Paclitaxel-loaded sodium deoxycholate-stabilized zein nanoparticles: characterization and in vitro cytotoxicity

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ABSTRACT

Paclitaxel (PTX) is one of the most successful antineoplastic drugs and is widely used for the treatment of many forms of advanced and refractory cancer. Unfortunately, various drawbacks including non-selective cytotoxicity, poor water solubility and low bioavailability limit its clinical use. The aim of this study was to characterize a novel colloidal system made up of the natural protein zein, that would be able to efficiently retain the anticancer compound and increase its in vitro pharmacological effects. In fact, zein has promising characteristics that render it a potential material to be used in drug delivery application. The influences of temperature, pH and serum incubation on the stability of these particles, entrapment efficiency of PTX and in vitro toxicity on different cancer cell lines were evaluated. The nanosystems containing PTX demonstrated suitable storage stability, and were not destabilized by temperatures of up to 50 °C, pH alterations, the freeze-drying process or serum proteins. The encapsulation of PTX did not destabilize the structure of the zein nanoparticles and a suitable drug entrapment efficiency resulted. PTX-loaded zein nanoparticles showed an increased toxicity on different cancer cell lines with respect to the free drug, confirming its potential application in preclinical and clinical investigations.

1. Introduction

The peculiar characteristics of paclitaxel (PTX), a tricyclic diterpenoid isolated from the Pacific or Western yew tree Taxus brevifolia, make this natural compound one of the most useful and effective chemotherapeutic drugs available, effective against a broad spectrum of tumors (Bernabéu et al., 2017). In particular, this compound plays a crucial role in the treatment of ovarian, cervical and breast cancers, as well as non-small cell lung cancer, Kaposi’s sarcoma and acute leukemia (Madaan et al., 2013). It is a microtubule-stabilizing compound that selectively disrupts microtubule dynamics, binding with high affinity to the β subunit of tubulin, which causes the distortion of the cytoskeletal structure and consequent mitotic arrest in the G2 and M phases of the cell cycle, triggering apoptosis in proliferating cancer cells (Ravar et al., 2016). The combination of both antiproliferative and cytotoxic properties contributes to the antitumor efficacy of PTX. Unfortunately, like many other anticancer drugs, its clinical use is greatly limited by various drawbacks, including poor water solubility, low bioavailability and poor tumor localization. To overcome these problems, PTX was formulated with Cremophor EL and dehydrated ethanol Taxol® for parenteral administration in patients, but the presence of the organic solvent induced severe side effects in 20–40% of them such as allergies, hypersensitivity and anaphylactic reactions, compromising the therapeutic index of the drug (Xu et al., 2015). Therefore, various biocompatible formulations were developed in order to avoid the use of organic solvents and Abraxane (PTX-loaded albumin nanoparticles) was approved in 2005 for human use (Kundranda and Niu, 2015). This formulation mitigated the side effects associated with the use of Cremophor EL and significantly enhanced the antitumor efficacy of PTX (Danhier, 2016). However, despite the biocompatibility of the human albumin protein it contains, this formulation has only moderately increased the bioavailability and safety of PTX because it continues to cause side effects such as hair loss, weakness, decreases in the red blood cell count and joint and muscle pain (Thomas et al., 2015). In addition to Abraxane, paclitaxel is also marketed on two other nanoplatforms for parenteral administration, liposomes (Lipusu®) and polymeric micelles, namely Genexol® PM (Ye et al., 2013), Nanoxel®.
Among these, biodegradable polymeric nanoparticles have aroused a considerable amount of interest due to their biocompatibility, their capacity to retain either hydrophilic or hydrophobic drugs, their significant stability and the possibility of being administered by way of different routes (Wang et al., 2017). Moreover, the colloidal size and ample surface area can promote their interaction with aqueous media, favoring the localization of the entrapped compounds inside the tumor by means of their enhanced permeation and retention (EPR) effects when they are circulating in the blood stream (Dong et al., 2016). In addition, nanoparticles can modulate the function of the drug efflux pumps of the cancer cells, promoting the decrease of multidrug resistance as was true in the case of polyalkylenoacrylates (Duan et al., 2012).

Among the polymeric nanoparticles, protein-based nanosystems have been used to entrap various active compounds with the aim of increasing their pharmacological activity and decreasing their side effects; in particular, plant proteins have been widely investigated because they are considered “environmentally economic” as compared to animal proteins, due to the minimum natural resources required for their preparation (Elzoghby et al., 2017).

