalents, suggesting the efficacy of NFX at low doses (Sales Junior et al., 2017). The mechanisms of action of these trypanocidal agents are still under analysis, suggesting that BNZ reduction leads to the formation of glyoxal, a cytotoxic compound (Hall and Wilkinson, 2012). In the case of NFX, its metabolism produces nitrile derivative, which exhibit the anti-parasitic activity (Hall et al., 2011). Unfortunately, in the last years, several reports described some kind of resistance of *T. cruzi* to both BNZ and NFX (Filardi and Brener, 1987; Veloso et al., 2001; Wilkinson and Kelly, 2009).

Although both drugs are widely used, one of its major disadvantages is the appearance of serious adverse effects. In the case of NFX, the patients may suffer different adverse effects including psychiatric and neurological disorders and gastrointestinal complications (Jackson et al., 2010). On the other hand, patients under treatment with BNZ may present cutaneous eruption, generalized edema, fever, polyneuropathy, polyneuritis and bone marrow complications (Bern, 2011).

4. Nanotechnology approaches for chagas disease

In general, the majority of the drugs used for the chemotherapy of

NTDs, are poorly soluble in water. In the case of BNZ, experimental data obtained by Maximiano et al. (2010) showed that the solubility of BNZ in distilled water or simulated gastric and enteric fluids is approximately 0.2 mg/ml. However, other reports informed that BNZ solubility value is 0.4 mg/ml (Kassim et al., 2004; Figueirêdo et al., 2018). In addition, determination of partition coefficient indicated that the Log P value was approximately 0.7. According to the Biopharmaceutical Classification System (BCS), BNZ belongs to Class IV drug (reduced solubility and permeability) (Kassim et al., 2004; Maximiano et al., 2010). However, it should be mentioned that BNZ is also included in the Class II of the BCS, which indicate a reduced drug solubility and high permeability (Lima et al., 2011; Ferraz et al., 2018). Moreover, The International Pharmacopeia (WHO, 2018) describes BNZ as practically insoluble in water. In accordance with all those evidences, a low and erratic bioavailability is usually expected. Due to aqueous solubility and drug dissolution rate are critical parameters to control the rate of absorption and further bioavailability, the presence of polymorphs can greatly modify the corresponding drug solubility and, then, its biopharmaceutical properties (Censi and Di Martino, 2015). Thus, Maximiano et al. (2011), decided to evaluate whether BNZ would exhibit polymorphism after the recrystallization from mixtures of different solvents. By thermal analysis, the authors confirmed the appearance of, at least, on polymorph of BNZ. Later, Honorato et al. (2014) identified by X-ray powder diffraction three different powder patterns corresponding to three different polymorphs of BNZ (Forms I, II and III). Solubility assay showed that the solubility in water of such structures were 0.22 mg/ml (Form I), 0.24 mg/ml (Form II) and 0.25 mg/ml (Form III). These findings were in accordance with the work of Maximiano et al. (2010). This information possess direct implications on the BNZ stability and aqueous solubility leading, then, to the formulation of the most convenient dosage form.

NFX is classified into the class III of BCS (Kasim et al., 2004) suggesting a high-solubility and low-permeability properties. However, in agreement with the International Pharmacopeia, it is considered practically insoluble in water (WHO, 2018). Unfortunately, data related with polymorphism, solid-state properties and drug stability are not yet available in the literature.

Concerning the BNZ and NFX physicochemical properties, the nanotechnology offers some innovative strategies based on the reduction of the particle size to nanometric scale, to overcome both solubility and permeability issues. In such scale, at least one dimension must be in the range of 10–1000 nm (1 nm = 10⁻⁹ m) in order to make use of size and structure dependent properties and phenomena, which vary at this reduced size because of the limited electrons movement, increase of superficial area, aggregation, among others (Desai, 2012). Taking into account, this novel technology can have a significant impact to improve several biopharmaceutical characteristics of lipophilic drugs including aqueous solubility, dissolution rate, biodistribution, stability during host-circulation, bioavailability, uptake mechanisms, specificity, efficacy, and sustained release (Pathak and Thassu, 2016). The design of nano-drug delivery systems includes liposomes, nanoemulsions, nanocrystals, polymeric, lipid and metallic nanoparticles. Such nanosystems are able to transport drugs to the intracellular target, reducing toxic effects due to its specificity and drug concentration achieved inside the target (Al-Kassas et al., 2017). Over the last decades, different techniques to produce nanomedicines were study to enhance the biopharmaceutical characteristics of several drugs for the treatment of NTDs (Islan et al., 2017).

