UNIVERSITY OF SÃO PAULO
School of Pharmaceutical Sciences
Graduate Program in Drug and Medicinal Products
Concentration area: Production and Control

Innovative pharmaceutical strategies for niclosamide repositioning

EDUARDO JOSÉ BARBOSA

Ph.D. Thesis to obtain the title of Doctor.

Supervisor: Prof. Dr. Nádia Araci Bou-Chacra
Co-Supervisor: Prof. Dr. Gabriel Lima Barros de Araujo

SÃO PAULO
2022

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Original Version

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Prof. Dr.
Supervisor/ President

1st examiner

2nd examiner

3rd examiner

4th examiner

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I

Dedication

I dedicate this study to my parents,

for all the support in my life

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To my family for their support in my life.

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Epigraph

(...)
– Dad?
– Yes?
– Do you like this hotel?
– Yes, I do. I love it. Don't you?
– I guess so.
– Good. I want you to like it here. I wish we could stay here for ever, and ever... and ever.
(...)

Danny and Jack Torrance dialogue in Stanley Kubrick's *The Shining*.

RESUMO

BARBOSA, E.J. **Estratégias farmacêuticas inovadoras para o reposicionamento da niclosamida**. 2022. 112p. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2022.

O antiparasitário niclosamida tem apresentado promissora atividade anticâncer em estudos pré-clínicos contra diversos tipos de câncer, como coloretal e próstata. Assim, o objetivo deste trabalho foi desenvolver formulações inovadoras para o reposicionamento da niclosamida como agente anticâncer. No capítulo I foi realizada revisão crítica da literatura sobre as propriedades físico-químicas do fármaco, além de resultados de estudos clínicos da niclosamida contra câncer de coloretal e de próstata. Além disso, foi feita revisão sobre estudos que desenvolveram formulações contendo esse fármaco, bem como hipóteses para melhorar o desempenho biofarmacêutico dessa molécula. No capítulo II foi realizado o desenvolvimento de dispersão solida amorfa contendo niclosamida. Soluções de fármaco/polímero foram levitadas em levitador acústico e caracterizadas por raios-X de luz síncrotron. Este conjunto permitiu medições rápidas e de alta qualidade, bem como identificação de recristalização da niclosamida. Plasdone® e Soluplus® demonstraram melhores propriedades para formar as dispersões amorfas, com o último apresentando aumento de solubilidade superior. O estudo mostrou que a formulação desenvolvida aumentou em duas vezes a solubilidade aparente de saturação da niclosamida em água. No capítulo III o objetivo foi o desenvolvimento, a caracterização físico-química e atividade anticâncer *in vitro* de uma nanoemulsão de niclosamida, tendo células HCT-116 como modelo celular. Resultados preliminares indicaram o Capmul® MCM C8 como o melhor lipídio líquido para o sistema, mas as primeiras nanoemulsões contendo este lipídio não foram estáveis para justificar seu uso. Por outro lado, Miglyol® 812 indicou ser um lipídio líquido adequado para o sistema. A nanoemulsão de niclosamida (~200 nm) com Miglyol® 812 e poloxâmero 188 foi estável por 56 dias, com distribuição monomodal do tamanho de partícula. O ensaio de viabilidade celular contra células HCT-116 demonstrou que a citoxicidade da niclosamida é dependente do tempo e da concentração. Os resultados aqui obtidos encorajam mais pesquisas para entender e otimizar o desempenho da niclosamida como uma substância anticancerígena.

Palavras-chave: niclosamida, reposicionamento, câncer de cólon, dispersão solida, levitação acústica, luz síncrotron, nanoemulsão.

ABSTRACT

BARBOSA, E.J. **Innovative pharmaceutical strategies for niclosamide repositioning**. 2022. 112p. Thesis (Ph.D.) – School of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2022.

The antiparasitic niclosamide has shown promising anticancer activity in preclinical studies against several types of cancer, such as colorectal and prostate. Thus, the objective of this work was to develop innovative formulations for the repositioning of niclosamide as an anticancer agent. In chapter I, a critical review of the literature on the physicochemical properties of the drug was carried out, in addition the results of clinical studies against colorectal and prostate cancer. Besides, a review was carried out on studies that developed formulations containing this drug, as well as hypotheses to improve the biopharmaceutical performance of this molecule. In chapter II, the development of solid amorphous dispersion containing niclosamide was carried out. Drug/polymer solutions were acoustic levitated and characterized by synchrotron X-ray light. This set allowed fast, high quality measurements, as well as the identification of niclosamide recrystallization. Plasdone® and Soluplus® demonstrated better properties to form amorphous dispersions, with the latter showing superior solubility enhancement. The study showed that the developed formulation increased the apparent saturation solubility of niclosamide in water by two times. In chapter III the objective was the development, physicochemical characterization and *in vitro* anticancer activity of a niclosamide nanoemulsion, having HCT-116 cells as a cellular model. Preliminary results indicated Capmul® MCM C8 as the best liquid lipid for the system, but the first nanoemulsions containing this lipid were not stable to justify its usage. On the other hand, Miglyol® 812 indicated to be a suitable liquid lipid for the system. The niclosamide nanoemulsion (~200 nm) with Miglyol® 812 and poloxamer 188 was stable for 56 days, with a monomodal particle size distribution. Cell viability assay against HCT-116 cells demonstrated that niclosamide cytotoxicity is time and concentration dependent. Results herein obtained encourage further research to understand and optimize niclosamide performance as an anticancer drug substance.

Keywords: niclosamide, repositioning, colon cancer, solid dispersion, acoustic levitation, nanoemulsion.

SUMMARY

Chapter I
Niclosamide repositioning for treating cancer: challenges and nano-based drug delivery opportunities

This study was published as Eduardo José Barbosa, Raimar Löbenberg, Gabriel Lima Barros de Araujo, Nádia Araci Bou-Chacra, with the title of "Niclosamide repositioning for treating cancer: Challenges and nano-based drug delivery opportunities", in *European Journal of Pharmaceutics and Biopharmaceutics*, 2019, 141, 58-69.

Abstract

Drug repositioning may be defined as a process when new biological effects for known drugs are identified, leading to recommendations for new therapeutic applications. Niclosamide, present in the Model List of Essential Medicines, from the World Health Organization, has been used since the 1960s for tapeworm infection. Several preclinical studies have been shown its impressive anticancer effects, which led to clinical trials for colon and prostate cancer. Despite high expectations, proof of efficacy and safety are still required, which are associated with diverse biopharmaceutical challenges, such as the physicochemical properties of the drug and its oral absorption, and their relationship with clinical outcomes. Nanostructured systems are innovative drug delivery strategies, which may provide interesting pharmaceutical advantages for this candidate. The aim of this review is to discuss challenges involving niclosamide repositioning for cancer diseases, and the opportunities of therapeutic benefits from nanosctrutured system formulations containing this compound.

Keywords: Niclosamide, repositioning, physicochemical properties, cancer, clinical trials, nanostructured systems.

1 Introduction

The discovery of a new drug and its way to the market can be a risky process that may cost billions to pharmaceutical companies. The compound may fail due to its toxicity or lack of efficacy in clinical trials, besides the long time involved in the process until reaching the market, which is estimated between 10 – 17 years [1]. In this context, to find new uses for existing drugs, generally referred to as drug repositioning, is a strategy that has been gaining attention. It can provide advantages compared to the traditional approach, for instance, the reduced risk of failure due to toxicity. In this case safety data and the pharmacokinetic profile of an approved drug are already known. It is also associated with lower costs and reduced timeline, as the drug has already been evaluated in early stages of clinical trials [1,2]. This process has an estimated cost of around $300 million and may last for about 6.5 years [2].

This strategy also provides an opportunity to develop innovative formulations, which may provide clinical benefits compared with those of older marketed drugs [3]. In this case, nanostructured systems are especially interesting. The reduction in particle size to the nanoscale promotes changes in physicochemical and pharmacokinetic properties, among them increasing dissolution rate and bioavailability. This is particularly important for problematic drugs with low solubility and absorption, allowing dose and toxicity reduction [4]. Hence, as different alternatives of creation of the formulation are associated with the development of nanostructured systems [4], it also brings the perspective of patent protection for innovative products [3].

In this context, it is noteworthy to mention that there are different terms to express the concept of "drug repositioning". In the study of Langedijk and colleagues, the authors analysed "repositioning" and other words used in the literature until 2013 [5]. In this study, a great increase was observed in the number of publications using terms related to drug repositioning, after 2010. In addition, equivalent terms have been adopted, including "reprofiling" or "redirecting", but "repurposing" has been the most common and interchangeable with "repositioning". However, a common definition was not identified. Among them, terms referring to new therapeutic applications, or new uses for "old" or approved drugs were adopted. Hence, examples of successfully repositioned drugs include sildenafil, previously designed to treat coronary artery disease in the 1980s, but then during Phase I clinical trials, it was discovered that it could treat erectile dysfunction. After its failure in Phase II studies for treating angina, it was approved in 1998 for erectile dysfunction [6]. Another example is thalidomide, initially designed to be used as a sedative, and then shown to treat morning sickness for pregnant

women in the 1960s, until its withdrawal from the market due to birth defects. Then, several studies in the subsequent decades reported its anticancer effects, which eventually led to its approval for multiple myeloma in 2006 [6].

Niclosamide is a drug present in the Model List of Essential Medicines from the World Health Organization [7], used since the 1960s for tapeworm infection [8]. For adults, the dose administered is 2 g orally, presenting efficacy and safety due to its local action in the gastrointestinal tract [9]. Its mechanism of action is attributed to uncoupling of oxidative phosphorylation in the mitochondria, which interferes with the metabolism of these organisms [10,11]. Recently, new biological activities related to cancer diseases have been attributed to this molecule, which perhaps make this drug one of the most promising candidates for repositioning from new therapeutic indications [12,13].

Nonetheless, such expectations also must be accompanied by rational and realistic evaluation of its potential, since the repositioning process still requires proof of efficacy and safety of the compound. This implies different challenges to be dealt with, such as the physicochemical properties of the drug, its oral absorption process and clinical outcomes. Therefore, this review aims to discuss challenges involving niclosamide repositioning for cancer diseases, in addition the prospect of therapeutic benefits that nano-based formulations may provide.

2 Mechanistic insights in the physicochemical properties of niclosamide

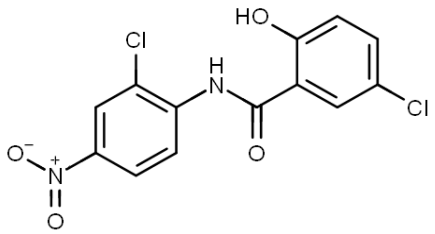
The development of innovative formulations aiming at drug repositioning requires knowledge of the physicochemical properties of the drug substance, since they are related to the complex drug absorption process, and its distribution in the tissues [14]. These properties greatly influence decisions about the design and development of pharmaceutical products aiming at efficient drug delivery. Their mechanistic understanding provides a scientific basis in drug product performance [14,15].

2.1 Crystal stability and relationship with poor aqueous solubility

Niclosamide is composed of yellow pale crystals, and it is practically insoluble in water, and moderately soluble in ethanol, chloroform and ether [16]. This compound presents 327.12 g/mol molecular weight (M.W.) (Fig. 1), and three crystal forms are described in the literature: a hygroscopic anhydrous form, and two monohydrates, H_A and H_B, with melting points in the

range 228-230 °C (Table 1) [17,18]. The existence of different hydrated forms implies differences in physicochemical properties, such as water solubility and intrinsic dissolution [17]. In aqueous medium, the anhydrous form rapidly tends to convert to H_A, which in turn converts to the most stable, and the least water-soluble, H_B [17,18]. Conventional analytical techniques, such as thermal analysis, infrared spectroscopy and X-ray diffraction, may be used to characterize these forms [17,18].

Fig. 1. Molecular structure of niclosamide (M.W. 327.12 g/mol).



Source: Barbosa et al., 2019.

Table 1. Physicochemical properties of niclosamide.

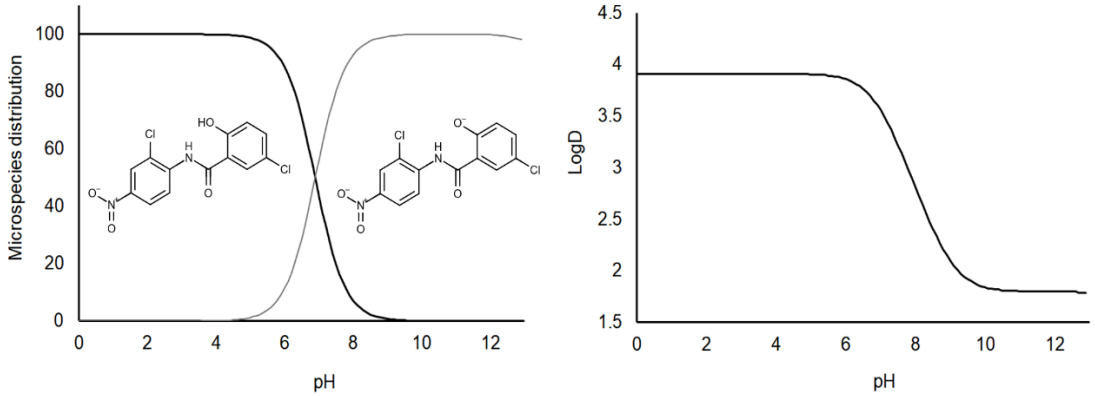
pKa	LogP	Crystal form properties			
		Crystal form	Dehydration [d] (ºC)	Melting point [d] (°C)	Water solubility [e] (µg/mL)
		Anhydrous	–	229	13.32
6.89 [a] 7.25 [b]	3.91 [a] 4.45 [c]	H_A	100	228	0.95
		H_B	173	230	0.61

a: *In silico* prediction by Chemicalize.com; b: determined at 25 ºC by capillary electrophoresis [19]; c: determined at 22 ºC in octanol/water partition [20]; d: Determined by differential scanning calorimetry (DSC) [17,18]; e: Measured at 25ºC [17].

According to the Chemicalize.com prediction, the molecule is a weak acid, with higher proportion of anionic species in blood pH (7.4) (Fig. 2). According to the Biopharmaceutical Classification System (BCS), solubility and permeability are two important factors associated with drug absorption by oral route: since the drug is in a solid state, it has to be dissolved in gastrointestinal fluids to be absorbed by intestinal tract into the blood stream [21,22].

Considering the dose of 2 g used for tapeworm infection, niclosamide is classified as a low solubility drug by BCS. In this case, its oral absorption is not an issue when the objective is the local action in the gastrointestinal tract, but obviously becomes a challenge when it is aimed at efficient systemic drug exposure.

Fig. 2. pKa and logD *in silico* predictions by Chemicalize.com.



At pH 6.89, distribution is 50% for neutral and anionic species, and at this pH LogD is 3.6.

Source: Barbosa et al, 2019.

Neutral molecules are able to permeate biological membranes, a phenomenon energetically unfavourable to charged species [23]. Then, oral absorption of niclosamide becomes a "double-edged sword": the compound is a weak acid; therefore it is neutral at low pHs (Fig. 2), which restricts its solubility in the gastric region. On the other hand, as the molecule enters the small intestine, although an increase of pH theoretically favours its solubilization, permeation through enterocyte membranes becomes less favoured with higher proportion of charged species. In addition, no significant difference in solubility was observed in acid medium (0.1 N HCl) and in buffered medium (6.8 pH) [24], which further increases the challenge for the absorption. In this context, based on its molecular and crystalline structure, and also considering the work of Bergström and colleagues [25], a hypothesis may be derived to explain the fundamentals involved in niclosamide's poor solubility in water.

In their study, the relationship between physicochemical properties and aqueous solubility were evaluated using statistical tools for a set of 15 poor soluble drug substances [25]. According to the authors, "brick dust" and "grease balls" are terms generally used to refer to poor soluble compounds: the former refers to those composed of a stable crystalline structure, marked by strong intermolecular bonds that prevent interaction with water, whereas the latter refers to high lipophilic compounds that do not interact with this solvent [25]. In brief, solid-

state properties (such as melting point, enthalpy and entropy of melting) showed low correlation, while properties such as lipophilicity and nonpolar surface area showed a correlation to aqueous solubility, indicating that these drugs would be “grease balls”, with solvation limited solubility [25].

For niclosamide, the existence of N–H···O intramolecular interaction favours a near planar conformation for the structure, whereas O–H···O (from hydroxyl to carbonyl group) align the molecules along an axis, being connected and stabilized by interactions between Cl···NO_2 groups and between aromatic rings, promoting the formation of layered molecular chains [26,27]. The presence of hydrogen-bonding sites (NO_2, OH, carbonyl groups), and two Cl atoms contribute to solvation by water, and the anhydrous crystal lattice allows the entry of water or other molecules in the cavities of the structure, forming hydrates or solvates [26,27].

Solvation and disruption of the crystal packing are among the processes related to solubilization of crystalline compounds, which requires energy to occur [28]. Besides, the presence of water molecules may provide stability to a crystal lattice, comparing to the anhydrous form [28]. Thus, a niclosamide crystalline structure might restrict interactions with water and breaking of the crystal, which is reflected in the low solubility of the anhydrous form (13.32 µg/mL). In addition, formation of stabilized hydrates might limit even more niclosamide interactions with water, reflecting the lower aqueous solubility for H_A and H_B monohydrates (0.95 and 0.61 µg/mL, respectively). Therefore, niclosamide water solubility could also be associated with a solvation limited phenomenon. However, since it presents a stable crystalline structure (M.P. 228-230ºC), its profile could be more similar to tolfenamic acid. As referred to by Bergström and colleagues, specifically for this also small molecule (261.1 g/mol), the combination of lipophilicity and stability of the crystal (M.P. 212 ºC) was likely related to its low solubility [25].

2.2 Lipophilicity: lipid-associated oral absorption and drug-likeness considerations

If niclosamide has poor aqueous solubility, on the other hand, a high lipophilicity profile is indicated by the *in silico* and experimental logP values (3.91 and 4.5, respectively). These values show that niclosamide solubility is thousands of times higher in the organic phase than in aqueous phase, reflecting a tendency to dissolve in lipids, in crossing biological membranes, and binding to proteins [29,30]. The LogD parameter also refers to lipophilicity, but the pH variable is included in the calculation. Hence, the value of 3.6 at pH 6.9 (Fig. 2) indicates a decreasing trend in its partition in the lipid phase due to a higher proportion of ionized species

at this pH. The high lipophilicity also might favour the development of lipid-based formulations [29]. However, not all poor water-soluble compounds may present good solubility in lipids, since the high stability may also be an obstacle to solubilization in these components [30]. Therefore, care should be taken with the development of the formulation, such as a rational approach for the selection of the excipients, and use of design of experiments (DOE) [30].

A possible mechanism by which lipid formulations might be associated with niclosamide oral absorption was studied by Alskär and colleagues, when lipolysis-triggered supersaturation and precipitation of eight compounds were evaluated, and correlated with their physicochemical properties [31]. Three types of formulations were prepared: one containing long-chain lipids (soybean oil and Maisine® 35-1), a second containing medium-chain lipids (Capmul® MCM EP and Captex® 355), and a third containing only surfactant and cosolvent (Cremophor® EL and Carbitol™). After *in vitro* lipolysis tests, using intestinal simulated fluid as medium, solubility and solid characterization were performed. In brief, niclosamide significantly precipitated as a crystalline hydrate after addition of pancreatic extract and, despite the decrease in solubility due to the precipitation, the compound remained in a supersaturated state during the tests [31].

As a comparison, according to the authors, the compound showed poor saturation solubility in the medium with enzymes (30 µg/ml). However, during lipolysis, the values related to the aqueous phase dropped from 250 to around 80 µg/ml after 60 minutes of test, using the medium-chain rich lipid-based formulation as drug carrier [31]. Obviously that solubility values may vary according to conditions of the test, but this range is clearly higher than a reported value for the most water-soluble anhydrate form (13.32 µg/ml) [17]. Therefore, although the possibility of solid formation and the subsequent dissolution-rate dependence for absorption, lipid-based formulations might improve niclosamide bioavailability due to the ability to maintain some degree of supersaturation in the gastrointestinal fluids [32]. This could be optimized if maintained as long as possible during the passage of the drug throughout the intestine [32]. Using multivariate analysis, Alskär and colleagues observed that compounds with low molecular weight (< 350 g/mol) and high melting point (> 200 ºC), among them niclosamide and tolfenamic acid, tended to precipitate in crystalline forms, also indicating some similarity between these two molecules related to the solid state [31].

During the digestion of a formulation, the lipids can be absorbed or partitioned into micelles or mixed micelles, composed of bile salts and/or lipidic excipients [33,34]. Then, the drug released is also subjected to partition into the micellar species formed [33,34]. Thus, supersaturation might not be the only phenomenon related to a possible enhancement of

niclosamide oral absorption. For instance, lipid particles can adhere to the membrane of the enterocytes, releasing the drug within the cells, which can be optimized by reducing the particle size, providing a higher surface area for adhesion [34]. Additionally, inhibition of drug efflux transporters by surfactants and other excipients, such as Tween® 80 and Cremophor® EL, were already proposed as a possible mechanism, increasing the permeability and oral absorption of drugs [35-37]. Therefore, these examples reflect the need for a better understanding of the phenomena that may be associated with oral absorption of niclosamide and other drugs.

Regarding the lipophilicity of drugs, increasing logP leads to an increase in the volume of distribution of the compound, and also in central nervous system (CNS) penetration [29]. Nonetheless, a high logP value (> 5) is not desirable for drug candidates because it may be reflected in poor aqueous solubility and absorption, and likelihood of *in vivo* toxicity, which could compromise approval in clinical trials [29]. Thus, because niclosamide is a drug candidate, the possibility of systemic action raises the question about its drug-like features, that is, if its physicochemical properties are related to likelihood of successful approval [38].

Based on molecular descriptors and physicochemical properties, different rules of thumb or guidelines have been proposed to guide the design and development of drug candidates [38,39]. One of the first, and most known, was the work of Lipinski, which proposed a cLogP (calculated logP) ≤ 5 for good oral candidates, based on a statistic evaluation of compounds that reached, at least, Phase II clinical trials [38,39]. Another example was the work of Gleeson, who used a set of compounds from GlaxoSmithKline (GSK) to evaluate the influence of molecular descriptors in ADMET properties (absorption, distribution, metabolism, excretion, toxicity). The author suggested that molecules with both M.W. < 400 and clogP < 4 have a better chance of presenting desirable ADMET properties [40]. Lastly, Waring proposed an optimum range between 1 and 3 and for lipophilicity, since high hydrophilic compounds also present undesirable properties, such as high renal clearance and low permeability and tissue distribution [41].

Hence, considering cLogP value (3.91), niclosamide would not be so distant from a desirable value for lipophilicity, further regarding LogD 3.6 at pH 6.9. However, obviously the experimental value (4.45), and its water solubility place this candidate in a “not ideal” drug-likeness region, compared to other compounds. This means that high doses might be required to achieve therapeutic blood levels. Hence, it justifies diverse pharmaceutical strategies, such as nanostructured systems, to improve its aqueous solubility and oral absorption.

3 The discovery of niclosamide anticancer activities

A study by MacDonald and colleagues, in 2006, perhaps may be the first that indicated a possible repositioning process of niclosamide for cancer diseases, when unexpected results were revealed for this compound [42]. In this study, protein-protein interactions in HEK 293 cells were evaluated by protein-fragment complementation assays (PCA). The objective was to observe hidden or not expected biological activities from 107 compounds, of different therapeutic classes. Using this approach, drug substances that could present unexpected antiproliferative activities were identified. The action of niclosamide was confirmed in five different cancer cell lines (PC3, A549, MiaPaCa, LOVO and U87MG), with a mean IC_{50} of 0.6 μM [42].

Then, several preclinical studies associated its anticancer activity with the inhibition of the signaling pathways Wnt/β-catenin [43-49], STAT3 [50-60], Notch [61-64], NFκB [65-68] and mTOR [69-72], which are cellular mechanisms related not only to different cell functions, but also to pathological conditions when deregulated. The effect in multiple signaling pathways contributes to its action against different types of cancer cells, among them colon, prostate, ovarian, breast and lung [12,13].