Zein, for example, a protein of corn, has been used for the development of nanoparticles because it is characterized by useful physico-chemical properties for drug delivery applications (Oliveira et al., 2018). It received the “GRAS” status thanks to its biodegradability, biocompatibility, low immunogenicity and its continually renewable and inexpensive source (Chang et al., 2017).

Zein is a highly hydrophobic protein with a peculiar bricklike shape able to retain lipophilic compounds such as essential oils (Parris et al., 2005), vitamin D3 (Luo et al., 2012), resveratrol (Huang et al., 2017), or carotenoids (Jiao et al., 2018). The encapsulation of anticancer compounds within zein nanoparticles can promote their accumulation in solid tumors by the enhanced permeability and retention effect related to the capillary fenestrations of leaky tumor vasculature (Elzoghby et al., 2017). Lai and coworkers demonstrated that 5-Fluorouracil-loaded zein nanosystems mostly accumulated in the liver following intravenous injection, increasing the drug localization by 31.33% (Lai and Guo, 2011). Recently, Dong et al. demonstrated that doxorubicin can be successfully encapsulated into zein nanoparticles reaching a loading capacity of 15.01 mg of drug per gram of protein (Dong et al., 2016). Our research team recently proved that it is possible to combine raw zein nanoparticles and various surfactants, demonstrating that sodium deoxycholate (SD) can dramatically stabilize the colloidal structure (Gagliardi et al., 2018). The aim of the present work was to encapsulate PTX within SD decorated-zein nanoparticles, characterizing the nanosystems as a function of storage period, temperature and pH. Moreover, the drug entrapment efficiency and the stability of the formulations after serum incubation and the freeze-drying process were evaluated. Finally, the toxicity of PTX-loaded zein nanoparticles on two cancer cell lines (MCF-7 and K562) was investigated with respect to the free form of the drug.

2. Materials & methods

2.1. Materials

Yellow zein, sodium deoxycholate monohydrate (SD), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (used for MTT tests), phosphate buffered saline (PBS) tablets, dimethyl sulfoxide, and amphotericin B solution (250 μg/ml) were all purchased from Sigma Aldrich (Milan, Italy). Paclitaxel (PTX) was kindly provided by Indena S.p.a. (Milan, Italy). ([H]cholestereryl hexadecylether ([H]CHCE, 40 Ci/mmol) was obtained from Perkin Elmer-Italia (Monza, Italy).

For the in vitro studies, DMEM (Dulbecco’s Modified Eagle’s Medium) and RPMI1640 enriched with Glutamax I, tripisin/EDTA, penicillin/streptomycin solution and fetal bovine serum (FBS) were obtained from Gibco (Life Technology, Milan, Italy). Human breast cancer cells (MCF-7) and chronic myelogenous leukemia (K562) were provided by the IRCCS Azienda Ospedaliera Universitaria San Martino – IST Istituto Nazionale per la Ricerca sul Cancro. All other materials and solvents used in this investigation were of analytical grade (Carlo Erba, Milan, Italy).

2.2. Preparation of zein nanoparticles

The zein nanoparticles were prepared according to the nano-precipitation method of the pre-formed polymer in an aqueous solution as previously described (Gagliardi et al., 2018). Briefly, 10 mg of zein were dissolved in 3 ml of an ethanol/water solution (2:1 v/v) at room temperature and added to 5 ml of aqueous phase made up of MilliQ water containing 1.25% w/v of SD, homogenized using an Ultraturrax® (model T25, IKA® Werke) at 2400 rpm for 1 min and then mechanically stirred at 600 rpm for 12 h on a magnetic plate to favor the evaporation of the organic solvent (Gagliardi et al., 2018).

Zein nanoparticles containing PTX were obtained by adding different amounts of the drug to the organic phase. Successively, the colloidal formulations were centrifuged at 90 k rpm for 1 h using a Beckman Optima™ Ultracentrifuge (Fullerton, Canada) in order to perform the analytical investigations, or suitably purified by means of Amicon® Ultra centrifugal filters (cut-off 10kDa, 4000 rpm for 120 min) before the cytotoxicity experiments in vitro.

