Copper complex nanoformulations featuring highly promising therapeutic potential in murine melanoma models

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Abstract

Aim: Pre-clinical evaluation of a cytotoxic copper(II) complex formulated in long circulating nanoliposomes for melanoma treatment.

Materials & Methods: Liposomal nanoformulations of the copper-complex were characterized in terms of thermodynamic behavior (differential scanning calorimeter), pH-sensitivity (spectrophotometry) and antiproliferative effects against murine melanoma B16F10 cells *in vitro*. Preclinical studies were performed in a C57BL/6 syngeneic melanoma model.

Results: Nanoformulations were thermodynamically stable, CHEMS-containing nanoliposomes were pH-sensitive and preserved the antiproliferative properties of the copper compound. These nanoformulations significantly impaired tumour progression *in vivo*, devoid of toxic side effects, compared to control mice or mice treated with the free metallodrug.

Conclusions: Copper-complex-containing nanoliposomes demonstrate high anticancer efficacy and safety, constituting a step forward to the development of more effective therapeutic strategies against melanoma.

Keywords

Melanoma; Copper 1,10-phenanthroline complex; Nanoliposomes; pH-triggered delivery; Syngeneic murine model.

Introduction

Melanoma derives from the malignant transformation of melanocytes. When detected and treated at an early stage, most melanoma cases are curable. However, once it progresses to the metastatic state, treatments often fail, resulting in 80% of deaths related to skin cancers. Moreover, an increasing incidence, combined with the limited effectiveness of current chemotherapeutic approaches, pose many challenges for the successful treatment of metastatic melanoma [1]. In this context, to expand the current repertoire of cancer treatments and to circumvent limitations associated with resistances to current therapies, the identification of new drugs with high potency and novel mechanisms of action is of outmost importance.

The widespread success of the Pt(II) complex cisplatin in the clinic for the treatment of various tumours has placed coordination chemistry of metal-based drugs in the frontline for fighting cancer [2]. In this field, copper-based complexes (mostly Cu^{2+}; Figure 1) have been investigated as new generation metallodrugs on the assumption that endogenous metals may be less toxic for normal cells with respect to cancer cells [3,4]. For example, the cytotoxic dinuclear Cu^{2+} complex $[Cu(phen)_2]^{+}$ (phen = 1,10-phenanthroline) has been reported to bind at the minor groove of DNA and to mediate single-stranded breaks through oxidation of deoxyribose [5]. Recent advances have seen copper complexes containing a phenanthroline-type ligand and with the general formula $[Cu(N^N)(O^N)]^{+}$ or $[Cu(N^N)(O^O)]^{+}$ – from the *Casiopeína* class – entering clinical trials [6]. For example, the Cu^{2+} complex named Casiopeína IIgly (Cas IIgly, Figure 1) is currently under investigation as a potential new anticancer drug, through apoptosis *via* a mitochondrial pathway, originated from reactive oxygen species (ROS)-mediated dysfunction [7,8]. More recently, Kellett and coworkers reported the complex [Cu(o-phthalate)(phen)] as an intracellular ROS-active cytotoxic agent [9] and on other Cu^{2+} complexes featuring phen ligands and endowed with antiproliferative effects. The latter were able to catalyze intracellular superoxide ($O_2^{\bullet -}$) and singlet oxygen ($^1O_2$) formation with radical species, mediating oxidative damage within nuclear DNA, in the form of double strand breaks, as well as in mitochondria, in terms of membrane depolarization [10].

Interestingly, Cu(II) complexes have also been recently described as able to inhibit the aquaporins [11], membrane water and glycerol channels often abnormally expressed in tumors of different origins [12], suggesting an additional anticancer mechanism of action for these metallodrugs.

Despite the numerous promising reports, the translation of metal-based compounds into clinical trials is frequently hampered by inherent toxic side effects [13]. Furthermore, metallodrugs are generally known to undergo ‘speciation’ in the biological environment, defined as the formation of different compound’s species from the original one upon its reaction with biomolecules, buffer components and the redox environment [14]. Such reactivity may lead to the de-activation of the pharmacologically active species and, therefore, should be limited and/or controlled. To overcome

these drawbacks and to increase the therapeutic benefit of metal-based compounds, new strategies come into play, namely the use of nanotechnological approaches for targeted delivery.

Nano lipidic systems show great potential as carriers for antitumour drugs following adsorption onto their surface or incorporation within their core. These nanocarriers are able to improve the stability of the associated compounds [15], and the inclusion of ligands at their surface may be accomplished, promoting a specific targeting towards the site of action, thus minimizing toxic side effects and, ultimately, increasing the therapeutic effect [16]. In this regard, nanoliposomes emerge as invaluable tools in drug delivery, being the most extensively studied and successful lipid-based nanosystem. A repertoire of advantageous features includes biocompatibility, biodegradability of their main constituents, low toxicity, and the ability to incorporate both hydrophilic and hydrophobic compounds [17–19]. Furthermore, several liposomal nanoformulations for the treatment of human diseases are currently in clinical use, and many more in clinical trials [20]. In the case of melanoma, the application of putative nanotechnological therapeutic approaches has been providing encouraging results (reviewed in: [21,22]).

