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PROJECT FINAL REPORT

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1. Publishable Summary Report

1.1. Final publishable summary report

1.1.1. Executive summary (not exceeding 1 page)

BERENICE (BEnznidazol and triazol REsearch group for Nanomedicine and Innovation on Chagas diseasE) is a solid and well-balanced consortium that gathers highly qualified expertise in all the different scientific, technological and clinical areas related to Chagas disease.

The aim of Berenice project was to develop a low-cost intervention with a high cost-effective impact. The original idea was to use nanotechnology in order to reduce the final dose of drug while improving its toxicity profile and therefore increase the efficacy of the current standard of cure, benznidazole. After having deepened in the knowledge of the pharmacokinetics of the drug based in both preclinical studies and phase I clinical trials, we have reach the necessary rational to demonstrate such hypothesis in larger clinical trial, although without using nanotechnology approach.

Through data obtained during the early stages of the project, we obtained the first results on pharmacokinetics and pharmacodynamics of the major trypanocidal drug, benznidazole, with a high bibliometric impact and therefore the ability to generate new lines of research. The first results of this project were focused on the better comprehension and control of nanostrcutures as the Small Unilamellar Vesicles (SUVs) and solid Lipid Nanoparticles (SLNs), mainly its behavior as drug delivery nanodevices for the specific APIs to be conjugated. Data regarding pharmacokinetics of the drug, bioavailability and biodistribution of the drug were also obtained, what it represents a crucial step-forward in the understanding of the response of the drug in humans.

From a clinical point of view, we have increase the knowledge about the toxicity mechanisms of benznidazole. We have correlated the presence of HLA B305 with the occurrence of serious side effects. Such information has been incorporated in the Clinical Trial carried out in 4 countries and 7 centers, with the Spanish coordinating center located at Vall d´Hebron University Hospital in Barcelona (Spain). Currently, Berenice is implementing the Clinical Trial that supports the hypothesis that modifying the therapeutic regimen of BNZ by reducing the final dose for treatment of Chagas disease in chronic phase, it may represent at least similar response rates compared to the standard scheme, with reduced toxicity and improved adhesion treatment.

1.1.2. Project context and objectives (not exceeding 4 pages)

Chagas disease is a chronic, systemic, parasitic infection caused by the protozoan Trypanosoma cruzi, which was discovered in 1909. Recognized by the World Health Organization (WHO) as one of the world's 13 most neglected tropical diseases, it is considered endemic in all countries of South America, Central America and the Southern United States, except in the Caribbean. With an estimate of between 8 and 10,000,000 people infected and approximately 14,000 deaths/year, Chagas represents the second highest burden of disease among Tropical Diseases in the Americas.

Although Chagas disease has been identified and described for more than 100 years, the therapeutic alternatives for treatment are limited: benznidazole and nifurtimox are the only 2 drugs available. Treatment is effective during the acute stage of the infection. Different studies show that approximately 60-85% of parasitological cure is achieved in treated patients. During the chronic phase, it is not so clear (Caryn Bern, 2011). Both benznidazole and nifurtimox have frequent side effects, especially in adults, requiring up to 30% of cases to discontinue the medication.

BERENICE (BEnznidazol and triazol REsearch group for Nanomedicine and Innovation on Chagas diseasE) is a solid and well-balanced consortium that gathers highly qualified expertise in all the different scientific, technological and clinical areas critical for the success and future valorization of the project's results.

The main objective and expected positive result is the development of a new, more effective, better-tolerated and cheaper drug formulation to cure Chagas disease. Initially was intended to apply nanotechnology to encapsulate benznidazole will generate a new drug delivery system that will allow the release of medication directly into the intracellular space, therefore increasing tissue drug concentration and avoiding side effects. A better toxic profile will be obtained because of the reduced amount of benznidazole used.

Secondary objectives of the project are the following:

- To obtain the firsts results of pharmacokinetics of Benznidazol and its new formulation.
- To collect the fragmented and dispersed selected knowledge to serve as a basis for new developments.
- To achieve a safer and optimized drug delivery through nanotechnologies.
- To improve the toxic profile of the main current treatment, Benznidazole.
- To assess trypanocidal activity of new formulations in vitro and in animal model.

- To assess trypanocidal activity of new triazole in humans.
- To assess trypanocidal activity of combined therapy against Chagas disease for the first time.
- To involve partners, research and industry in EU and in endemic countries.
- To promote technology transfer and foster in-site solutions at a lower cost.
- To get the registration of final product.
- To concrete an exploitation plan to increase access to the treatment.

BERENICE is also expected to have an impact at the following levels:

Scientific Community

BERENICE's results on benznidazole's pharmacokinetics and pharmacodynamics, together with the evaluation of the usage of a combination of imidazole and benznidazol against Chagas disease, will generate new lines of research.

Furthermore, developments in Nanomedicine will lead to the creation of new nanotechnological drug delivery systems (NDDs). This would mean lower medicine doses, safer medications and increase in efficacy of the treatment.

Socio-economic impact

The infected population in Latin-America (LA) is estimated to be between 10 and 15,000,000 people. Due to migratory flows, in the EU, Latin American people infected in their countries of origin represents a total population of about 90.000 people - most of them situated in Spain, France and Portugal. The new treatment's cost and adverse effects will be considerably lower if compared to those of the older one; thus, it will allow for more patients to be cured and more money to be saved.

Health Care Systems and Public Health

A cheaper, safer and more effective reformulation of the drug used to treat Chagas will have a great impact on the life of Chagas patients. It will ensure access to curative treatment to a greater number of people while reducing side effects and morbidity associated with the evolution of the disease and preventing deaths attributed to cardiomyopathy.

Additionally, the new medicine will have a positive impact on the health systems that support Chagas patients. A more effective and safer treatment would increase the number of patients treated and therefore cause a significant reduction or even a definitive elimination of parasitaemia. This would reduce new acute cases acquired through vector-borne transmission (anthropozoonosis) and those transmitted from mother to child (vertical transmission).

Industry

The results obtained in BERENICE will enhance European competitiveness through the transformation of research into commercially successful products in the field of Neglected Diseases.

1.1.3. Main S&T results/foregrounds (not exceeding 25 pages)

The work performed since the beginning of the project can be summarized as follows:

TECHNOLOGICAL AND INNOVATION RESULTS
Partners and Work Packages Involved: **WP3 Innovative Galenic Development (2 CIBER, 9 BIOPRAXIS)** **WP6 Scaling up of Technologies ((2 CIBER, 7 Praxis, 8 Elea, 9 BIOPRAXIS)**

WP3 Innovative Galenic Development

The design of this work package was based in some assumptions. Benznidazol (BNZ), current drug of choice for the treatment of Chagas disease, is poorly soluble in water. That fact might be related with its low efficiency. This aforementioned issue, together with other unfavorable pharmacokinetic properties such as short terminal half-life and limited tissue penetration, could lead to an irregular oral absorption and promote an erratic bioavailability. In view of these hypotheses, the general objective of WP3 was the development of highly performing galenic formulations based on the use of sublingually delivered nanomedicines for the improvement of the current chemotherapies employed in Chagas disease treatments. More specifically, WP3 aimed to develop:

- Novel benznidazole (BNZ) lipid-based drug delivery systems: 1) Solid Lipid Nanoparticles (SLNs), as particulate drug carrier systems able to achieve a sustained release of the drug minimizing its adverse effects, 2) Small Unilamellar Vesicles (SUVs) with tailored size, morphology, supramolecular structure and response to external stimuli, in order to improve the pharmacological properties of the API.
- Alternative drug administration path: 1) New formulations of the nanostructured API for its sublingual administration to reach directly blood stream and obtain an optimum concentration.

Despite the great efforts, neither SUVs (manufactured using the DELOS-susp technology, patent EP1843836B1) nor SLNs and NLCs (obtained by the emulsification/solvent evaporation and the hot melt homogenization techniques, respectively) accomplished with these general objectives of the project. In the case of SUVs, the encapsulation efficiencies of BNZ were too low and, thus, the activity against parasite was much reduced. SLNs were discarded, on the basis of the in vitro assays (cytotoxicity and activity). Thus, NLCs loaded with 30% of BNZ were

used to develop sublingual tablets. Direct compression and wet granulation were selected to process the suspension as well as the lyophilized powder of NLC formulations. All in all, none of these strategies were suitable to overcome the problem of the water content of the formulations. In addition, the lipidic composition of the NLC nanoparticles complicated the compression of the NLCs because the nanoparticles got attached to the punches. Besides of the difficulty in developing this sublingual dosage form with the NLC, in vitro efficacy studies revealed that higher NLC doses than free BNZ were needed to reach intracellular parasite forms[2].