4 Repositioning clinical studies for colon and prostate cancer

From 2009 to subsequent years, preclinical studies that evaluated niclosamide against colon and prostate cancer cells provided encouraging results. For colon cancer, its activity was related to inhibition of Wnt signaling, which is an important mechanism for stem cells, associated with self-renew and homeostasis of tissues in adults, but also related to several diseases when aberrantly active [73,74]. Niclosamide then showed its potential for use against colon cancer, including metastatic conditions [43,44,46].

For prostate cancer, promising results were reported by studies that evaluated niclosamide combined with drugs approved by the Food and Drug Administration (FDA) agency. Abiraterone (Zytiga®) is an androgen synthesis inhibitor approved in 2011, while enzalutamide (Xtandi®) is an androgen receptor (AR) antagonist and inhibitor approved in 2012 [75-77]. The main strategy for treating metastatic prostate cancer is castration by surgical or chemical intervention, which reduces androgen levels and, consequently, tumour growth [78,79]. However, in most cases, cellular adaptations involving AR signaling still promote disease progression [78,79]. The use of abiraterone or enzalutamide provides clinical benefits, but

therapy resistance may arise and can be associated with AR variants [78-80]. Hence, inhibition of the STAT3 pathway by niclosamide not only provided anticancer effects, but also helped overcome resistance to these drugs [55-57]. Taken together, these results supported and prompted a series of clinical trials that began in 2015 (Table 2), with the first results being published in 2018 (Table 3).

The first study completed was from the University of Washington (NCT02532114), in which relevant findings were published by Schweizer and colleagues [81]. In this study, niclosamide was administered in soft gelatin capsules of 500 mg, for patients with castration-resistant prostate cancer (CRPC), previously treated with abiraterone. Two dose levels were assessed: 500 and 1000 mg, three-times-daily (TID) each. According to the authors, due to the poor oral bioavailability typically reported for niclosamide, the objective was to administer higher doses of the compound than those used for tapeworm infection (2 g/day).

Briefly, combined treatment was well tolerated with the 500 mg dose regimen, but the two patients treated with 1000 mg dose level presented adverse events, among them nausea, colitis and diarrhea, which were to some extent already predicted, as this dose level was clearly higher than those normally used for helminthic infection [81]. As previously mentioned, for adults the treatment is 2 g in a single dose, and in the case of *Hymenolepis nana* infection, it is followed by 1 g daily for 6 days [9]. Particularly, for one of the patients, symptoms began on day 26, suffering from diarrhea lasting > 72 hours, whereas for the other, abdominal pain and diarrhea started on day 8, leading to hospitalization and medical care. Thus, the 500 mg dose was considered the maximum tolerated dose [81]. Complementary studies showed that even after administrating high doses of niclosamide no clinical evidence of activity is observable [81]. This fact can be assigned to the inability to overcome the poor bioavailability and consistently achieve the plasma concentrations necessary to inhibit tumour growth, based on CRPC models [55-57]. However, despite the clinical failure of the compound, the authors recognized some limitations of the study, such as the small number of patients and non-evaluated drug-drug interaction and the need for the development of alternatives with improved oral bioavailability [81]. In this context, as long as niclosamide shows striking anticancer activity, a better understanding of the potential for repositioning can be obtained from the ongoing clinical trials.

Table 2. Niclosamide repositioning clinical studies for cancer diseases. For this search, the keyword “niclosamide” was used at the ClinicalTrials.gov website.

Start	Phase	Sponsor (Identifier)	Drug substances	Type of cancer	Estimated patients	Treatment	Outcome measures	Estimated conclusion
2017	I	Duke University USA (NCT02687009)	Niclosamide	Colon	18	Niclosamide (PO QD), during 7 days, prior to surgical resection of primary tumour.	Dose limiting toxicity, niclosamide blood levels	2022
2017	I	University of California, Davis USA (NCT03123978)	Niclosamide, enzalutamide	Prostate	12	Niclosamide (PO BID) and enzalutamide (PO QD) on weeks 1-4. Treatment repeats every 4 weeks in the absence of disease progression or unacceptable toxicity.	Dose limiting toxicity, adverse events, OS, PFS, PSA response, time to treatment failure	2021
2016	II	University of California, Davis USA (NCT02807805)	Niclosamide, prednisone, abiraterone acetate	Prostate	40	Niclosamide (PO BID), prednisone (PO BID), and abiraterone acetate (PO QD). Treatment repeats every 4 weeks in the absence of disease progression or unacceptable toxicity.	Dose limiting toxicity, OS, PFS, PSA response, overall response	2021
2015	II	Charite University Germany (NCT02519582)	Niclosamide	Colon	37	Niclosamide (PO QD) until disease progression or toxicity.	OS, PFS, TP, disease control rate, adverse events.	2020
2015	I	University of Washington USA (NCT02532114)	Niclosamide, enzalutamide	Prostate	5	Niclosamide (PO TID) and enzalutamide (PO) for 28 days, until disease progression or unacceptable toxicity.	Dose limiting toxicity, niclosamide blood levels, PSA response.	2017 (concluded)

PO: by mouth, QD: once a day, BID: twice a day, TID: three-times-daily, OS: overall survival, PFS: progression-free survival, PSA: prostate-specific antigen, TP: time to progression.

Table 3. Treatments and first results from clinical trials for niclosamide repositioning. The study from the University of Washington was completed in 2017 and published in 2018 [81]. University of California, Davis [82] and Charité University [83] published preliminary results in 2018.

Study (niclosamide treatment)	Drug substances	Patients (ages)	Niclosamide			Results
			Blood levels (ng/mL)		Adverse effects	
			Target	Achieved		
University of Washington (500 – 1000 mg/PO TD)	Niclosamide, enzalutamide	5 (60-84)	> 163.5	35.7-182 *	Nausea, diarrhea, colitis	No PSA reduction
University of California, Davis (1600 mg/PO TD)	Niclosamide, abiraterone, prednisone	6 (74-83)	> 32.71	100-162 **	Nausea, diarrhea	PSA reduction for at least four patients
Charité University (2000 mg/ PO QD)	Niclosamide	5	–	429-1777 *	No drug related toxicity reported	Possibility of longer time until disease progression

* Corresponds to C_{max} values. ** Corresponds to trough levels. PO: by mouth, QD: once a day, BID: twice a day, TID: three-times-daily; PSA: prostate-specific antigen.

What reinforces this argument is that different findings were described in preliminary results from the University of California, Davis (NCT02807805) (Table 3), when niclosamide was evaluated in combination with abiraterone and prednisone [82]. In a preliminary phase, CRPC patients were evaluated, with dose escalation of niclosamide from 400 mg PO BID to 1,600 mg PO TID (Table 3). According to results, the 1,600 mg PO TID regimen was well tolerated in five patients, considering the recommended dose for Phase II trial, which is, intriguingly, even higher than those assessed in the previous study from the University of Washington, with nausea and diarrhea being reported.

Although the niclosamide blood levels are in a range comparable to the failed study from the University of Washington (Table 3), the trial from the University of California used "trough level" as a pharmacokinetic parameter, which refers to the lowest blood concentrations during therapeutic drug monitoring, obtained at the end of a dose interval [84]. Thus, these Universities adopted different strategies to address the relationship between the anticancer activity and niclosamide blood levels. The University of California trial emphasized that therapeutic blood

levels are achievable. Hence, this might indicate that higher concentrations can be obtained, that would be necessary for some anticancer effect. An indication for this is that, in this study, PSA reduction was reported for two patients (<0.01 ng/mL). In addition, two other patients presented reduction of ≥ 50% in PSA response [82]. In summary, the authors considered that the combination of niclosamide with abiraterone and prednisone presented promising preliminary safety and efficacy results [82].

For colon cancer, initial results from the Cherite University trial (NCT02519582) (Table 3) were described in the study of Burock and colleagues (2018) [83]. Patients with metastasized colorectal cancer received 2 g of niclosamide in tablets (Yomesan®), once a day, until observation of disease progression or toxicity. In brief, no toxicity was reported. In addition, C_{max} values were clearly higher than values from the University of Washington trial (Table 3), which further indicates that higher niclosamide blood levels might be achieved. A patient with the highest median plasma level (598 ng/mL) showed stable disease at 4 months and, according to the authors, preliminary results indicated that those with higher plasma concentrations might present longer time until disease progression, justifying more investigation [83].

In summary, niclosamide showed consistent anticancer activity in preclinical studies, which justified initiating clinical trials for its repositioning. Initial clinical results might show the importance of the blood levels to present some effect against colon and prostate cancer. However, high doses were administered to achieve target concentrations, as predicted by its physicochemical properties. This raises the question, in the case of approval, as to which doses will be therapeutically applied. Although it is well tolerated when used for tapeworm infection, generally a single dose is administered in these cases. Thus, the impact of its administration during longer periods for patients with cancer diseases has still not been clarified. In this case, nausea, diarrhea and gastrointestinal irritation will likely be commonly observed adverse effects. Therefore, taken together, these considerations further justify the need for nano-based drug delivery systems.

5 Nanostructured systems: fundamentals, types, and methods of production.

According to FDA guidelines for the industry, materials in the nanoscale range can exhibit different physical or chemical properties, or biological effects, differing from their larger counterparts [85]. This guidance defines these systems as 1) those that have, at least, one of their dimensions in the range from 1 to 100 nm; or 2) if the dimensions are in the range from 100 to 1,000 nm, different properties or effects are attributed to their size [85]. Another feature

commonly described in the literature is that they have a higher surface to volume ratio [86]. This means that the reduction of size provides a higher surface area for the system, compared to the bulk material, which also means that a higher proportion of atoms or molecules are present on the surface of the particle [86]. Hence, at the nanoscale, altered properties, such as optical, electrical, magnetic and thermal, can emerge, which can be explored for pharmaceutical or biomedical applications, for instance as therapeutic or diagnostic purposes [86-88].

One example is related to drug substances in their pure solid state, when the reduction of particle size provides physicochemical improvements supported by the Noyes-Whitney equation (1) [89]:

$$\frac{dC}{dt} = \frac{DS}{Vh}(Cs - C) \qquad (1)$$

According to this model, *dC/dt* is the dissolution rate, *S* is the surface area, *D* is the diffusion coefficient, *h* is the diffusion layer thickness, *V* is the volume of dissolution, *Cs* is the saturation solubility, and *C* is the concentration at time *t* [89]. Then, an increase of the surface area enhances dissolution rate. Another commonly reported effect is that particle size also affects the diffusion layer thickness and saturation solubility [89,90]. Thus, increased saturation solubility and dissolution rate are two important features of solid particles at the nanoscale, which is a valuable strategy to improve absorption and bioavailability of poor water-soluble drugs.

Other advantages are usually attributed to nanomaterials. One example is that a higher surface area contributes to adhesiveness of the particles to biological membranes, which can improve their uptake by cells [91]. In addition, incorporation of drugs into nanocarriers can reduce degradation and toxicity [92]. Drug targeting, in turn, consists of coating the surface of nanocarriers with ligands that bind to specific receptors, generally overexpressed by target cells, which is useful for cancer diseases [93]. This strategy, therefore, can provide specificity for treatment, with the prospect of reducing damage to normal tissues [93].

However, if nanoparticles provide interesting advantages for pharmaceutical products, on the other hand one of the biggest challenges related to their development is the stability of the system. The higher surface to volume ratio also means that the higher proportion of chemical species on the surface are not surrounded by their counterparts (comparing to the inner species of the particle), being in contact with the external medium [86]. Therefore, these systems present high total surface energy, being thermodynamically unstable, and phenomena like

agglomeration and Ostwald ripening reflect the tendency of the particles to aggregate, increasing the particle size [94]. Thus, the use of polymers (to promote steric hindrance) and/or surfactants (to provide electrostatic repulsion or reduction of interfacial tension) are strategies usually adopted to preserve the stability of the system [94].

Different strategies have been adopted to develop nanostructured systems. Using nanocrystals is the simplest approach. As previously mentioned, this consists of reducing the particle size of the micronized compound to the nanoscale, being stabilized in water by the presence of polymers or surfactants [95]. Among the advantages related to them is the simplicity of the formulation, and the fact that established techniques for large-scale production are used for their preparation, which supports their approval for clinical use [90,96]. In fact, different products are already on the market [89]. One marked feature is related to nanocrystals: the formulation does not require incorporation of the drug into a matrix system, providing then 100% drug loading to the nanoparticle [95].

The other types may be defined as matrix systems or nanostructured carriers. These structures are classified differently, such as lipid-based, polymeric-based, carbon or magnetic based [96]. Briefly, lipid-based formulations include nanoemulsions, composed of mixture of two immiscible liquids, with submicron droplets of a liquid dispersed in another liquid, stabilized by the presence of surfactants [97]. The two types of nanoparticles that are solid at ambient temperature are: solid-lipid nanoparticles, composed of a solid lipid matrix containing the drug; and the nanostructured lipid carriers, composed of a mixture of solid and liquid lipids [98,99]. Polymeric nanoparticles include the use of natural or synthetic polymers to encapsulate drugs in vesicular reservoirs or in solid polymeric matrix [100], whereas polymeric micelles are composed of amphiphilic polymers (two or more in the case of mixed micelles) which self-assemble into nanocarriers [101]. Still considering polymers, the application of a strong electric field is used to produce nanofibers, which can also act as drug carriers [102]. Examples of carbon derived nanomaterials include nanotubes, graphene and fullerenes, which can also be functionalized with ligands [103]. Magnetic nanostructures are composed of metal or metal oxides, which are directed to the targeted tissue by an external magnetic field [104].

Two types of methods of production are used to prepare nanostructured systems: bottom-up and top-down. A third method is a combination of these two [105,106]. In bottom-up techniques, the drug are dissolved in a solvent, and the production of nanoparticles are carried out by its precipitation, by adding an antisolvent. The advantages include the low cost and use of low energy for preparation. On the other hand, drawbacks include the use of toxic solvents, and the challenge of large-scale production [105,106]. In contrast, top-down techniques start

from the micronized drug, and application of mechanical forces reduces the particle size to the nanoscale. Therefore, these are high energy techniques [105,106]. The disadvantages include the use of high energy equipment, and possibility of alteration of the crystal form of the starting compound. Among the advantages, established techniques (media milling and high pressure homogenization) have good reproducibility, with the prospect of industrial production. The combination of these techniques is generally done by preparing nanoparticles through a bottom-up technique, and then reducing the size by a top-down method [105,106].

6 Niclosamide nano-based drug delivery formulations and prospective therapeutic benefits

Table 4 presents research findings on nano-based formulations containing niclosamide. From a total of 17 studies, distribution is: 3 included nanocrystals [116, 122, 123]; 6 included polymeric nanoparticles [108, 110, 117, 119, 120, 123]; 3, lipid nanoparticles [107, 111, 121]; 3, nanofibers [112, 114, 118]; 2, micelles [109, 115]; and 1, carbon nanoparticles [113]. In these studies, niclosamide also presented *in vitro* and *in vivo* anticancer activity when present in formulations. However, promising improvements in pharmacokinetic parameters were described in some of these studies. In addition, considering niclosamide's diverse anticancer activity, the possibility of synergy with other compounds and drug targeting were also explored.

Examples of pharmacokinetic improvements were reported by Zhang and colleagues when nanoemulsions were designed for evaluating niclosamide pharmacokinetic properties in rats [121]. In this study, two types of nanoemulsions were prepared: with and without poly(ethylene glycol) monooleate (PEGM), in order to assess the ability of this polymer to provide "stealth" properties, that is, to avoid lipolysis by digestive enzymes. Among the results, no significant differences were observed in the lyposis test, considering the presence of PEG, which the authors associated with the insufficient molecular mass of the polymer (~1200) in to promote "stealth" properties.

Table 4: Nanostructured systems containing niclosamide.

Year	Type of nanoparticle (method of preparation)	Components	Nanoparticle characterization				Performance		Ref.
			Size (nm)	PDI	Z.P. (mV)	E.E. (%)	*In vitro* IC_{50} (µM) (cell)	*In vivo*	
2018	Solid Lipid (Solvent evaporation)	Stearylamine, polysorbate 80, pluronic F-68	112.18 ± 1.73	0.417 ± 0.026	+23.8 ± 2.7	82.21 ± 0.62	~ 18 * (MDA-MB231)	–	[107]
2018	Polymeric (solvent evaporation)	PEGCE, PS-b-PAA, anti-CD44-peptide	100 ± 25	–	–	–	~ 2 (MCF-7, MDA-MB231)	Regression in tumour growth from MCF-7 cells in mice	[108]
2017	Self-assembly polypeptidic micelles (conjugation synthesis)	Elastin-like polypeptide	30-81	–	–	–	0.94 (HCT116)	Reduced tumour volume from HCT116 cells in mice	[109]
2017	Polymeric (desolvation)	Chitosan, polysorbate 80, glutaraldehyde, sodium sulfate, sodium metabisulfite	100-120	–	+24	> 90	7.5 (MCF-7), 8.75 (A549)	–	[110]
2017	Solid lipid (micro-emulsion)	Stearic acid, polysorbate 80, PEG400	204.2 ± 3.2	0.328 ± 0.02	–33.16 ± 2	89.1 ± 0.03	–	11.08-fold increase in bioavailability in rabbits, compared to marketed drug	[111]
2016	Nanofiber (electrostatic spinning)	PEO, Ag poly(e-caprolactone)	632	–	–	–	1.24 (A549), 1.21 (MCF-7)	–	[112]

Table 4: Nanostructured systems containing niclosamide. Continuation.

Year	Type of nanoparticle (method of preparation)	Components	Nanoparticle characterization				Performance		Ref.
			Size (nm)	PDI	Z.P. (mV)	E.E. (%)	*In vitro* IC_{50} (μM) (cell)	*In vivo*	
2016	Pristine carbon nanoparticles (Facile hydrothermal)	Agave nectar, cucurbit[6]uril	88 ± 5	–	–18 ± 5	–	21 ± 2 (MCF-7)	Reduction of 50% in tumour size in mice, comparing to control	[113]
2016	Nanofibers crosslinked with magnetic nanoparticle (electrostatic spinning)	bPEI, PEO, Fe3O4, folic acid, glutaraldehyde	655 ± 76	–	–	–	– **	–	[114]
2016	Mixed micelles (thin-film hydration)	Pluronic®, biotin	31.8 ± 1.7	0.131	–3.37 ± 1.08	91.9 ± 1.9	< 0.9 (A549)	–	[115]
2016	Nanocrystals (electrospray)	PVA	105 ± 21	–	–	–	3.59 (CP70), 3.38 (SKOV-3)	Reduced tumour growth from CP70 and SKOV-3 cells in mice	[116]
2015	Polymeric (desolvation)	Albumin, glutaraldehyde	199.9	–	–34.2	92.36	5 (A549), 2.6 (MCF-7)	–	[117]
2015	Nanofiber (electrostatic spinning)	bPEI, PEO, glutaraldehyde	430-576	–	+6 to +12	–	3.129 (A549), 2.147 (U87MG)	–	[118]

Table 4: Nanostructured systems containing niclosamide. Continuation.

Year	Type of nanoparticle (method of preparation)	Components	Nanoparticle characterization				Performance		Ref.
			Size (nm)	PDI	Z.P. (mV)	E.E. (%)	*In vitro* IC_{50} (µM) (cell)	*In vivo*	
2015	Polymeric (solvent evaporation)	Hyperstar polymers, amonafide	90 ± 10	–	–	–	1 ± 0.5 (MDA-MB231), 30 ± 5 (MCF-7), 20 ± 5 (SKBR-3), 5 ± 1 (BT549)	–	[119]
2015	Polymeric (solvent evaporation)	PEGCE, PS-b-PAA	~ 69	0.2	–12	86.9	12 ± 2 (MDA-MB231), 5 ± 1 (MCF-7), 1 ± 0.5 (C32)	–	[120]
2015	Nanoemulsions – a: PEG; b: without PEG (melt dispersion / HPH)	PEGM, oleic acid, MCT, egg lecithin, soybean lecithin, polysorbate 80, glycerin.	a: 162.2 ± 3.8 b: 307.8 ± 5.2	< 0.30	a: –21.8 ± 0.24 b: –20.8 ± 0.45	a: 9.12 *** b: 9.06 ***	–	~5-fold increase in bioavailability, compared to drug solution, in rats	[121]
2015	Nanocrystals (wet milling)	Polysorbate 80, poloxamer 188, PVP, SDS, TPGS	235.6	< 0.30	> +25	–	4.62 (EC9076)	Delayed tissue distribution, compared to drug solution, in rats	[122]
2013	a: nanocrystals b: polymeric (electrospray)	a: PVA b: PLGA ****	a: 105 ± 21 b1: 662 ± 121 b2: 584 ± 110	–	–	–	a: 2.44 (CP70), 5.52 (SKOV-3); b1: 1.37 (CP70), 7.81 (SKOV-3); b2: 5.55 (CP70), 7.45 (SKOV-3)	–	[123]

PDI: Polydispersity index; Z.P.: zeta potential; E.E.: encapsulation efficiency; PEGCE: Polyethylene glycol cetyl ether; PS-b-PAA: poly(styrene)-block-poly(acrylic) acid; bPEI: branched poly(ethylenimine); MCF-7, MDA-MB231, SKBR-3 and BT549: breast cancer cells; HCT116: colon cancer cells; A549: lung cancer cells; PEG400: Polyethylene glycol 400; AUC: area under the curve; PEO: poly(ethylene oxide); PBS: phosphate-buffered saline; PVA: poly(vinyl alcohol); CP70 and SKOV-3: ovarian cancer cells; U87MG: brain cancer cells (glioma); C32: skin cancer cells (melanoma); HPH: High Pressure Homogenisation; PEGM: Polyethylene glycol monooleate; MCT: medium-chain triglyceride; PVP: polyvinylpyrrolidone; SDS: sodium dodecyl sulfate; TPGS: Tocopheryl polyethylene glycol succinate; EC9076: esophageal cancer cells (carcinoma); PLGA: poly lactic-*co*-glycolic acid. *: Measured as cytotoxic concentration (CTC50); **: Percentage of L132 and KB cell viability were compared with formulations, not as a function of the concentrations. ***: Refers to drug loading. ****: b1 refers to single and b2 refers to dual-capillary electrospray system.

Regarding pharmacokinetic evaluation, niclosamide was administered by oral gavage in Sprague-Dawley rats (250 ± 20 g), at the dose of 20 mg/kg. Then, C_{max} values for nanoemulsions were 0.726 and 0.432 µg/mL, for with and without PEGM, respectively, whereas 0.195 µg/mL for the suspension [121]. Despite differences in particle size between the nanoformulations, similar bioavailability values were observed: ~2.5 (AUC_{0-t} µg.h/mL) for nanoemulsions, whereas ~0.5 for the suspension, resulting in a 5-fold increase. Hence, considering the possible mechanisms related to niclosamide oral absorption, these results support the prospect of increasing bioavailability and reducing the dose by lipid-based matrix systems.

The authors still note that while blood levels in rats are not directly related to humans, a low dose (20 mg/kg) was administered to the animals, which for them could be an indication that target blood levels in humans could be easily achieved. In this context, the pharmacokinetic of niclosamide had also been evaluated in a preclinical study from Duke University [44]. In this study, niclosamide was mixed into a polyethylene glycol system (90% polyethylene glycol-300, 10% 1-methyl-2-pyrrolidone) for oral administration of 200 mg/kg, using NOD/SCID mice (23-25 g) as in *in vivo* model. Thus, C_{max} was 0.893 µg/mL (t_{max} = 15 minutes), whereas blood levels ranged from ~0.04 to ~0.08 µg/mL (0.5 to 12h). According to the authors, high doses were administered to the animals, which for them could be associated with complex absorption behavior of the compound in different regions of the gastrointestinal tract [44].

Lipid-based nanoformulations were also designed and prepared in Rehman et al. [111]. In this study, solid lipid nanoparticles were prepared by micro-emulsion technique, using stearic acid (SA), polysorbate 80 and polyethylene glycol (PEG). Four variables were evaluated: concentrations of SA, polysorbate-80 and PEG, and stirring time. Then, optimized formulations were prepared varying concentrations of the drug. The objective was to evaluate the phamacokinetic profile of the compound by oral route in rabbits (2 ±0.3 kg), comparing with marketed drug (Mesan®). In brief, C_{max} values for the nanoparticles and marketed drug were 3.97 and 1.84 µg/mL, respectively, whereas reported bioavailability (AUC_{0-t} µg.h/mL) were 16.74 for nanoformulation, and 1.51 for Mesan® [111].