It is well-known that nanoliposomes are able to accumulate in regions of enhanced vascular permeability, as those found in inflammatory [23] and tumour pathologies [17,18,22,24]. This effect has been described as the enhanced permeation and retention (EPR) effect [25,26]. For taking advantage of this effect, long blood circulation times are required, and the drug should remain associated within nanoliposomes [27]. Moreover, the microenvironment of solid tumours, as in the case of melanoma, is slightly acidic, exhibiting pH values around 6, in contrast to pH 7.4 displayed by healthy tissues [28,29]. Combining these two features, a pH-triggered Cuphen release from long circulating nanoliposomes constituted one of the goals of the present work.

In our previous work [30], the Cu^{2+} complex, Cuphen [$Cu(phen)Cl_2$] (Figure 1), was successfully incorporated in long circulating nanoliposomes, demonstrating antiproliferative effects towards different tumour cell lines and absence of *in vivo* toxicity after parenteral administration in healthy mice [30]. In the present study, we designed Cuphen long circulating nanoliposomes and tested their therapeutic effect in a syngeneic murine melanoma model, following an optimization of experimental conditions. Overall, this study provides a robust basis in the area of nanomedicine using metal-based compounds as an improved therapeutic strategy against melanoma.

Material and methods

Chemical products

Cuphen and 1,10-phenantroline were purchased from Sigma and the clinical grade 5-Fluorouracil (5-FU) was kindly supplied by Hospital de Santa Maria, Lisboa, Portugal. The pure phospholipids, phosphatidylcholine (PC), dimyristoyl phosphatidylcholine (DMPC), cholesteryl hemisuccinate (CHEMS) and distearoyl phosphatidylethanolamine covalently linked to poly(ethylene glycol) 2000 (DSPE-PEG), used for the preparation of liposomal nanoformulations, were purchased from Avanti Polar Lipids (AL, USA). Cholesterol (Chol) was acquired from Sigma (Sigma-Aldrich, MO, USA). Deionized water (Milli-Q system; Millipore, Tokyo) was used in all experiments. Nuclepore Track-Etch Membranes were purchased from Whatman Ltd (NY, USA). Culture media and antibiotics were obtained from Invitrogen (Life Technologies Corporation, NY, USA). Reagents for cell proliferation assays were purchased from Promega (WI, USA). All the remaining chemicals used were of analytical grade.

Animals

Male C57Bl/6 mice 8–10 weeks old were purchased from Charles River (Barcelona, Spain). Animals were kept under standard hygiene conditions, fed commercial chow, and given acidified drinking water *ad libitum*.

All animal experiments were conducted according to the animal welfare organ of the Faculty of Pharmacy, University Lisbon, approved by the competent national authority *Direção-Geral de Alimentação e Veterinária* (DGAV) and in accordance with the EU Directive (2010/63/UE) and Portuguese laws (DR 113/2013, 2880/2015 and 260/2016).

Cell line culture conditions

Murine melanoma B16F10 (ATCC#CRL-6475) cell line was maintained in Dulbecco's Modified Eagle's medium (DMEM) with high-glucose (4500 mg/l), supplemented with 10% fetal bovine serum and 100 IU/ml of penicillin and 100 µg/ml streptomycin (Invitrogen) (hereafter designated complete medium). Cells were kept at 37°C, under a 5% CO_2 atmosphere. Maintenance of cultures was performed every 2-3 days, until cells reached a confluence of about 80%.

Nanoliposomes preparation

Nanoliposomes composed of the selected phospholipids were prepared by the dehydration–rehydration method [30,31]. Long circulating nanoliposomes were produced by including DSPE-PEG. Chol or CHEMS were also included in the lipid compositions. For all the nanoformulations, an initial lipid concentration of 20 µmol/ml was used. Briefly, the selected phospholipids were dissolved in chloroform and the lipid solution was evaporated (Buchi R-200 rotary evaporator, Switzerland) to obtain a thin lipid film in a round-bottom flask. The lipid film was then dispersed with a Cuphen aqueous solution (750 µM), and the so-formed suspension was frozen (-70°C) and lyophilized

(Freeze dryer, CO, USA) overnight. The rehydration of the lyophilized powder was performed in PBS buffer, pH 7.4 in two steps, to enhance the Cuphen incorporation [32]. The so-formed liposomal suspension was then filtered, under nitrogen pressure (10–500 lb/in2), through polycarbonate membranes of appropriate pore size until an average vesicle size of 0.1 µm was obtained, using an extruder device (Lipex: Biomembranes Inc., Vancouver, Canada). The separation of non-incorporated Cuphen was performed by ultracentrifugation at 250,000×g, for 120 min, at 15°C in a Beckman LM-80 ultracentrifuge (Beckman Instruments, Inc, CA, USA). Finally, the pellet was suspended in PBS buffer. Unloaded nanoliposomes were also prepared with the same lipid compositions.