Additionally, and in agreement with several studies, nanomedicines may successfully to overcome the resistance to multiple antimicrobial agents (AlMatar et al., 2017; Baptista et al., 2018). Then, it could be possible to postulate herein that similar nanocarriers might be able to avoid the mechanisms of resistance to BNZ, NFX and other nitroheterocyclic drugs including the mutation of a nitroreductase and the P-glycoprotein efflux pump (Guedes et al., 2004). Following similar mechanistic pathway than other nanoformulated systems, BNZ or NFX

nanoparticles might cross the cell membrane by specific mechanisms and release the drugs at the target site in a more efficient manner than the untreated drugs, potentially avoiding the drug-resistance (Murta et al., 1998; Garcia-Salcedo et al., 2016). However, to the best of our knowledge, research studies are still lacking, so far, to confirm the suitability of nanoformulated BNZ or NFX to reduce such resistance.

Considering this scenario, the development of a suitable pharmaceutical systems to improve the physicochemical properties (solubility, dissolution rate and stability) as well as the biopharmaceutical performance of both BNZ and NFX to achieve a more effective therapy with reduced adverse effects is highly necessary (Figueirêdo et al., 2018; Santos Souza et al., 2017; Ferraz et al., 2018).

5. Nifurtimox nanoparticles

In agreement with the literature, the advantages of the design of polymeric nanosystems for drug delivery are, mainly related with the capacity of targeting specific cell, tissues and organs (Choi and Han, 2018). Depending on the type of polymer selected, such nanostructures may be able to modulate the release of the encapsulated drug as well as to modify nanoparticle surface and circulation time. However, in some cases, intermediate byproducts can be formed through biodegradation process increasing the toxicity of those nanocarriers (Nicolas et al., 2013). In agreement with it, a deep knowledge about the mechanisms of drug uptake, cellular trafficking and distribution as well as binding to specific receptors is required. In this regard, González-Martin et al. (1998) published the formulation of NFX polyalkylcyanoacrylate nanoparticles as targeted delivery systems for the treatment of Chagas disease. Ethylcyanoacrylate nanocarriers were formulated by means of emulsion polymerization process. Different concentrations of NFX, polymers and nonionic Tween® 20, as surfactant agent, were assayed to prepare such nanosystems. NFX nanoparticles with a particle size less than 200 nm, as measured by scanning electron microscopy and cytometry, were obtained. The NFX encapsulation efficiency was nearly 34%, however, it was decreased as the amount of drug was increased. Analysis of drug release patterns indicated that, at pH 1.2 and pH 7.4, 20% and 65% of NFX was released from nanoparticles, respectively. This finding could be related with the erosion surface of the polyalkylcyanoacrylate carrier by increasing the pH, as described in 1995 by Cicek et al. *In-vitro* studies using cultures of *T. cruzi* epimastigotes, isolated from a chronic chagasic patient, indicated an enhanced anti-parasitic efficacy compared to a standard solution of raw NFX. Thus, the parasitism rate was, markedly reduced up to 94% after 2 h incubation with 0.001% of the NFX nanoparticle suspension. Microscopy evaluation displayed intracellular damage, degeneration of the kinetoplast, and lysis of membrane of the parasite. In addition, similar cellular alterations were observed in treated isolated amastigotes. A non-expected trypanocidal activity was found when parasites were treated with unloaded nanoparticles. Even though no mechanism was described to elucidate such finding, it could be postulated that a potential degradation of polyalkylcyanoacrylate may produce formaldehyde, which is toxic for the parasites. Later, Sánchez et al. (2002) reported both the formulation of polyethylcyanoacrylates nanoparticles loaded with NFX and *in-vitro* trypanocidal activity on cell-culture-derived trypomastigotes and on intracellular amastigotes. NFX nanosystems were prepared by means of an emulsion polymerization process with ethylcyanoacrylate and Tween® 20 in dimethylsulfoxide. The characterization of the nanoformulations showed particles with average size of 200 nm and an encapsulation capacity of 8.2 µg of NFX per milligram of nanoparticles. *In-vitro* trypanocidal studies on free trypomastigote forms were performed using eight different concentrations of both NFX encapsulated and non-encapsulated. A 100% trypanocidal effect was obtained when NFX nanoparticles were used at a concentration of 1.85 µg ml⁻¹. To reach a similar trypanocidal activity, non-encapsulated NFX was used at a concentration of 5.55 µg ml⁻¹. In particular, at low concentration of NFX (0.21 µg ml⁻¹), the corresponding trypanocidal

activity of loaded nanoparticles and free drug were 83.1% and 53.9%, respectively. The IC₅₀ of NFX nanoparticles was 0.17 µg ml⁻¹ whereas the IC₅₀ of free NFX was remarkably lower (0.008 µg ml⁻¹) and cytotoxicity studies on Vero cell line confirmed that both NFX loaded and non-loaded formulations presented a cytotoxic effect. On the other hand, comparison of the trypanocidal activity on trypomastigote forms between the NFX nanoparticles and the respective solution indicated that at lower concentrations (0.21 and 0.62 µg ml⁻¹), nanoparticles were more active than the drug in solution. In agreement with the results of both publications, NFX nanoparticles exhibit *in-vitro* trypanocidal activity on both free trypomastigotes and intracellular amastigotes, by means of degeneration and lysis processes of the membrane of *T. cruzi*. However, more studies are required to improve the encapsulation efficacy and confirm the detectable toxic effects of the polymeric carrier. According the literature survey, no further studies are available, to date, related with the formulation and *in-vivo* evaluation alternative NFX nanosystems.