One of the justifications for this study was that the solid lipid nanoparticles could improve absorption of niclosamide to the lymphatic system, reducing the first-pass effect by the liver. This possibility is attributed to lipid formulations in general, and it is based on the way that lipids from the diet are digested, and then absorbed in the intestine [124]. This phenomenon is mediated by the synthesis and secretion of chylomicrons by enterocytes, which secrete the lipoproteins to the lymphatic capillaries. The hypothesis for the absorption and transport of

drugs by lymphatics is that, after the digestion of the lipid components of the formulations (a combination of lipids, surfactants and or co-solvents), drug and lipids (fatty acids and monoglycerides) are uptaken by the enterocytes [124-126]. Then, long-chain triglycerides are synthetized in the cell and usually incorporated into chylomicrons, along with the drug and proteins. Thus, after secretion by the enterocytes, the size of these large lipoproteins limits their entry into blood vessels, which favours the entry into lymphatic capillaries. Hence, it is believed that drug and chylomicrons follow the unidirectional flow of the lymphatic vessels, reaching the systemic blood circulation by subclavian vein [124-126]. Therefore, despite a possible crystalline solid precipitation, lipid-based formulations might enhance niclosamide oral absorption not only due to phenomena such as supersaturation in intestinal fluids, but also due its partition in the lipid phase. In this case, the possibility of solubilization in triglycerides and incorporation into chylomicrons could favour its absorption toward the lymphatic system. This could be a strategy to achieve therapeutic benefits, as increasing bioavailability, reduction of the first pass-effect by the liver and drug-drug interaction.

Still considering lipid-based formulations, a pharmaceutical advantage that might be achieved could be related to the crystal transformations of niclosamide. This phenomenon might be critical to nanocrystals, since the lack of a matrix allowed the nanosized crystalline structure to be in contact with water, being subject to hydration. As previously discussed, this implies changes in physicochemical properties, such as saturation solubility and intrinsic dissolution. Considering the *in silico* and experimental logP values (3.89 and 4.45, respectively), niclosamide has a much higher tendency to dissolve in lipid phase than in aqueous phase. Hence, incorporation and solubilization of this drug substance in a lipidic matrix system could diminish the influence of niclosamide crystal transformations (anhydrous to monohydrate H_A, and then to H_B) in stabilizing the nanoparticles. On the other hand, possible disadvantages include lower drug loading, compared with nanocrystals, and the required compatibility among the excipients. In the case of solid lipid nanoparticles, polymorphic changes of the solid lipid may be a critical factor in stabilizing the system [99].

Considering nanocrystals, this strategy was presented in the work of Lin and co-workers, when niclosamide nanoparticles were prepared by electrospray technique to evaluate its anticancer activity against ovarian cancer cells [116]. The nanosuspension contained 1% polyvinyl alcohol (PVA) in a phosphate-buffered saline solution, and formulation test presented an average particle diameter of 105 nm. Among the results, the nanosuspension was able to suppress the metabolism and the *in vitro* growth of CP70 and SKOV3 cells, with IC_{50} (μM) of 3.59 and 3.38, respectively. Besides, using NOD/SCID mice, and after administration of 100

mg/kg of nanoniclosamide by oral gavage, reduced tumour growth was observed in the animals [116].

In this study, they also evaluated the pharmacokinetic profile of niclosamide. Using Sprague-Dawley rats, nanocrystals dispersed in PBS solution (0.46 mg/mL) were administered by oral gavage (5 mg/kg) or intravenous injection (2 mg/kg). In brief, the estimated bioavailability by oral route was 25%. According to the authors, despite the better results compared to a reported 10% value, they emphasized the need for improvement, considering its potential for ovarian cancer treatment [116]. Nonetheless, if this difference was maintained in humans, target blood levels could be more feasible to achieve, allowing the prospect of reducing the dose. Thus, strategies based on nanocrystals might work as if they "brought" the compound closer to a desirable drug-likeness region.

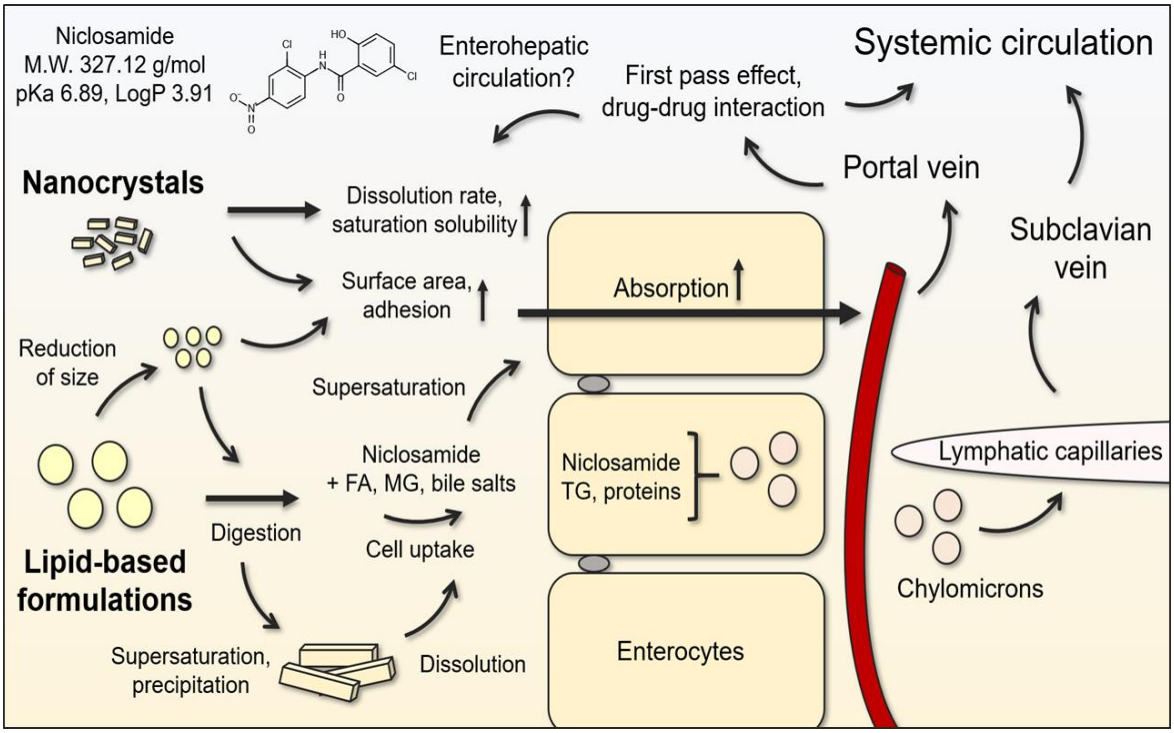
Still considering their study, a second peak was observed in the plasma concentration profile by oral and IV route, which for them could be associated with an enterohepatic circulation phenomenon [116]. In the study of Duke University, the authors associated the oral absorption of the compound in rats with complex behavior in different regions of the gastrointestinal tract, but which was less likely to be related to enterohepatic circulation [44]. Lastly, in the study of Zhang and colleagues, a similar pattern, with a second peak, is also observed in the pharmacokinetic profiles of the nanoemulsions, although a hypothesis for this phenomenon was not discussed [121].

Drugs that reach the bloodstream are eliminated mainly by two pathways: by the kidneys or the liver. In the latter case, compounds may be removed in an unchanged form or as metabolites, and some of them may be excreted via bile [127]. Hence, enterohepatic circulation is the elimination of drugs or other substances from the bile to the small intestine, which are available for reabsorption, being subject to new elimination by the liver or reaching systemic circulation [127,128]. Examples of cases already observed in humans include the drugs diltiazem, irinotecan, nevirapine, meloxicam and piroxican [127,128]. Molecules that undergo this phenomenon usually show multiple peaks in plasma concentration versus time profiles. However, obviously drug metabolism in animals is not directly related to humans, and it is difficult to attribute a single cause for multiple peaking in pharmacokinetic profiles [128].

Therefore, it is still not clarified if niclosamide is subject to enterohepatic circulation. If it is another challenge for its oral absorption, bioavailability improvements provided by nanostructured systems might be achieved by two different strategies. Nanocrystals may promote a higher amount of dissolved drug that reaches the portal circulation, that might provide therapeutic concentrations more easily. The other strategy is the drug delivery based

on lipid formulations. This could contribute to niclosamide absorption not only due to supersaturation, but also due to the transport by the lymphatic system, reducing the first-pass effect by the liver. These considerations are summarized in Fig. 3.

Fig. 3. Summary of nanocrystals and lipid-based strategies for niclosamide.



Nanocrystals may improve the oral absorption due to an increase in dissolution rate and saturation solubility, and the enhanced surface area that favours adhesion to cell membranes. It does not prevent metabolization by the liver, but provides a higher amount of drug that reaches systemic circulation. Reduction of particle size of lipid-based formulations may improve adhesion to cell membranes and, after digestion, supersaturation provides a higher amount of solubilized drug available for absorption. This strategy is also associated with the hypothesis of targeting the lymphatic system, which is based on the incorporation of drugs into chylomicrons that, after secretion by enterocytes, enter lymphatic capillaries and follow the unidirectional flow of the vessels, reaching systemic circulation by the subclavian vein. M.W.: molecular weight; FA: fatty acids; MG: monoglycerides; TG: triglycerides. M.W., pKa and logP values obtained by Chemicalize.com. Source: Barbosa et al., 2019.

A particular feature of niclosamide that may be explored, and which does not involve oral absorption necessarily, is its diversity for anticancer activity. As previously shown, this compound presents actions in different signaling pathways. Hence, this might provide synergistic effects with other compounds, which was observed not only with abiraterone and enzalutamide, but also with erlotinib, a tyrosine kinase inhibitor used for lung cancer [51,52]. The therapeutic benefits include overcoming of drug resistance and reduction of dose [129,130].

Besides, this may reinforce the argument for the repositioning process, further considering that a combination therapy is a common approach for serious diseases like cancer [130,131].

This idea was evaluated in the study of Misra and colleagues, when polymeric nanoparticles were prepared to encapsulate niclosamide, a STAT3 blocker, and amonafide, a topoisomerase-II inhibitor, using them against triple negative breast cancer (TNBC) cells [119]. The strategy was to prepare "hyperstar polymers" (HSP) as nanocarriers, when hyperbranched macro-initiators were polymerized to produce a core-shell structure to contain the two compounds. The spherical nanostructure was composed of protonable tertiary amine groups in the shell (for water dispersion), and acid-degradable acetal groups in the core (for drug release under acid conditions). Then, the nanoparticles were compared with the compounds alone, and with a conventional formulation containing the two drugs substances, in MTT assays. Among the results, the combination were able to produce synergistic effect against TNBC cells (IC_{50}) (~5 µM for BT549, and ~1 µM, for MDA-MB231) compared to MCF-7 and SKBR-3 cells (~30 and ~20 µM, respectively) [119].

Later, Misra and colleagues also adopted the strategy of drug targeting for breast cancer stem cells, which could be also associated with synergy [108]. The concept of cancer stem cells (CSC), briefly, is based on evidence that, from a population of cancer cells, a small percentage present stemness features, which can self-renew or differentiate into rapidly proliferating ones [132,133]. Thus, conventional chemotherapy would have an effect against the majority of the cells, but the remaining unaffected CSCs would be responsible for repopulation, therapy resistance and metastasis formation [133,134]. In the study, polyethylene glycol cetyl ether (PEGCE) and poly(styrene)-block-poly(acrylic) acid (PS-b-PAA) were used to prepare polymeric nanoparticles containing niclosamide, being marked with a CD44-targeting peptide. Among the results, from a population around 10% of CSC MCF-7 cells ($CD44^+$), maximum *in vitro* reduction was observed with targeted nanoparticles: around 60%, comparing with 20-30% using niclosamide alone or encapsulated in non-targeted structures. In addition, the reduction of tumour growth in xenograft mice, the targeted nanostructures reduced the $CD44^+/CD24^-$ CSC population *in vivo* and downregulated stemness marker genes. Mechanistically, the authors attributed the anticancer effects to inhibition of STAT3 phosphorylation by niclosamide [108].

Different strategies against CSCs include inhibition of cellular mechanisms (Wnt, Hedgehog, Notch, NFκB) [134]. Niclosamide may block three of these pathways (Wnt, Notch, NFκB) [12,13]. Besides, Misra and colleagues attributed its action by inhibition of STAT3 signaling. Hence, since this compound may present activity against CSCs, which can be

optimized with nanostructured systems, treatments including the combination of this molecule with conventional drugs could provide better results considering recurrence, therapy resistance and metastasis.

7 Conclusion

The challenges involving niclosamide repositioning for cancer diseases begin with its physicochemical properties, since its stable crystalline structure and its lipophilicity restricts solubility in water, which implies undesirable drug-like features that affect oral absorption. In fact, this was reflected by the high daily doses administered in preliminary clinical studies, sometimes much higher than the usually used for tapeworm infection. This raises concerns of whether the dose will be different if applied therapeutically, and what would be the impact of administering it for long periods. Initial results indicated that the blood levels might be critical for some effect against prostate and colon cancer. Thus, it also raises the question of whether the activity will be confirmed in ongoing clinical trials. An unsuccessful performance does not exclude this candidate, since niclosamide already showed effects against different types of cancer cells, but further highlights the challenges involving its repositioning. The drawbacks of the physicochemical properties bring the opportunity for the development of nano-based formulations, which were not evaluated in the clinical studies. Nanocrystals are the simplest strategy, as the increase in saturation solubility and dissolution rate might improve oral absorption and provide therapeutic blood levels. Despite the possibility of precipitation, drug supersaturation by lipid-based formulations might also improve absorption, and are associated with the hypothesis of targeting the lymphatic system, preventing metabolization by the liver as well. Niclosamide diverse activity may be explored by the combination with other compounds, in order to achieve synergistic effects. Approaches that are more sophisticated may include the use of ligands for drug targeting. In this case, treatments including a combination with conventional chemotherapy against CSCs might provide therapeutic benefits related to drug resistance and recurrence.

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Chapter II

Acoustic levitation and High-resolution Synchrotron X-Ray powder diffraction: a fast screening approach of niclosamide amorphous solid dispersions

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Abstract

The levitation of samples in an acoustic field has been of interest in the preparation and study of amorphous solid dispersions (ASD). Here, niclosamide-polymer solutions were levitated in a multi-emitter single-axis acoustic levitator and analyzed for 10 minutes at a High-resolution synchrotron X-ray powder diffraction beamline. This assembly enabled high-quality and fast time-resolved measurements with microliter sample size and measurement of solvent evaporation and recrystallization of niclosamide (NCL). Polymers HPMCP-55s, HPMCP-50, HPMCP-55, Klucel®, and poloxamers were not able to form amorphous dispersions with NCL. Plasdone® and Soluplus® demonstrated excellent properties to form NCL amorphous dispersions, with the last showing superior solubility enhancement. Furthermore, this fast levitation polymer screening showed good agreement with results obtained by conventional solvent evaporation screening evaluated for five days in a stability study carried out at 40 ºC/75% RH. The study showed that acoustic levitation and high-resolution synchrotron combination opens up a new horizon with great potential for accelerating ASD formulation screening and analysis.

Keywords: Acoustic levitation, synchrotron light, niclosamide, polymer screening, amorphous solid dispersion.

1 Introduction

The levitation of objects using ultrasonic fields (i.e., acoustic levitation) is based on the acoustic radiation force phenomenon [1], in which small objects are trapped at the pressure nodes of a standing wave field [2]. The number of applications of acoustic levitation is continuously growing and spreading across different areas such as chemistry [3] and analytical chemistry [4], aerospace [5], polymers [6], environmental [7], biology [8], genetics [9] and nanotechnology [10].

In pharmaceutical sciences, this technology offers a field of great opportunities. Experiments using the acoustic technology revealed a powerful new tool for optimization of mini-tablets coating process [11], or excipient selection for dry powder vaccines [12]. The free levitation of microsamples prevents interactions with container surfaces and allows rheological properties analysis of liquid drops [13] and structural characterization of proteins [14] or drug substances [15]. Furthermore, creative applications by combining analytical techniques have been able to follow *in situ* chemical reactions in a levitated drop [16] and monitor malarial pigments in live malarial-infected levitated red blood cells [17] .

A promising application of acoustic levitation is to accelerate the development of amorphous solid dispersions (ASD) of drug substances. This is accomplished by a combination of acoustic levitation with X-ray diffraction methods [15]. ASDs development has become an important method to overcome the poor water solubility of drugs. The lack of long-range structural organization and the absence of crystalline lattice lead to a high free energy state of the system [18]. Allied to surface activity properties of polymers, ASDs have the potential to increase apparent water solubility and extend the time of supersaturation levels in the gastrointestinal tract. These phenomena contribute to drug absorption and, consequently, increase oral bioavailability [19].

However, the current strategies for carrier screening and ASD preparation involve multiples steps before analysis. Spray drying, rotary evaporation, and melting techniques (e.g., hot-melt extrusion) are examples of this strategy [20]. These techniques require transferring a test sample from the container to recover the specimens. This transfer process sometimes involves scratching and can be related to yield issues. Any loss of drug may represent a challenge in preformulation studies when only a few milligrams of the drug candidate are available. Besides, those procedures may trigger crystallization and introduce artifacts to the tests. The success of ASD formulations relies on selecting carriers, which can maintain the drug in the amorphous state and slow down the recrystallization kinetics during shelf-life. Hence,

the presence of residual crystallinity should be detected and avoided since it can initiate and accelerate crystallization in formulations [21].

For this application, acoustic levitation in ASD screening is advantageous since it provides a surface-free environment; also, it allows *in situ* X-ray analysis with a reduced amount of drug. Benmore and Weber demonstrated the potentiality of using high-energy X-ray diffraction to study several amorphous drugs prepared by laser melting. Using the method of quenching molten droplets suspended in an acoustic levitator, they monitored the shelf-life over several months [22]. The high energy of synchrotron sources enables high-quality time-resolved measurements to be performed during physical transformations or synthesis processes. Also, it provides a high real-space resolution, which allows the separation of overlapping peaks (commonly found in drug substance and excipient mixtures) and the detection of small crystalline amounts (<1%) [6].

In this study, we introduce drop evaporation under an acoustic field screening approach, which may simulate a standard small-scale solvent evaporation screening process of ASD preparation. To demonstrate the method, the BCS class II drug niclosamide [23] was used as the model compound in combination with pharmaceutical polymers approved for oral solid dosage forms. This anthelmintic drug showed promising results in preclinical trials investigating its repurposing. Findings include activities against cancer cells, besides antimicrobial and antiviral activity [24]. Recently its effect against COVID-19 has been proposed due to its known effect against several viruses [25,26]. However, one of the challenges for niclosamide repurposing is its low water solubility, reflecting its low oral absorption and high doses administered in clinical trials [27].

Reports of solid dispersions including niclosamide can be found in the literature [28,29,30]. These studies adopted the general approach of developing the formulation and studying the proof of concept in performance tests. Besides, they used conventional characterization techniques to study the formulation. Here we highlight the feasibility of the acoustic levitation/synchrotron radiation technique for screening ASD of compounds like niclosamide which are prone to crystallization. To the best of our knowledge, the presented approach for this kind of compound has not been addressed in the literature. Therefore, the aim of this study was to study the amorphization capacity of polymers by synchrotron radiation/acoustic levitation under a drop evaporation approach, using niclosamide as the model drug.

2 Materials

Niclosamide (purity >99%, Cayman Chemical USA) was choose as the model drug since it is a poorly water-soluble compound prone to crystallization. To assess the feasibility of using acoustic levitation/synchrotron radiation in screening ASD, we choose polymers with diversified molecular structures, commonly used in ASD development [21,31]. Polymers tested were poloxamers Kolliphor® P188 and Kolliphor® P407 (polyethylene-propylene glycol copolymers) and Soluplus® (polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer), obtained from BASF (Brazil); Plasdone™ S-630 (poly(1-vinylpyrrolidone-co-vinyl acetate)) and Klucel™ (hydroxypropyl cellulose, LF Pharm, molecular weight 95,000 daltons), obtained from Ashland (Brazil); hydroxypropyl methylcellulose phthalate (HPMCP) derivatives HPMCP 50, HPMCP 55 and HPMCP 55S, obtained from Shin-Etsu (Japan). Ethanol (99.5%, 0.005% maximum water) and acetone (99.5%, 0.5% maximum water content) (Sigma Aldrich) were used as solvents. All samples were used as acquired.

3 Method

3.1 Polymer screening by synchrotron radiation/acoustic levitation

Ethanol:acetone mixture (50:50 w/w) was used to prepare solutions of niclosamide raw material and its 1:3 drug-polymer combinations. The polymers tested in the screening were: poloxamers Kolliphor® P188 and P407, Soluplus®, Plasdone™ S-630, Klucel™, HPMCP, HPMCP 50, HPMCP 55, and HPMCP 55S. Droplets of 20 µL of the solutions were levitated in an acoustic field generated by a multi-emitter single-axis acoustic levitator [32], operating at 40kHz and mounted in the XRD2 beamline at the Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil), as shown in Fig. 1. A 10 keV monochromatic beam energy (wavelength =1.23687(3) Å) was used for the high-resolution X-ray experiments, and the scattered X-rays were detected using a Pilatus 300k area detector (Dectris). The sample-detector distance of 286.8 mm was calibrated using aluminum oxide (Al_2O_3) powder sample. The diffraction angle range and time analysis were set to 15-30° and 600 s, respectively, and 2D diffraction patterns were recorded every 12 s, with a 10 s integration time. In the case of niclosamide:soluplus droplet, the only measurement recorded was at the end of ten minutes due to a failure in the data record system. The detector calibration and azimuthal integration of the

2D images were performed with the pyFAI software package [33]. Diffraction profiles were plotted with Origin software (version 9.6, OriginLab Corp., Northampton, MA).

Fig 1. Left: experimental design at the XRD2 beamline station

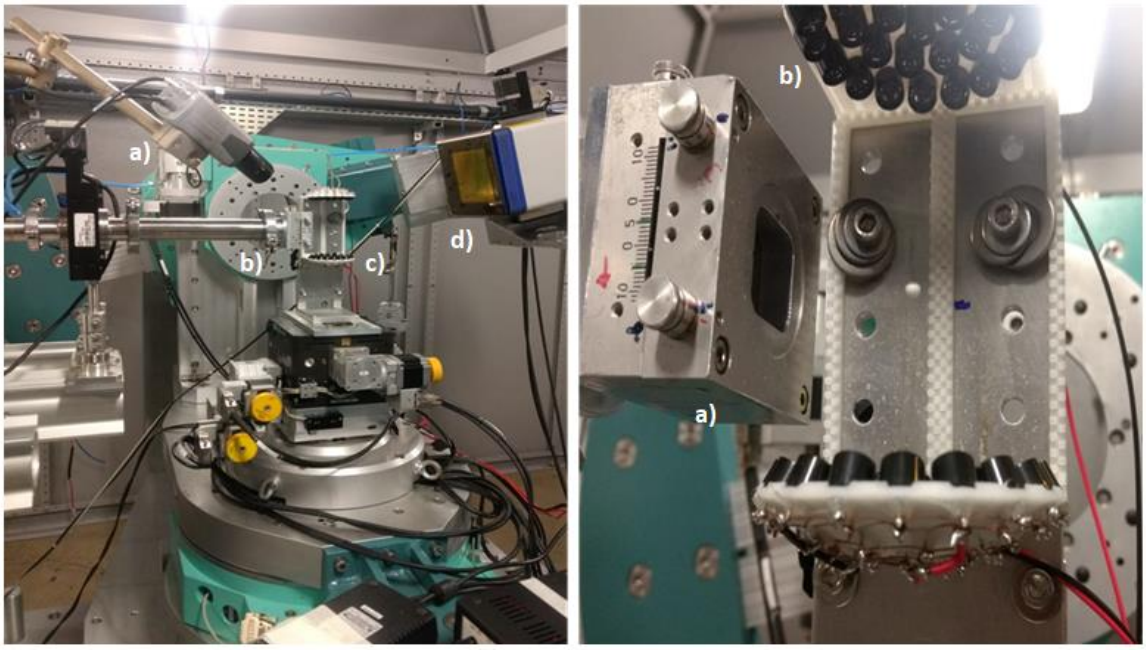


Fig 1. Left: a) Camera used to align the levitating sample to the X-ray beam, b) X-ray source, c) TinyLev acoustic levitator, d) 2-D detector. Right: a levitating sample in front of the beam source: a) X-ray source, b) set of 36 transducers (up and down, resulting in 72), which convert electrical signals into an acoustic standing wave field. Source: from the own author. Source: Barbosa et al., 2021.