As a consequence of these aforementioned results with lipid-based drug delivery systems, it was decided to start as a contingency plan the preparation of a novel family of nanostructured benznidazole drug delivery systems based on the use of BNZ : hidroxy-propyl-β-cyclodextrin (CD) complexes, hereafter BNZ:CD. The complexation with cyclodextrins (CD) together with the use of efficient technologies that lead to nanostructured materials with controlled characteristics could provide a way of increasing the drug solubility and improving its performance, as well as obtain nanostructured systems with high BNZ loadings, and with good compressibility and wettability properties. Therefore, the impact of the preparation of BNZ as nanostructured BNZ:CD complexes on the physico-chemical and pharmacological characteristics of this active against Trypanosoma cruzi was evaluated. Three different prototypes presenting 12%, 24% and 50% loading of BNZ (% BNZ/total mass) were synthesized using a supercritical CO_2-based process called Precipitation with a Compressed Antisolvent (PCA). The resulting samples were fully characterized in terms of physico-chemical parameters –such as particle size, morphology, composition and phase behavior- and also in terms of in vitro and in vivo toxicity, and efficacy. BNZ:CD complexes were obtained as fine white powders (the size distribution was centered on 7-10 microns) with low density and good wettability properties. Their nanostructure depended on their BNZ:CD proportion and changed from a homogeneous nanoparticulate solid in the case of the less loaded sample to an intimate mixture of needle-shape particles and nanostructured areas for the complex with the highest content in BNZ. In all cases, certain BNZ-CD molecular interactions were observed by DSC, which might be responsible of the meliorated wettability and dissolution rates of the complexes with respect to pure BNZ. These advantages together with the absence of cytotoxicity and a comparable in vitro activity encouraged the performance of in vivos studies. The nanostructured complexes were found to be well tolerated in vivo in male and female Swiss mice and efficacy studies using a murine model of disease were designed.

Due to restrictions in size (mass) of the sublingual tablets that would be used, in a first instance, in ulterior in vivo studies, the consortium decided that BNZ:CD (50%) complex,

[2] Vinuesa T et al., Benznidazole Nanoformulates: A Chance to Improve Therapeutics for Chagas Disease. Am J Trop Med Hyg. 2017 Nov;97(5):1469-1476.

which contained the highest content in BNZ and, thus, would lead to the smallest tablets, was the best option for starting the scalability study in order to get enough quantities for performing such experiments. An ex vivo experimental study of sublingual mucosa penetration was also carried out using Franz cells, to support the selection of BNZ: CD complexes, which showed the best flux rate through the tissue for these complexes.

The viability of scaling up the production of this potentially eligible final formulation (nanostructured BNZ:CD (50%) complex), was performed by producing the complex in a 7-fold bigger PCA plant which allowed to obtain a final batch of around 30g per experiment. The full physico-chemical characterization of the resulting scaled-up BNZ:CD (50%) samples revealed that these batches had the same properties than those obtained at smaller scale. Moreover, the reproducibility between the experiments performed at large scale allow also the possibility of mixing batches for the obtaining of larger quantities of this complex if needed. This first step towards the final scaling-up for the preparation of clinical batches was very encouraging since it revealed that assuring the precipitation conditions the resulting product maintained its properties regardless the scale. This process was also designed at the industrial scale for its implantation at Biopraxis GMP facilities in Miñano (Spain).

Despite these findings, preliminary in vivo studies inside the consortium revealed that the sublingual route was not crucial as alternative to the current treatment administration route to obtain an optimum plasma concentration since first pass metabolism was not detected in mice neither in dog. Therefore, due to the high oral bioavailability data found in mice and dog models, and the efficacy results in animal moldel, the consortium considered interesting to prepare oral tablets containing a reduced dose of BNZ:CD (50%) as an important breakthrough in the treatment of Chagas disease.

In vivo efficacy studies using a murine model were designed by FIOCRUZ (Partner 3) to prove the possibility of administering orally BNZ:CD formulations at reduced doses for the obtaining of similar efficacy results as the current treatment. It was demonstrated that BZ:CD complexes presented therapeutic failure when they were administered during the acute phase of the infection, while parasitological cure occurred when the BNZ:CD (50%) complex prototype was administered at the dose of 40mg/kg (less dose than the one approved for the treatment of Chagas disease, which is 100 mg/kg) during the chronic phase of the infection. Nevertheless, in this preliminary in vivo efficacy study it was found that at the same administered BNZ:CD dose, the commercialized BNZ showed comparable efficacy, indicating a no superior anti-parasite activity of BNZ:CD complexes with regard to the current BNZ therapy.

WP6 Scaling up of technologies:

Within the Berenice project, we faced the challenge of obtaining an inmediate-release tablet formulation of a nanostructured benznidazole complex (developed by CIBER Partner 2) that

meets the quality requirements for this pharmaceutical form and that provides the desired therapeutic effect. In addition, the design of a manufacturing method that will be reproducible and that ensures the quality, safety and efficacy of the product, as well as its productive performance for the large-scale production of the medication.

ELEA (Partner 8) and BIOPRAXIS (Partner 9) started with the pharmaceutical development, for example if there were any patent publish or reference product medication. In relation to patent studies, to the date no relevant documentation was found that prevents the development and registration of the product. There is no innovative reference product, therefore the previous knowledge and experience gained in the development, scaling and production of our Beznidazole 50 and 100 mg tablets, and the information of the drug obtained in the preformulation was used for this development.

The excipients to be used in the formula under study were selected based on previous knowledge of formulations of Benznidazole 50 and 100 mg, as well as taking into account the functionality of each excipient and the requirements of the drug for a formulation.

For each desired function, two possible excipients were selected, which after resulting compatible with the active in an active-excipient compatibility study, will be submitted to formulation studies to verify that their use, selected concentration and characteristics result in the expected effect.

Based on the characteristics of the co-processed active and the information obtained in the manufacture of other doses of benznidazole, it was decided that the functional excipients required to obtain an acceptable formula were:

- Diluents: To obtain a tablet of acceptable dimensions and an optimal integral process. Lactose monohydrate was pre-selected; microcrystalline cellulose; Bicalcic phosphate anhydrous.
- Disintegrants: To ensure the release of the drug. Crospovidone was pre-selected; croscarmellose sodium.
- Binders: In this case the experience with Benznidazole 50 and 100 mg, indicates that the use of binders is recommended, to improve the granulation, compression and final conditioning process by forming more robust granules, less friable tablets and consequently less dust in the conditioning.
- Polyvinylpyrrolidone K30 and Hipromelose were pre-selected.
- Particle-particle lubricant: it is required, due to the poor flow properties of the drug. And its static charge. In this case, only one excipient was selected, since it is the most widely used one with greater success for this functionality: colloidal silicon dioxide.
- Lubricants: to avoid sticking in the tooling. Magnesium stearate, sodium stearyl fumarate were pre-selected.

After several tests, and the manufacturing of pilot batches for being tested in animals sended to Brazil, this co-processed API was ruled out from the clinical standpoint, due to the poor performance obtained in animals.

Taking into account the results obtained from the preclinical assays, the objectives of the project were restructured and optimized. The new aim was to obtain a novel dosing esqueme for Benznidazol. The main challenge was to achieve large-scale production, considering that a new dosing scheme more friendly to the patient will improve adhesion to it, and mainly will generate a new look at the treatment that results in an increase of the prescriptions, and consequently of the productivity. Although the doses studied of Benznidazole, were within ELEAS' product portfolio, ELEA (Partner 8) took care of consistent optimization of our product and our manufacturing processes. So at the beginning ELEA (Partner 8) had to identify the critical points of the formulation and process:

As it is an immediate realease tablet, produced by wet granulation, the critical points to consider are:

- Weighing of active and excipients
- Granulation Final point
- Residual humidity
- Granulometry
- Uniformity of content
- Compression:
- Disintegration
- Dissolution
- Friability
- Uniformity of weight
- Uniformity of hardness

All these parameters WERE affected in some way or another by the selected excipients and the parameters defined for the selected process.