6. Benznidazole nanocrystals

Nanocrystals, in a form of nanosuspensions, are a very attractive approach for administration of poorly-water soluble drugs through various routes, such as oral, parenteral, ophthalmic, transdermal and pulmonary delivery. These nanoformulations improve both physicochemical and biopharmaceutical properties of the drugs including solubility, dissolution rate, absorption, bioavailability, potential site-specific drug delivery, and suitability for drugs with a narrow absorption window (Müller et al., 2001). Nanocrystals of lipophilic drugs are obtained through well-known methodologies named: (1) the bottom-up process (nanoprecipitation) and (2) the top-down process (milling). The bottom-up or down-up technique belongs to the molecular nanotechnology. It focuses on the construction of structures and objects from their atomic and molecular components (Merisko-Liversidge et al., 2003). Particularly, the main goal of the 'bottom up' technology is to join small structures to make larger structures. This process starts with the molecules in solution and its further aggregation to form the solid particles. Nucleation and crystal growth are the two main steps for nanocrystal formation. Precipitation or recrystallization may be produced through different technologies including liquid solvent-antisolvent addition, supercritical fluid, solvent elimination and high-energy processes (Sinha et al., 2013). However, it is necessary to mention that this alternative also may exhibit a low stability of drugs due to the absence of a protective carrier material (polymer, lipid). Regarding Chagas disease, a solvent-antisolvent precipitation (bottom-up technique), for preparing BNZ nanosuspensions was reported by Scalise et al. (2016) for the first time. Nanocrystals were formulated through the nanoprecipitation technique using as stabilizer poloxamer 188, a nonionic copolymer with a central hydrophobic chain of poly(propylene oxide) and two hydrophilic chains of poly(ethylene oxide), with an average molecular weight of 8400 Daltons. BNZ was dissolved in ethanol (solvent), and added dropwise to a water/poloxamer 188 solution (antisolvent). Due to the nanoprecipitation process is a phenomenon of high surface energy, the particles may form aggregates or agglomerates and, therefore, is it necessary to add one or more stabilizers. In this work, poloxamer 188 stabilized the BNZ nanocrystals by means of steric hindrance avoiding the respective particle aggregation and growth. The average particle size of the BNZ nanocrystals was in a range of 60–65 nm with a size distribution (PDI) of 3.35 ± 0.1 and a ζ-potential value of -18.30 ± 1.0. In agreement with those results, the nanoprecipitation process was a very promising approach to formulate BNZ in nanoparticles. Once characterized, nano-BNZ was evaluated *in-vitro* for cytotoxicity using a Vero cell cultures. The results of the MTT assay indicated slightly differences between the cultures treated with 10, 25 and 50 µg/ml of BNZ nanocrystals suspended in PBS and untreated drug. Moreover, no morphological modifications of the cell membrane were microscopically observed suggesting the safety of such

nanoformulations in terms of cell viability. Then, blood compatibility was examined to analyze the potential application of nanoformulated BNZ as chemotherapeutic agent. In contrast to other nanosystems that produce lysis of the red blood cells, this assay demonstrated no hemolysis of the red blood cells after incubation with both BNZ nanoparticles and raw BNZ. Having on mind that the parasite infects the heart tissues, the effects of BNZ nanocrystals were evaluated in infected myocytes. The results showed a remarkable inhibition of amastigote growth in cardiac cells using low concentrations of drug. It could suggest that nanoformulated BNZ may penetrate the heart tissues and increase its activity against the intracellular growth of *T. cruzi* amastigotes. To evaluate the trypanocidal activity of nanoformulated BNZ, an assay in the acute phase *T. cruzi* infected mice was carried out. Thus, a 100% survival for at least 50 days was observed after treatment during 30 days with BNZ nanosystems (50, 25 and 10 mg/kg/day). Then, the trypanocidal *in-vivo* assay was performed during 15 days and the results indicated a 100% survival (50 days) for infected mice treated with 50 and 25 mg/kg/day, suggesting that this nanosystem presents *in-vivo* trypanocidal activity in dose dependent manner. Later, the same research group evaluated the efficacy of nanoformulated BNZ, in different concentrations, administered during the acute phase in infected mice that were immunosuppressed during the chronic phase. Production of *T. cruzi*-specific antibodies, heart cells inflammation and ROS production by Vero cells, were also evaluated. *In-vivo* assay showed a reduction in *T. cruzi*-specific antibodies in comparison with those of the untreated infected mice, after treatment with nanoformulated BNZ (25 mg/kg/day). No antibodies were found in 50% of mice after 90 days and 100% after 180 days. However, treatment with 50 mg/kg/day showed the absence of antibodies after 90 days. In contrast, both mice treated with raw BNZ and untreated mice displayed no differences in antibody titer. In addition, heart tissue inflammation produced by the parasite was diminished after treatment with nano-BNZ (25 and 50 mg/kg/day) (Rial et al., 2017). According these results, BNZ nanocrystals would be able to reach intracellular parasites, probably due to the improved solubility and permeation of the drug when nanoformulated. However, more research is required to develop a stable and secure nano-BNZ, taking into account that this new strategy may result in a novel chemotherapeutic treatment for Chagas disease.