Nanoliposomes characterization

Nanoliposomes were characterized in terms of incorporation parameters, mean size, and surface charge. Loading capacity was defined as the final Cuphen to lipid ratio (Cuphen/Lip)f and the incorporation efficiency (I.E.) in percentage was determined according to the following formula:

$$\text{I.E. (\%)} = \frac{\left(\frac{\text{Cuphen}}{\text{Lip}}\right)_f}{\left(\frac{\text{Cuphen}}{\text{Lip}}\right)_i} \text{x } 100$$

Cuphen was quantified spectrophotometrically at 270 nm (ε = 33,000 M^{-1} cm^{-1}) after disruption of the nanoliposomes with ethanol [30]. Linearity of calibration curves was ensured from 2.5 to 25 µM (R^2 = 0.9997; Slope: 0.0309 ± 0.0002; Y-intercept [x = 0]: -0.0078 ± 0.0010). Lipid content was determined using an enzyme-linked colorimetric method , Phospholipids Choline oxidase-Peroxidase (Spinreact, Spain).

Differential Scanning Calorimetry (DSC)

DSC measurements were performed in a calorimeter DSC Q200, TA Instruments, USA. Thermal analysis was carried out using heating and cooling rates of 3°C/minute, ranging from 15 to 40°C for DMPC:Chol:DSPE-PEG (**F4**) and DMPC:CHEMS:DSPE-PEG (**F6**). Thermal cycles were repeated on different samples to ensure constancy and reproducibility of data. The reference pan was empty. Data analysis was performed using TA Universal Analysis program. The transition temperature (Tc) of phospholipids was taken at the interception of the baseline with the tangents of the right and left side of the heating curve. The enthalpy of Tc was obtained from the area under the peaks and normalized to phospholipid content [31].

Assessment of pH-sensitive properties

Cuphen nanoformulations, DMPC:Chol:DSPE-PEG (**F4**) and DMPC:CHEMS:DSPE-PEG (**F6**), were incubated in PBS buffer at three different pH values, 4.5, 6 and 7.4, for 90 min, at 37ºC, under stirring. At the end of the incubation period, the separation of released Cuphen was performed by ultracentrifugation at 250,000×g, for 120 min, at 15°C in a Beckman LM-80 ultracentrifuge. Nanoliposomes were suspended in PBS buffer according to the initial volume, and Cuphen and phospholipid contents determined spectrophotometrically as described above. The stability was

defined as the ratio in percentage between Cuphen to lipid ratio after incubation at pH 4.5, 6 or 7.4 and the Cuphen to lipid ratio before incubation at pH 7.4, according to the following formula:

$$\frac{\left(\frac{\text{Cuphen}}{\text{Lip}}\right)\text{f pH (4.5 / 6 / 7.4)}}{\left(\frac{\text{Cuphen}}{\text{Lip}}\right)\text{i pH 7.4}} \text{ x } 100$$

Cytotoxicity evaluation

B16F10 murine melanoma cell viability was evaluated in the absence (control) or presence of increasing concentrations of Cuphen, in free and liposomal forms, by the MTS assay [30].

Cells at a concentration of 7.5×10^3 cells/ml were placed in 96-well plates (200 µl) for 24 h in culture conditions specified above [30]. Afterwards, complete medium was replaced, and cells treated with Cuphen in free or liposomal forms, at concentrations ranging from 0.2 to 12 µM, or no addition (control). Unloaded nanoliposomes constituted another control group, using the same lipid concentrations as in Cuphen nanoformulations. After 72 h, complete medium was removed and replaced with 100 µl of incomplete medium plus 20 µl of MTS. Absorbance was measured at 490 nm in a microplate reader Model 680 (Bio-Rad, CA, USA) after an incubation period of 2 h at 37ºC. The background absorbance (incomplete medium) was subtracted from all the absorbance readings. Cell proliferation analysis was carried out in GraphPad Prism®5 (GraphPad Software, CA, USA). Values were plotted and fit to a standard inhibition log dose-response curve to generate the IC_{50} values. A total of three independent experiments, with six replicates *per* condition, were carried out.

Tumour syngeneic mouse model

For syngeneic tumour induction, a total of $5x10^5$ or 1×10^5 B16F10 murine melanoma cells were suspended in PBS (100 µl) and injected subcutaneously (s.c.) in the right flank of C57Bl/6 male mice [33,34]. Tumours became palpable around 8 days after s.c. injection of B16F10 cells and treatment schedule was initiated. Mice were randomly divided in groups of five and received the formulations under study by intravenous (i.v.) route, three times a week, for two weeks. Negative control group received PBS (Control); positive control group received 5-FU at 40 mg/kg of body weight (5-FU); and Cuphen in the free form (Free Cuphen) or incorporated in long circulating nanoliposomes (**F1**, **F4** and **F6**) was administered at 2.5 mg/kg body weight. Mice were monitored every day for pain or distress and body weight was registered two times a week. Tumour size was regularly measured using a digital caliper and respective volumes were calculated according to the formula: $V\ (mm^3) = (L \times W^2)/2$, where L and W represent the longest and shortest axis of the tumour, respectively. Relative tumour volumes (RTV) were determined for each animal, as the ratio between volumes at the indicated day and volumes at the beginning of treatment. Three days after the final treatment, mice were euthanized, blood was collected, and primary tumours were excised, weighed and a portion was

stored at -80°C for further analysis. Spleen, liver, kidneys and lungs were excised and weighed. Tissue index was calculated according to the formula:

$$\text{Tissue index} = \sqrt{\frac{\text{organ weight}}{\text{animal weight}}} \times 100$$

Hepatic biochemical parameters

Serum was isolated from the blood, and serum aspartate transaminase (AST) and serum alanine transaminase (ALT) were measured using a commercially available Kit (Spinreact, Spain).