In Benznidazole 50 and 100 mg, it is observed that the granulation process must be optimized, since large mixing agglomerates are formed, which make it difficult to detect the final point and the discharge of the product. By not having a binder, the point of granulation and granule formation is obtained by overgranulation the mixture, which complicates the calibration of the wet granulate and prolongs the drying times.

ELEA (Partner 8) manufactured a scale lot of 40 KG, and evaluated:

- 2 mixing times, before the addition of magnesium stearate, and Uniformity of the mixture was analyzed.
- Variation of the machine speed: High, medium, low. It was determined in the 3 cases Uniformity of weight, Uniformity of hardness, disintegration and friability.

- Loader speed: setting the speed of the machine on average. It was determined in the 2 cases Uniformity of weight, Uniformity of hardness, disintegration and friability.

Once the optimum loader speed and machine speed has been determined, the compression force is evaluated: high and low and Uniformity of hardness, friability and disintegration is determined. Looking foward the results, ELEA (Partner 8) concluded that the formula and process of Benznidazol were adecuate for producing large scale batch.

PRE-CLINICAL RESULTS

Partners and Work Packages Involved:

WP2 Treatment Map (1 ICS-HUVH, 3 FIOCRUZ)

WP4 In vitro analysis of biological activity (4 UNL, 6 UB)

WP5 Preclinical Trials in Murine Model (3 FIOCRUZ, 4 UNL, 6 UB)

Benznidazole (BNZ) despite being the reference drug for the treatment of Chagas disease is not the ideal drug due to its high toxicity and efficacy dependent on the strain, and the infection phase. Previously studies showed that BNZ has low solubility and low permeability, little was known about the pharmacokinetic and biodistribution of this drug and current treatment regimen might be overdosed. On this basis, different experiments were carried out by FIOCRUZ (Partner 3):

1) **PHARMACOKINETICS AND BIODISTRIBUTION OF BENZNIDAZOLE**
   a. SINGLE DOSE IN MICE MODEL: In this study, BNZ tissue biodistribution in a murine model and its pharmacokinetic profile in plasma were monitored. A bioanalytical high-performance liquid chromatography method with a UV detector (HPLC-UV) was developed and validated according to the European Medicines Agency for quantification of BNZ in organs and plasma samples prepared by liquid liquid extraction using ethyl acetate. The developed method was linear in the BNZ concentration, which ranged from 0.1 to 100.0 g/ml for plasma, spleen, brain, colon, heart, lung, and kidney and from 0.2 to 100.0 g/ml for liver. Validation assays demonstrated good stability for BNZ under all conditions evaluated. Pharmacokinetic parameters confirmed rapid, but low, absorption of BNZ after oral administration. Biodistribution assays demonstrated different maximum concentrations in organs and similar times to maximum concentration and mean residence times, with means of 40 min and 2.5 h, respectively. Therefore, the biodistribution of BNZ is extensive, reaching organs such as the heart and colon, which are the

most relevant organs affected by Trypanosoma cruzi infection, and also the spleen, brain, liver, lungs, and kidneys. Simultaneous analyses of tissues and plasma indicated high BNZ metabolism in the liver. Our results suggest that low bioavailability, instead of inadequate biodistribution, could be responsible for therapeutic failure during the chronic phase of Chagas disease.

b. SINGLE DOSE IN DOG MODEL: The aim of our study was to investigate the pharmacokinetics (PK) of benznidazole and also to evaluate the influence of intrinsic and extrinsic factors on the BNZ PK in dog model. The oral PK parameters showed remarkable consistency between female in anestrus phase and male dogs. After BNZ administration in different dosing-time, the PK parameters were not different during the dark phase and light phase. Multiple oral doses of BNZ do not influence the single intravenous dose PK of BNZ in dogs. This finding suggests that there is no inhibition or induction in distribution and elimination process of BNZ in dog model. No significant differences were observed between BNZ PK parameters of different routes of administration (intravenous, intraperitoneal and oral), consequently the bioavailability of BNZ was 100%. These results confirm that BNZ is completely absorbed by intestine and that the hepatic metabolism is not important for the oral absorption in dog model. Take together the BNZ solubility and its bioavailability in dogs, we suggest that BNZ might be classified as Class 3 in the Biopharmaceutical Classification System (Low solubility and high permeability), and therefore new formulations to improve the oral absorption of BNZ might not be an important therapeutic approach to optimize the BNZ dosage regimens.

c. PHARMACOKINETICS OF BENZNIDAZOLE IN INFECTED MICE (experiment in progress): Chagas' disease is characterized by an inflammatory state, being associated with alterations in the expression of cytokines. Cytokines can alter the expression and activity of metabolic enzymes (CYP450) and membrane proteins (P-gp) by altering the bioavailability, distribution, and clearance of drugs in clinical use. These changes may contribute to the variability of the therapeutic response and incidence of adverse reactions of benznidazole treatment. This study is investigating the influence of experimental chronic T. cruzi infection on the pharmacokinetics of benznidazole in the murine model.

d. PHARMACOKINETICS AND BIODISTRIBUTION OF BENZNIDAZOLE MULTIPLE DOSE IN MICE (experiment in progress): The broad and enlightening determination of the parameters that characterize the absorption and kinetic arrangement of BNZ in animal models is essential for the design of strategies of better efficacy, safety and cost-effectiveness in the pharmacotherapy of Chagas' disease, such as dose adjustment and / or regimen of dose of BNZ alone. The present study is evaluating the pharmacokinetics, tissue distribution and dose proportionality of BNZ in different multiple dose regimens in mice.

2) EFFICACY OF DIFFERENT BENZNIDAZOLE FORMULATIONS AND SCHEMES

a. NANOSTRUCTURED BENZNIDAZOLE**:** Part of the inefficiency of treatment may be related to low solubility and low permeability. Cyclodextrin is a pharmaceutical excipient used to improve the solubility and bioavailability of hydrophobic substances. Thus, the objective of this study was to test the efficacy of nanostructured BNZ and HPβCD complex (BNZ:HPβCD) in curing Swiss mice infected with the Y strain of Trypanosoma cruzi during acute phase of Chagas disease. We evaluated complexes with different concentrations of BNZ (12, 24, and 52% BNZ / total weight) at doses of 40 and 20 mg/kg/day administered by gavage for 20 consecutive days. In addition to untreated and treated with BNZ 100 mg/kg/day (reference treatment) control groups. To evaluate the treatment were measured: parasitaemia curve, survival, and Fresh Blood Examination (FBE) and Real-Time Polymerase Chain Reaction (qPCR) after immunosuppression. All animals treated with BNZ:HPβCD at dosages of 40 and 20 mg/kg/day, despite being able to control the parasitemia had 100% positivity already in the FBE. Only the mice treated with BNZ 100 mg/kg/day obtained cure after FBE e qPCR tests (50%). Thus, our results demonstrate that treatment with BNZ 100 mg/kg/day remains the best way to treat Swiss mice infected with Trypanosoma cruzi.

b. BENZNIDAZOLE LOW-DOSE: We investigated the in vivo activity of Benznidazole low-dose against T. cruzi Y strain, using Swiss mice in the acute and chronic phase of Chagas disease. The animals were treated for 20 days with doses of 20, 40 and 100 mg/kg/day. The results showed that treatment in the acute phase with a dose of 20 and 40 mg/kg/day is able to suppress parasitaemia and preventing death in infected animals but do not exhibit parasitological cure capacity. However, in the chronic phase 100 % of the mice treated with BNZ 40 mg/kg/day and 89% of

animals treated with BNZ 100 mg/kg/day achieved cure. An experimental low-dose treatment of 40 mg/kg/day is as effective as the usual dose of 100 mg/kg/day in the mice model. The chronic model of Chagas disease retrieved better results than the acute model. Colon appears to be a key tissue to evaluate treatment efficacy according to its high proportion of qPCR positivity (20%) in mice with negative qPCR in blood. Our results demonstrate that it is possible to achieve the same therapeutic effect in the chronic phase using lower dosages of benznidazole, decreasing toxicity of the treatment. Based on all this, the current therapy strategy and efficacy assessment should be questioned and re-thought.

c. TIME AND DOSE-DEPENDENCE OF BENZNIDAZOLE TREATMENT: In this sense, we evaluated the efficacy, both on the acute and chronic phases of the disease, using mice infected with strains that have different BNZ susceptibilities. The following treatments were tested: (1) infected and non-treated; (2) infected and treated with BNZ 100 mg/kg/day 20 days; (3) Infected and treated with BNZ 100 mg/kg/day 40 days; (4) Infected and treated with BNZ 40 mg/kg/day 20 days; (5) Infected and treated with BNZ 40 mg/kg/day 40 days. Our results show that the group of animals infected by Be-78 strain (susceptible), when treated in the chronic phase with a lower dose for a longer period of time (40 mg/kg/day for 40 days) presented better treatment efficacy than with the standard protocol (100 mg/kg/day for 20 days). In acute infection by the Y and VL-10 strains of T. cruzi (respectively partly susceptible and resistant), the treatment with a lower dose but standard time of treatment (40 mg/kg/day 20 days) or a lower dose with longer time (40 mg/kg/day 40 days) were both unable to promote cure. Our results indicate that the theory of proportionality of the BNZ, in relation to dose and time, does not apply to the Be-78 strain in contrast to the observed in the infection by Y and VL-10 strain. In this way, new approaches for therapeutic effectiveness could include: strains characterization, discovering of new biomarkers of resistance that will permit individualized treatment, or even though discovering of new drugs.