7. Benzimidazole nanoparticles

The polymeric encapsulation of the non-polar BNZ at the nanometer scale changes its specific surface properties. This could improve the ability to cross biological barriers and targeting the affected tissues (Silva-dos Santos et al., 2017). Thus, Seremeta et al. (2019) described the nanoencapsulation of BNZ using Eudragit® by nanoprecipitation and freeze-drying process using a single polymer (Eudragit® RL PO or Eudragit® RS PO) or a blend (Eudragit® RL PO/Eudragit® RS PO, 1:1 wt ratio). The production yield after the process was nearly 86% and the encapsulation efficiency was around 95%. The freeze-drying process did not greatly modify the particle size. The range of the hydrodynamic diameter, before and after freeze-drying, was between 201–250 nm and 242–303 nm, respectively, with polydispersity index value below 0.2 indicating size homogeneity. Freeze-dried nanoparticles storage at $4 \pm 2^\circ\text{C}$ and then dispersed in water exhibited a stable behavior. After 70 days, the particle size was varied between 270–321 nm. *In-vitro* release studies in medium of pH 1.2 at 37°C showed enhanced BNZ dissolution rate from nanoparticles with respect to raw drug due possibly to a reduction of the both size and drug crystallinity after process. Values of drug dissolution efficiency at 30 min (DE_{30}), 60 min (DE_{60}) and 120 min (DE_{120}) for BNZ-loaded nanoparticles were 20.5, 36.9 and 63.9, respectively. While in the case of the untreated drug these values were 5.5, 12.3 and 27.2 respectively. This indicates a statistically significant increase of ED with respect to free BNZ ($p < 0.001$).

On the other hand, Nhavene et al. (2018) designed a nanosystem comprising mesoporous silica nanoparticles (MSNs) with chitosan-

succinate covalently attached to their surface pore to act as anchor for BNZ. Chitosan is a versatile biomaterial produced by the alkaline deacetylation of natural chitin. It is a biocompatible and biodegradable polymer and possess low immunogenicity characteristics, while MSNs are solid inorganic compounds with specific physicochemical properties due to it is possible to control its pore and particle size. Thus, the combination of chitosan with MSNs, using trimethoxysilane as covalent crosslinker, might help to formulate a novel carrier with particular properties that may facilitate the cellular uptake. Thus, the transmission electron microscopy characterization of these nanocarriers showed the presence of spherical nanoparticles with highly ordered mesoporous structures and hexagonal arrays of uniform pores. Then, the ζ -potential analysis gave as a result a negative value, probably related to the silanol groups of the silica, in agreement with other reports. However, by adding chitosan to the nanocarrier, the ζ -potential changed to a positive value, probably due to the positively charged amino group of chitosan. In any case, this modification confirmed the functionalization of the silica. By solid-state nuclear magnetic resonance was also possible to confirm the functionalization of the MSNs with chitosan succinate and, then, the drug loading on the surface of the MSNs. In particular, the reaction of chitosan to mesoporous silicate using the crosslinking agent is through a nucleophilic attack of the amino groups of chitosan to the less steric carbon of the epoxide. After the covalent attachment of chitosan to the mesoporous carbon chain, new signal peaks related with specific carbon atoms of the main chain were detected. Finally, coupling of BNZ to the silicate-chitosan structure was also confirmed by solid-state nuclear magnetic resonance. *In-vitro* assay using epimastigotes of *T. cruzi* CL Brener strain indicated that the free-BNZ carriers were not toxic against parasites and a normal grow of the strain was detected. In contrast, nanoformulated BNZ are cytotoxic and inhibit the growth of the epimastigote forms. Thus, a remarkable decrease of the rate of live parasites in presence of encapsulated BNZ was observed when compared to untreated BNZ. The findings of this research indicated that nanoformulated BNZ at low concentration exhibit a similar inhibitory antiparasitic effect than high concentrations of drug suggesting that this “nano” approach is a very useful tool to reduce the infection. In another work, Tessarolo et al. (2017) described the preparation of CaCO_3 nanoparticles for the delivery of BNZ. The nanoparticles were obtained by mixing a solution of BNZ in ethanol:water, with calcium chloride, sodium carbonate and sodium citrate solutions using two surfactants: sodium dodecyl sulphate and poloxamer 188. The analysis by atomic force microscopy indicated that particle size of spherical empty CaCO_3 nanoparticles were within the range of 27–64 nm. The loading amount of BNZ in CaCO_3 nanoparticles was determined by infrared absorption spectroscopy and after the corresponding interpolation of the drug absorbance in different diluted solutions the concentration of encapsulated BNZ was around 25%. Incubation of BNZ nanoparticles with epimastigote forms showed a growth inhibition at 24, 48 and 72 h, in dose-dependent manner. At 24 h, IC_{50} of encapsulated BNZ and untreated BNZ were $8.72 \mu\text{g mL}^{-1}$ and $56.7 \mu\text{g mL}^{-1}$, respectively, while at 72 h, IC_{50} values were $4.8 \mu\text{g mL}^{-1}$ and $4.3 \mu\text{g mL}^{-1}$, respectively. The inhibition effects of nanoformulated BNZ were more pronounced in the first 24 h of the assay, probably due to a fast drug release from the CaCO_3 nanocarrier. The evaluation of the viability of trypomastigotes showed that encapsulated BNZ was more effective than the untreated drug in all assayed concentrations. Thus, at 24 h, the LC_{50} of BNZ nanoparticles was $1.77 \pm 0.58 \mu\text{g mL}^{-1}$, while the LC_{50} of raw drug was $66.9 \pm 20.3 \mu\text{g mL}^{-1}$. Finally, the *in-vitro* assay against amastigote forms, demonstrated that BNZ nanoparticles exhibited a similar antiparasitic effect than the untreated BNZ, confirming that BNZ loaded in CaCO_3 nanoparticles is a suitable approach against the epimastigote forms as well as against both blood trypomastigotes and intracellular amastigotes (Tessarolo et al., 2017).