3.2 Preparation of solid dispersions by rotary evaporation and X-Ray Powder Diffraction (XRPD) characterization

Solid dispersions were also prepared by rotary evaporation and characterized to compare the results with the screening by synchrotron radiation/acoustic levitation. Drug-polymer solutions were prepared by varying w/w proportion to identify the highest possible drug concentration in the ASD. Drug-polymer mixtures were 1:3, 1:1 and 3:1, for a total formulation weight of 400 mg were dissolved in ethanol:acetone mixture (50:50 w/w). Solutions were rotary evaporated in a Büchi Rotavapor® R-215 equipment. The bath temperature was set to 50 ºC, under the pressure of 175 mbar, and the evaporation process lasted for 1 hour. Samples were dried at room temperature (25 ºC).

Conventional XRPD diffraction measurements were performed in a Bruker diffractometer (D8 Focus), using CuK$\alpha_{1/2}$ radiation (λ = 1.5418 Å). Scanning was performed in

the 2-40º range (2Ɵ), with a step size of 0.07º (2Ɵ) and a 5 sec/step scanning speed. Data were collected in the reflection mode.

3.3 Exploratory stability study

The best polymers identified were selected to prepare 1:3 amorphous solid dispersions (drug:polymer). To assess how long the system would keep its amorphous state, formulations were exposed to 40 ºC/75% RH conditions for 5 days in a stability chamber (Nova Ética, São Paulo, Brazil), disposed in watch glass without covering it. Afterward, samples were then evaluated by XRPD. In order to verify the best drug:polymer proportion, we increased the amount of drug to 1:1 and 3:1 (drug:polymer). The best proportion was then evaluated under the same conditions previously described.

3.4 Exploratory dissolution studies

Dissolution exploratory studies of the pure drug and the most stable dispersions were performed by adding the equivalent of 4 mg of niclosamide in 40 mL of phosphate buffer (pH 6.8) with 1% SDS, at a temperature of 37 °C (± 0.5 °C), and under magnetic stirrer (300 rpm). The temperature was controlled using a bath and measured with a sensor coupled with the magnetic stirrer. The concentration of niclosamide in solution was monitored every 5 s for 120 minutes in a 400 Series UV/Vis Spectrophotometer (Spectral Instruments, Tucson, Arizona), at 345 nm, with a 0.5 cm pathlength dip probe.

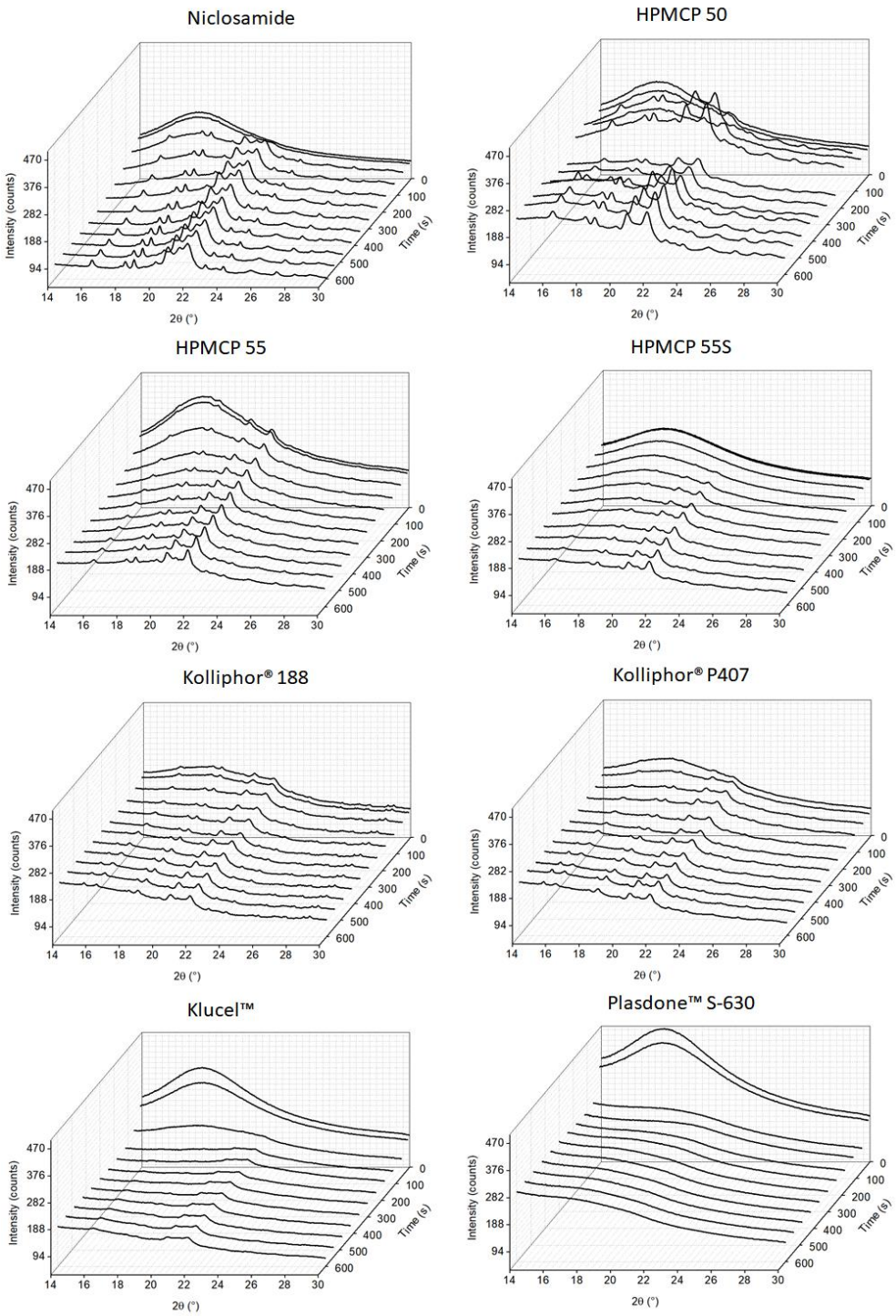
4 Results and discussion

4.1 Polymer screening by synchrotron radiation/acoustic levitation

Fig.2 shows the high-resolution XRPD patterns of levitated droplets for niclosamide alone and with each polymer combination. The containerless condition of acoustic levitation combined with high-resolution synchrotron data allowed to differentiate the behavior of niclosamide amorphization in different polymers. For niclosamide raw material, the peaks with the highest intensity were found in the region between 20–22 °(2θ) (Fig.2). These peaks were selected to identify possible crystalline domains in the samples.

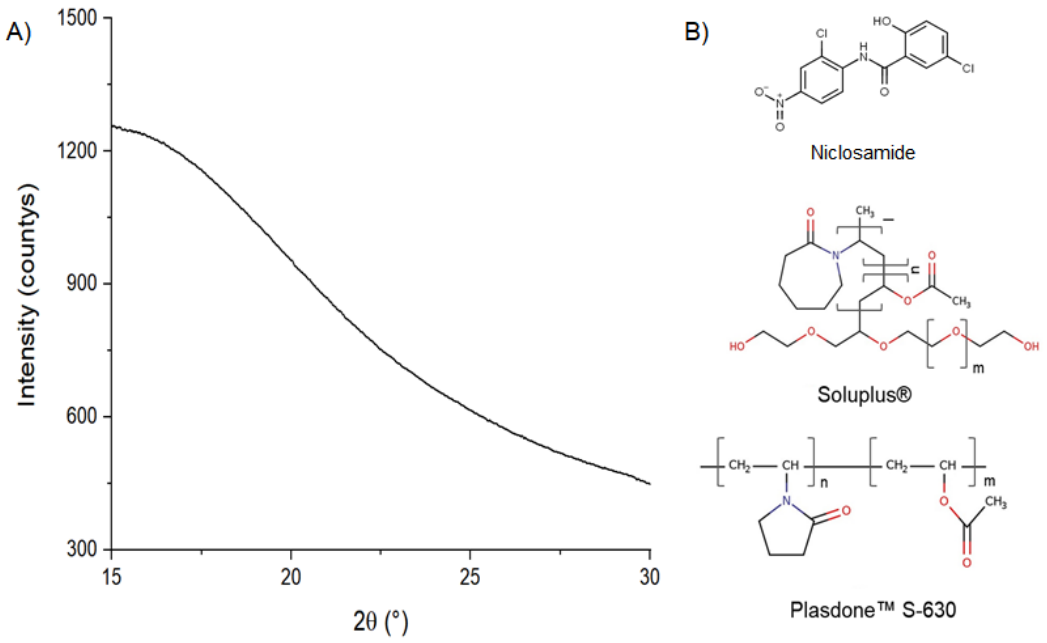
In the beginning, all the samples showed the profile predominantly amorphous ("halo pattern"), as expected due to solubilization. As the droplet evaporated, yielding a more concentrated solution, more peaks appeared during the analysis, increasing in intensity, and the peaks became more defined, indicating partially sample recrystallization. For the ASD systems prepared using HPMCP 55, HPMCP 55S, HPMCP 50, Kolliphor® P188 and Kolliphor® P407, it was possible to notice a fast crystallization behavior, since at the very beginning small niclosamide peaks were visible through the whole diffractogram. In the mixture with HPMCP 55S the crystallization peak were absent in the first readings, showing a better capacity for stabilization. However, in the ASD system prepared with Klucel™, only peaks with the highest intensity are present. In this case, Bragg peaks appear after 200 s, but an increase in intensity in the region between 20° to 22° (2θ) was not observed. The sample prepared with Plasdone™ S-630 maintained niclosamide amorphization, since in this system Bragg peaks are completely absent during the whole experiment. Similarly, niclosamide crystallization peaks disappeared completely in the mixture with Soluplus® at the end of the process (10 minutes) (Fig.3A). Unfortunately, only the last reading of this Soluplus®-niclosamide sample was stored after 10 minutes due to software problems. In summary, Plasdone™, Soluplus®, Klucel™ and HPMCP 55S promoted niclosamide amorphization. However, only Plasdone™ and Soluplus® could maintain the niclosamide in the amorphous state, at least during the analysis.

Fig. 2 High-resolution XRPD of niclosamide raw material and drug-polymer mixtures in screening experiments for ASD development.



Drops of 20 µL (1:1 ethanol:acetone) were levitated. Crystallization peaks emerge due to the increase in niclosamide concentration as the drop evaporates, aiding to select polymers for ASD development. Source: Barbosa et al, 2021.

Fig. 3. A – Hi-resolution XRPD of niclosamide:soluplus mixture in the drop at the end of 10 minutes. B – Molecular structures of niclosamide, Soluplus® and Plasdone™ S-630.



Even after the end of the analysis it is not possible to observe niclosamide Bragg peaks, indicating the amorphous state of the drug. Source: Barbosa et al., 2021.

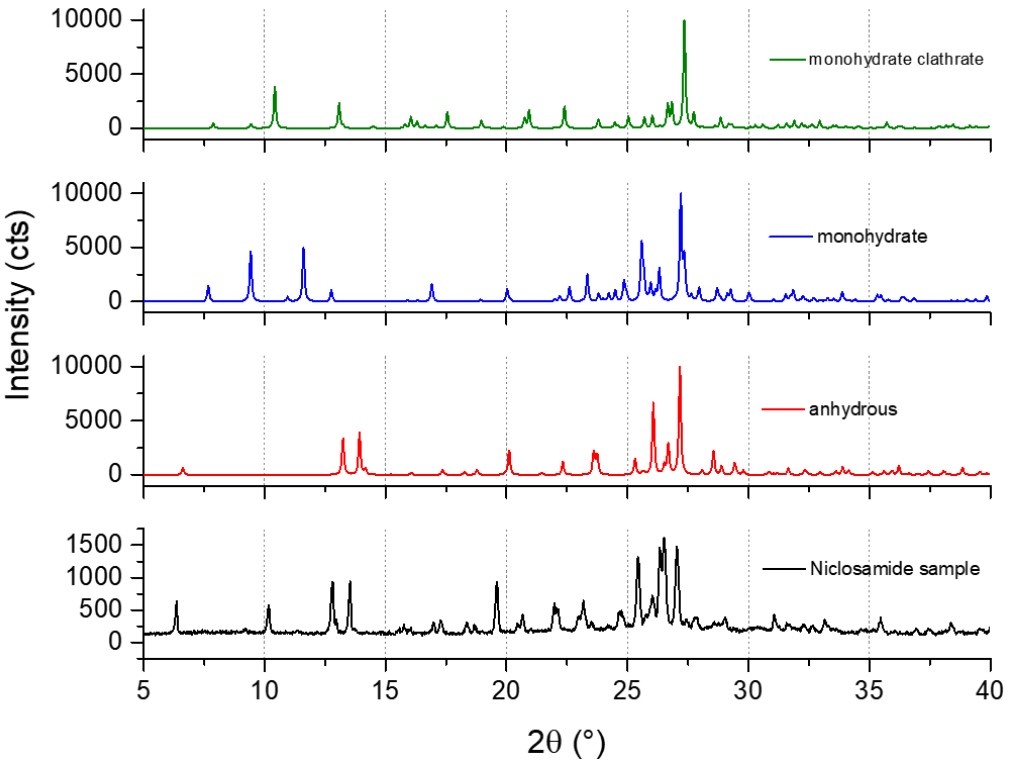
Although not explored in this investigation, the formation of hydrogen bonding interactions and viscosity of the solution may be the most probable mechanisms involved in amorphous stabilization efficiency. The presence of hydrogen atoms covalently bound to a more electronegative atom in niclosamide favors ASD stabilization by hydrogen bonding interaction with Soluplus® and Plasdone™ S-630. Both polymers are well known to promote drug stabilization in amorphous dispersions through hydrogen bonding interactions. For instance, Liu et al. (2020) reported that FTIR analysis and molecular docking indicated strong intermolecular interaction between the hydroxyl group of a natural saponin and the Soluplus® carbonyl group (Fig 3B) in diosgenin ASD, prepared by rotary evaporation-freeze-drying-microwave methods [34]. Jara et al. (2021) showed by FTIR and NMR analysis interaction between the carbonyl of the pyrrolidone group in PVP-VA and the hydroxyl group of niclosamide in ASD prepared by hot-melt extrusion [28]. However, in the case of spray-dried acetaminophen/Plasdone™ S-630 ASDs reported by Zhao et al. (2012), no clear indication of hydrogen bonding was observed to explain Plasdone™ S-630 effectiveness [35]. The authors proposed that the time related to the water loss from the drops during the manufacturing process could explain the crystallization tendency. A hypothesis raised by them is that solutions with

the lowest viscosities (and that with Plasdone™ S-630 presented the lowest value) dried rapidly, contributing to drug amorphization [35].

4.2 Preparation of solid dispersions and XRPD characterization

Three polymorphs of niclosamide, that agreed with the raw material peaks in the pattern, were found in the Cambridge Structural Database (CSD)® under the codes HEBFUR (anhydrous), OBEQAN01 (monohydrate) and OBEQAN (monohydrate clathrate). These structures were used to simulate the diffraction patterns and compare them to the measured drug pattern (Fig. 4). The niclosamide molecule adopts a near planar conformation due to NH···O intramolecular interaction. In the crystal structure, the molecules are disposed in layered chains, which are stabilized by interactions such as OH···O, Cl···NO2 and between aromatic rings [36]. The formation of the hydrate forms can be related to the exposure of the anhydrous phase to the atmospheric conditions and by the potential residual presence of water in ethanol.

Fig. 4: Qualitative analysis of the possible phases present at the niclosamide sample.

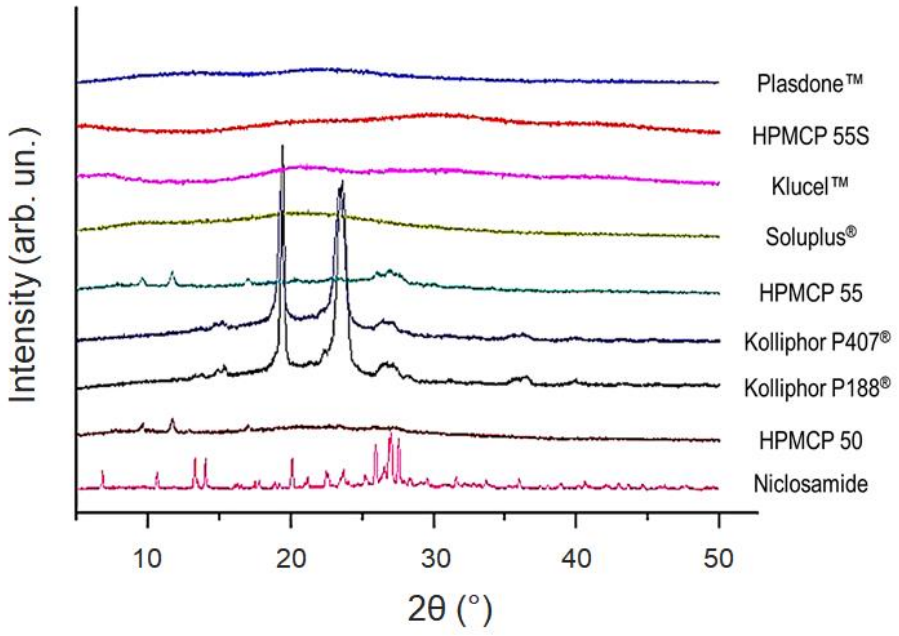


Source: Barbosa et al., 2021.

The diffraction patterns of the samples obtained by rotary evaporation are shown in Fig. 5. In niclosamide raw material profile, Bragg peaks with more intensity appear in the 25-27° range. This result is in accordance with literature reports [36,37]. Considering this finding, Plasdone™ S-630, Soluplus®, Klucel™ and HPMCP 55S were most effective in promoting niclosamide in its amorphous state. This result is comparable to those identified using synchrotron radiation fast screening experiments, except that the mixtures with Klucel™ and HPMCP 55S revealed a certain degree of crystallinity at the end of the reading. This crystallinity was not observed by the conventional XRPD analysis in the rotary-evaporated samples (Fig. 5).

Acoustic levitation requires a small amount of liquid sample, and molecules in this system have sufficient mobility to arrange themselves for nucleation and crystal growth. Besides, the acoustic waves may contribute to overcome the energetic barrier to allow nucleation. The sample is then characterized with a high-resolution X-ray source. This setup allows real-time monitoring of the crystallization degree as the drug concentration increases in the drop. In conventional XRPD, a higher amount of drug and polymer is already dispersed in the solid-state. Hence, changes in the molecular arrangement are not expected during the reading. Along with the lower resolution of the X-ray source, small differences in crystallinity may be hidden and crystallization trends may require a longer time to be detected with this technique.

Fig. 5. XRPD from niclosamide and formulations in the 1:3 proportion (drug:polymer).

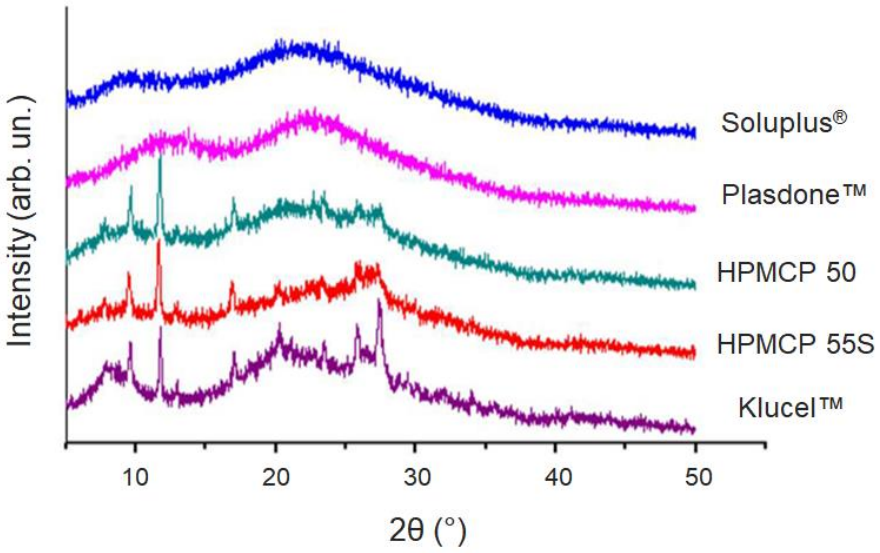


Source: Barbosa et al., 2021.

4.3 Exploratory stability study

According to the results shown in Fig. 6, Bragg peaks with more intensity appear in the 9-13° and 25-30° range for all drug:polymer dispersions, except those with Soluplus® and Plasdone™ S-630. It is worth noting that after five days of stability study, crystallization peaks start to appear in dispersions with Klucel™ and HPMCP 55S. This tendency had been real-time detected in synchrotron radiation/acoustic levitation, but it was possibly "hidden" in conventional XRPD. It means that the results from the first technique may not only be evaluated to assess the sample amorphization degree, but they also can potentially be related to the stability of the dispersion over time. In the case of niclosamide, crystallization peaks in mixtures with Klucel™ and HPMCP 55S were detected by conventional XRPD only after five days of stability study. Other factors might influence this finding such as the drug, organic solvent and amorphization method. Hence, Soluplus® and Plasdone™ S-630 were the most effective polymers in maintaining niclosamide amorphization during the exploratory stability study. Therefore, this result is essentially the same as that obtained with synchrotron radiation/acoustic levitation fast screening experiments. The findings herein obtained are especially useful for the development of formulations with new molecules, when small amounts of drug are available for experiments. Table 1 summarizes the differences between the two X-ray techniques herein observed:

Fig. 6 XRPD from the formulations after five days of stability study (40 °C/75% RH) with the 1:3 proportion (drug:polymer).



Source: Barbosa et al., 2021.

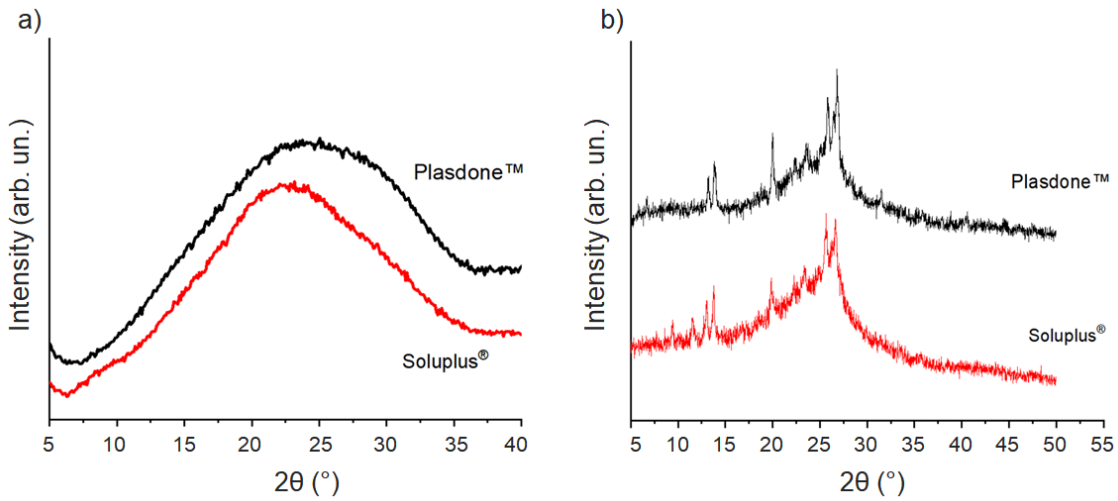
Table 1. Differences between synchrotron/acoustic levitation and conventional XRPD in polymer screening for niclosamide ASD development.