3) ASSESSMENT OF NEW COMPOUNDS

a. OLEYLPHOSPHOCHOLINE (OlPC)- Dafra Pharma Research & Development (Turnhout, Belgium): Oleylphosphocholine (OlPC) is administered orally,

has excellent bioavailability, high half-life, do not have severe adverse effects and has good results in the treatment of other protozooses. Thus, the objective of this work was to evaluate the therapeutic efficacy of OlPC against T. cruzi as well as its combination with BNZ. The animals were distributed in 9 groups: (1) Untreated; (2) Treated with BNZ 100 mg/kg/day for 20 days; (3) Treated with BNZ 100 mg/kg/day for 10 days; (4) Treated with BNZ 40 mg/kg/day for 20 days; (5) Treated with OlPC 40 mg/kg/day for 20 days; (6) Treated with OlPC 20 mg/kg/day for 30 days; (7) Treated with BNZ 40 mg/kg/day for 20 days + OlPC 40 mg/kg/day for 20 days; (8) Treated with the BNZ combination 100 mg/kg/day for 10 days + OlPC 20 mg/kg/day for 30 days; (9) Treated with BNZ combination 100 mg/kg/day for 20 days + OLPC 40 mg/kg/day for 20 days. Animals that presented Fresh Blood Examination and qPCR from blood and tissue negative, following immunosuppression protocol, were considered cured. Thus, monotherapy with OlPC or the combination with BNZ did not show good results for the treatment of T. cruzi infections; however, the control group of BNZ 40 mg/kg/day for 20 days in the chronic phase presented results that should be better analyzed and explored.

WP4 IN VITRO ASSAYS:

UB (Partner 6) and UNL (Partner 4)'s efforts have been mainly focused on testing the different products generated by galenic partners and looking for some alternative molecules of bacterial origin to fight against the parasite. The screening of the biological activity of nanoparticles received using the transfected CLB strain has been performed. In vitro cytotoxicity utilizing two mammalian cell lineages has been performed, the first one being of murine origin: L929 (line also used for infecting cell to obtain trypomastigotes) and the second one being of human origin with high metabolic activity: Hep G2

From month 1 to month 18, efforts of partners involved in WP4 were mainly focused on the setting up a laboratory completely devoted to the analysis of the products generated by CIBER-BBN (Partner 2). UB (Partner 6) optimized the parasitological techniques with the best performing strain of the parasite, combined with use of the mammalian cell lineages that gave the best performance related to parasitisation percentage and evaluation of cytotoxicity.

For the whole of the screening assays a strain transfected with E.coli beta-galactosidase (CL B5 strain, F Buckner) was gently provided by the group of Dr. José A. Escario from the Parasitology Department, of the University Complutense of Madrid. Various parasite strains were obtained including clinical isolates with different biological characteristics (tissue

tropism, acute and chronic form of disease and drug sensitivity to the current specific chemotherapy). Colombian strain was obtained from FIOCRUZ (Partner 3):

Collection parasite strains obtained:

- CL B5 strain transfected with lacZ gene (Buckner et al. 1996)
- Y strain (Silva and Nussensweig, 1953).
- Colombian strain, resistant to Bnz.
- Maracay strain (B582)
- Clinical isolates: BCN 590 Bolivia. Tc I
- BCN 848 Chile. Tc I
- Triatoma isolates: BCN 567 Mexico Tc II

The biological characterization of the strains was determined in UB (Partner 6)'s lab conditions: the growth curves of epimastigotes, their susceptibility to BNZ and their infectivity to mammalian cell lines.

The BNZ susceptibility was determined and the IC_{50} determined using Prism. Graphpad Prism software. (CL B5 strain IC_{50} to BNZ 7.96).

The mammalian cell lines used to produce trypomastigotes were Mus musculus fibroblasts (L-929) with CL B5 strain and Vero (african green monkey kidney cells) for Maracay strain.

For citotoxicity two cell lines were choosen. One was the same used to infect with trypomastigotes (fibroblasts L- 929), and the second, a highly metabolic active cell line Hep G2 (Human liver hepatocellular carcinoma).

Empty SUV nanoformulates made by cholesterol and cationic surfactants (Quatsomes) presented a remarkable toxicity when tested at high concentrations declining at lower concentrations.The empty lipid nanoparticles presented a toxicity profile related to the concentration. Even the existence of minor variations it was seen that all the products appeared as non-toxic (< 10% toxicity).The efficacy of each compound was estimated by determining the epimastigote growth percentage (%AE value).

Benznidazole was used as reference drug. Each concentration was tested in triplicate and each experiment was performed three times separately.Trypomastigote/amastigote susceptibility: The screening was performed with the transfected CL-B strain expressing the Escherichia coli □-galactosidase gene. The results were expressed as percentage of T. cruzi growth inhibition in treated infected cells vs untreated infected cells.

The loaded nanoparticles of the first lot of Nanobiocel didn't appear more efficient than BNZ probably due to a drug release problem.The NLCs 20% of the 2ond lot improved a little their activity nevertheless none of them appear as a good candidate. In the range of the concentrations of BNZ available with liposomes it was not feasible to determine the IC 50.

With the cyclodextrine PCA2 the IC50 was in the range of the IC50 of free BNZ; this formulation enhanced the solubility of the drug. This strategy was considered the more adequate. The test where performed with cyclodextrines with a 12% of BNZ. The first results obtained with trypomastigotes/amastigotes also showed that cyclodextrines with BNZ presented good activity against the parasite.

From month 19 to month 36 the efforts of UB (Partner 6) were focused on testing the two different lots of products generated by CIBER (Partner 2) and looking for some alternative molecules of bacterial origin to fight against the parasite.

UB (Partner 6) performed the screening of the biological activity of nanoparticles received using the transfected CLB strain of the parasite and evaluated the in vitro cytotoxicity. A new strain of a T.cruzi collection was obtained (Tulahuen strain).

The ability of infection of neuronal/glial primary cultures was tested at different stages of differentiation by all strains. Infections were performed at 4, 8 and 15 days. All the results were negative as no one of the used strains infected the obtained primary cultures.The results obtained with all of the received products gave a very low profile of toxicity (always inferior to 15%).

Regarding to the biological activity against the parasite the NLC 20 nanoparticles did not appear more efficient than free BNZ giving a IC50 of 53.9 µM in front of the IC50 of 26.6 µM of free BNZ used as control, assuming difficulties in the release of the molecule.

Respect to the activity of cyclodextrines against epimastigotes, loaded cyclodextrines showed an IC50 similar to free BNZ (16.9 µM, 21.9 µM, 36.9 µM vs 26.8 µM). It seemed that these results were significant enough to encourage the research in order to test their in vivo activity against the parasite in murine model of experimental infection.

In order to check the feasibility of the absorption of cyclodextrines sublingually it was decided to broad the scope of UB (Partner 6) participation with the study of the ability of cyclodextrines to cross the sublingual epithelium. In collaboration with the Faculty of Pharmacy of the University of Barcelona (Dr. Ana Calpena) sublingual permeation studies in pig oral mucosa were accomplished.

Sublingual permeation studies were realized with two types of cyclodextrines, benznidazole and NLC 20. Concentration of the BNZ in the permeation samples was quantified by using high performance liquid chromatography in the Services of Chemical Analysis (Servei d'Anàlisi Química UAB (SAQ). The methodology used has been developed according to the utilized at the FIOCRUZ (Partner 3) laboratory in Brazil for the determination of the BNZ concentration in plasma and tissues.