8. Benzimidazole lipid nanovesicles

Lipid nanovesicles represent one of the most interesting supramolecular assemblies due to several reasons including convenient size, surface properties, high drug-loading capacity, chemical modification of the surface, biocompatibility and biodegradability (Romero and Morilla, 2013). In particular, liposomes are amphiphilic, biodegradable and biocompatible spherical vesicular structures constituted by, at least, one lipid bilayer formed by the dispersion of phospholipids and other lipid derivatives into water (Sercombe et al., 2015). These lipid vesicles display an excellent capacity to carry hydrophilic or lipophilic molecules as well as an improvement of the therapeutic efficacy of the encapsulated drug. Moreover, liposomes are very useful for targeting specific cellular targets (Allen and Cullis, 2013). However, these lipid nanosystems exhibit some disadvantages including fast elimination from the bloodstream, short half-life, oxidation and hydrolysis depending on the lipid composition, and high production costs (Akbarzadeh et al., 2013). Then, its development should be evaluated in any particular case.

Morilla et al. (2002) prepared liposomes with the aim to increase BNZ permeability, reduce its degradation in liver and intestines and, finally, to reach specifically infected cells. Nanocarriers were prepared using soybean:cholesterol:distearoyl-phosphatidylglycerol (molar ratio 2:2:1, mol/mol/mol) and BNZ dissolved in dimethyl sulfoxide. Drug loading obtained was of 2 g BNZ/100 g total lipids at a total lipid concentration of 20–30 mM. Release of drug upon dilution assays (450-folds) in 10 mM buffer Tris–HCl (pH 7.4) at 37 °C showed a fast drug loss at the beginning (65% drug lost/min), followed by a second step where stopped leaking (0.4% drug lost/min) (Morilla et al., 2002). In 2004, the researchers performed an *in-vivo* assay treating non-infected rats, by intramuscular, subcutaneous, and intravenous routes to evaluate whether the new nano-BNZ would improve the biopharmaceutical properties of the drug including biodistribution and bioavailability. In the case of intravenous route, after a dose of 0.2 mg drug/kg, a major amount of BNZ liposomes was detected in liver compared to the free drug. Even though it could be a promising result, after the repeated intravenous administration of liposomal BNZ (0.4 mg/kg, twice a week from day 5 to day 22, post-injection), it was not observed a reduction of the parasitemia in infected mice. In agreement with the authors, after the phagocytosis process, the intracellular pathway of the encapsulated BNZ and its further release could be due to the permeation capacity of the drug across the membrane. The chemical constitution of the liposomes might also improve the BNZ delivery to the site of action (Morilla et al., 2004).

Recently, Vinuesa et al. (2017) described the *in-vitro* trypanocidal effect and cell viability of both BNZ liposomes and quatsomes, non-liposomal vesicles constituted by quaternary ammonium surfactants and cholesterol. Liposomes were formulated following the traditional technique using three different mixtures of cholesterol and dipalmitoylphosphatidylcholine, and quatsomes were prepared using mixtures of cholesterol-benzalkonium chloride or cholesterol-myristalkonium chloride. The procedure was carried out using compressed CO₂. BNZ and the lipid components of the carriers were dissolved in ethanol and the resulting solution was poured into a high-pressure autoclave and pressurizing it with compressed CO₂. The homogeneous cholesterol-based nanostructures were obtained after depressurizing the resulting CO₂-expanded solution in an aqueous phase. Unloaded quatsomes were within the size range of 46–124 nm, depending on their composition while unloaded liposomes appeared in a size range of 103–117 nm. On the other hand, liposomes loaded with BNZ (0.3%) exhibited a size of 118 ± 26 nm and a ζ-potential of -9.7 ± 0.8 mV. *In-vitro* evaluation of the cell viability indicated that all unloaded quatsomes were toxic, being unsuitable as BNZ carriers. On the other hand, unloaded liposomes display a very low toxicity in contrast to the BNZ loaded liposomes, which displayed toxic effects even at low concentration. *In-vitro* trypanocidal activity of the unique BNZ loaded nanovesicle showed a