2.3. Physico-chemical characterization

A Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom, a dynamic light scattering apparatus), was used to evaluate the mean diameter, size distribution and Z-potential of the formulations. The formulations were centrifuged at 90 k rpm for 1 min and then mechanically stirred at 600 rpm for 12 h on a magnetic plate to favor the evaporation of the organic solvent (Gagliardi et al., 2018).

2.4. Effect of pH, temperature and serum on the stability of nanosystems

The nanoformulations (1 ml samples contained in glass Pyrex test tubes) were incubated in a water bath for 1 h at fixed temperatures ranging from 30 to 50 °C. Successively, the particle sizes, polydispersity indexes and zeta-potentials were evaluated. Moreover, the influence of pH on the modulation of the physico-chemical properties of various zein-based formulations was studied by dispersing the samples (dilution 1:50) in deionized water at different pH values (4.0, 6.0, 8.0, 10.0, by a pHmeter SevenCompact S210 Mettler Toledo), using 1 mol/l NaOH or HCl, for 1 h under slow, continuous stirring at room temperature.

The stability of PTX-loaded zein nanoparticles in serum was investigated following their incubation in 70% of FBS (Cosco et al., 2017). In detail, 200 μl samples of the formulations were added to 1 ml of FBS and incubated at 37 °C for 48 h, while undergoing stirring at 600 rpm. The samples were analyzed at different incubation times (0,5, 1, 2, 3, 4, 6, 24 and 48 h) and their average size was measured as previously reported.

2.5. Entrapment efficiency of PTX

The amount of PTX retained by each type of colloidal structure was evaluated through HPLC analysis using a Thermo Scientific (Rodano, MI, Italy) Dionex Ultimate 3000, equipped with a 25 cm × 4.6 mm Thermo Scientific Hypersil GOLD C18 column packed with 5 μm particles.
Table 1
Composition and physico-chemical properties of zein nanoparticles.

| Sample       | Code | Zein (mg/ml) | Stabilizer (% w/v) | Mean sizes (nm) | Polydispersity Index | Zeta potential (mV) |
|--------------|------|--------------|--------------------|-----------------|----------------------|---------------------|
| Surfactant free | A 0.2 | –            | 450±12             | 0.22±0.15       | -15±1                |
|              | B 0.4 | –            | 317±12             | 0.27±0.01       | -3±1                 |
|              | C 0.6 | –            | 146±1              | 0.24±0.01       | 6±2                  |
|              | D 0.8 | –            | 135±1              | 0.25±0.01       | 9±1                  |
|              | E 1   | –            | 126±2              | 0.21±0.01       | 14±1                 |
|              | F 2   | –            | 101±1              | 0.18±0.01       | 21±1                 |
|              | G 3   | –            | 151±5              | 0.58±0.04       | 28±1                 |
| T80*         | H 2   | 1.25         | 289±22*            | 0.55±0.07       | 10±1**                |
|              | I 2   | 2.5          | 131±1**            | 0.18±0.01       | 12±1**                |
|              | L 2   | 5            | 125±3**            | 0.27±0.01       | 11±2**                |
| PLX188*      | M 2   | 1.25         | 102±3              | 0.24±0.03       | 22±1                  |
|              | N 2   | 2.5          | 100±2              | 0.19±0.01       | 34±5*                 |
|              | O 2   | 5            | 90±1               | 0.15±0.01       | 21±2                  |
| SD*          | P 2   | 1.25         | 105±1              | 0.15±0.01       | 32±2**                |
|              | Q 2   | 2.5          | 320±106*           | 0.46±0.05       | -40±1**               |
|              | R 2   | 5            | >1000**            | 0.76±0.17       | -35±5**               |

*p < 0.05, **p < 0.001 (with respect to the formulation F).

* Tween 80®

Poloxamer 188®

Polysorbate 80®

2.6. Freeze-drying of nanosystems

PTX-loaded zein nanoparticles were lyophilized by freeze-drying (VirTis SP scientific sentry 2.0) equipped with a vacuum pump (Carpanelli S.p.a, B14 model). Briefly, 0.5 ml of formulation was enriched with various concentrations (5 and 10% w/v) of different cryoprotectants as previously described, transferred to pyrex glass vials and frozen in liquid nitrogen for 2 minutes (Gagliardi et al., 2018). Successively, samples were placed in the freeze-drying chamber and subjected to a cryo-drying process for 24 hours. The temperature of the condenser was approximately -55°C, while the pressure was approximately 30–50 mTorr. At the end of the procedure the resulting powder was rehydrated with the same volume of sublimated water by manual shaking and then the sizes and surface charges were evaluated through dynamic light scattering (see section 2.3.).