Caspase 3/7 activity in melanoma

Caspase 3/7 activity was measured using the Caspase-Glo 3/7® Assay (Promega Corp., Madison, WI, USA), according to the method previously described [35]. Briefly, 15 µg of total protein extracts was incubated with the reagent 30 min at room temperature and protected from the light. Luminescence was measured for 2 h, with 30 min of interval, using the GloMax®-Multi Detection System (Promega Corp.).

Statistical analysis

Results are expressed as mean ± standard deviation (SD) or standard error mean (SEM). Statistical analysis was performed using One-way ANOVA followed by Tukey Post-Hoc test and independent samples *t*-test using GraphPad Prism®5 for Windows (GraphPad Software, CA, USA). $p<0.05$ was considered statistically significant.

Results

Physicochemical characterization of Cuphen nanoliposomes

Cuphen (Figure 1) was the selected metal complex for the current study, due to its antiproliferative effect against several cancer cell lines, including melanoma [30]. Long circulating nanoliposomes encapsulating Cuphen were prepared using the dehydration-rehydration method, followed by an extrusion step to reduce the mean size of the so-formed nanoformulations [30,31]. Owing to its hydrophilic properties, Cuphen was added after formation of the lipid film. The so-formed liposomal suspension was lyophilized and, afterwards, rehydration was performed in two steps with PBS buffer to increase Cuphen incorporation [32], following a well-established methodology that has been described since 1984 (Kirby and Gregoriadis, 1984). For the preparation of Cuphen nanoliposomes, phospholipids with different transition temperatures (Tc) were used, aiming to evaluate the influence of Tc on Cuphen incorporation parameters. The three main phospholipids tested were PC, DMPC and HPC, presenting Tc values of -6ºC, +23ºC and +41ºC, respectively. All Cuphen nanoliposomes contained DSPE-PEG at a 5% molar ratio in the lipid composition, as our purpose was to develop formulations with long circulating properties. In fact, although some other polymers have been successfully tested as alternatives to PEG, this polymer still remains the golden standard for preparing long circulating liposomes [36-38]. Several studies have been performed to evaluate the influence of PEG molecular weight, the phospholipid used to be linked to the polymer and the molar ratio used in the lipid composition on the morphology of lipid structure formed. When liposomes were prepared with PEG at 8 and 12 % molar ratio, discs and mixed micelles were observed, respectively. Moreover, we have already assessed the in vivo profile of PEG liposomes using other compounds [23, 31].

In the first series of Cuphen nanoformulations (**F1**, **F2**, **F3** and **F4**), Chol was included in the lipid composition. A second series of pH-sensitive nanoformulations (**F5**, **F6** and **F7**) was developed to explore Cuphen release at the slightly acidic tumour microenvironment [28,29]. To attain this goal, CHEMS was included in the lipid composition since, when in contact with an acidic milieu, it becomes protonated, leading to bilayer disruption and subsequent drug release [29,39–41].
In Table 1, the influence of the lipid composition on Cuphen incorporation parameters, namely final Cuphen to lipid ratio (loading capacity) and Incorporation Efficiency (I.E.), as well as mean size and zeta potential values are depicted.

Lipid composition influences nanoliposomes permeability and, consequently, the stability of the incorporated drug. Bilayers constituted by phospholipids with a moderate Tc, such as DMPC (Tc = +23ºC), are less prone to drug leakage than those containing PC (Tc = -6ºC). In PC-based nanoliposomes, Chol is frequently included to enhance the incorporation parameters of low molecular weight hydrophilic compounds [42,43].

Cuphen nanoformulation **F1** displayed an I.E. of 42% and a loading capacity of 26 nmol/µmol of lipid. Keeping the same molar ratio, the use of a more rigid phospholipid, DMPC, markedly reduced the incorporation parameters of Cuphen, with an I.E. of 9% and loading capacity of 3 nmol of Cuphen *per* µmol of lipid (**F2**). The reduction of Chol molar ratio in the lipid composition from 33 mol% **(F2**) to 16 and 13 mol % resulted in increased incorporation parameters for **F3** and **F4**, respectively. Moreover, no statistically significant differences, in terms of loading capacity, were observed between **F4** (DMPC) and **F1** (PC), 19±4 and 26±8 nmol of Cuphen/µmol lipid, respectively. Overall, the use of DMPC as the main phospholipid instead of PC does not require the inclusion of a high molar ratio of Chol to stabilize Cuphen within nanoliposomes.