Technique	Type of sample	Sample contact	Relative molecular mobility*	Reading time	Resolution*	Time to detect crystallization
synchrotron/ acoustic levitation	1 drop (20 µL)	containerless (levitating)	high	seconds, minutes	high	real-time
conventional XRPD	solid (100 mg)	on a surface	low	minutes, hours	low	up to five days

* Comparison between the two techniques.

Aiming to maximize drug load in solid dosage forms in future formulations, we tried to increase the amount of niclosamide in solid dispersions with Soluplus® and Plasdone™ S-630. Fig. 7 shows the diffraction patterns of the tested proportions. As observed previously, Bragg peaks with more intensity appear in the 25-30° range in the 3:1 proportion. Hence, with only 1:1 proportion we achieved drug amorphization in the solid dispersion at an initial analysis just after preparation. Since solid dispersion with these polymers provided the overall best results, we chose them to perform comparative dissolution measurements.

Fig. 7: XRPD from formulations with the proportion (drug:polymer): (a) 1:1 and (b) 3:1.



Source: Barbosa et al., 2021.

4.4 Exploratory dissolution studies

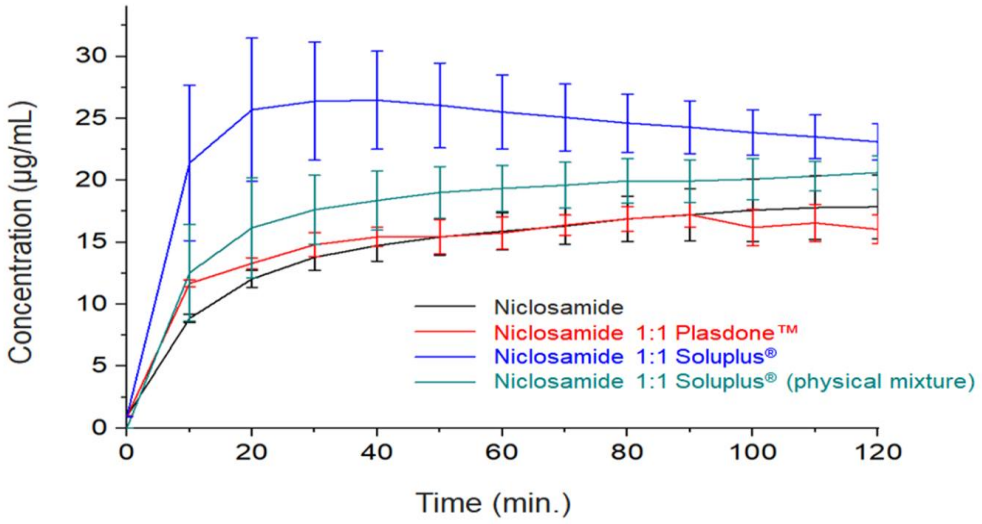
Soluplus® and Plasdone™ S-630 showed an excellent and equivalent capacity to amorphize niclosamide; this fact was confirmed by two X-ray techniques: synchrotron radiation and conventional XRPD. Thus, both were expected to have potentially the same ability to increase the aqueous solubility of niclosamide compared to other polymers. Unexpectedly, the solid dispersion with Soluplus® provided a much higher concentration than the dispersion with Plasdone™, reaching an average niclosamide concentration of 26 µg/mL (Fig.8). This value represents approximately a 73% increase compared to pure crystalline niclosamide (15 µg/mL), and a 100% increase in comparison with the most soluble anhydrous form reported in the literature (13 µg/mL) [37]. It is noteworthy that the niclosamide sample used in this study may contain another crystalline phase (Fig.4), even in small fractions, which may be considered when evaluating the dissolution results. However, no phase mixture characterization study has been reported so far, and further investigation is needed to evaluate the impact of phase mixtures on the dissolution performance.

We consider three hypotheses that could be tested in future studies to explain the dissolution results. The first is the surfactant capacity of the polymers. Soluplus® has an amphiphilic characteristic, with a hydrophilic polyethylene glycol moiety and a lipophilic vinyl-caprolactam-vinyl-acetate moiety [38]. Plasdone™, in turn, has a hydrophilic poly-vinylpyrrolidone vinyl-acetate backbone [31], which might have promoted drug:polymer interactions and stabilized niclosamide in the amorphous state, as reported when using niclosamide and Kollidon® VA64 in ASD [28]. However, it lacks a lipophilic part, which diminishes its surfactant ability and might explain the poor dissolution performance compared with Soluplus®. The second is the possibility of SDS:polymer interaction in the dissolution medium, mainly in the case of Plasdone™. The interaction of this polymer with sodium lauryl sulfate (SLS) has been reported, forming PVP-VA/SLS complexes in solution. Authors found that increased concentrations of SLS hindered maintaining sorafenib supersaturation in FaSSIF medium [39]. In our case we used 1% SDS and, therefore, this hypothesis may be considered for our system. The third is to verify whether nanoparticles are formed from the ASD during dissolution. In the study of Jara et al. (2021), DSC and X-ray diffraction indicated the amorphous state of the hot-melted niclosamide:Kollidon® VA64 ASD [28]. However, the apparent solubility enhancement was surprisingly attributed to the presence of nanoparticles, rather than supersaturation itself. Herein the release profile with Plasdone™ was unexpected as

well, which justifies verifying whether nanoparticles are formed or not to better understand the dissolution phenomenon.

Further, to test the hypothesis that the improvement was due to surfactant capacity from the polymer, we measured the solubility of niclosamide 1:1 Soluplus® physical mixture (Fig.8). The result was smaller to that achieved with the amorphous solid dispersion, showing that this unexpected positive effect occurred not only due to the ability of the polymer to solubilize, but also due to the amorphization.

Fig.8 Dissolution profiles of pure crystalline niclosamide and drug:polymer amorphous dispersions (1:1) in phosphate buffer (pH 6.8) containing 1.0% SDS at 37 ±0.5 °C.



Source: Barbosa et al., 2021.

5 Conclusion

In this study we presented the acoustic levitation method combined with high-resolution synchrotron X-Ray powder diffraction as a fast tool for the polymer screening of ASD pharmaceutical formulations. The Class II model drug niclosamide was characterized in its crystalline form and mixed with polymers, commonly used for oral formulations. The lack of diffraction peaks indicates the capacity of the polymer to mix with niclosamide and shows the potential for preparing the formulation in a fast 10 minute experiment. In this context, Soluplus® and Plasdone™ S630 were the best polymers to promote and maintain niclosamide amorphization, being potential candidates for futher formulation development. Both synchrotron/acoustic levitation and conventional XRPD provided the same screening result,

excepting that the first technique allows real-time characterization of the crystalline structure, whereas conventional method detected crystallization after five days of a stability study. The acoustic levitation and high-resolution synchrotron combination approach opens up a new horizon with great potential for accelerated formulation screening and analysis.

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Chapter III

Niclosamide nanoemulsion: preparation, physicochemical characterization and *in vitro* anticancer activity

This study will be submitted as Eduardo José Barbosa, Cláudio Fukumori, Sarah de Araujo Sprengel, Gabriel Lima Barros de Araujo, Luciana Biagini Lopes, Nádia Araci Bou-Chacra, with the title of "Niclosamide nanoemulsion: preparation, physicochemical characterization and *in vitro* anticancer activity", in *European Journal of Pharmaceutics and Biopharmaceutics* or *International Journal of Pharmaceutics*.

Abstract

Colorectal cancer – the second most lethal type of neoplasm, responsible for 940,000 deaths worldwide in 2020 – is characterized by the appearance of polyps from the abnormal proliferation of colon and rectum cells. When the disease is diagnosed, common treatments are based on surgery and drug interventions. However, these two types have unpleasant consequences for the patient, such as difficulties during recovery from the surgical procedure or nausea after chemotherapy sessions. New forms of treatments and drugs substances have become the theme of numerous lines of research. This study aimed at the development, physicochemical characterization, and *in vitro* anticancer activity of a niclosamide nanoemulsion, with HCT-116 as the cell model. During the nanoemulsion development, preliminary results indicated Capmul® MCM C8 as the best liquid lipid for the system. However, first nanoemulsions containing this lipid were not stable to justify its usage. On the other hand, Miglyol® 812 proved to be a suitable liquid lipid for the system. Niclosamide nanoemulsion (~200 nm) with Miglyol® 812 and poloxamer 188 was stable for 56 days, with monomodal particle size distribution during the period. The cell viability assay with the human colorectal carcinoma cell line HCT-116 demonstrated that niclosamide citoxicity is both time and concentration dependent.

Keywords: Niclosamide, Miglyol® 812, nanoemulsion, colon cancer, lipidic system, HCT-116.

1 Introduction

In 2020, 1.93 million new cases of colorectal cancer (CRC) were diagnosed, while 940,000 caused deaths were globally reported. It is estimated that 3.2 million new cases will be worldwide reported until 2040 [1]. Most CRC develop from a sporadic and benign abnormal proliferation of colon and rectum cells called polyps [1,2]. Usually, these polyps can remain years without neoplastic evolution, whose discovery can occur only when symptoms appear – such as change in bowel habits, weight loss, abdominal pain and anemia [3,4,5]. Patients are often diagnosed when the disease is advanced, and common treatments are based on surgery and drug interventions, through the use of fluoropyrimidines, capecitabine, UFT and TAS-102 in chemotherapies. However, these two types have unpleasant consequences for the diagnosed, such as difficulties during recovery from the surgical procedure or nausea after chemotherapy sessions [6].

Due to the aforementioned adverse effects, new forms of treatments and drugs substances have become the theme of numerous lines of research. Since the cost of development and registering new drugs are exorbitant, repositioning alternatives arise, which consists of identifying new uses for approved or under development molecules, different from the original therapeutic indication [8].

An example of a drug that has recently become the target of numerous researches for its repositioning is niclosamide, indicated for the treatment of infections by *Taenia sp.* since the 1960s [9]. Its mechanism is described by stopping parasitic infections by uncoupling the oxidative phosphorylation that occurs in the parasite's mitochondria. Besides, it has been described that the drug also inhibits oncogenic signaling pathways – such as Notch, mTOR, NF-kB, STAT-3 and Wnt/β-catenin – which, when unregulated, contribute to cases of breast, prostate and colon and rectal cancer, which justifies the objective for repositioning [10,11,12].

Its structure has a melting point around 230°C, in addition to low water solubility and dissolution, being classified as a BCS class II drug, which implies low water solubility and compromises intestinal absorption and oral bioavailability [13,14,15], major challenges faced by researchers who want to find innovative treatments using niclosamide as an active ingredient.

Nanoemulsions, in turn, are a constituent part of the area of nanostructured systems. This type of technology is characterized by a dispersion of two immiscible liquids that are stabilized by surfactant and other components that help to maintain the stability of the system. The administration route can be by oral, parenteral, and topical [16,17]. Due to their very low

average diameter particles (< 500 nm), nanoemulsions can increase the bioavailability and absorption of low water-soluble drugs due to the higher saturation solubility and dissolution rate, besides contact the higher contact surface between the nanoparticles and biological membranes. In addition, they allow the controlled and targeted release of drugs, which reduces the toxicity of the substances involved in the formulation and improves the pharmaceutical efficacy [18].

Therefore, this study aims at the development, physicochemical characterization and in *vitro* anticancer activity of a niclosamide nanoemulsion, with HCT-116 as the cell model.

2 Materials

Miglyol® 812, Miglyol® 840, Captex® 300, Captex® 355, Captex® 8000, Captex® GTO, Capmul® MCM C8, oleic acid, and the oils: olive, mineral, corn, cotton, soybean, sesame and safflower were the liquid lipids tested. Poloxamer 188 used as a hydrophylic surfactant, and niclosamide as the drug. Other materials were: purified water, absolute ethanol, dimethyl sulfoxide (DMSO); magnetic sterrer bars, vials; polystyrene cuvette, glass flasks, cover glasses, and Amicon® Ultra-0.5 10 kDa centrifugal filter.

3 Method

3.1 Niclosamide differential scanning calorimetry (DSC) and thermogravimetric (TG) analysis

Niclosamide DSC thermal behavior was characterized in a DSC-60 equipment (Shimadzu) in a closed Al crucible. Sample mass was ~2 mg, and the scanning was performed with heating rate of 10 °C/min., temperature range from 25 to 350 °C, under a dynamic nitrogen atmosphere (50 mL/min). Thermogravimetric analysis was carried out in a TGA-60 thermobalance (Shimadzu) in platinum crucible, with sample mass of ~2.8 mg, under a N_2 cycle atmosphere (100 mL/min), set for 25-600 °C temperature range and heating rate of 10 °C/min.

3.2 Liquid lipid selection

For this test Crystal16 (Crystal Pharmatech Inc., USA) was used [19]. One gram of each lipid was weighted and added in 2 mL vials. Then, 10 mg of niclosamide were added, along

with magnetic sterrer bars. The samples were submitted to heating from 25°C to 80 °C, which was kept for 1h, followed by cooling until 25 °C. The equipment was set for 0.5°C/min heating rate and 700 rpm. The turbidity of the sample (%) indicated the solubilization degree of the drug. Table 1 shows the number of the vial and its respective liquid lipid.

Table 1 – Vial (2 mL) number and respective liquid lipid (1 g) containing 10 mg of niclosamide.

Vial	Liquid lipid	Vial	Liquid lipid
1	Miglyol® 812	9	Miglyol® 840
2	Mineral oil	10	Olive oil
3	Captex® 300	11	Corn oil
4	Captex® 355	12	Cotton oil
5	Captex® 8000	13	Soybean oil
6	Captex® GTO	14	Sesame oil
7	Oleic acid	15	Safflower oil
8	Capmul® MCM C8		

3.3 Surfactant selection

From liquid lipid selection, the lipid chosen was used to prepare blank nanoemulsions for choosing the best surfactant for the system. For this purpose, four surfactants were tested according to Table 2:

Table 2 – Composition of blank formulations to select the surfactant for the nanoemulsion. The liquid lipid and purifed water were fixed to 5 % and 94% (w/w), respectively.

Formulation	Surfactant	% (w/w)
1	Poloxamer 188	1
2	Kolliphor® RH 40	1
3	Tween™ 80	1
4	Gellucire® 44/14	1

3.4 Nanoemulsion preparation

The nanoemulsions were prepared according to the following protocol: the components were weighted and transferred to a beaker: one for the aqueous phase (containing surfactant) and the other for the lipidic phase (liquid lipid and drug). They were heated until 80°C, kept under this temperature and stirred at 250 rpm. Once the components were solubilized, the aqueous phase was added to the oil one. The system was then subjected to premixing using Ultraturrax® at 13.000 rpm for 5 min. The formulation was then submitted to high pressure homogenization (NanoDeBEE, BEE International, Inc., USA), set for 10,000 psi and 5 cycles/min. Then, the formulations were store in glass flasks and kept under refrigeration at 4°C and room temperature.

3.5 Nanoemulsion optimization

Following the preliminary tests to select the best formulation for niclosamide nanoemulsion, design of experiments was used to otpimize the formulation. Experimental design by surface response was performed using MiniTab® 20 software. Two independent variables were considered: the percentage (w/w) of liquid lipid and surfactant, according to Table 3. The particle size (hydrodynamic diameter) was the response for the statistical analysis, which was performed using MiniTab® 20 software.

Table 3 – Statistical design for nanoemulsion optimization.

Independent variable	% (w/w) Level	
	-1	+1
Liquid lipid	5	10
Surfactant	1.5	3

3.6 Hydrodynamic diameter (HD), polydispersity index (PI) and zeta potential (ZP) determination

For these tests Zetasizer Nano ZS90® equipment (Malvern Instruments) was used. Average particle size (hydrodynamic diameter) was determined by photon correlation

espectroscopy (PCS), at 25°C and 90° angle. Zeta potential (ZP) was determined by electrophoretic mobility, according to Henry's equation:

$$U_E = \frac{2\,\varepsilon\,z\,f(\kappa a)}{3\eta}$$

where: U_E = electrophoretic mobility, z = zeta potencial, ε = dielectric constant, η = viscosity and f(κa) = Henry's function. For ZP determination the field strength was 20V/cm and purified water used as dispersant. Samples were diluted using 10 µL of the formulation to 2000 µL of purified water.

3.7 Stability study

The formulations were stored in climatic chamber (ClimaCell Eco-Line, Germany), set for 30°C/75% RH, and also stored under refrigeration at 4°C. At the end of each period, the particle size, ZP and PDI were determined using Zetasizer Nano ZS90® (Malvern Instruments), using same condictions described in section 3.6.

3.8 UV-visible spectrophotometry linearity

Spectrophotometer Evolution Series 201® (ThermoFisher, USA) was used for this test. First, a 800 µg/mL niclosamide stock solution in absolute ethanol was prepared. Ultrasonication of 10 min. was carried out to solubilize the drug in the medium. Then, a 200 – 400 nm UV scanning was performed to select the best wavelength to obtain the linearity in ethanol. Linearity was obtained in the range (µg/mL): 3, 5, 11, 14, and 17, each point measured in triplicate, with respective dilutions from the stock solution to reach the concentrations.

3.9 Drug content

For drug content determination, samples diluted in absolute ethanol were prepared. The components were weighted and 30 min of ultrasonication was carried out for complete dissolution. Samples were prepared according to Table 4:

Table 4 – Samples prepared in absolute ethanol and niclosamide reading concentration for UV drug content determination.

Sample	Niclosamide (mg)	Nanoemulsion (mL)	Flask (mL)	Stock solution (µg/mL)	Reading concentration (µg/mL)
Standard	40	–	50	800	12
Nanoemulsion	–	0.480	20	500*	12

*Theoretical concentration based on nanoemulsion composition.

3.10 Encapsulation efficiency

For determination of the free drug concentration, 0.5 mL from the nanoemulsion was transferred to Amicon® Ultra-0.5 10 kDa centrifugal filter and submitted to centrifugation (Cetrifuge 5424 R, Eppendorf, Germany), set for 5000g for 30 min. The filtrate was withdrawn, diluted in absolute ethanol and the absorbance was read in UV-visible spectrophotometer to determine the free drug concentration. Encapsulation efficiency (EE%) was determined according to the following equation:

$$EE\% = \frac{TD-FD}{TD} \text{ X } 100$$

where TD e FD corresponds to total drug (from drug content) and free drug concentrations (mg/mL), respectively.

3.11 HCT-116 cell viability assay by MTT test

For this test, HCT-116 cells (American Type Culture Collection, Manassas, VA) were used as the cell model (human colorectal carcinoma). DMEM – F12™ medium (Gibco, Carlsbad, CA) with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptus were used for the cell culture in 96-well culture plates containing $3x10^4$ cells/well. Culture plates were treated with samples diluted in culture medium, and cells were handled in laminar flow and incubated in ovens maintained at 37°C and 5% CO^2.

To evaluate the HCT-116 cellular viability, samples containing 1.53 µmol/mL of niclosamide were used: free drug in DMSO (NCL-DMSO), nano formulation (Nano-NCL, 200 nm), placebo formulation (Blank, 200 nm) and a conventional emulsion (Micro-NCL, >1 µm,

PDI >0.6) with the same composition as Nano-NCL. The preparation of this sample involved the same steps as the preparation of Nano-NCL, except that after mixing the aqueous and lipid phases, the system was just shaken in a UltraTurrax shaker at 13,000 rpm for 5 min. The presence of this control was not only to compare the nano x micro emulsion performance, but also to verify the need for high pressure homogenization. In this case, the premixing was not sufficient to achieve nanoscale. Samples were diluted in culture medium at concentrations of 15 to 0.12 µmol/mL of niclosamide, added to the wells with cells, and the plates were incubated for 24, 48 and 72h.

Three hours before the end of the incubation period, the medium was replaced with culture medium containing 0.5 mg/ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich), and the plates were incubated for 3h. Then, the medium was removed and, after drying, DMSO was added and the reading was performed on a plate spectrophotometer at 595 nm wavelength. The negative control was cells treated with DMSO, whereas the positive control was doxorubicin (Sigma-Aldrich).

Data were processed using the Microsoft Excel software, discounting the absorbance value of DMSO and considering the negative control as 100% viability. Then, the results were analyzed by a four-parameter nonlinear regression mathematical model using the GraphPad Prism 7 program (GraphPad Software, Inc., San Diego, CA, USA) and graphs were created using Origin 2019.

4 Results and discussion

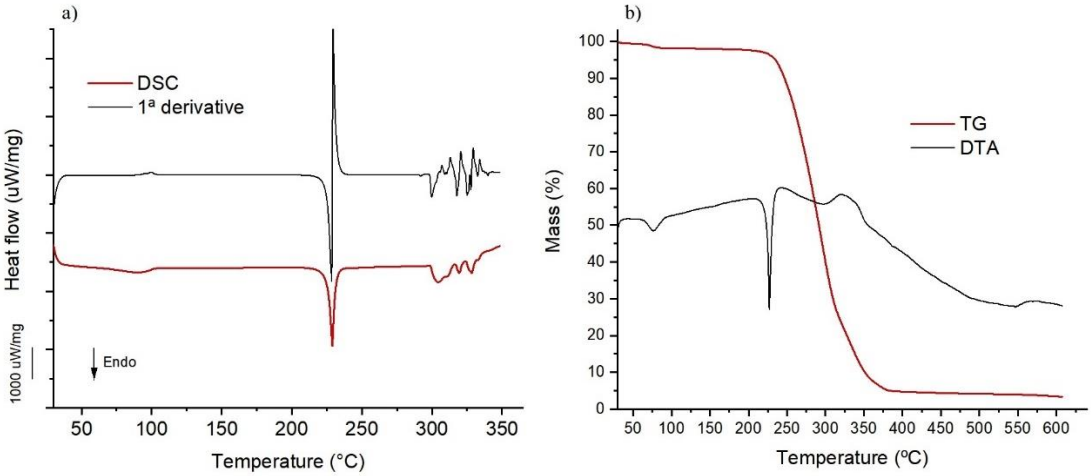
4.1 Niclosamide differential scanning calorimetry (DSC) and thermogravimetric (TG) analysis

Fig. 1 shows the DSC (a) and TG (b) from niclosamide sample. The endothermic event in the region 80-100 in DSC curve possibly is attributed to the evaporation of the water present in the sample. This is due to anhydrous niclosamide is prone to hydration to the monohydrate form, as described in the literature [13,14]. TG curve indicates that the hydration degree was close to 2% due to the reduction of this valor at 85 °C. Thus, it is possible that some hydration degree was present in the sample.

According to Fig. 1, niclosamide melting point (MP) peak occurred at 228 °C, which is in accordance with literature findings [13,14,20]. This confirms niclosamide thermal stability, which indicates its suitability for the development of nanoemulsion by high pressure

homogenization, since this process requires high pressure and temperature to prepare the formulations. However, along with its molecular weight (327.12 g/mol), the result is also an indication of niclosamide propensity for recrystallization, considering its glass forming ability (GFA) classification [21]. In this case, niclosamide is considered a class I molecule, which refers to drug substances that are highly prone to precipitate in a crystalline, and most stable, form [22,23].

Fig. 1. Niclosamide DSC (a) and TG (b) curves.



Source: Author's own elaboration.