The results indicated that the best permeation was obtained with BNZ/CD 22%.

	BNZ/CD 12%	BNZ/CD 22%	BNZ	MIXTURE	NLC'S 13%
J (µg/h) (Flux)	2.00	3.92	2.48	2.18	2.30
J (µg/h/cm2)	3.07	6.03	3.82	3.35	3.54
Latency (h)	1.4	0.5	2.1	1.86	0.6
BZN (µg/cm2)	18.62	27.69	19.38	15.85	13.38

Taking into account the results with the nanoformulations based on the effect of BNZ, this team started an exploration of different products of bacterial origin that were investigated in UB (Partner 6) facilities as antimicrobial and antitumoral agents. Preliminary results were reported. prodigiosin, as well as some derivatives and related molecules such as obatoclax, resulted to have a powerful antitrypanocide effect.

From month 37 to month 54 the task realized by UB (Partner 6) was focused on finishing the evaluation of the products generated by CIBER (Partner 2), on the parasitic form trypomastigotes, as the laboratory had problems with the in the mass production of parasite. Moreover, during this period UB (Partner 6) was looking for some alternative molecules of bacterial origin, as well as some synthetic derivates. (Biological Prodigiosin produced by Serratia marcescens 2170 ATCC strain, synthetic Prodigiosin and Obatoclax both synthetized by R. Quesada Chemistry Department, University of Burgos, and lately several tambjamines, synthetized by the same chemistry laboratory, and based on the structure of the natural prodigiosins). Since the results obtained with the use of nanoencapsulated Benznidazol were not as satisfactory as expected, the trypanocidal activity of these new compounds was determined as well as their perspectives in the use in Chagas treatment.

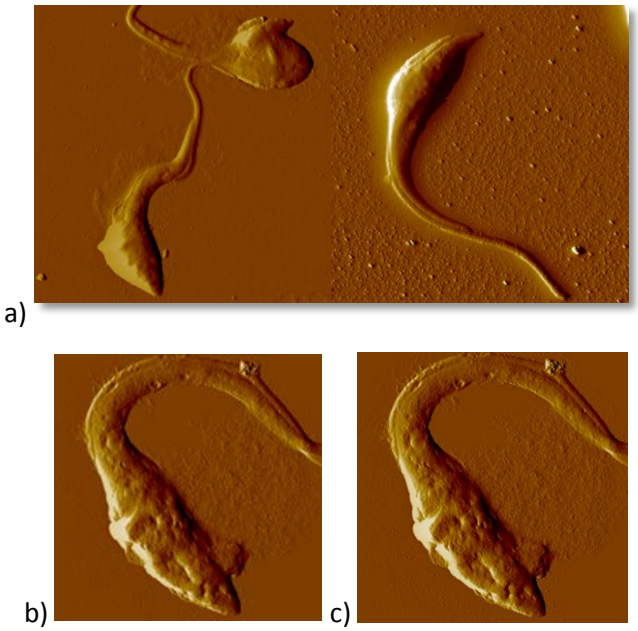
Concerning the activity against trypomastigotes, it should be stated that in preliminary studies the activity for trypomastigotes/amastigotes of both loaded cyclodextrines and NLCs was found to be satisfactory. Nevertheless, due to the scarcity of parasite forms the tests were performed only once. When the assays with trypomastigotes were repeated in triplicate, none of the nanoparticles previously selected (low cytotoxicity and biological activity against epimastigotes higher than or similar to that of free BNZ), showed an activity higher than free-BNZ. The least active lipid particles were NLC20.

From the month 55 to month 72 new experiments where developed with benznidazole and new extracted and purified natural prodigiosin.The extraction method and purification of natural prodigiosin was improved.

The effect of both benznidazole and obtained prodigiosins against the parasite was evaluated by means of atomic force microscopy analysis (AFM) of epimastigotes exposed and

unexposed what allowed to visualize the injuries produced and to measure the cell damage (lowering of height and enhance of surface nano-roughness of the treated epimastigotes. The alterations induced in membranes by prodigiosin were analyzed by examining its channel-forming capacity in black lipid bilayers via electrophysiological measurements. We conclude that there was no pore-forming activity of prodigiosin in protein-free environments.

Figure 1 Epimastigote forms of T. cruzi treated with prodigiosin



AFM amplitude images obtained at a 20 μm^2 scan size, of: (a) untreated T. cruzi epimastigotes; (b) T. cruzi epimastigotes after 72 h of treatment with PG 3, 1.26 µM: (c) T. cruzi epimastigotes after 72 h of treatment with benznidazole, 48 µM.

CLINICAL ADVANCES

Partners and Work Packages Involved:

WP7 Clinical Trial (1 ICS-HUVH, 3 FIOCRUZ, 5 ANLIS-INP, 8 Elea, 9 BIOPRAXIS)

CORRELATION THE PRESENCE OF HLA B305 WITH THE PRESENCE OF SERIOUS SIDE EFFECTS

A prospective observational study was performed by ICS-HUVH (Partner 1). It included adult Chagas disease patients accepting to receive benznidazole (100mg/8 hours for 60 days).

The objective was to characterize the skin toxicity of benznidazole in patients with Chagas disease, determine the serum cytokine profile, and evaluate the potential association with specific HLA alleles and benznidazole concentration. Serum cytokine levels were measured at day 0, 15 and 60 of treatment. Class I and II HLA alleles were determined. When cutaneous reaction was detected, a skin biopsy was performed. Serum benznidazole concentration was determined at the time of cutaneous reaction, or at day 15 of treatment.

Fifty-two patients were included, 20(38.5%) had cutaneous reaction and median time of appearance was 9 days. Skin biopsies showed histopathological findings consistent with drug eruption. Patients with cutaneous drug-reaction had higher proportion of eosinophilia during treatment, and higher IL-5 and IL-10 serum concentrations at day 15 of treatment than those without cutaneous reaction. Treatment interruption (that included moderate-severe cutaneous reactions) was more frequent in patients carrying HLA-B*3505 allele (45.5% vs 15.4%,p=0.033). No differences in benznidazole serum concentration were found.

Data obtained from this experiment represented an important breakthrough in the treatment of Chagas disease and such information was incorporated in the Clinical Trial carried out.

CLINICAL TRIAL

A Clinical Trial was performed by ICS-HUVH (Partner 1), FIOCRUZ (Partner 3) and ANLIS-INP (Partner 5).

Based on the results obtained by the preclinical groups, where lower benznidazole doses achieved the same efficacy ratio than standard dose, a clinical trial was designed in order to corroborate such important results. Moreover, we take advantage of such opportunity to rethink the current benznidazole scheme taking in to account the recent discoveries.

A. Background

Currently, recommended BNZ dosage and duration regimen for CD treatment is 5-7 mg/kg/day for 60 days. This recommendation is based on studies carried out in the 70s (Barclay et al, 1976; Coura et al, 1978). However, nowadays both the dose and duration of treatment are under discussion predicated in findings from CD murine models, PK/PD studies

and studies from patients who discontinued the treatment. In this way, it seems clear that BNZ dose may be optimized.

Lower dose: Two population pharmacokinetic studies have shown that lower dosage with the same duration would have the same efficacy. One of them was carried out in children and the other one in adults. In the pediatric study, children were treated with standard dose of BNZ. Although significantly lower concentrations of the drug were achieved compared to those reported in adults, the treatment was effective in all patients who completed the treatment course. Moreover, data from a second study carried out in adults, revealed that a dose of 5 mg/kg/day might lead to overexposure in the majority of patients, and

BNZ dose of 2.5 mg/kg/day is enough to adequately keep BNZ trough plasma concentrations within the recommended target range according to previous pharmacokinetic studies.

Higher dose: Recent in vitro assays that quantify the time necessary to eliminate the parasites (time-to-kill assays), showed that nitroheterocyclic compounds, such as BNZ, are dose-dependent. In fact, treatment schemes with higher dose of BNZ than standard (400 mg daily) with the same duration have been already used (STOP-CHAGAS study), without observing a higher proportion of side effects. Furthermore, another study of 54 patients treated with BNZ tried to establish the correlation between the serum concentrations of the drug and the appearance of AEs. Fifty-three patients (98%) experienced at least one AE during follow-up, but no relationship was found between the drug serum concentration and the occurrence of AEs.