For the pH-sensitive nanoliposomes, the results show that the increase on the Tc of the main phospholipid also led to a systematic reduction of Cuphen loading on **F5**, **F6** and **F7**: 43, 34 and 28 nmol of Cuphen/µmol lipid, respectively. All liposomal nanoformulations presented a mean size around 0.13 µm and a polydispersity index under 0.15, demonstrating the high homogeneity of all nanoformulations. Regarding surface charge, all Cuphen nanoliposomes presented a zeta potential close to neutrality. Based on the above results, DMPC-based nanoformulations **F4** and **F6** were selected for further studies, as both presented suitable incorporation parameters.

Thermotropic behavior of Cuphen nanoliposomes: drug-lipid interactions

The raw materials for the preparation of nanoliposomes are mainly phospholipids, and the respective Tc is an important parameter that will determine the packing of the phospholipid acyl chain order and, consequently, the fluidity of nanoliposomes. In addition, the thermodynamic behavior of lipid bilayers may be influenced by the incorporated drug leading to changes in the physical stability of the liposomal nanoformulation [44,45].

In the present work, the influence of Cuphen on the thermodynamic behavior of nanoliposomes was assessed by differential scanning calorimetry (DSC). The enthalpy change (ΔH) and the Tc were evaluated for both loaded and unloaded nanoliposomes, and the results are depicted in Table S1. Calorimetric studies were performed for **F4** and **F6** as these were the selected formulations for *in vivo* studies.

In terms of ΔH, no changes were observed between unloaded and loaded nanoliposomes. This effect may be explained by the hydrophilic properties of the copper complex and possibly reduced interaction with the lipid bilayer. The range of values obtained, 4.6 to 6 kJ/mol, was lower when comparing to pure DMPC systems, with a ΔH around 14 kJ/mol [46]. The reduction on the enthalpy observed might be due to the presence of Chol, CHEMS or DSPE-PEG at 13, 38 and 5 mol% in the lipid composition, respectively. Regarding the Tc, for the tested liposomal formulations, the values were quite similar ranging from 21.9 and 23ºC. Further studies to evaluate separately the influence of each component of the lipid composition may clarify the above observations.

Cuphen pH-sensitive nanoliposomes

Tumours' microenvironment, as in the case of melanoma, is slightly acidic, presenting pH values around 6, in contrast to the normal physiological pH of 7.4 [28,29], which heavily impacts cancer development [47,48], and it can be explored as stimuli for triggering local liposomal drug release. In the current work, we developed long circulating pH-sensitive nanoliposomes by including CHEMS in the lipid composition [29,39-41]. To validate pH-sensitive properties of **F6**, the quantification of the metallodrug still associated to nanoliposomes was assessed upon exposure to different pH conditions. In paralel, the stability of **F4** was also evaluated as control. These studies aimed to mimic the pH conditions of the tumor microenvironment and to prove that, once **F6** reaches tumor sites, Cuphen will be released. Both nanoformulations were incubated in PBS buffer at pH 4.5, 6 and 7.4, and the experiments were performed as described in the materials and methods section. Results are displayed in Figure 2.

According to the results, a systematic reduction on Cuphen retention in nanoliposomes was observed for **F6** when incubated in decreasing pH conditions. While at pH 7.4 the Cuphen percentage was approximately 90%, at pH 6 and 4.5 the respective values were 63 and 24%. Conversely, **F4** did not display such behavior, as no statistically significant differences were observed for the three tested pH values, with Cuphen to lipid ratios ranging from 65-70%. These results validate our assumption: only **F6**, featuring CHEMS in the lipid composition, displays pH-sensitive properties, enabling Cuphen release as a function of pH. According to the literature, CHEMS p*Ka* was found to be 5.8 when associated to palmitoyl phosphatidyl choline (POPC) (50:50) [49] and 5.4 in bilayers of DMPC:CHEMS (70:30) [50]. Moreover, the protonation of CHEMS at pH ranging from 4.5 to 6.5 has been described [39]. These data support the rational for using **F6** as a tool for liposomal Cuphen release at the mildly acidic tumour microenvironment. The same effect has been reported for the widely described pH-sensitive lipid composition based on a fusogenic phospholipid, dioleoyl phosphatidyl ethanolamine (DOPE), in combination with CHEMS [39,41].

Cuphen antiproliferative effects against murine melanoma cells

Cell viability of the B16F10 murine melanoma cell line was evaluated in the absence (control) or in the presence of increasing concentrations of Cuphen, in both free and liposomal forms (**F1**, **F4** and **F6**), using the MTS assay. Figure 3 illustrates the dose-response curves and the IC_{50} values for B16F10 cellular viability following incubation with Free Cuphen, **F1**, **F4** and **F6**. All Cuphen formulations presented similar IC_{50} values in the low micromolar range as for Free Cuphen (IC_{50} ca. 3-5 μM). In a previous work we have already evaluated the antiproliferative effect of this compound in the presence of a nontumorigenic human keratinocyte cell line, HaCaT [30]. The obtained results showed that both Cuphen and its liposomal formulations were similarly toxic towards HaCaT cells (IC_{50} in the range 3-5 μM). This result is in accordance with the relevant expression for aquaporin-3 described for this human cell line [51]. In addition, unloaded nanoliposomes with the respective lipid composition were

tested, revealing no cytotoxicity towards B16F10 cells (data not shown). Although no differences concerning the *in vitro* antiproliferative effects were observed for all tested Cuphen formulations, the use of long circulating nanoliposomes for *in vivo* delivery would be advantageous for improving the biodistribution profile and, particularly, the accumulation at tumour sites [18,27, 36-38].