4.2 Liquid lipid selection

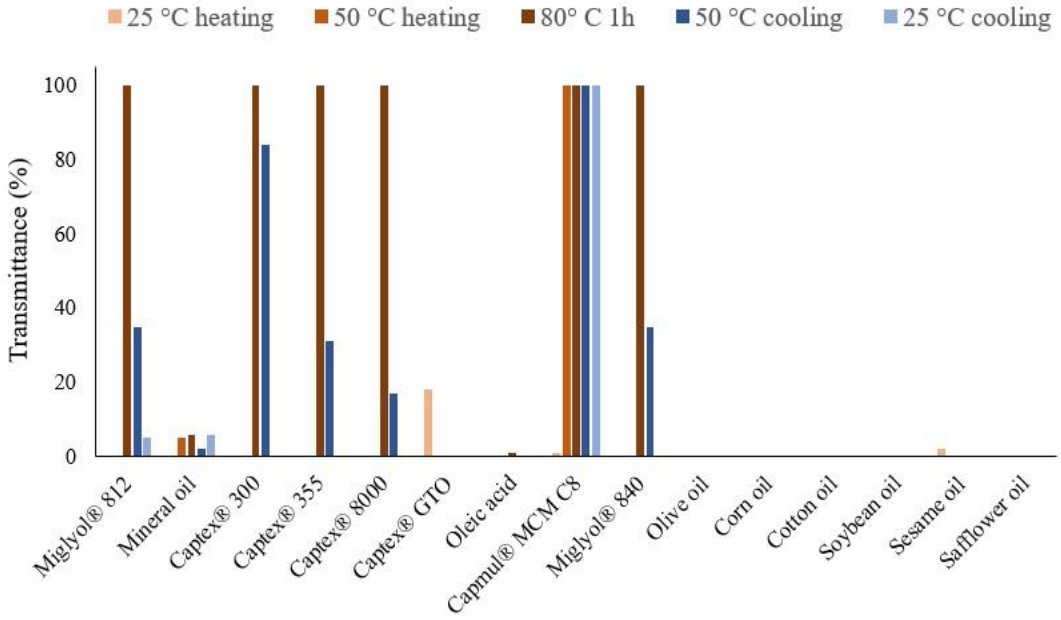
Fig. 2 shows the samples before and after the heating/cooling cycle to estimate the lipid capacity to solubilize niclosamide. Just before the test (Fig. 2A), Capmul® MCM C8 (sample 8) indicated that could be a possible candidate since niclosamide quickly dispersed in this lipid, turning the lipid medium yellowish. For the other samples, most of the drug settled in the bottom of the vial, including olive oil (sample 10), whose yellowish color is a characteristic of the lipid. After the test, only in Capmul® MCM C8 (sample 8) niclosamide was completely dissolved, while for the other samples most of the drug was suspended or settled in the bottom of the vial (Fig. 2B).

Fig. 3 shows the reached transmittances (%) of the samples during the heating/cooling cycle. As hypothesized, Capmul® MCM C8 presented the best performance among the lipids. Besides, the transmittance at the end of the test is in accordance with the visual aspect of the samples.

Fig. 2. Vials just before (A) and after (B) the heating/cooling cycle in Crystal16 equipment.

Author's own elaboration.

Fig. 3. Transmittance (%) of the samples during heating/cooling cycle in Crystal16 to estimate the lipid capacity to solubilize niclosamide.



Source: Author's own elaboration.

Therefore, the lipids were grouped according to their estimated capacity to solubilize niclosamide, as indicated in Table 5. Capmul® MCM C8 was considered the best since, even in the end of the test, the transmittance was 100%, indicating a clear and homogeneous medium.

The average group include lipids that, despite their capacity to solubilize niclosamide at high temperature (80 °C, 100% transmittance), they were not able to maintain the drug solubilization during the cooling phase. This is undesirable since after preparing the nanoemulsion, the drug could precipitate as the system reaches room temperature. Finally, the worst group include lipids that were not able of even solubilize niclosamide at 80°C, which was therefore discarded as candidates.

Table 5 – Liquid lipids groups according their estimated capacity to solubilize niclosamide.

Best	Average	Worst
Capmul® MCM C8	Miglyol® 812	Mineral oil
	Captex® 300	Captex® GTO
	Captex® 355	Miglyol® 840
	Captex® 8000	Oleic acid
		Olive oil
		Corn oil
		Cotton oil
		Soybean oil
		Sesame oil
		Safflower oil

4.3 Surfactant selection

From the liquid lipid selection, blank nanoemulsions were prepared to determine the surfactant for the nanomulsions. Table 6 shows results from the four tested formulations, using Capmul® MCM C8 as the liquid lipid:

Table 6 – Composition and physicochemical characterization of blank formulations to select the surfactant for the nanoemulsions. Capmul® MCM C8, surfactant and purifed water were fixed to 5, 1 and 94% (w/w), respectively. PDI: polydispersity index; ZP: zeta potential.

Formulation	Surfactant	Size (nm)	PDI	ZP (mV)
1	Poloxamer 188	220.4 ± 0.7	0.029 ± 0.024	-31.7 ± 0.8
2	Kolliphor® RH 40	–	–	–
3	Tween™ 80	–	–	–
4	Gellucire® 44/14	–	–	–

Formulations 2, 3 and 4 readly presented phase separation and presence of precipitate.

From the four formulations, only 1 did not present phase separation or precipitate. Besides, particle size (220.4 ± 0.7 nm), PDI (0.029 ± 0.024) and ZP (-31.7 ± 0.8) indicated adequate nanoescale and stabilization mechanism by electrostatic repulsion. Hence, these results showed that poloxamer 188 could be an adequate choice for the lipidic system with Capmul® MCM C8 and niclosamide.

4.4 Nanoemulsion preparation

Following liquid lipid and surfactant selections, nanoemulsions containing Capmul® MCM C8 (5-10% w/w), poloxamer 188 (1-3% w/w) and niclosamide (10 mg/1 g of liquid lipid) were prepared. To be considered adequate nanoemulsions, we stablished desirable quality criteria that formulations would have to meet: particle size <300 nm, PDI <0.2, and agglomerate/aggregate-free preparations determined by visual inspection. Preliminary tests showed that despite the homogeneous appearance soon after preparing the formulations, all of them presented a yellow precipitate after a short time of refrigerated (4°C) or room temperature, and inadequate particle size (0.5-1.0 µm). We considered that niclosamide was not encapsulated as we expected. To address this issue, some hypotheses were raised and tested.

First, the amount of niclosamide should be reduced. We reduced to half the amount of niclosamide, but results were the same after few days on refrigerated (4°C) or room temperature. Second, we prepared new formulations including surfactants not tested yet (lecithin and labrasol) to stabilize the system; and third, we increased the pressure during high pressure homogenization process. However, again formulations presented phase separation and inadequate particle size.

We choose Capmul® MCM C8 considering the great difference to the other lipids tested in Crystal16 analysis, aiming not only niclosamide solubilization but also to optimize its drug load in the formulation. Since this component is a semi-solid lipid at room temperature, variations on temperature could alter its consistency, compromising niclosamide encapsulation and the stability of the system. We also raised the hypothesis that possible incompatibility of the components with this lipid could also be related to the physical instability. For instance, Negi et al. developed a nanostructured lipid carrier for an anticancer agent with Capmul® MCM C8, which was selected as the best liquid lipid for the system. However, solid lipids gelucire 39/1 and glyceryl mono stearate (GSM) showed low compatibility (miscibility) with Capmul® MCM C8 [24]. We then considered using another lipid from the liquid lipid selection. According to the average group (Table 4), we understand that lipids from this group had

equivalent perfomance on solubilizing niclosamide. This is due to their possible inability to maintain the drug substance solubilized as the formulation reaches room temperature (Fig. 3). Then we choose Miglyol® 812, for at least presenting some transmittance (%) on cooling phase, and Captex® 8000, maitaining the drug:lipid proportion (10 mg:1 g), according to Table 7.

Table 7: Composition, size and PDI measurements of the formulations obtained. Percentages referring to w/w, considering 50 g of formulation. The concentration of Poloxamer 188 was fixed at 3%. Water was added to 100%.

	Formulation				Stability (days)*			
	Miglyol (%)	Capmul (%)	Captex (%)	Niclosamide (%)	Size (nm)		PDI	
					4	11	4	11
1	7.500	–	–	–	192.0 ± 1.8	194.2 ± 2.9	0.144 ± 0.033	0.144 ± 0.015
2	3.750	3.750	–	–	511.5 ± 13.8	521.7 ± 21.7	0.054 ± 0.049	0.051 ± 0.033
3	2.500	2.500	2.500	–	1,792 ± 359	1,773 ± 240.3	0.157 ± 0.136	0.607 ± 0.102
4	7.500	–	–	0.075	189.3 ± 5.8	199.6 ± 1.1	0.194 ± 0.060	0.217 ± 0.024
5	3.750	3.750	–	0.075	447.1 ± 7.6	519.1 ± 28.1	0.117 ± 0.046	0.327 ± 0.140
6	2.500	2.500	2.500	0.075	2,021 ± 622	4,572 ± 1053	0.159 ± 0.125	0.674 ± 0.317

*Time between preparation and measurement.

According to our findings, formulations containing lipid mixtures (such as Miglyol® 812 and Capmul® MCM C8) did not meet the quality criteria. In addition, formulations showed physical stability <7 days. We again considered the hypothesis of possible interaction between lipids and insufficient amount of surfactant to maintain the stability of the nanoemulsion.

On the other hand, formulations containing only Miglyol® 812 met the quality criteria. The combination of Miglyol® 812 and poloxamer 188, with addition of sodium azide, allowed the development of colloidal lipid carriers containing cannabidiol [25]. Besides, Real et al. (2021) employed a full factorial design to develop Lecithin/Span®80 based nanoemulsions containing Miglyol® 812 [26]. They used model drugs with different logP values (including

niclosamide, logP 4.45 [12]) to assess the influence of factors on the particle size and stability of the nanoemulsions. Among the results, they found that Miglyol® 812 concentration and logP of each drug had strong impact on the stability of the nanoemulsion. In this case, the higher the lipophilicity, the greater the association efficiency between the nanosystem and the drug substance [26]. Therefore, we expected that nanoemulsions with Miglyol® 812, poloxamer 188 and niclosamide would be viable.

Based on the literature and on preliminary experimental results, thirteen new formulations were prepared using the MiniTab® 20 software. The formulations were prepared using Miglyol® 812 as the only lipid. The independent variables were % of lipid and surfactant concentrations (w/w) (Table 8).

4.5 Nanoemulsion optimization

Once Miglyol® 812 and poloxamer 188 were chosen as liquid lipid and surfactant, respectively, their influence on the nanoemulsion, along with niclosamide, was studied in an experimental design. Hence, Table 8 shows size and PDI values from formulations of the experimental design:

Table 8 – Composition and size parameters from the formulations of the experimental design. Poloxamer 188 concentration was fixed at 3% (w/w) and purified water was added to reach 100% (w/w), considering a batch of 50 g.

	Formulation			Stability (days)			
	Miglyol	Poloxamer	Niclosamide	Size (nm)		PDI	
	(%)	(%)	(%)	1	8	1	8
1	7.5	2.25	0.075	210.7 ± 3.9	204.5 ± 0.9	0.214 ± 0.029	0.179 ± 0.044
2	7.5	2.25	0.075	214.4 ± 2.6	210.5 ± 1.5	0.165 ± 0.030	0.156 ± 0.043
3	5.0	1.50	0.050	226.8 ± 3.0	219.9 ± 2.8	0.202 ± 0.020	0.169 ± 0.028
4	10.0	2.25	0.100	232.5 ± 1.3	230.2 ± 1.9	0.155 ± 0.010	0.160 ± 0.021
5	10.0	3.00	0.100	213.7 ± 4.8	210.4 ± 1.4	0.192 ± 0.024	0.187 ± 0.007
6	7.5	3.00	0.075	201.2 ± 2.9	203.1 ± 1.7	0.208 ± 0.015	0.204 ± 0.007
7	7.5	2.25	0.075	208.8 ± 2.1	206.6 ± 1.0	0.178 ± 0.017	0.168 ± 0.015
8	7.5	1.50	0.075	241.2 ± 2.2	241.1 ± 1.2	0.186 ± 0.035	0.202 ± 0.025
9	7.5	2.25	0.075	211.2 ± 1.2	208.3 ± 2.3	0.173 ± 0.010	0.165 ± 0.030
10	10.0	1.50	0.100	266.5 ± 3.5	262.0 ± 1.1	0.168 ± 0.027	0.191 ± 0.023
11	7.5	2.25	0.075	209.5 ± 0.7	205.4 ± 0.7	0.178 ± 0.023	0.162 ± 0.019
12	5.0	2.25	0.050	196.3 ± 4.0	193.6 ± 0.8	0.211 ± 0.037	0.192 ± 0.006
13	5.0	3.00	0.050	179.3 ± 2.1	179.1 ± 2.5	0.184 ± 0.009	0.188 ± 0.015

*Time between preparation and measurement.

To be considered well-adjusted to the experimental values, the regression must be significant ($p < 0.05$; $\alpha=0.05$), with a non-significant lack of fit ($p > 0.05$; $\alpha=0.05$) [27]. Table 9 presents the Analysis of variance using particle size as response:

Table 9 – Analysis of variance using particle size as response, to test the significance of the regression for data obtained of the formulations from the experimental design.

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	3	5602.14	1867.38	215.91	0.001
Linear	2	5308.36	2654.18	306.88	0.001
Miglyol® 812	1	2027.68	2027.68	234.44	0.001
Poloxamer 188	1	3280.68	3280.68	379.32	0.001
Square	1	293.77	293.77	33.97	0.001
P188*P188	1	293.77	293.77	33.97	0.001
Error	9	77.84	8.65		
Lack-of-fit	5	59.09	11.82	2.52	0.196
Pure error	4	18.75	4.69	*	*
Total	12	5679.98			

DF: degrees of freedom; Adj SS: adjusted sum of squares; Adj MS: adjusted mean squares; F-value: F statistics; P-value: significance level.

The non-significant P-value of lack-of-fit (0.196) indicates that the model is statistically well suited and with significance. Besides, the proportions of Miglyol® 812 and Poloxamer 188 influence the characteristics of the formulations due to significant P-values (both equal to 0.001). To confirm the significance and adjustment of the proposed regression, R^2 and adjusted R^2 are analyzed. Both demonstrate the overall performance of the model and ensure that only terms that are meaningful to the data are included. They also evaluate the noise of the experimental environment and variables that can invalidate the model. Table 10 presents the R^2 and adjusted R^2 values, in addition the values of the significance tests of the coded coefficients.

Table 10: Significance test of regression coeficients and R^2 values.

Term	SD	SD of Coef.	T-value	P-value	VIF
Constant	211.91	1.11	190.65	0.001	
Miglyol® 812	18.38	1.20	15.31	0.001	1.00
P188	-23.38	1.20	-19.48	0.001	1.00
P188*P188	9.54	1.64	5.83	0.001	1.00
R^2 = 98.63%		Adj R^2 = 98.17%		R^2 (pred.) = 96.75%	

R^2: determination coefficient; Adj R^2: adjusted determination coefficient; R^2 (pred.): predictive determination coefficient of the fitted model; SD: standard deviation; T-value: T estatistics; P-value: significance level.

Both R^2 and Adj R^2 values do not differ significantly (0.46%). There is also no significant difference between the R^2 (pred) and R^2 and Adj R^2 values. Additionally, the three results had values greater than 80%, which indicates the probability that the model's predictions are close to the results found experimentally. Therefore, the model can be used to optimize the obtaining of the nanoemulsion. In this way, the first-order global model presented in the following equation is considered adequate.

$$\text{Hydrodynamic diameter (nm)} =$$
$$312.7 + 7.353\ \text{Miglyol}^{®}\ 812 - 107.5\ \text{P188} + 16.95\ \text{P188*P188}$$

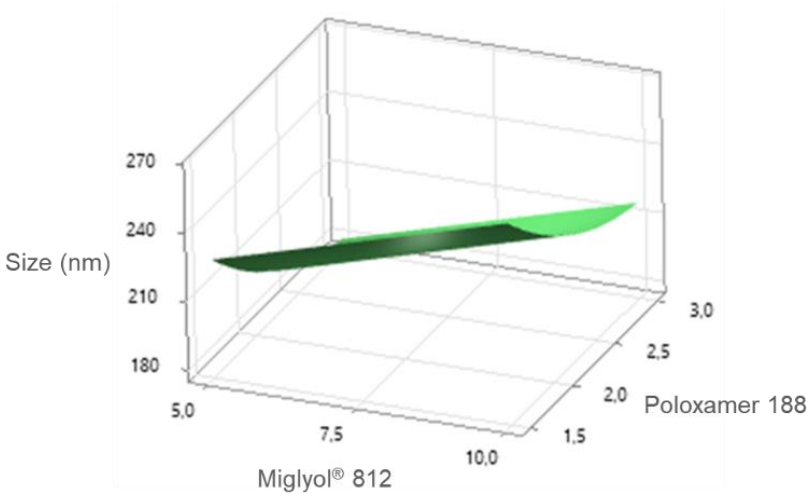
The equation describes the influences of the components on the particle size, where each variable refers to the proportion in the formulation (% w/w). Hence, poloxamer 188 (P188) has the major contribution to reduce hydrodynamic diameter due to its negative coefficient (-107.5). Miglyol® 812 (7.353) and the quadratic function of the surfactant proportion (16.95) favor the increase of the particle size. We tested this model with two formulations (A and B), according to Table 11:

Table 11 – – Tested formulations to verify the mathematic model for prediction of particle size. Components described according to proportion in the formulation (%, w/w), with purified water added to 100%.

Formulation	Miglyol® 812 (%)	Poloxamer 188 (%)	Niclosamide (%)	Size (nm)		PDI
				Predicted	Experimental	
A	5.25	2.50	0.05	180-200	189.7 ± 2.8	0.217 ± 0.004
B	5.50	2.75	0.05	180-200	186.9 ± 2.8	0.197 ± 0.016

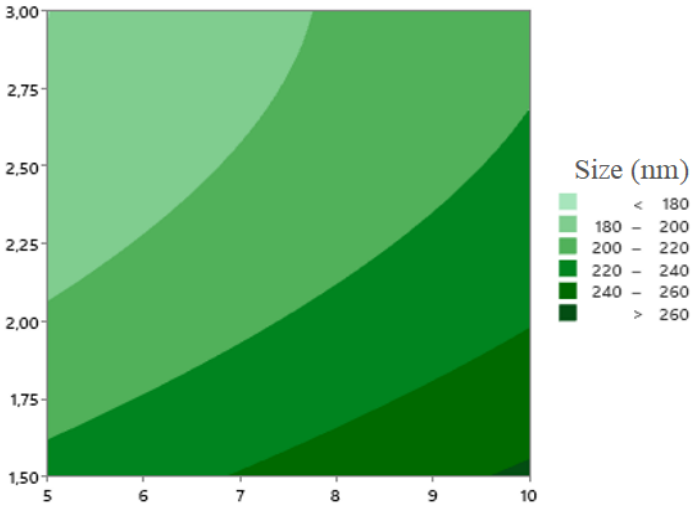
Through the response surface and contour graphs (Fig. 4 and 5) it was possible to understand the optimal region for particle size as a function of the lipid and surfactant concentrations. The lowest values of particle size (up to 200 nm) were observed in formulations with higher poloxamer 188 concentrations (1.6-3.0% w/w). Differently, formulations with surfactant concentration lower than 1.6 % showed higher values of particle size (200-260 nm) as the concentration of Miglyol® 812 increased.

Fig. 4. Surface response plot relative to the particle size of the nanoemulsion containing niclosamide.



Source: Author's own elaboration.

Fig. 5. Contour plot of particle size according to the proportion of Miglyol® 812 and poloxamer 188 in the formulation (% w/w)



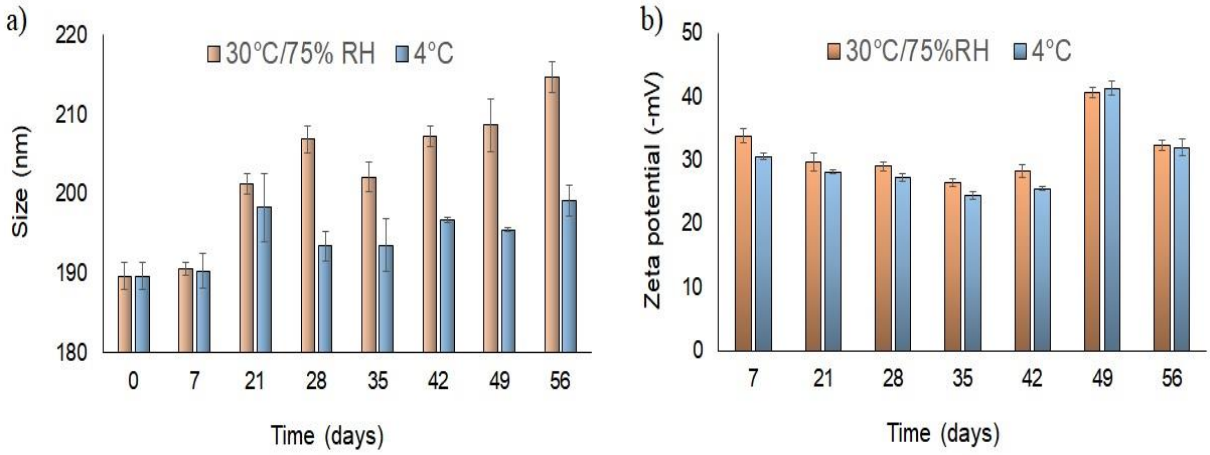
Source: Author's own elaboration.

4.6 Stability study

Due to adequate quality criteria, formulation 12 (5% Miglyol® 812, 2.25 % poloxamer 188, 0.05% niclosamide, w/w) from the experimental design was selected for the stability study and efficacy tests. Fig. 6 shows the stability of the nanoemulsion according to a) particle size and b) zeta potential (-mV). According to results, particle size ranged from 190 to 217 nm at 30 °C/75% RH, and from 190 to 200 nm at 4°C, after 56 days of stability. These results show that Miglyol® 812 was a suitable as the liquid lipid, despite not being the first choice from lipid selection. In addition, results also show that it was not obvious to obtain the formulation, considering preliminary evidence for using Capmul® MCM C8. As a comparison, formulation with Miglyol® 812 was at least 7-fold more stable (56 days) than with Capmul® MCM C8 (8 days).

The zeta potential (Fig.6b) results indicate that the highly negative values contribute to the stabilization of the nanoparticle through an electrostatic repulsion mechanism. Average zeta potential was -31.5 ± 4.7 and -29.9 ± 5.7 mV, at 30ºC/75%RH and 4°C, respectively.

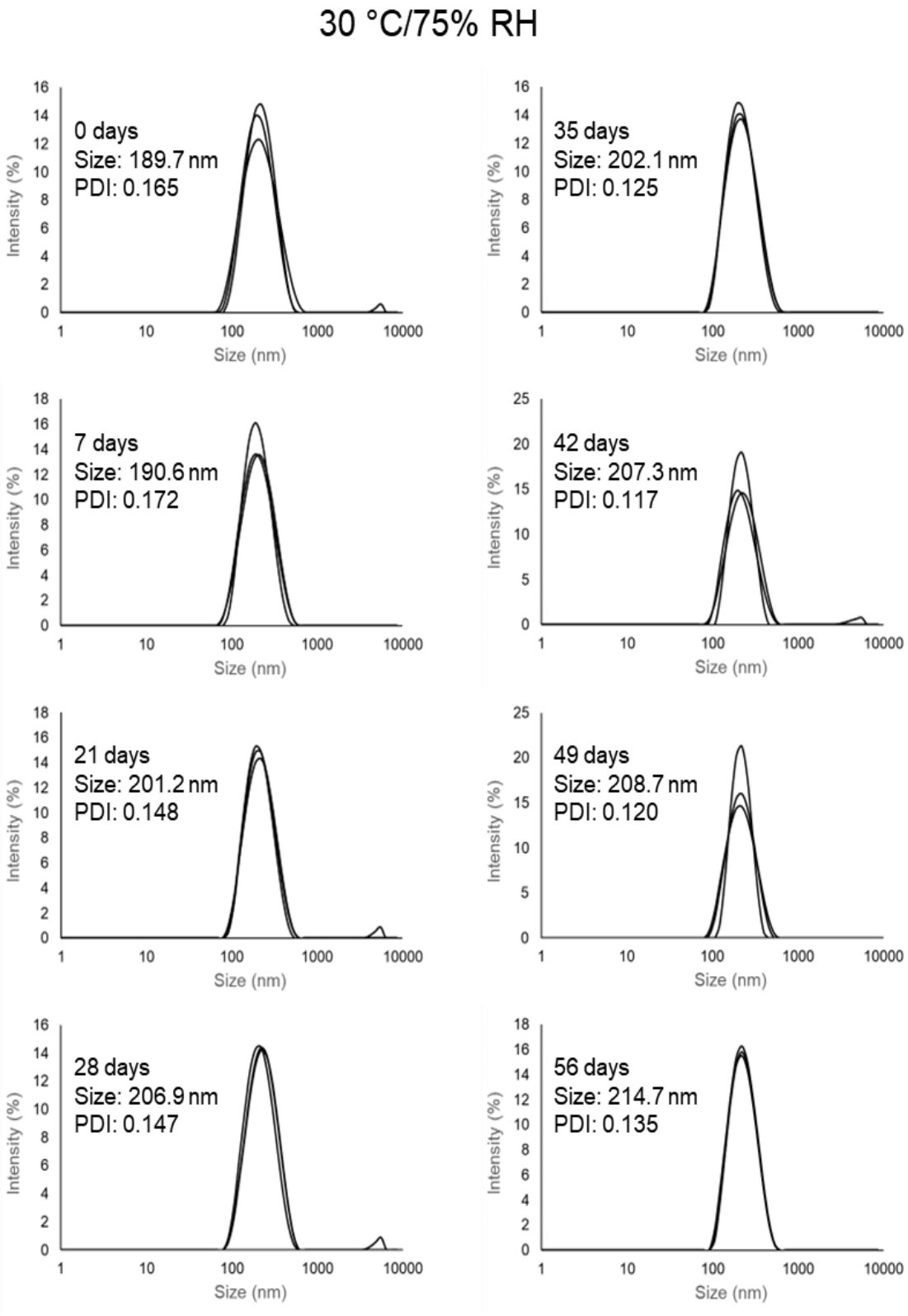
Fig. 6. Stability study of the niclosamide nanoemulsion.