Shorter regimens: Finally, regarding the duration of treatment recent studies in animal models have shown that shorter schemes (25% of standard duration) achieve the same cure rate. This is not yet experimented in clinical trials, but findings from one study showed an important cure rate in patients who had to abandon the treatment due to severe AEs.

B. Study design

MULTIBENZ is a phase II non-inferior design, three arms randomized, double blind, multicenter and international clinical trial for assessing the efficacy and safety of three different BNZ dosification schemes for the treatment of CD in chronic phase. It will be carried out in four different countries: Argentina, Brazil, Colombia and Spain.

Objectives: The primary objective of MULTIBENZ is to evaluate the efficacy of different BNZ regimens at 12 months postrandomization in patients with CD in the chronic phase. The primary efficacy outcome is defined as the proportion of patients with sustained parasitic load suppression in peripheral blood measured by polymerase chain reaction (PCR) during the first 12 months of follow-up after randomization.

The secondary objectives are: to evaluate the parasitic kinetics by detecting parasitic DNA measured by PCR in peripheral blood at different time points, to evaluate the serological

response by ELISA methods, to assess the tolerability and safety of the different BNZ regimens, to correlate BNZ levels with the therapeutic response and AEs, to correlate the presence of HLA-B*3505 with the presence of severe AEs, and to correlate the different discrete type units (DTUs) of *T. cruzi* with the therapeutic response.

Patient eligibility: Patients aged ≥18 years having any combination of at least 2 positive serologic tests against *T. cruzi* (indirect immunofluorescence, indirect hemagglutination or ELISA) and not having previously received treatment with BNZ or nifurtimox (either completely or partially).

Randomization and follow-up: Patients are randomly assigned to receive BNZ 150 mg/day for 60 days, 400 mg/day for 15 days, or the standard scheme (300mg/day for 60 days). To avoid bias, randomization will be centralized. Patients will be randomized 1:1:1 to every treatment arm and balanced by country.

Scheduled follow-up visits will occur at 7, 15, 28, 60 days and up to 4,6,8 and 12 months after initiation treatment.

Sample size and data analysis: For the sample size calculation, we considered a non-inferiority design with a reduction of 50% of the total number of patients who either present a positive PCR during the follow-up or have to discontinue the treatment due to AEs has been taken into account as a hypothesis. It is estimated that in the standard arm of treatment 40% of patients are evaluated as failure according to the intention-to-treat principle. The study will try to find differences between countries considering 15% among them as acceptable.

For a unilateral type I error (p = 0.05) and a power of 80% the estimated effect size would be 204 individuals. Considering 15% of defaulted patients, the total number of patients that should be included in the study is 240, which will be 60 patients per country.

The categorical data will be presented as absolute numbers and proportions, and the continuous variables will be expressed as means and standard deviation (SD) when normal distribution is demonstrated (using the Kolmogorov-Smirnov test), or as median and interquartile (IR) range when it is not.

For comparing the distribution of categorical variables the $\chi 2$ or Fisher test will be use and Mann-Whitney or Student´s t-test for continuous variables, respectively, depending on the presence or not of normal distribution. The analysis of the proportion of patients with positive PCR for *T. cruzi* will be analyzed and expressed by a survival curve. A comparative analysis of the main variable among the three groups will be carried out, as well as an analysis of the time until the PCR positivization.

Finally, the sensitivity, specificity and positive and negative predictive values of the presence of HLA-B*3505 and its relationship with the occurrence of severe AEs will be calculated. The

effectiveness analysis will be carried out through the per protocol and intention-to-treat principle.

Laboratory procedures:

Serology: Two different anti-*T. cruzi* serology tests based on different antigens were used for assessing the patient eligibility. To avoid inter-laboratory variability, serum samples collected at the times indicated in the protocol will be sent at the end of the study to a centralized laboratory that will process them using two techniques in parallel: Architect Chagas (Abbott laboratories, Wiesbaden Germany) and ORTHO *Trypanosoma cruzi* ELISA Test System (Ortho diagnostics). In order to give greater robustness to the results, samples from external quality control sent by the National Program of Quality Control of Brazil (PNCQ) will be included.

PCR: Laboratories included in the project will carry out the same RT-PCR protocol following the instructions included in the Laboratory Manual agreed among all of them. To carry out this technique, 5 ml of whole blood will be collected and mixed with 5 ml of guanidine hydrochloride 6M-EDTA 0.2M for a minimum of 72 hours at room temperature. Three DNA extractions will be performed using the manual column method (High Pure PCR Template Preparation Kit, Roche, USA) except in Spanish centers (HVH and RyC) where DNA extraction will also be done in triplicate using an automated extraction method (NucliSens easyMAG, Biomerieux, France).

The consensual PCR protocol consists of a real-time multiple PCR (Duffy et al 2013) that allows the amplification of a T.cruzi satellite DNA region and a linearized recombinant plasmid used as an internal amplification control. The RT-PCR will be carried out in duplicate from each of the extractions. At least one amplification of the six performed with an amplification cycle (Ct) of T.cruzi below 40 and a correct value of IAC will be interpreted as positive. To be correct, the values of the IAC must meet Tukey's criteria.

To assess the homogeneity of the results obtained by the different laboratories, a harmonization panel consisting of 10 tubes containing blood with uninfected guanidine and infected with 1, 10 and 100 parasitic equivalents / mL of strains Tc V and Tc VI was processed. The samples were processed blindly by the different laboratories and the results were evaluated by an external center in charge of providing the panel and analyzing the results (INGEBI, Buenos Aires). On the other hand, and following the same work scheme, four external quality control panels will be analyzed during the study period.

HLA typing: The typing of HLA-B alleles is carried out from the dried blood samples on paper (Dried blood spots; DBS). For this, DNA is extracted using the DNA Elution Solution reagent (Qiagen 159994) and the concentration and quality are evaluated by measuring the absorbance at 260 and 280 nm using the Colibri microvolume spectrometer (Titertek-Berthold). The characterization of the HLA-B alleles is carried out by PCR-SSO / LUMINEX, following the instructions of the LIFECODES HLA Typing kit (Immucor, Longwood Diagnostic).

Briefly, the PCR-SSO / Luminex consists of the amplification with biotinylated primers of the most polymorphic regions of the HLA-B gene followed by the hybridization of the amplified product with specific probes for each allele located on the surface of Luminex microspheres and revealed with conjugated streptavidin with phycoerythrin. Finally, it is analyzed by a fluoroanalyzer xMAP100 (Luminex).

Benznidazole serum concentration: Quantification of BNZ is done from dried blood samples on paper (Dried blood spots, DBS). The quantification is performed by liquid chromatography (ACQUITY UPLC HSS T3 C18, 2.1x50 mm (Aquity, Waters)) coupled to triple quadrupole mass spectrophotometry (Xevo TQ, Waters).

Study organization: The MULTIBENZ study network includes 4 countries and 7 centers, with the Spanish coordinating center located at Vall d´Hebron University Hospital in Barcelona (Spain). The study contemplates a total of three stages:

1. Recruitment sites:

Countries involved in the recruitment will be Spain (University Hospital Vall d'Hebron, Barcelona and University Hospital Ramón y Cajal, Madrid); Argentina (Instituto Nacional de Parasitología Dr. Mario Fatala Chaben, Buenos Aires and Instituto de Cardiología Juana Francisca Cabral, Corrientes); Brasil (Centro de Pesquisas René Rachou - Fundação Oswaldo Cruz, Belo Horizonte and Hospital Universitário Clemente de Faria, Montes Claros); Colombia (Fundación Cardioinfantil - Instituto de Cardiología

2. Selection phase

Patients with CD in the chronic phase who come to the study centers will be evaluated in order to assess if they meet the inclusion criteria. For those who accept to participate and sign the informed consent, a detection of parasitic DNA by PCR in peripheral blood will be performed. Patients with PCR negative for *T. cruzi* will be withdrawn from the study. The screening procedure must occur up to 90 days or less before the initiation of treatment. Serology and DNA determination by PCR will be accepted as valid and will not need to be repeated if there were a positive result obtained in a previous period of 3 months.

All patients will undergo a clinical history and physical examination. Peripheral blood extraction will be performed for analysis: hemogram, biochemistry and HLA study, and a negative pregnancy test (either in urine or blood) is mandatory in case of women in childbearing age. The evaluation of the visceral involvement of CD will be completed with a chest X-ray and electrocardiogram. The performance of other complementary tests will be carried out according to the clinical investigator decision, but they will not be considered necessary for the inclusion of the patient.