Therapeutic effects of Cuphen against melanoma – influence of lipid composition and *in vivo* safety

PC-based long circulating nanoliposomes

Taking into account the promising cytotoxic effects against several cancer cell lines, and the absence of *in vivo* toxicity revealed by Cuphen nanoformulation **F1** in previous studies [30], we proceeded with the evaluation of its anticancer activity in a syngeneic murine melanoma model, in comparison to Free Cuphen. As positive control, 5-FU, a clinically approved pro-apoptotic drug for the treatment of various types of cancer, including melanoma [52,53], was used. The therapeutic effect was evaluated in terms of tumour volume progression, expressed as relative tumour volume (RTV), and survival rate, as shown in Figure 4. The results show that a similar progression on tumour volume was observed for Cuphen nanoformulation **F1** and 5-FU treatments over 15 days (Figure 4A), while Free Cuphen was markedly less effective. It should be mentioned that, due to the aggressiveness and exponential growth of melanoma mass tumours observed in this model, mice bearing tumours with an elevated volume had to be sacrificed before the end of the experimental protocol (Figure 4B). In this regard, mice treated with either Free Cuphen or 5-FU presented survival rates superior to 50%. Most importantly, mice receiving i.v. injections of Cuphen nanoliposomes were the only group with 100% of survival, as shown in Figure 4B. In addition, the ligand 1,10-phenanthroline (phen) was also administered in its free form as a control in the same syngeneic murine melanoma model. The results demonstrated that the therapeutic action of Cuphen was due to the presence of the Cu^{2+} complex (Figure S1, Supplementary data).

DMPC-based long circulating nanoliposomes

Aiming to eliminate the high morbidity observed in the previous model for the control group and to minimize animals' suffering, a lower number of B16F10 cells ($1x10^5$) was used for melanoma tumour induction, that allowed to extend the experimental protocol. In the previous animal model (Figure 4), no relevant therapeutic benefit was achieved for mice treated with **F1**, when comparing to the positive control 5-FU. Hence, to further investigate the therapeutic activity of Cuphen nanoformulations, the two selected DMPC-based lipid compositions, **F4** and **F6**, were tested in comparison to Free Cuphen. Thus, one week after tumour induction, mice received i.v. injections of the formulations under study, at a dose of 2.5 mg/kg of body weight, three times a week, for two weeks. The therapeutic effect was evaluated in terms of tumour volume (Figure 5A), RTV (Figure 5B), tumour weight (Figure 5C), and

caspase 3/7 activity (Figure 5D). Animal weight was also assessed during the experimental protocol (Figure 5E).

A major therapeutic effect was observed for **F4** and **F6** Cuphen nanoformulations, which significantly delayed tumour progression when comparing to Control and Free Cuphen mice groups. As depicted in Figure 5A, at the end of treatment schedule, **F4** and **F6** groups presented the smallest tumour volumes, below 1000 mm^3, while 2- to 3-fold higher values were observed for Free Cuphen and Control groups, respectively. The therapeutic effect of Cuphen nanoliposomes was confirmed by RTV analysis (Figure 5B) that evaluates the tumour progression of each animal over time. While mice treated with **F4** and **F6** reached the lowest values (RTV ca. 8), Control and Free Cuphen groups presented RTV values around 24 and 13, respectively. In terms of both tumour volume and RTV, no statistically significant differences were observed between **F4** and **F6** at the end of the treatment, suggesting that, higher doses may be required to select the most effective one. In relation to tumour weight at the end of the treatment protocol (Figure 5C), mice receiving Cuphen nanoliposomes displayed the lowest values among all tested groups. Moreover, we investigated if Cuphen mechanism of action could involve apoptosis by determining the caspase 3/7 activity in protein extracts from tumour samples (Figure 5D). The results suggest that, comparing to Control group, the therapeutic benefit observed for mice receiving Cuphen formulations may not involve cell death by apoptosis, as no statistically significant differences among all tested groups were observed. Finally, body weight of all animals was steady over the course of the experiment and did not differ among the experimental groups. It is also important to highlight the fact that all groups presented a survival rate of 100%, comparing to the previously established model.

To evaluate the safety of the Cuphen formulations at the administered dose (2.5 mg/kg), tissue index and hepatic biomarkers were assessed, as depicted in Table 2. In terms of tissue index, no changes for the analyzed organs were observed among tested groups. Tissue index provides information about organ development, which is constant under normal circumstances. While an increased tissue index indicates organ hypertrophy, congestion or edema, a decreased ratio indicates organ atrophy and degenerative changes [54–57].