Source: Author's own elaboration.

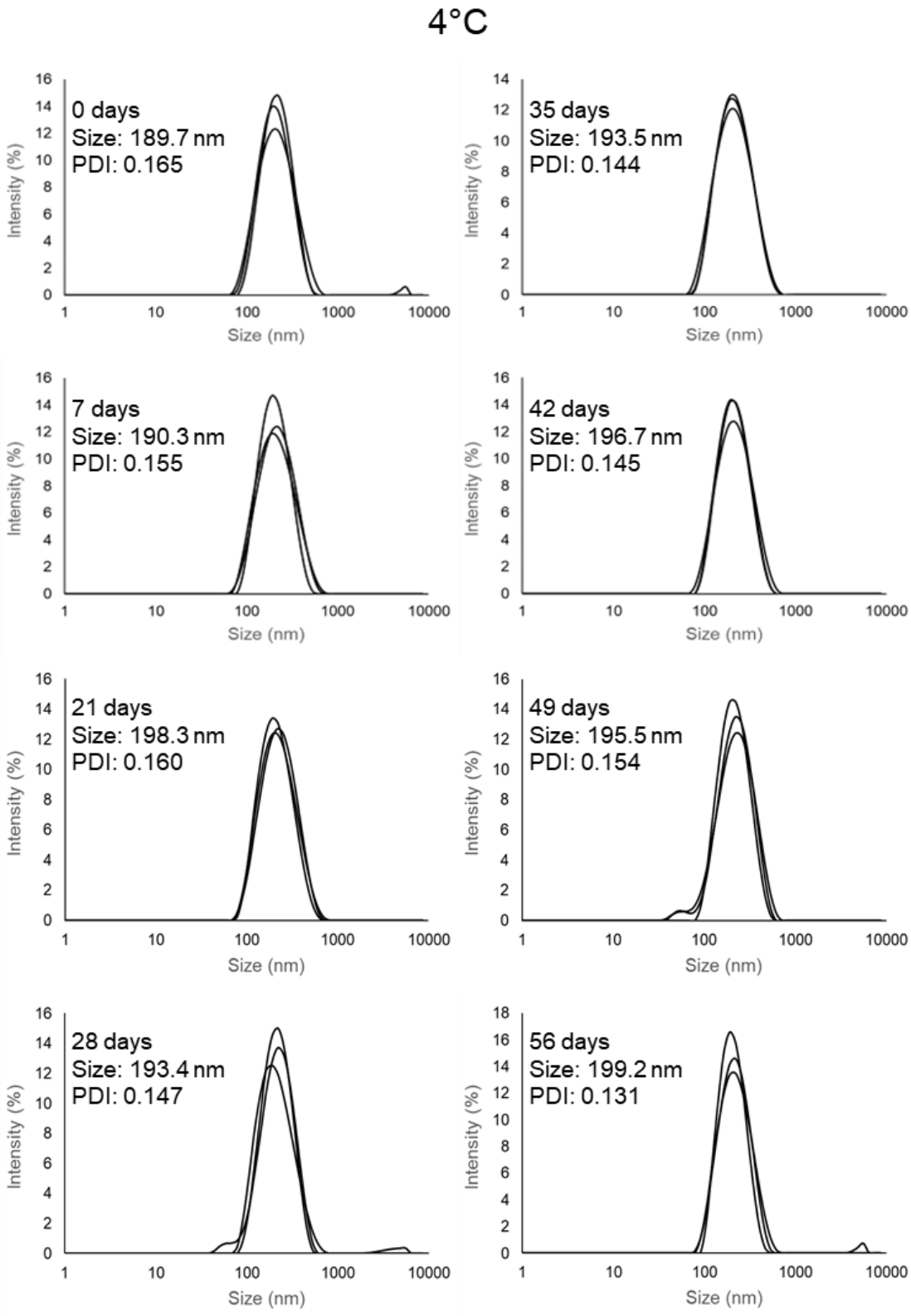
Fig. 7 and 8 shows particle size distribution of the formulations during the stability study. It is possible to observe a monomodal distribution for the particle size, with average PDI of 0.14 and 0.15 for samples kept in 30 °C/75% RH and 4°C, respectively.

Fig. 7. Particle size distribution of niclosamide nanoemulsion during the stability study in climatic chamber (30 °C/75% RH).



Source: Author's own elaboration.

Fig. 8. Particle size distribution of niclosamide nanoemulsion during the stability study under refrigerated temperature (4 °C).



Source: Author's own elaboration.

4.7 UV-visible spectrophotometry linearity

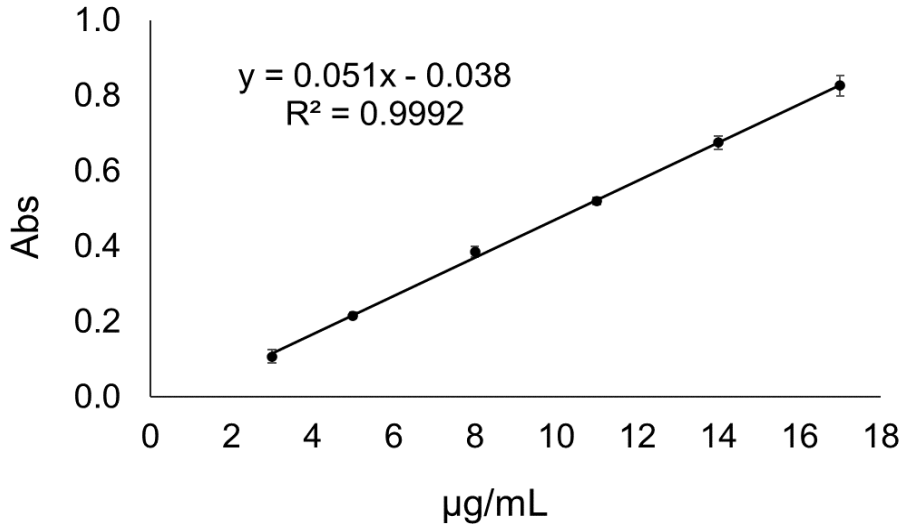
Table 12 presents the reading results for niclosamide concentration linearity in ethanol, while Fig. 9 shows the graph obtained. The UV-scan in 200-400 nm is in accordance with literature, including maximum absorbance (λ = 333 nm) selected for the measurements [20].

Table 12 – Reading results (λ = 333 nm) for niclosamide concentration linearity in ethanol.

µg/mL	Abs			Mean	SD	RSD (%)
3	0.093	0.103	0.127	0.108	0.017	16.230
5	0.216	0.208	0.225	0.216	0.009	2.931
8	0.369	0.394	0.393	0.385	0.014	3.673
11	0.510	0.525	0.525	0.520	0.009	1.665
14	0.696	0.664	0.667	0.676	0.018	2.616
17	0.856	0.801	0.823	0.827	0.028	3.349

SD: standard deviation; RSD: relative standard deviation.

Fig. 9. Niclosamide linearity curve in ethanol



Source: Author's own elaboration.

4.8 Drug content

In Table 13 are indicated the abs readings from the standard solution and sample, both diluted in ethanol. To determine drug content, we considered the mean of the sample readings (n = 3), which was equal to 0.696 ± 0.008. Then, we determined sample reading concentration comparing with the standard solution (0.688; 12 µg/mL), obtaining the value of 12.14 µg/mL.

Then we applied a dilution factor (41.66) to determine niclosamide drug content of 0.505 mg/mL (~1.54 µmol/mL, 327.12 g/mol). When compared theoretical and experimental values (0.500 and 0.505 mg/mL, respectively), we concluded that the obtained nanoemulsion did not present drug loss during the stages of the development process. The small difference may be attributed to intrinsic experimental errors, such as weighing, water evaporation and analytical factors.

Table 13 – Readings (abs) from the samples prepared and diluted with absolute ethanol to determine drug content.

	Standard (12 µg/mL)	Sample
	0.689	0.704
	0.695	0.689
	0.680	0.696
Mean	0.688	0.696
SD	0.008	0.008
RSD (%)	1.097	1.078

4.9 Encapsulation efficiency

Following sample preparation with mild centrifugation, we measured the abs of the filtrate using the linearity in ethanol. However, the clear and homogenous solution obtained presented negligible abs. Then, since abs value was below the lowest point of the linearity curve, we estimated EE (%) applying the formula:

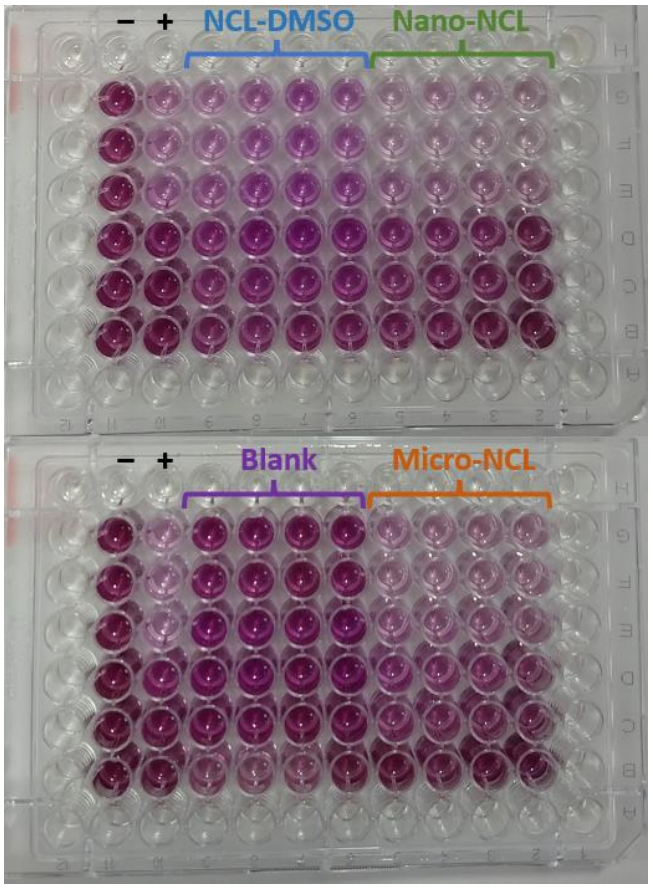
$$EE\% = \frac{TD-FD}{TD} \text{ X } 100$$

considering 0.505 and 0.05 mg/mL as TD (from drug content) and FD (free drug), respectively. Hence, we estimated EE (%) as at least ~90%. Factors such as possible niclosamide crystallization and its retention at the filter might be related to the result. However, what reinforces EE (%) herein estimated is the stability of the nanoemulsion, with PDI ~0.15 and monomodal distribution throughout at least 56 days.

4.10 HCT-116 cell viability assay by MTT test

Fig. 10 shows a 72h treatment plate used for the MTT assay. The pink color indicates MTT metabolism by the viable cells. The darker the well, the greater the number of viable cells. The cell viability assay with the human colorectal carcinoma cell line HCT-116 demonstrated that niclosamide citoxicity is both time and concentration dependent. Table 14 shows the IC_{50} values calculated according to the model, while Fig. 11-13 show the cell viability (%) in function of formulation (A) and niclosamide (B) concentration for 24, 48 and 72 h of treatment. Taken together, Nano-NCL IC_{50} (48h, 1.259 µM) is in accordance with literature (1-2 µmol/L), for HCT-116 as cell model [28].

Figure 10: A 72h treatment plate used for MTT test.

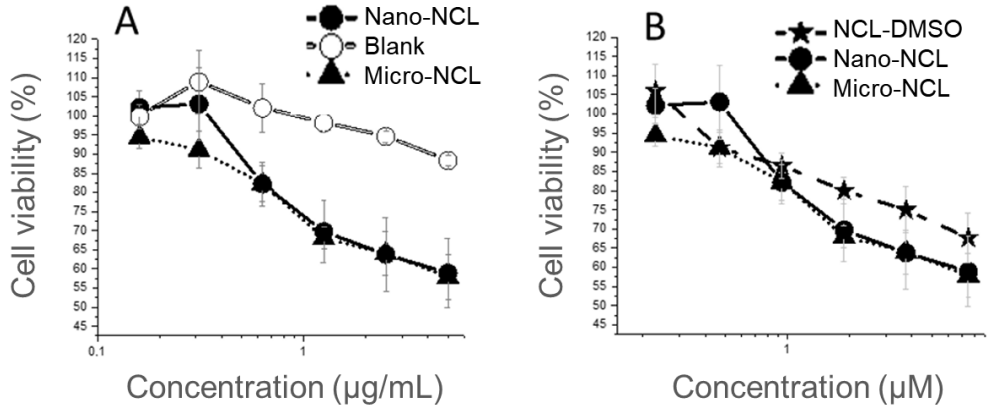


-: negative control (DMSO); +: positive control (doxorubicin); NCL-DMSO: free drug; Nano-NCL: niclosamide nanoemulsion (200nm); Blank: placebo nanoemulsion (200nm); Micro-NCL: coarse niclosamide emulsion (>1 µm). Source: Author's own elaboration.

Table 14 – IC_{50} values calculated according to the model. Values in mg/mL refers to sample concentration, considering formulation density as 1 g/mL.

Sample	Time (h)					
	24		48		72	
	µM	mg/mL	µM	mg/mL	µM	mg/mL
NCL-DMSO	NC	–	5.460	–	1.321	–
Micro-NCL	9.936	6.609	4.504	NC	1.676	1.080
Nano-NCL	8.775	5.842	1.259	0.844	1.054	0.699
Blank	–	15.82	–	NC	–	4.909

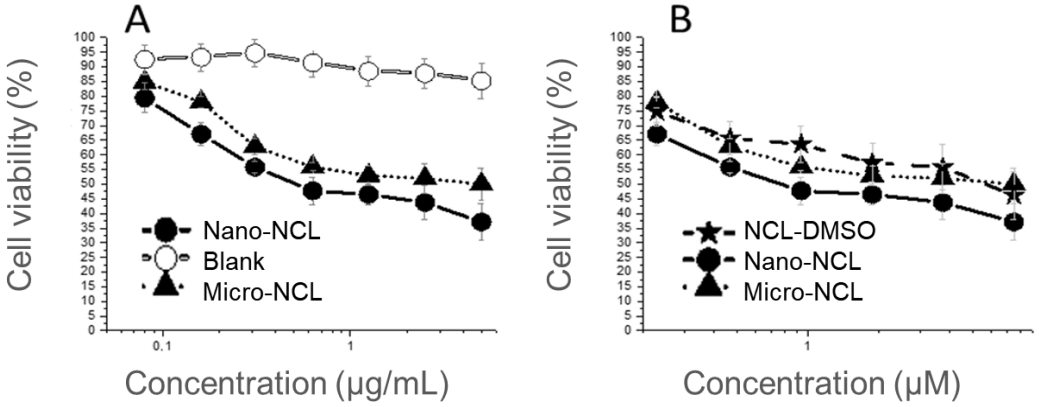
Fig. 11 – Cell viability at 24 h of treatment according to (A) formulation concentration and (B) niclosamide concentration. Data represented as mean ± sd, n = 8-10, in 3 independent experiments.



Source: Author's own elaboration.

In 24h, the Nano-NCL IC_{50} (8.775 µM) is slightly lower than of Micro-NCL (9.936 µM), showing that the presence of the lipid system is important for the drug substance's activity, but without great difference in relation to its size. This can also be evidenced by similar curves (Figure 11 ▲ and ●). IC_{50} of Blank sample (15.82 mg/mL) is almost three times higher than Nano-NCL (5.842 mg/mL), showing that the components of the formulation have cytotoxicity that increases with concentration (Figure 11A), however niclosamide is critical for system activity; this is also evidenced by the right shift curve of the white nanoemulsion in relation to the other two (Figure 11A).

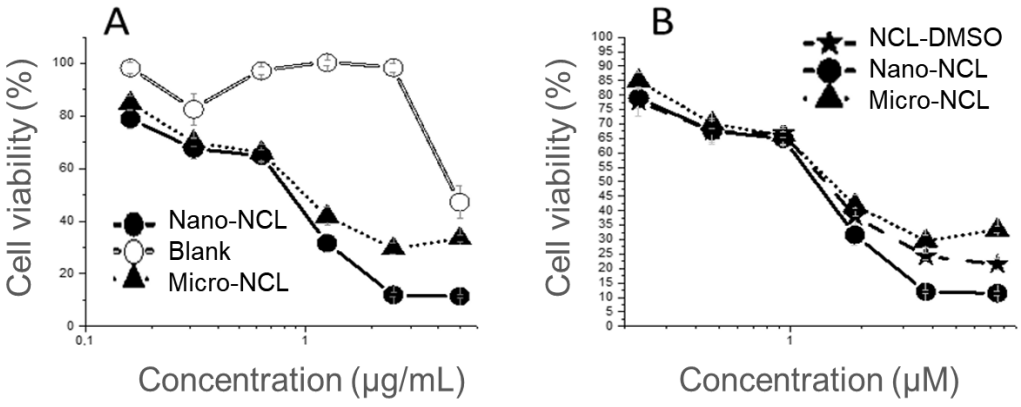
Fig. 12 – Cell viability at 48h of treatment according to formulation (A) or niclosamide (B) concentration. Data represented as mean ± sem, n = 8-10, in 3 independent experiments.



Source: Author's own elaboration.

In 48h it is possible to observe that NCL-DMSO (IC_{50} = 5.460 µM) has an IC_{50} higher than the Micro-NCL (IC_{50} = 4.504 µM), evidencing that the emulsion influences the cytotoxicity in this treatment period. Nano-NCL (IC_{50} = 1.259 µM), in turn, has an IC_{50} 3.6x lower than the Micro-NCL, demonstrating the importance of the size of the system for potentiating the cytotoxic effect of the drug substance. Blank nanoemulsion does not show sufficient cytotoxicity to calculate the IC_{50} in this model (Table 14), also evidenced by the curve (Fig. 12A), again indicating that niclosamide is essential for the activity of the system.

Figure 13 – Cell viability at 72 h of treatment according to formulation (A) or niclosamide (B) concentration. Data represented as mean ± sem, n = 8-10, in 3 independent experiments.



Source: Author's own elaboration.

At 72h we observed that NCL-DMSO IC_{50} (1.321 µM) is similar to that of the Micro-NCL (1.676 µM), indicating little influence of the system at that time. Nano-NCL (1.054 µM) has IC_{50} 1.6x lower than Micro-NCL (1.676 µM), demonstrating that the nanoscale had little influence on the potentiation of niclosamide cytotoxicity in this treatment period. Besides, when comparing the values of NCL-DMSO and Nano-NCL, the drug encapsulation in the lipid matrix did not affect its anticancer effect; on the contrary, a small increase in activity was observed (IC_{50} of Nano-NCL 1.25x lower than NCL-DMSO). This represents a gain, since it is inappropriate to administer the free drug in an organic solvent such as DMSO, while the nanoemulsion represents a viable way to deliver the drug. Blank sample has the highest IC_{50} (4.909 mg/mL) demonstrating the lowest cytotoxicity (Table 14).

Niclosamide cytotoxicity increases in DMSO as treatment time increases, since IC_{50} is 4x lower in 72h (1.321 µM) than to 48h (5.460 µM). This trend is also observed in the other treatments, especially with Nano-NCL, when IC_{50} in 24h (8.775 µM) is almost 7x greater than in 48h (1.259 µM), which in turn is 1.2x greater than in 72h (1.676 µM). This also allows decreasing the treatment time from 24 to 48h, which is important for the activity of the nanoemulsion in this model, but the increase from 48 to 72h did not have much influence.

A hypothesis to explain the difference between Nano-NCL and Micro-NCL results is the stability of the lipidic system. In this case, at 72h both nano and microemulsions might has destabilized, releasing the drug substance in the medium, which would have equalized both treaments in this period. Another possibility is the limitation of the assay. The hypothesis is that in MTT test the lack of relevant biological barriers, the close contact of the drug substance with the cell monolayer, and the sensitivity of the cell model to niclosamide might have contributed to the small difference between Nano-NCL and Micro-NCL as well.

5 Conclusion

This study aimed at the development, physicochemical characterization, and *in vitro* anticancer activity of a niclosamide nanoemulsion, with HCT-116 as the cell model. During the nanoemulsion development, formulations with Capmul® MCM C8 were not sufficiently stable to justify the usage of this lipid, despite the evidence in lipid selection. On the other hand, Miglyol® 812 proved to be a suitable liquid lipid for the system. Niclosamide nanoemulsion (~200 nm) with Miglyol® 812 and poloxamer 188 was stable for 56 days, with monomodal particle size distribution during the period. The *in vitro* viability assay showed that niclosamide

toxicity against HCT-116 cell model was both time and concentration dependent. Results herein obtained encourage further research to understand and optimize niclosamide performance as an anticancer drug substance.

6 Acknowledgments

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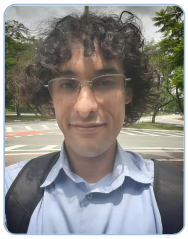
Appendix:

Curriculum Lattes



Eduardo José Barbosa

Endereço para acessar este CV: http://lattes.cnpq.br/2730692379141606
ID Lattes: **2730692379141606**
Última atualização do currículo em 17/07/2022

Possui graduação em Farmácia e Bioquímica pela Universidade de São Paulo (2014), e mestrado pelo programa Fármaco e Medicamentos, da Universidade de São Paulo (2017). Atualmente cursa o doutorado, com pesquisa que envolve estratégias farmacêuticas para o reposicionamento de fármacos na terapêutica. Possui experiência em desenvolvimento farmacotécnico, com ênfase em formas farmacêuticas sólidas para administração oral. Atuou durante dois anos na indústria (Fundação Para o Remédio Popular - FURP) em setor de desenvolvimento farmacotécnico. Possui experiência com estudos de pré-formulação, incluindo determinação da solubilidade, do pKa e do logP de moléculas (método potenciométrico Cheqsol), bem como estudo de reometria de pó úmido por medição de torque (Mixer Torque Rheometer), e levitação acústica de materiais para aplicação farmacêutica. Atualmente trabalha com desenvolvimento e caracterização de sistemas nanoestruturados (matriciais lipídicos e nanocristais) e dispersões sólidas amorfas. **(Texto informado pelo autor)**

Identificação

Nome Eduardo José Barbosa
Nome em citações bibliográficas BARBOSA, E. J.;BARBOSA, EDUARDO JOSÉ;JOSÉ BARBOSA, EDUARDO;BARBOSA, EDUARDO J.
Lattes iD http://lattes.cnpq.br/2730692379141606
Orcid iD ? https://orcid.org/0000-0003-3892-2193

Endereço

Endereço Profissional Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Departamento de Farmácia.
Av. Lineu Prestes, 580 - Bloco 15
Butantã
05508900 - São Paulo, SP - Brasil
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Formação acadêmica/titulação

2018 Doutorado em andamento em Fármacos e Medicamentos (Conceito CAPES 4).
Universidade de São Paulo, USP, Brasil.
Título: Innovative pharmaceutical strategies for niclosamide repositioning,
Orientador: Nádia Araci Bou Chacra.
Coorientador: Gabriel Lima Barros de Araujo.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.
Palavras-chave: Niclosamide; Repositioning; colon cancer; amorphous solid dispersion; acoustic levitation; nanoemulsion.
Grande área: Ciências da Saúde
Grande Área: Ciências da Saúde / Área: Farmácia / Subárea: Tecnologia farmacêutica.
Grande Área: Ciências da Saúde / Área: Farmácia / Subárea: Nanotecnologia.
Setores de atividade: Pesquisa e desenvolvimento científico; Fabricação de produtos farmoquímicos e farmacêuticos; Atividades de atenção à saúde humana.