3. Treatment phase

In this phase, the patient will be randomized to one of the three arms of treatment, whose duration will be 60 days independently of the arm assigned. Initially a baseline visit will be performed in which patient will be trained on how to take the drug and identify adverse events. After that, a total of four scheduled visits will occur during the treatment period, at 7, 15, 28 and 60 days after treatment initiation.

During this phase, any patient may consult spontaneously due to the eventual occurrence of any AE. The decision to interrupt the treatment will be according to the discretion of the clinical researcher treating the patient, taking into account the severity, intensity and extent of these AEs. In addition, the patient may decide unilaterally to suspend or not the medication at any time of treatment period.

4. Follow-up phase

Once the patient has taken the last dose of treatment, which may be at the end of the therapeutic scheme (day 60), when a severe AE that obliges to suspend the medication appear or when the patient decided to suspend unilaterally the medication, this phase will begin, and it will last up to 12 months after randomization.

1.1.4. The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results (not exceeding 10 pages)

Potential impact (including the socio-economic impact and the wider societal implications of the Project so far) and the main dissemination activities and exploitation of the results (not exceeding 10 pages).

A global overview

The aim of Berenice project was to develop a low-cost intervention with a high cost-effective impact.

The original idea was to use nanotechnology in order to reduce the final dose of drug while improving its toxicity profile and therefore increase the efficacy of the current standard of cure, benznidazole.

After having deepened in the knowledge of the pharmacokinetics of the drug based in both preclinical studies and phase I clinical trials, we have reach the necessary rational to demonstrate such hypothesis in larger clinical trial.

We reached the scientific impact through the sum of efforts and knowledge of several prestigious academic institutions both in Europe and Latin America. Given the high expected impact of the project, would have been impossible to deal it without the creation of this international multidisciplinary consortium. The technology development needed, the different in vivo animal models or the degree of expertise required, are not concentrated in any single centre or in one country. The international consortium, bringing together European and active research and field partners in Latin America led towards highly innovative and new approaches to prevention, treatment, diagnosis and management of this disease.

The importance of the impact of BERENICE project lies on the variety of levels that aims to influence: scientific advancements, technology development level, pharmaceutical industry, therapeutic level and therefore on the health of patients, individually and on the control of Chagas diseases in endemic and non-endemic countries.

Therefore, the potential impact of this initiative aimed, in a concrete, realistic and cost effective manner, to have bear on the treatment of Chagas' disease which became nowadays one of the most neglected tropical diseases.

Detailed Impacts

Burden of Chagas Disease

Chagas disease is still nowadays serious health problem in Latin America and consequently in Europe and the United States, as a result of the migratory movements. Endemic in 21 Latin American countries, its prevalence is estimated around 6 million

people infected and approximately some 12,000 deaths / year. In terms of morbidity, a 2006 World Bank report stated that the disease caused 867.000 DALYs annually. Even if this burden of disease is adjusted in the context of currently available epidemiological information, Chagas disease still represents the second highest burden of disease (BOD) among Tropical Diseases in the Americas. In the EU, the increased presence of LA people due to immigration phenomena, infected in their original countries, represents a total population of about 90.000 persons infected, most of them in Spain, Italy and Portugal. To demonstrate the efficacy of a new therapeutic scheme based on a significant reduction of the dose with a decrease of the proportion of patients suffering from serious adverse events could represent an increase of patients treated.

The greater patients treated with a major completion ratio would represent more patients cured and in the case of women at child-bear-age more cases of vertical transmission interrupted, which represents an important step for the elimination of the disease.

Scientific advancements

Through data obtained during the early stages of the project, we obtained the first results on pharmacokinetics and pharmacodynamics of the major trypanocidal drug, benznidazole, with a high bibliometric impact and therefore the ability to generate new lines of research.

The first results of this project were focused on the better comprehension and control of nanostrcutures as the Small Unilamellar Vesicles (SUVs) and solid Lipid Nanoparticles (SLNs), mainly its behavior as drug delivery nanodevices for the specific APIs to be conjugated.

Data regarding pharmacokinetics of the drug, bioavailability and biodistribution of the drug were also obtained, what it represents a crucial step-forward in the understanding of the response of the drug in humans.

From a clinical point of view, we have increase the knowledge about the toxicity mechanisms of benznidazole. We have correlated the presence of HLA B305 with the occurrence of serious side effects, representing an important breakthrough in the treatment of Chagas disease. Such information has been incorporated in the Clinical Trial carried out in 4 countries and 7 centers, with the Spanish coordinating center located at Vall d´Hebron University Hospital in Barcelona (Spain).

Based on the results obtained by the preclinical groups, where lower benznidazole doses achieved the same efficacy ratio than standard dose, a clinical trial was designed in order to corroborate such important results. Moreover, we take advantage of such opportunity to rethink the current benznidazole scheme taking in to account the recent discoveries.

Impact on Nanomedicine development

In the frame of BERENICE project, innovative CO_2-based manufacturing platforms have been used to develop new benznidazole (BNZ) nanomedicines: the Depressurization of an expanded Liquid Organic Solution-SUSpension (DELOS-SUSP) and the Precipitation

with a Compressed Antisolvent (PCA) technologies. Both manufacturing processes have been proved to be scalable and can accomplish GMP requirements. Concretely, the use of PCA has resulted in an efficient technology to process BNZ:Cyclodextrin (CD) complexes, leading nanostructured materials with controlled characteristics and high active loadings, suitable for the in vivo administration of the required doses. With this achievement, we have strategically contributed to the adoption of Key Enabling Technologies (KETs), such as nanotechnology, advanced materials, and advanced manufacturing technologies in Europe, through the demonstration of the capacity to get greener, safer and efficient therapies. Despite the BNZ:CD complexes were discarded as candidate treatment to be used in clinical trials since no evidences of superior efficacy were found in a preliminary preclinical in vivo study, it would be worthy to perform further studies in the frame of future research projects. It would be interesting to explore whether these nanostructured complexes present other favourable properties such as longer terminal half-life and enhanced tissue penetration, which could lead to regular oral absorption and less erratic bioavailability of BNZ, and a reduction of the side effects when administered chronically.

Besides that, Solid Lipid Nanoparticles (SLNs) and Nanostructured Lipid Carriers (NLCs) containing different doses of BNZ have been successfully prepared by the emulsification solvent evaporation technique and by the hot melt homogenization technique, respectively. Although they have not shown ideal properties to allow for the implementation of these nanoparticulate formulations in clinics, as SLNs were less active than free BNZ and NLCs were slightly less trypanosomal and more toxic than CDs, they show great potential for the encapsulation of drugs.

Nanomol group of CIBER-BBN has translated the complexation of actives with cyclodextrins(CD) concept and the PCA production process to obtain nanostructured medicines in the frame of other projects, such as UNDERLIPIDS project (RTC-2015-3303-1), financed by the Spanish Ministry MINECO. This project has clearly benefited from the innovative findings, developed under BERENICE, to get novel products to the market, objective that is reflected by the presence of the pharmaceutical company named Pharmamar.

Similarly, NanoBioCel group of CIBER-BBN has applied the encapsulation of drugs in SLNs in NLCs successfully in different applications, supported by the Ministry of Economy and Competitiveness of Spain through projects obtained in competitive calls , such as: NANOTEG. (SAF2013-42347-R), IGRALZHEIMER (RTC-2015-3542-1), UNDERLIPIDS (RTC-2015-3301-1) and BIOTAPE (RTC-2016-4770-1), among others.

The significant results obtained with the innovative BNZ formulations have been disseminated in the frame of congresses, seminars, etc, as well as they have been used to publish the scientific article Vinuesa T et al., Benznidazole Nanoformulates: A Chance to Improve Therapeutics for Chagas Disease. Am J Trop Med Hyg. 2017 Nov;97(5):1469-1476, and to prepare another one (in progress), which is going to describe the development and physico-chemical characterization of nanostructured BNZ:CD complexes for the Chagas disease treatment.