In addition to tissue index, and according to the FDA agency guidelines, we also evaluated hepatic aminotransferases – alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [58]. Alterations in these enzymatic biomarkers allow the detection or prediction of potential harmful hepatic effects upon exposure to a drug or derived from metastatic conditions [59,60]. Lower values for AST were observed for mice receiving Cuphen nanoformulations in comparison to control group or mice treated with Free Cuphen. Despite all values were within the reference intervals reported by the mice provider, [61] these differences may demonstrate the safety advantages of the copper complex after incorporation in nanoliposomes. In other melanoma mice model, high values of plasmatic ALT have been reported indicating liver damage. However, the tumour induction was performed by

injecting B16 cells directly into the spleen leading to metastatic melanoma [62]. Data in Table 2 shows no alterations for all treated groups, demonstrating that Cuphen formulations are safe for i.v. administration.

Discussion

Although several metallodrugs display *in vitro* cytotoxic effects towards tumor cells, their *in vivo* therapeutic activity and progression into clinical trials is frequently hampered by low specificity to biological targets or de-activation of the pharmacologically active species mostly due their speciation in physiological conditions. To overcome these drawbacks and increase the therapeutic benefits, the use of a safe and efficient delivery system is of pivotal importance. It is worth mentioning that, recently, in the field of metallodrugs development, strategies for developing nanoformulations of the clinically approved anticancer platinum drugs in form of liposomes and polymers have made it to clinical studies. For example, a liposomal cisplatin formulation (Lipoplatin™) is already evaluated in several phase III studies and has been considered as a "novel cisplatin" [63, 64, 65].

In the present work, liposomes were the selected lipid-based system aiming to preserve the therapeutic activity of the incorporated copper-complex while ensuring a preferential accumulation at tumor sites. When designing copper-complex nanoformulations, several pharmaceutical technological properties must be taken into consideration such as high metallodrug loadings, preservation of cytotoxic activity towards tumor cells and, most importantly, a significant *in vivo* therapeutic efficacy.

Regarding the incorporation efficiency of Cuphen in liposomes, changing the initial Cuphen concentration from 500 to 750 nmol/ml resulted in an almost 2-fold increase in the loading capacity. *In vivo,* these results translated into the administration of a much lower amount of lipid for the same Cuphen therapeutic dose.

In vitro, the antiproliferative effect of Cuphen formulations towards B16F10 melanoma cells is comparable to the one of Free-Cuphen, with IC_{50} in the low micromolar range. *In vivo*, the therapeutic effect of the Cuphen-formulations towards a melanoma model, evaluated in terms of tumour volume, RTV, tumour weight and caspase 3/7 activity, are extremely of note. The choice of the main phospholipid component in the lipid composition was an important factor for selecting the most effective Cuphen nanoformulation *in vivo*. In fact, DMPC-based Cuphen nanoliposomes demonstrated a superior antitumor activity in the murine melanoma model, an achievement that may be correlated with the higher Tc of DMPC in comparison to PC (as in formulation **F1**) and of course, Free Cuphen. After systemic administration, DMPC nanoliposomes are able to stabilize loaded Cuphen and, consequently, increase the amount of the metallodrug that reaches tumor sites. On the other hand, the low Tc for PC might favour a premature release of Cuphen loaded liposomes while in circulation, justifying the modest antitumor effect observed in the present work.

Finally, the differences in therapeutic effect exerted by the pH sensitive formulation **F6** with respect to the non-pH sensitive **F4** were negligible, and more studies are necessary to explore the advantage of using different lipid compositions. Further studies to clarify the mechanism of action should also be

performed, including AQP3 inhibition and AQP3 gene and protein expression in frozen tumor samples, as well as a dose response effect for this metallodrug formulation.

Overall, the successful results obtained from this proof of concept study, devoid of hepatic toxic effects, renders DMPC-based Cuphen nanoliposomes an attractive candidate for melanoma treatment.

Conclusions

In this study, we successfully developed and explored an alternative therapeutic strategy featuring high potential against melanoma. Specifically, we examined the antitumour activity of the Cu(II) complex featuring a 1,10-phenanthroline ligand, Cuphen, in a well-established syngeneic murine melanoma model. Cuphen delivery to melanoma tumours was accomplished using long circulating nanoliposomes. *In vivo* data demonstrated that DMPC-based Cuphen nanoliposomes significantly impaired melanoma progression devoid of toxic side effects for a dose of 2.5 mg/kg of body weight. Higher therapeutic doses may be envisioned in further studies. Future studies should also include the evaluation of the kinetics of metallodrug release from the liposomes.

To the best of our knowledge, despite the numerous reports on the anticancer effects of copper complexes featuring N^N donor ligands, this is the first publication that reports the potential therapeutic effect against melanoma of the copper complex Cuphen following its incorporation in long circulating nanoliposomes. Such promising anticancer effects might provide a strong basis for a successful bench to bedside research, ultimately benefiting melanoma patients.