2014 - 2017 Mestrado em Fármacos e Medicamentos (Conceito CAPES 4).
Universidade de São Paulo, USP, Brasil.
Título: Aplicação da goma gelana como aglutinante em formulações de pellets contendo teofilina,Ano de Obtenção: 2017.
Orientador: Humberto Gomes Ferraz.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.
Palavras-chave: Goma gelana; pellets; teofilina; aglutinante; PVP; extrusão/esferonização.
Grande área: Ciências da Saúde
Grande Área: Ciências da Saúde / Área: Farmácia / Subárea: Tecnologia farmacêutica.
Grande Área: Ciências da Saúde / Área: Farmácia / Subárea: Polímeros.
Setores de atividade: Pesquisa e desenvolvimento científico.

2007 - 2013 Graduação em Farmácia e Bioquímica.
Universidade de São Paulo, USP, Brasil.
Título: Causas e consequências da obesidade na infância e adolescência: efeitos na farmacocinética.
Orientador: Sandra Helena Poliselli Farsky.

Formação Complementar

2021 - 2022 Inovação para Cientistas. (Carga horária: 25h).
Emerge Brasil, EMERGE, Brasil.

2020 - 2020 Publons Academy Practical Peer Review Course. (Carga horária: 15h).
Publons Academy, PUBLONS, Grã-Bretanha.

2019 - 2019 Writing in the Sciences. (Carga horária: 30h).
Stanford University, STANFORD, Estados Unidos.

2018 - 2018 Fundamentos de Estatística Aplicada à Avaliação de Processos Farmacêuticos. (Carga horária: 16h).
Fundação Instituto de Pesquisas Farmacêuticas, FIPFARMA, Brasil.

2014 - 2014 Aspectos Práticos da Implementação do QbD na Indústria Farmacêutica. (Carga horária: 16h).
Sindicato da Indústria de Produtos Farmacêuticos no Estado de São Paulo, SINDUSFARMA, Brasil.

2013 - 2013 Sirius T3: teoria e aplicação da medição do pKa, logP e da solubilidade. (Carga horária: 40h).
Sirius Analytical, SA, Inglaterra.

2013 - 2013 Dissolução de Formas Farmacêuticas Sólidas: Interpretando a RDC 31. (Carga horária: 16h).
Fundação Instituto de Pesquisas Farmacêuticas, FIPFARMA, Brasil.

2012 - 2012 Curso de Formação de Auditores. (Carga horária: 16h).
União Farmacêutica de São paulo - Unifar, UNIFAR, Brasil.

Atuação Profissional

Universidade de São Paulo, USP, Brasil.

Vínculo institucional
2018 - Atual Vínculo: Bolsista, Enquadramento Funcional: Aluno de doutorado, Carga horária: 40, Regime: Dedicação exclusiva.
Vínculo institucional
2020 - 2020 Vínculo: Estágio, Enquadramento Funcional: Aluno de doutorado, Carga horária: 6, Regime: Dedicação exclusiva.
Outras informações Estágio no Programa de Aperfeiçoamento de Ensino - USP, disciplina FBF0434 - Tecnologia Farmacêutica, do Departamento de Farmácia, da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo.

Vínculo institucional
2014 - 2017 Vínculo: Bolsista, Enquadramento Funcional: Aluno de mestrado, Carga horária: 40, Regime: Dedicação exclusiva.
Vínculo institucional
2015 - 2015 Vínculo: Estágio, Enquadramento Funcional: Aluno de mestrado, Carga horária: 6, Regime: Dedicação exclusiva.
Outras informações Estágio no Programa de Aperfeiçoamento de Ensino - USP, disciplina FBF0342 - Desenvolvimento Farmacotécnico, do Departamento de Farmácia, da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo.

Vínculo institucional
2013 - 2014 Vínculo: Bolsista, Enquadramento Funcional: Prática profissionalizante, Carga horária: 30, Regime: Dedicação exclusiva.
Atividades
02/2018 - Atual Pesquisa e desenvolvimento, Faculdade de Ciências Farmacêuticas.
Linhas de pesquisa
Farmacotécnica e tecnologia farmacêutica
02/2018 - Atual Pesquisa e desenvolvimento, Faculdade de Ciências Farmacêuticas.
Linhas de pesquisa
Nanotecnologia

Drogaria Pague Menos, PM, Brasil.

Vínculo institucional
2013 - 2013 Vínculo: Estágio, Enquadramento Funcional: Estagiário, Carga horária: 30

Fundação para o Remédio Popular, FURP, Brasil.

Vínculo institucional
2011 - 2012 Vínculo: Bolsista, Enquadramento Funcional: Estagiário, Carga horária: 30
Outras informações Atribuições em setor de Desenvolvimento Farmacotécnico: manipulações de lotes-piloto de formas farmacêuticas sólidas orais e acompanhamento de etapas da produção. Elaboração e acompanhamento de estudos de estabilidade. Elaboração de documentação técnica para setor de Registro. Pesquisa em base de dados e em compêndios oficiais. Execução de testes físicos com produtos e matérias-primas.

Instituto de Assistência Médica ao Servidor Público Estadual, IAMSPE, Brasil.

Vínculo institucional
2007 - 2010 Vínculo: Servidor Público, Enquadramento Funcional: Auxiliar Adminstrativo, Carga horária: 30
Outras informações Atribuições administrativas em laboratório de Análises Clínicas: cadastro de exames no sistema e atendimento ao público quanto a resultados de exames.

Linhas de pesquisa

1. Farmacotécnica e tecnologia farmacêutica
2. Nanotecnologia

Projetos de pesquisa

2018 - Atual Reposicionamento de fármacos para novas indicações terapêuticas.
Descrição: O reposicionamento de fármacos é uma abordagem interessante para se obter novas opções terapêuticas para o tratamento de doenças crônicas/infecciosas. Essa estratégia tem como vantagem o conhecimento prévio de dados sobre eficácia e segurança do fármaco. Isso traz a perspectiva de redução do tempo de avaliação do candidato até sua aprovação. Porém, as propriedades físico-químicas dos fármacos podem oferecer obstáculos para a eficácia terapêutica. Isso é especialmente relevante para compostos com baixa solubilidade, de acordo com o Sistema de Classificação Biofarmacêutica. Estratégias farmacêuticas oferecem soluções para esse desafio. Dispersões sólidas amorfas mantém o fármaco em seu estado amorfo. Isso permite um aumento da solubilidade aparente do composto e, consequentemente, um aumento da biodisponibilidade. Sistemas nanoestruturados permitem uma maior eficácia devido à redução do tamanho de partícula do fármaco à escala nano. Além disso, a possibilidade de incorporação do compostos em sistemas matriciais lipídicos pode melhorar sua absorção oral por meio de fenômenos como a supersaturação. Entre os benefícios dessas estratégias estão a maior absorção, redução de dose e da toxicidade de fármacos. O objetivo da pesquisa é desenvolver formulações contendo fármacos candidatos a reposicionamento na terapêutica.
Situação: Em andamento; Natureza: Pesquisa.
Alunos envolvidos: Doutorado: (1) .

Integrantes: Eduardo José Barbosa - Integrante / Nádia Araci Bou Chacra - Coordenador.
2014 - 2017 Avaliação da goma gelana como aglutinante em formulações de pellets contendo teofilina.
Descrição: A goma gelana é um polissacarídeo usado pela indústria alimentícia em produtos como geleias, bolos e iogurtes. Esse composto possui a capacidade de atuar como geleificante e espessante nesses produtos, além de possuir características físico-químicas interessantes como estabilidade química e enzimática. Trabalhos na literatura relatam o uso desse composto em formulações farmacêuticas, atuando como aglutinante em comprimidos, ou como sistema matricial de liberação controlada de fármacos. Contudo, poucos trabalhos adotam uma abordagem aplicada, visando a produção industrial do produto. Para isso, uma possibilidade é desenvolver formulações de pellets pela técnica de extrusão/esferonização, e posterior secagem por leito fluidizado. Essa técnica é utilizada pelas indústrias farmacêuticas, o que permite obter produtos mais realistas visando o mercado. Portanto, o objetivo deste trabalho é desenvolver formulações de pellets contendo teofilina, utilizando o polissacarídeo gelana como aglutinante. Serão utilizados os diluentes celulose microcristalina, starch 1500 e maltodextrina para produção dos pellets. Inicialmente, a goma gelana será avaliada em comparação com o PVP. Por meio de delineamento experimental, serão analisadas as variáveis aglutinante e diluente. Os pellets serão caracterizados quanto aos aspectos físicos (friabilidade, esfericidade e granulometria) e quanto à dissolução da teofilina. Espera-se poder avaliar a influência da gelana nas características físico-químicas dos pellets comparando com o PVP, que é um agente aglutinante já utilizado pela indústria farmacêutica.
Situação: Concluído; Natureza: Pesquisa.
Alunos envolvidos: Mestrado acadêmico: (1) .

Integrantes: Eduardo José Barbosa - Integrante / Humberto Gomes Ferraz - Coordenador.
2013 - 2014 Measurement of aqueous solubility of thiabendazole: correlation between shake-flask and cheqsol method.

Descrição: De acordo com o Sistema de Classificação Biofarmacêutica, a solubilidade e a permeabilidade são fatores críticos relacionados à absorção oral de fármacos. O uso de métodos inovadores para determinação da solubilidade pode trazer benefícios à indústria farmacêutica e à comunidade acadêmica. Por meio do método potenciométrico Cheqsol pode-se determinar a solubilidade, o pKa e o logP de compostos. Entre as vantagens estão a automação da análise e maior obtenção de informações da molécula, como a estabilidade físico-química e a lipofilicidade. Além disso, essa técnica permite um menor tempo de análise e economia de materiais. Isso é especialmente interessante para moléculas novas e candidatos a fármaco, quando a quantidade de amostra é um fator limitante para a caracterização do composto. Contudo, metodologias inovadoras também requerem um maior entendimento de suas variáveis e de seus resultados. O objetivo desse projeto é medir a solubilidade do antiparasitário de classe II tiabendazol, determinada por método potenciométrico Cheqsol e por método convencional por saturação (shake-flask).
Situação: Concluído; Natureza: Pesquisa.
Alunos envolvidos: Graduação: (1) / Mestrado acadêmico: (1) .

Integrantes: Eduardo José Barbosa - Integrante / Humberto Gomes Ferraz - Coordenador / Leandro Giorgetti - Integrante / Rebeca Ruiz - Integrante.

Revisor de periódico

2020 - Atual Periódico: BIOMEDICINE & PHARMACOTHERAPY
2021 - Atual Periódico: ACS Infectious Diseases

Áreas de atuação

1. Grande área: Ciências da Saúde / Área: Farmácia / Subárea: Farmacotécnica e tecnologia farmacêutica.
2. Grande área: Ciências da Saúde / Área: Farmácia / Subárea: Nanotecnologia.

Idiomas

Inglês Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Espanhol Compreende Bem, Fala Razoavelmente, Lê Bem, Escreve Razoavelmente.
Alemão Compreende Pouco, Fala Pouco, Lê Pouco, Escreve Pouco.

Produções

Produção bibliográfica

Citações

Web of Science

Total de trabalhos:14 Total de citações:73 Fator H:4

Barbosa, E.J. Data: 12/05/2022

SCOPUS

Total de trabalhos:12 Total de citações:78

Barbosa, Eduardo José Data: 12/05/2022

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. MONTEIRO, LIS MARIE ; LÖBENBERG, RAIMAR ; **BARBOSA, EDUARDO JOSÉ** ; DE ARAUJO, GABRIEL LIMA BARROS ; SATO, PAULA KEIKO ; KANASHIRO, EDITE ; DE ARAUJO ELIODORO, RAISSA H. ; ROCHA, MUSSYA ; DE FREITAS, VERA LÚCIA TEIXEIRA ; FOTAKI, NIKOLETTA ; BOU-CHACRA, NÁDIA ARACI . Oral administration of buparvaquone nanostructured lipid carrier enables in vivo activity against Leishmania infantum. EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES JCR, v. 169, p. 106097-106097, 2022.

2. M. C. DE ASSIS, JOÃO ; **BARBOSA, EDUARDO J.** ; BEZZON, VINÍCIUS D.N. ; LOURENÇO, FELIPE R. ; CARVALHO, FLAVIO M.S. ; MATOS, J.R. ; ARACI BOU-CHACRA, NADIA ; BENMORE, CHRIS J. ; BYRN, STEPHEN R. ; COSTA, FANNY N. ; L. B. DE ARAUJO, GABRIEL . Hot-melt extrudability of amorphous solid dispersions of flubendazole-copovidone: An exploratory study of the effect of drug loading and the balance of adjuvants on extrudability and dissolution. INTERNATIONAL JOURNAL OF PHARMACEUTICS JCR, v. 614, p. 121456-1, 2022.

3. DA ROCHA, NATALY PAREDES ; **BARBOSA, EDUARDO JOSÉ** ; BARROS DE ARAUJO, GABRIEL LIMA ; BOU-CHACRA, NÁDIA ARACI . Innovative drug delivery systems for leprosy treatment. Indian Journal of Dermatology Venereology & Leprology JCR, v. 1, p. 1-6, 2022.

4. ANALI BAZÁN HENOSTROZA, MIRLA ; DINIZ TAVARES, GUILHERME ; NISHITANI YUKUYAMA, MEGUMI ; DE SOUZA, ALINE ; **JOSÉ BARBOSA, EDUARDO** ; CARLOS AVINO, VALDIR ; DOS SANTOS NETO, EDSON ; REBELLO LOURENÇO, FELIPE ; LÖBENBERG, RAIMAR ; ARACI BOU-CHACRA, NÁDIA . Antibiotic-loaded lipid-based nanocarrier: a promising strategy to overcome bacterial infection. INTERNATIONAL JOURNAL OF PHARMACEUTICS JCR, v. 621, p. 121782-121782, 2022.

5. ☆ MACEDO, LUIZA DE O. ; **BARBOSA, EDUARDO J.** ; LÖBENBERG, RAIMAR ; BOU-CHACRA, NADIA A. . Anti-inflammatory drug nanocrystals: state of art and regulatory perspective. EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES JCR, v. 158, p. 105654-105654, 2021.

Citações: WEB OF SCIENCE 10

6. ROCHA, EMMILY DANTAS ; FERREIRA, MARCIA REGINA SPURI ; DOS SANTOS NETO, EDSON ; **BARBOSA, EDUARDO JOSÉ** ; LÖBENBERG, RAIMAR ; LOURENÇO, FELIPE REBELLO ; BOU-CHACRA, NADIA . Enhanced In Vitro Antimicrobial Activity of Polymyxin B-Coated Nanostructured Lipid Carrier Containing Dexamethasone Acetate. Journal of Pharmaceutical Innovation JCR, v. 16, p. 125-135, 2021.

Citações: WEB OF SCIENCE 5

7. ☆ **BARBOSA, EDUARDO J.**; ANDRADE, MARCO A.B. ; GUBITOSO, MARIANA R. ; BEZZON, VINÍCIUS D.N. ; SMITH, PAMELA A. ; BYRN, STEPHEN R. ; BOU-CHACRA, NÁDIA A. ; CARVALHO, FLAVIO M.S. ; DE ARAUJO, GABRIEL L.B. . Acoustic levitation and high-resolution synchrotron X-ray powder diffraction: A fast screening approach of niclosamide amorphous solid dispersions. INTERNATIONAL JOURNAL OF PHARMACEUTICS JCR, v. 602, p. 120611-9, 2021.

8. FAGIONATO MASIERO, JÉSSICA ; **BARBOSA, EDUARDO JOSÉ** ; DE OLIVEIRA MACEDO, LUIZA ; DE SOUZA, ALINE ; NISHITANI YUKUYAMA, MEGUMI ; ARANTES, GERALDO JOSÉ ; BOU-CHACRA, NÁDIA ARACI . Vegetable oils in pharmaceutical and cosmetic lipid-based nanocarriers preparations. INDUSTRIAL CROPS AND PRODUCTS JCR, v. 170, p. 113838, 2021.

Citações: WEB OF SCIENCE 4

9.

DE SOUZA, ALINE ; YUKUAMA, MEGUMI NISHITANI ; **BARBOSA, EDUARDO JOSÉ** ; MONTEIRO, LIS MARIE ; FALOPPA, ANA CRISTINA BREITHAUPT ; CALIXTO, LEANDRO AUGUSTO ; DE BARROS ARAÚJO, GABRIEL LIMA ; FOTAKI, NIKOLETTA ; LÖBENBERG, RAIMAR ; BOU-CHACRA, NÁDIA ARACI . A new medium-throughput screening design approach for the development of hydroxymethylnitrofurazone (NFOH) nanostructured lipid carrier for treating leishmaniasis. COLLOIDS AND SURFACES B-BIOINTERFACES JCR, v. 193, p. 111097, 2020.

Citações: WEB OF SCIENCE 2

10. NISHITANI YUKUYAMA, MEGUMI ; DE ARAUJO, GABRIEL LIMA BARROS ; DE SOUZA, ALINE ; LÖBENBERG, RAIMAR ; **JOSÉ BARBOSA, EDUARDO** ; ANALI BAZÁN HENOSTROZA, MIRLA ; PAREDES ROCHA, NATALY ; DE OLIVEIRA, ISABELA FERNANDES ; RABELO FOLCHINI, BEATRIZ ; MIDORI PERONI, CAMILLA ; FAGIONATO MASIERO, JESSICA ; ARACI BOU-CHACRA, NÁDIA . Cancer treatment in the lymphatic system: a prospective targeting employing nanostructured systems. INTERNATIONAL JOURNAL OF PHARMACEUTICS JCR, v. 587, p. 119697-119697, 2020.

Citações: WEB OF SCIENCE 3

11. FERNANDES DE OLIVEIRA, ISABELA ; **JOSÉ BARBOSA, EDUARDO** ; CHRISTINA CAMASMIE PETERS, MARIA ; ANALI BAZÁN HENOSTROZA, MIRLA ; NISHITANI YUKUYAMA, MEGUMI ; DOS SANTOS NETO, EDSON ; LÖBENBERG, RAIMAR ; BOU-CHACRA, NÁDIA . Cutting-edge advances in therapy for the posterior segment of the eye: solid lipid nanoparticles and nanostructured lipid carriers. INTERNATIONAL JOURNAL OF PHARMACEUTICS JCR, v. 589, p. 119831-119831, 2020.

Citações: WEB OF SCIENCE 14

12. **BARBOSA, EDUARDO JOSÉ**; FERRAZ, HUMBERTO GOMES . Gellan gum and polyvinylpyrrolidone (PVP) as binding agents in extrusion/spheronization pellet formulations. Acta Pharmaceutica JCR, v. 69, p. 99-109, 2019.

Citações: WEB OF SCIENCE 10

13. **BARBOSA, EDUARDO JOSÉ**; LÖBENBERG, RAIMAR ; DE ARAUJO, GABRIEL LIMA BARROS ; BOU-CHACRA, NÁDIA ARACI . Nidosamide repositioning for treating cancer: Challenges and nano-based drug delivery opportunities. EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS JCR, v. 141, p. 58-69, 2019.

Citações: WEB OF SCIENCE 27

14. DUQUE, M. D. ; ISSA, M. G. ; SILVA, D. ; **BARBOSA, E. J.** ; LOEBENBERG, R. ; FERRAZ, H. G. . In Silico Simulation of Dissolution Profiles for Development of Extended-Release Doxazosin Tablets. DISSOLUTION TECHNOLOGIES JCR, v. 25, p. 14-21, 2018.

Citações: WEB OF SCIENCE 4

Resumos publicados em anais de congressos

1. SPRENGEL, S. A. ; **BARBOSA, E. J.** ; BOU-CHACRA, N. A. . Nanoemulsão contendo niclosamida: preparação, caracterização físico-química e avaliação da atividade anticâncer in vitro. In: Simpósio Internacional de Iniciação Científica e Tecnológica da USP/SIICUSP (29. 2021 São Paulo), 2021, São Paulo. Simpósio Internacional de Iniciação Científica e Tecnológica da USP/SIICUSP. São Paulo: Pró-Reitoria de Pesquisa/USP, 2021.
2. COMPRI, J. C. Z. ; FELLI, V. M. A. ; **BARBOSA, E. J.** ; FERNANDES, F. P. ; FERRAZ, H. G. ; LOBENBERG, R. ; CHACRA, N. A. B. . Physical chemical caracterization of orotic acid: the first challenge to enable its use as an active pharmaceutical ingredient. In: L Supfab - Semana Farmacêutica de Ciência e Tecnologia da Faculdade de Ciências Farmacêuticas da USP, 2015, São Paulo. Brazilian Journal of Pharmaceutical Sciences, 2015.
3. COMPRI, J. C. Z. ; FELLI, V. M. A. ; FERRAZ, H. G. ; **BARBOSA, E. J.** ; FERNANDES, F. P. ; TAKATSUKA, T. ; Uramatsu, S ; LOBENBERG, R. ; CHACRA, N. A. B. . Orotic acid nanocrystals stabilized with Povacoat®: toward an innovative treatment of malaria. In: AAPS, 2015, Orlando. AAPS, 2015.
4. **BARBOSA, E. J.**; SOUZA, N. V. ; GIORGETTI, L. ; DUQUE, M. D. ; ISSA, M. G. ; FERRAZ, H. G. . Evaluation of the granulation process using flow and particle size analysis parameters. In: 7th International Granulation Workshop, 2015, Sheffield. 7th International Granulation Workshop, 2015.
5. **BARBOSA, E. J.**; GIORGETTI, L. ; RUIZ, R. ; FERRAZ, H. G. . Measurement of aqueous solubility of thiabendazole: correlation between shake-flask and cheqsol method. In: XIX Semana Farmacêutica de Ciência e Tecnologia, 2014, São Paulo. Brazilian Journal of Pharmaceutical Sciences, 2014. v. 50.
6. FERRAZ, H. G. ; **BARBOSA, E. J.** ; QUEIROS, A. R. ; ISSA, M. G. . Obtenção e caracterização de sistemas multiparticulados para a associação dos fármacos olmesartana medoxomila e hidroclorotiazida. In: Simpósio Internacional de Iniciação Científica da Universidade de São Paulo - SIICUSP, 2013, Ribeirão Preto. Simpósio Internacional de Iniciação Científica da Universidade de São Paulo - SIICUSP, 2013.

Apresentações de Trabalho

1. **BARBOSA, E. J.**. A Universidade de São Paulo. 2016. (Apresentação de Trabalho/Outra).

Eventos

Participação em eventos, congressos, exposições e feiras

1. Princípios básicos de liofilização. 2019. (Seminário).
2. Moleculary Imprinted Polymers as tools for the detection of antimicrobial resistance. 2018. (Seminário).
3. XIX Semana Farmacêutica de Ciência e Tecnologia.Determinação da solubilidade aquosa do tiabendazol: correlação entre método shake-flask e cheqsol. 2014. (Outra).
4. Simpósio Internacional de Iniciação Científica da Universidade de São Paulo - SIICUSP.Obtenção e caracterização de sistemas multiparticulados para a associação dos fármacos olmesartana medoxomila e hidroclorotiazida. 2013. (Simpósio).

Organização de eventos, congressos, exposições e feiras

1. HUBNER, A. A. ; SOUZA, A. D. F. ; **BARBOSA, E. J.** ; CUNHA, M. R. ; GONZAGA, R. V. . II Escola de Inverno: Pesquisa e Inovação em Fármacos e Medicamentos (EPIFAR). 2016. (Outro).