very low biological effect, probably due to the low concentration of drug in the liposomes. Therefore, although these studies are a valuable approach for improving the BNZ delivery, further studies are required to formulate BNZ liposomes with an effective biological activity against *T. cruzi* infection (Vinuesa et al., 2017).

9. Benzimidazole solid lipid nanostructures

One of the most widely explored approaches to formulate particles of sub-micron dimensions loaded with both hydrophobic and hydrophilic drugs are the solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) (Almeida and Souto, 2007; Shidhaye et al., 2008). These colloidal nanocarriers, constituted by a mixture of one or more lipids and a water-surfactant solution exhibit several advantages including small size, composition, drug controlled release, biocompatibility and biodegradability (Huynh et al., 2009). It is worth mentioning that lipid nanostructures are attractive pharmaceutical alternative to polymeric nanoparticles in terms of stability, toxicity and manufacturing issues and drug release. These lipid-based nanosystems are prepared by different methodologies including solvent emulsification/evaporation, melt homogenization, ultrasonication/high speed homogenization, supercritical fluid and spray-drying (Mishra et al., 2018). However, depending on the selected methodology it is necessary to consider the negative impact of the residual organic solvents used to formulate such lipid nanostructures. Other disadvantages are the low drug loading efficiency and an initial fast release. Additionally, the samples are prepared using the high-pressure homogenization technique, it is possible to observe drug degradation by the high temperature applied. Furthermore, it is common to observe certain drug expulsion due to the recrystallization during storage (Ghasemiyeh and Mohammadi-Samani, 2018).

Vinuesa et al. (2017) formulated SLNs and NLCs for the delivery of BNZ by emulsion solvent evaporation and melt homogenization, respectively, followed by freeze-drying using trehalose (15%) as cryoprotectant. The physicochemical characterization of the nanoformulations showed that BNZ loaded SLNs exhibited an average size of 166 nm, before lyophilization, and a ζ-potential of -21 mV. The encapsulation efficiency was between 83 and 95%, depending on the drug loading. The analysis of the loaded NLCs showed particles with an average diameter of 202 nm and a ζ-potential of -26 mV. The encapsulation efficiency was almost quantitative (98–99%). *In-vitro* studies using epimastigote forms (CL strain) indicated a major trypanocidal activity displayed by raw BNZ compared to loaded SLNs and loaded NLCs. This finding would be due to the low and incomplete release of BNZ from the nanocarriers. According to these results, further studies are needed to improve both the drug loading and the corresponding release rate in order to potentially reduce the growth of *T. cruzi* in a more effective manner (Vinuesa et al., 2017).

10. Benzimidazole cyclodextrin nanometric complexes

Cyclodextrins (CDs) are cyclic oligosaccharides (cycloamyloses) that comprise (α-1-4)-linked α-D-glucopyranose units. They are doughnut-shaped molecules or bucket-like or truncated cone with hydrophilic outer surface and a hydrophobic central cavity. Thus, CDs can form non-covalently bonded inclusion complexes (host-guest complexes) with a wide variety of compounds increasing the aqueous solubility and physicochemical stability of the guest molecule. Therefore, CDs are widely used in the pharmaceutical industry to improve solubility and oral bioavailability while diminishing toxicity (Lakkakula and Krause, 2014). It is possible, also, to control drug release from stable complexes and increase drug permeability through biological barriers (Conceição et al., 2018).

Regarding Chagas disease, Lyra et al. (2012) obtained and characterized inclusion complexes of BNZ and β-CD or randomly 2-methylated-β-cyclodextrin (RM-β-CD) in aqueous solution. Results of phase-