Impact on Production Costs

The partners involved in the BERENICE project has made a first outline of business plans to ensure a good exploitation and impact of project results. A detailed business plan was developed by consortium partners in order to describe how the Consortium intends to use the outcomes of the project in order to create and market new processes and products related to Chagas disease treatment, aligned with the scope ”Low-cost interventions for disease control in resource poor settings ”(medical devices included). In the case of the proposal and integrating the main results achieved to this date in the BERENICE project it has been considered that there are different exploitable results:

- **A new therapeutic scheme that represents a significant reduction of the drug. We can expect similar cure ratio when compared with the standard dose, besides a reduction in the final cost and probably an improvement in to the toxic profile.**

Currently, Berenice is implementing the Clinical Trial that supports the hypothesis that modifying the therapeutic regimen of BNZ for treatment of Chagas disease in chronic phase in its indeterminate form or symptomatic, it may represent at least similar response rates compared to the standard scheme, with reduced toxicity and improved adhesion treatment. Nevertheless, we describe a Business Plan based in the proposition whether the treatment regimens to be evaluated are advantageous vs the standard regimen of BNZ 300mg/day for 60 days.

New arms:

• Benznidazol 150mg/d for 60 days

• Benznidadol 400mg/day for 15 days

As shown in the financial forecasts of the project, an effective, low cost and safe oral treatment for the chronic and acute forms of Chagas disease, including new BNZ regimens, in addition to new biomarkers that monitor diagnosis and prognosis of the disease are expected to emerge from the results of the project. The cost of this proposed clinical practice in chronic patients could be significantly reduced (when comparing to current clinical schemes).

Dissemination and/or exploitation of project results

In the Frame of BERENICE project, work related to dissemination tasks has been carried out properly, achieving the expected results through the proper collaboration of all the implied partners.

The partners involved in the WP8 of the BERENICE project have made a first outline of Business Plan to ensure a good exploitation and impact of project results.

The major results obtained have been used to disseminate in several scientific events, especially in Latinamerica. The different events, workshops, channels and tools have allowed reaching the entire different stakeholder involved in Chagas disease and identified in the stakeholder's database. Several stakeholders meetings, three successful Workshops carried out in Brazil and Colombia, dissemination materials elaborated as well as presence in Social media channels where the Berenice project results (news, related activities to Chagas disease) have been frequently updated including Linkedin, Slideshare, Twitter and Facebook.

Academic Exploitation

Exploitation at this level deals with scientific dissemination (scientific journals, conferences) as well as with protecting the generated IP through patents, copyright or secrecy agreements, in order to license later the results to industrial partners. To ensure that results and innovations are optimally used and exploited, the members of the Consortium was educated in technology transfer and protection of intellectual property rights. For instance, all researchers are aware of the absolute requirement for maintaining strict confidentiality in order to make patenting of innovations possible. In this sense the partners undertook to communicate data to be published to the Consortium 30 days before publication. The consortium members have right to object to the publication if data to be published jeopardize the protection or the ownership of results. This was managed through the Steering Committee that can take measures to postpone publication.

The Project led to generation of new knowledge that can be used by these partners as input to further studies or projects or, when appropriate, in education at all levels: BSc, MSc, and PhD where aspects of the proposed research can be integrated. Concretely, an open access monograph with an updated overview of the recent discoveries in the field of Chagas disease treatment was published within Berenice Project. This special collection gathered 12 outstanding articles on Chagas disease with the newest challenges in the management of this field, and proposed contributors are key opinion leaders in their area of expertise, who will contribute with new and relevant information very useful for the Chagas disease scientific community.

In addition, the following 13 articles and altogether, 7 MSc and PhD thesis were produced from the outcomes of Berenice project results:

Scientific articles:

- Evaluation of cytokine profile and HLA association in benznidazole related cutaneous reactions in patients with Chagas disease; publishing date: 11.08.2015 at Oxford University Press
- Pharmacokinetic and tissue distribution of Benznidazole after oral administration in mice.
- Pharmacokinetic of benznidazole in healthy volunteers: Implications in future clinical trials; publishing date: 06.02.2017 at American Society for Microbiology

- Experimental and Clinical Treatment of Chagas Disease: A Review; publishing date: 06.02.2017 at American Society of Tropical Medicine and Hygiene
- Benznidazole Nanoformulates: A Chance to Improve Therapeutics for Chagas Disease; publishing date: 06.02.2017 at American Society of Tropical Medicine and Hygiene
- Course of serological tests in treated subjects with chronic Trypanosoma cruzi infection: A systematic review and meta-analysis of individual participant data; publishing date: 01.08.2018 at Elsevier
- Analogs of the Scorpion Venom Peptide Stigmurin: Structural Assessment, Toxicity, and Increased Antimicrobial Activity; publishing date: 01.04.2018 at Toxins Editorial Office
- Efficacy and safety assessment of different dosification of benznidazol for the treatment of Chagas Disease in chronic phase in adults. Randomized clinical trial (MULTIBENZ-BERENICE project). Pending for publication.
- Low-dose of benznidazole promotes therapeutic cure in experimental chronic Chagas disease with absence of parasitism in blood, heart and colon. Pending for publication.
- Nanostructured Benznidazole:Cyclodextrin complex for the treatment of Chagas Disease. Pending for publication.
- Benznidazole treatment: time and dose-dependence varies with the Trypanosoma cruzi strain. Pending for publication.
- Pre-clinical acute toxicological evaluation of Benznidazole nanoformulations. Pending for publication.
- Evaluation of toxicological parameters of BNZ and nanoformulations of BNZ in murine model. In progress. Pending for publication.

MSc and PhD thesis:

- "Development and validation of a bioanalytical method for pharmacokinetic studies and biodistribution of anti-Chagas Benznidazole drug in mice" Supervised by Principal Investigator Dra Claudia Martins Carneiro; presented at Fundacao Oswaldo Cruz
- “Interaction of Trypanosoma cruzi and nervous tissue, new therapeutic approaches for Chagas disease”. Line of research: infectious disease and transplantation" Supervised by Principal Investigator: Dra Teresa Vinuesa; presented at Universitat de Barcelona
- “New therapeutically options in the Treatment of Chagas disease”. Line of research: infectious disease and transplantation. Supervised by p Dra Teresa Vinuesa; presented at Fundacao Oswaldo Cruz
- Establishment of murine model for the modulation of benznidazole toxicity during treatment of Chagas disease (Trypanosoma cruzi).” Supervised by Principal Investigator Marcelo Sousa Silva presented at Instituto de Higiene y Medicina Tropical

- TFG Ciències biomèdiques. 2014-15 UB Mar Bonany, "Avaluació de l'efecte antiparasitari de productes d'origen bacterià“ Supervised by Principal Investigator: Dra Teresa Vinuesa, presented at Universitat de Barcelona
- TFM: Máster Master en Recerca Clinica. 2014-2015 UB. Laura Oliver Hernández. Producción y caracterización de sustancias antimicrobianas producidas por Serratia marcescens Supervised by Principal Investigator: Dra Teresa Vinuesa; presented at Universitat de Barcelona
- TFM: Máster en Microbiología avanzada 2016-2017 UB. Anna Mur Suñé. Producción y purificación del pigmento Prodigiosina y su efecto antibacteriano. Supervised by Principal Investigator: Dra Teresa Vinuesa; presented at Universitat de Barcelona

1.1.5. The address of the project public website, if applicable as well as relevant contact details

Web address: www.berenice-project.eu

The website has been inspired by the logo of the project. Therefore apart from presenting website structure, design and accessibility, we also submit the logo, which represents the Berenice project thought the different elements that compose it.

BERENICE Public/ Private Website

The public website is available at www.berenice-project.eu, to be used as the main vehicle of dissemination and interaction with the different classes of audience who seeks information about the BERENICE Project and its areas of work.

Project logo

The project logo is a fundamental vehicle to make the project easily recognized in Europe and endemic countries. All dissemination material produced within the project will use the logo and will be produced according to the agreed standards, in order to ensure that BERENICE reveals a clear identity and compliance with FP7 Guidelines.

The text

In the text within the logo we can read the word "Berenice". Berenice is not only the acronym of the project name, but also the name of the first person who was found to have the disease.

The line

It represents the "Tripanosoma cruzy" which is the protozoan that transmits Chagas disease which taken out of context might not mean anything to us, however within the context of the Berenice project, it represents one of the main project purpose: the fight against this protozoan and what it represents.

The balloons

The "Berenice" entails a research and innovation in nanotechnology. The logo highlights this process throught the representation of the idea of nanotechnology in a succession of balls all together, this idea evokes this item.

The combination of these three elements is a summary of the key words that we have considered essential in the project (Berenice, disease, Trypanosoma and nanotechnology).

Video

Main Contacts

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