Future perspective

The increasing incidence, aggressive malignancy and high mortality of metastatic melanoma associated to the lack of effective therapies demands the identification of new therapeutic targets. Certainly, copper compounds as Cuphen are likely to act *via* different mechanisms, including ROS formation and DNA damage. Furthermore, the recent report of the inhibitory effects of Cuphen towards the aquaporins (AQPs) may also shed light into new targets for this family of metallodrugs, associated to tumor cell growth, proliferation and metastasis. Taking into account that AQPs are also expressed in healthy tissues their modulation in pathological situations needs to be highly specific. The use of lipid-based systems, particularly liposomes, owning to their efficiency and ability to incorporate a wide array of compounds, constitutes a key strategy to control copper compounds' speciation and reactivity at tumor site.

In the present work we took advantage of the properties that pegylated liposomes present: long circulation times in bloodstream, extravasation into tumor interstitial space and accumulation at those pathological sites due to impaired lymphatic drainage, through a passive mechanism. In the future, the development of alternative Cuphen nanoformulations featuring specific ligands at their surface will allow a preferential targeting to receptors overexpressed at tumor sites. However, to

successfully achieve such strategy a delicate balance of selected ligands content and surface exposure, minimizing immunologic recognition and clearance should be accomplished.

In the present work proof-of-concept preclinical studies were performed in immunocompetent mice using murine melanoma cells. The future *in vivo* evaluation in a model that mimics the human metastatic progression of melanoma, should be envisioned. Overall the herein described therapeutic might provide a strong basis for translating preclinical findings to clinical trials.

Summary points

- *in vitro* and *in vivo* evaluation of a copper complex with high anticancer potential using a nanotechnological approach to enhance its therapeutic efficacy
- Copper-complex nanoformulations displayed suitable incorporation parameters as well as thermodynamic stability preserving antiproliferative properties towards melanoma cells.
- DMPC-based copper-complex nanoformulations markedly reduced tumour progression in murine melanoma models, in comparison to the metallodrug in the free form, and are devoid of toxic side effects;
- These preclinical studies pave the way to the emergence of innovative and more effective therapies against melanoma.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations.

Financial & conflicts of interest disclosure

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Legends of figures

Figure 1. Chemical structure of the Cu^{2+} complexes: Casiopeína IIgly (Cas IIgly) and Cuphen.

Figure 2. Stability of Cuphen-loaded nanoliposomes: influence of pH conditions. Cuphen nanoformulations (**F4** and **F6**) were incubated in PBS buffer at pH 4.5, 6 or 7.4, for 90 min, at 37ºC, under stirring. The stability was evaluated as the ratio in percentage between Cuphen to lipid ratio after incubation at pH 4.5, 6 or 7.4 and the Cuphen to lipid ratio before incubation, at pH 7.4. Data are

expressed as mean ± SD of two independent experiments with three replicates each. ** $p<0.01$ *vs* pH 6; *** $p<0.001$ *vs* pH 4.5.

Figure 3. Antiproliferative effect of Cuphen towards B16F10 cells. **(A)** Dose-response curves for cell viability of B16F10 in the presence of Cuphen formulations and **(B)** respective IC_{50} values, expressed as mean percentage (%) of control ± SD of three independent experiments with six replicates each. Results correspond to cellular viability after 72 h incubation with different Cuphen concentrations (0.2 to 12 μM), in free and liposomal forms (MTS assay).

Figure 4. Therapeutic evaluation of Cuphen nanoformulation **F1**, Free Cuphen and 5-FU in a syngeneic murine melanoma model. **(A)** Relative tumour volume (expressed as mean ± SEM of at least 5 animals *per* group) and **(B)** Kaplan-Meier survival curve. Tumour induction was performed by a s.c. injection of $5x10^5$ B16F10 cells. 10 days after tumour induction, mice received i.v. injections of the formulations under study (Cuphen and 5-FU at 2.5 and 40 mg/kg of body weight, respectively), three times a week, for two weeks. Control group received PBS. RTV was determined for each animal, as the ratio between volumes at the indicated day and volumes at the beginning of treatment.

Figure 5. Therapeutic effect of Cuphen formulations in a syngeneic murine melanoma model. **(A)** Tumour volume evolution, including representative tumour images of each treated group on day 22, **(B)** Relative tumour growth (RTV), **(C)** Tumour weight of each mouse on day 22, **(D)** Caspase 3/7 activity (Fold Change to Control), and **(E)** Animal weight. Tumour induction was performed by a s.c. injection of $1x10^5$ B16F10 cells. One week after tumour induction, mice received i.v. injections of the formulations under study, at a dose of 2.5 mg/kg of body weight, three times a week, for two weeks. Control group received PBS. RTV was determined for each animal, as the ratio between volumes at the indicated day and volumes at the beginning of treatment. Results are expressed as mean ± SEM of at least 5 animals *per* group. * $p<0.05$ Control *vs* **F4**/**F6**; ** $p<0.01$ Control *vs* **F6**; £ $p<0.05$ Control *vs* **F6**; § $p<0.05$ Control *vs* Free Cuphen/**F4**/**F6**; # $p<0.05$ Control *vs* **F4**/**F6** and Free Cuphen *vs* **F4**.