solubility diagram showed that BNZ solubility increases linearly with CD concentration and linear regression analysis suggested the formation of complexes with 1:1 stoichiometry. Phase-solubility diagrams in water at 25 °C showed that RM- β -CD formed complexes were more stable than the natural CD (β -CD) with enhanced complexation and solubility in the presence of methylated groups that enlarge the CD cavity. Photostability assays indicated that RM- β -CD slowed down the photodegradation of BNZ, compared to that of the “free” molecule, making it more stable in the presence of light. The results of this study on epimastigote forms of *T. cruzi*, DM28c clone, indicated that the complexation of BNZ with RM- β -CD did not interfere with trypanocidal activity, preserving the integrity of the molecule. The growth inhibition performance of both was similar and dose- and time-dependent, reaching 92% of growth inhibition at 0.2 mM. According to cytotoxicity assay in mammalian cells, complexes exhibited minor cytotoxic effects compared with the BNZ. It could be due to the higher hydrophilicity properties of complexes compared to the free drug. As reported, lipophilic substances are capable to interact and greatly modify the cell membranes producing, therefore, important damages to the cells. Thus, the authors concluded that CD inclusion complexes are a promising alternative for the development of a liquid formulation, safe and stable of BNZ in order to treat the Chagas disease (Lyra et al., 2012). In other study, Leonardi et al. (2013) investigated the influence of stoichiometric and non-stoichiometric BNZ-CDs complexes on the solubility, dissolution rate and drug bioavailability. The complexes were formulated at 1:1 and 1:2 drug-CD molar ratio, by the solvent evaporation technique using water and ethanol as solvents. It was found that BNZ solubility increased by increasing the carrier concentration, suggesting a formation of complexes with a stoichiometry of 1:1. BNZ solubility increased from 0.70 mM to nearly 2.90 mM by increasing CD ratio, probably due to intermolecular hydrogen bonds between the host and the drug, which could lead to the formation of water-soluble complexes. Particularly, Me- β -CD was the most effective carrier to enhance the drug solubility compared to HP- β -CD and β -CD carriers. This finding could be due to the higher hydrophobic character of the CD cavity by the presence of methyl groups, which could increase the affinity with hydrophobic drugs. Dissolution studies showed that both the type and ratio of the carrier had a direct impact on the BNZ release. At 10 min, 21% of non-complexed BNZ was dissolved whereas BNZ-Me- β -CD complexes dissolved 72% (1:1 M ratio complexes) and 90% (1:2 M ratio complexes) of drug at the same time. Such differences could be due to several factors including the affinity of the carrier by the drug, modification of the thermal behavior of BNZ after complexation, reduction of the drug crystalline patterns, and modification of the morphology, size and surface of drug particles when complexed. As described by Carrier et al. (2007), the complexation of drugs may increase or decrease its absorption and further bioavailability, having a direct impact on the biological activity. In this case, *in-vivo* studies demonstrated that no significant differences were observed in the drug absorption from the stoichiometric and non-stoichiometric BNZ complexes. In addition, it was found that pharmacokinetic parameters showed that complexes of Me- β -CD were the most effective to deliver BNZ. Overall, BNZ complexed with CDs may be able to reduce drug lipophilicity and also improve its oral absorption, confirming that this approach is valid for the development of oral dosage forms.

Later, Vinuesa et al. (2017) developed hydroxypropyl- β -cyclodextrin (HP- β -CD) complexes loaded with BNZ by an antisolvent precipitation method with compressed CO₂. Complexed drug was completely amorphous and showed homogeneous nanostructure revealed by differential scanning calorimetry and scanning electron microscopy. Cytotoxicity assay in mammalian cells (L 929 and Hep G2) showed that complexes had a significantly lower toxicity than free BNZ. Results of biological activity against epimastigotes of *T. cruzi* (CL strain, clone B5) indicated that the IC₅₀ of the inclusion system was higher than the IC₅₀ of free BNZ, values being 18.4 ± 4.7 and 14.8 ± 4.9, respectively, and with sonicated HP- β -CD a lower IC₅₀ was obtained than with free

BNZ after sonication (16.7 ± 4.6 and 26.8 ± 1.6, respectively). In addition, biological activity of nanostructured HP- β -CD against trypomastigotes/amastigotes of *T. cruzi* had an IC₅₀ of 4.0 ± 0 whereas the IC₅₀ of BNZ was 0.8 ± 0.4. Therefore, the adequate balanced anti-trypomastigote/toxicity of these systems may offer improved therapeutics for Chagas disease (Vinuesa et al., 2017).

Often the association of a third compound in these inclusion complexes has demonstrated higher complexation efficiency. These systems are denominated ternary complexes and generally are composed of a drug, CDs, and a third component (Srivalli and Mishra, 2016). For example, diverse polymers were used to modulate drug release from inclusion complexes with CDs. Soares-Sobrinho et al. (2012) also developed inclusion complexes by loading BNZ in binary systems with randomly methylated- β -cyclodextrin (RM- β -CD) and in ternary systems with hydrophilic polymers, HPMC or polyvinylpyrrolidone (PVP). After selecting the type and concentration of polymer (0,1% HPMC), the systems BNZ:RM- β -CD and BNZ:RM- β -CD:HPMC were obtained by two methods, kneading and evaporation. The corresponding analysis of the complexes suggested that the system with 1:0.17 proportion BNZ:RM- β -CD was the most adequate for the development of a formulation. *in vitro* dissolution assay showed the HPMC had not a significant influence on the release of BNZ and would not justify its use since the percentage of the drug dissolved was higher than 90% within 60 min in binary systems. Regarding to methods, the systems obtained by evaporation enabled total dissolution of the BNZ at 60 min with dissolution efficiency after 15 min (DE₁₅) of 48.9. The systems obtained by kneading also achieved satisfactory rates of dissolution with an average percentage of 94.4% of drug dissolved at 60 min and DE₁₅ value of 41.6. The kneading process is the most commonly used in the pharmaceutical industry since it has the advantages of being simple, high yielding and easy to scale up. Therefore, the use of this method is justified, in view of the possibility of scaling this up to industrial levels reducing the cost of production of a formulation with low concentration of CD (Soares-Sobrinho et al., 2012). Later, Sá-Barreto et al. (2013) were able to control the release of BNZ from solid inclusion complexes with HP- β -CD by using the hydrophilic hydroxypropyl methylcellulose (HPMC) polymer. The evaluation of such ternary complexes showed that the system containing HPMC presented a very slow dissolution rate, with a dissolution efficiency at 30 min (DE₃₀) of only 11.4. It could be due to when HPMC is in aqueous medium, a viscous gelatinous layer is formed, which delays the release of the drug from the polymer matrix. In contrast, the binary system without HPMC showed a dramatic improvement in drug dissolution rate compared to the dissolution rate of free BNZ. The possibility of combining both binary and ternary complexes in order to achieve an initial fast drug delivery followed by a sustained release could be a strategy towards improving the drug bioavailability (Sá-Barreto et al., 2013).

11. Future perspectives

The development of nanometric pharmaceutical systems represent the latest approach to improve the chemotherapeutic action as well as to avoid the undesirable biological effects of medicines. Specifically, nanotechnology is, probably, the best alternative to provide new insights on the treatment of Chagas disease. The particular properties of nanostructures allow their application in different dosage forms administered by oral route. However, one the main drawbacks of these promising delivery systems are the physical and chemical stability issues. Other limitations are the low encapsulation capacity, particle aggregation and/or agglomeration, precipitation, and recrystallization possess a remarkable impact on the release and permeability of drugs, which can lead to final products with different quality attributes, including low or erratic bioavailability and variable biological activity (Flühmann et al., 2019).

Furthermore, the pathway to more rational pre-clinical studies and further clinical applications is still far from being executed. To improve

the clinical translation process from the bench to the bedside, it is mandatory to address different experimental steps, including a detailed evaluation of those nanosystems with the blood, tissues and organs of non-infected and infected human host (Nehoff et al., 2014; Hua et al., 2018). It is also important to consider that one of critical step related with the development to “nano” drug delivery systems are the toxicity issues. Handling materials in a nanometric scale for drug delivery may result in the appearance of serious and/or unexpected side effects, due to such nanomaterials are in the size range of biomacromolecules and cells and may interact with them modifying negatively cell permeability and/or affecting normal cellular functions (Septiadi et al., 2018). Then, the corresponding nanocarriers might be inherently more toxic than the original untreated materials. Taking into account, more efforts are needed to prevent and detect both environmental impact and side effects in living organisms based on the manipulation of nanomaterials and use of nanomedicines, respectively.

12. Conclusions

BNZ and NFX are the unique available treatments for Chagas disease, to date. Nevertheless, none of them are capable to cure the infection in both phases of the disease. Additionally, high doses required and long-term treatments as well as the existence of serious adverse effects and drug-resistant parasites confirm an urgent need to develop more innovative and effective chemotherapeutic alternative to treat, successfully, Chagas disease.

In this regard, the purpose of this review was to summarize the latest advances related with the application of nanotechnologies to improve the delivery and biological activity of BNZ and NFX. Thus, according to the literature, only two research works published in 1998 and 2002 explored the nanoencapsulation of NFX into poly-ethylcyanoacrylate carrier. Since then, no any other work was published, suggesting the lack of interest to find a more suitable formulation of NFX against *T. cruzi* infection. On the other hand, a more extensive research to develop novel BNZ delivery systems by means of different nanocarriers is ongoing. Thus, the preparation of BNZ liposomes was described for the first time in 2002 and, recently nanocrystals, polymeric nanoparticles, and lipid nanostructures were developed. Some of these new approaches exhibited a remarkable *in-vitro* and *in-vivo* efficacy in infected murine model indicating that the application of the detailed nanotechnologies may be an attractive approach to improve the solubility and dissolution of BNZ and NFX leading to a potential reduction of the doses and, perhaps, novel treatment schemes. Therefore, it would be possible to reduce the appearance or incidence of side effects, avoiding the interruption of the treatment.

As demonstrated herein, BNZ and NFX nanostructures are novel drug delivery systems, which have many advantages over the available medicines, including their biocompatibility, biodegradability, superior solubility and fast or controlled release profiles. In conclusion, the novel strategies to improve the delivery of BNZ and NFX are crucial steps to reach novel treatments with less side effects and with therapeutic efficacy in the chronic phase of the disease.

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