2.7. Cell cultures and in vitro cytotoxicity

MCF-7 (breast cancer cell lines) and K562 cells (human chronic myelogenous leukemia cells) were incubated in plastic culture dishes (100 mm × 20 mm) in a water-jacketed CO2 incubator at 37 °C (5% CO2) using a DMEM with glutamine and RPMI1640, respectively, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (250 μg/ml) and FBS (10%, v/v) as previously described (Cosco et al., 2011; Cosco et al., 2012a,b).

MTT-assay was used to evaluate cell viability after incubation with PTX as free form (solubilized in ethanol) or encapsulated in zein nanoparticles and the investigation was performed as a function of the drug concentrations (0.01–1 μM) and incubation times (24, 48 and 72 h). The untreated cells were used as control while the cells treated with empty zein nanosystems or ethanol (at the same concentration as the formulations containing PTX) were used as the blank. Successively, 20 μl of tetrazolium salt solubilized in phosphate buffered solution (5 mg/mL) were added to each well, the plates were incubated again for 3 hours and then analyzed using a microplate spectrophotometer (xMARK™–BIORAD) at a wavelength of 540 nm with reference at 690 nm. Cell viability, expressed as a percentage, was reported as the mean of five different experiments ± standard deviation and was obtained through the following equation, in which AbsT is the absorbance of treated cells and AbsC is the absorbance of control (untreated) cells:

\[
\text{Cell viability} \% = \frac{\text{AbsT}}{\text{AbsC}} \times 100
\]

2.8. Interaction of tritiated zein nanoparticles with cells

The rate of interaction between the SD-stabilized zein nanoparticles and the cell lines (K562 and MCF-7) as a function of incubation times was investigated as previously described (Cosco et al., 2012b). Briefly, [³H]
2.9. Statistical analysis

The statistical analysis of the various experiments was performed by ANOVA and results confirmed by a Bonferroni t-test, with a p value of <0.05 considered statistically significant.

3. Results and discussion

3.1. Physicochemical and technological characterization

Zein nanoparticles were recently developed and characterized by our research team. These colloidal systems had a protein concentration of 2 mg/ml, an absence of aggregates and a suitable mean diameter (Gagliardi et al., 2018). Moreover, the use of SD (1.25% w/v) significantly increased the stability of the nanoparticles with respect to other hydrophilic surfactants, modulated the surface charges of the nanosystems (from positive to negative values) and favored a good degree of retention of the various compounds, showing properties of colloidal systems suitable for possible systemic administration (Table 1) (Gagliardi et al., 2018).

Considering this data, the initial step of the present work was focused on the evaluation of the physico-chemical parameters of SD-stabilized zein nanoparticles prepared with various concentrations of PTX. Table 2 shows the results of the DLS analysis; in detail, the zein nanoparticles had an average diameter of less than 200 nm, a low polydispersity index representing a narrow size distribution of the nanosystems, and a surface charge of about -30 mV, ideal for the stability of the colloids over time (Bhattacharjee, 2016; Celia et al., 2011). The addition of PTX to the organic phase resulted in a slight increase of the average diameter and size distribution of the zein nanoparticles which was proportional to the concentration of the drug used, probably because the encapsulation of the lipophilic compounds present in the zein nanosystems enhances intra-particle attraction and aggregation (Chen and Zhong, 2015). Contrarily, the surface charges of the nanosystems were not strongly affected by the anticancer drug, probably because the active compound did not significantly modulate the protein arrangement.

3.2. Influence of heating, serum incubation and pH on the particle stability

The knowledge of the effects of temperature on the stability of zein-based nanoformulations is an important parameter to be investigated because the systems may be subjected to heat during the manufacturing procedures, storage and administration (Chen and Zhong, 2015). The protective feature provided by SD was previously described by our research team, showing that surfactant-free zein nanoparticles dramatically increase their mean sizes at temperatures higher than 30 °C (Gagliardi et al., 2018). For this reason the nanosystems containing PTX were incubated at temperatures up to 50 °C and mean sizes, size distribution and surface charges were evaluated. Table 5 shows that the average diameter of the particles remained relatively small but it is interesting to note the increased mean sizes of the particles prepared with the highest concentration of PTX. The evaluation of the polydispersity index confirmed this trend probably as a consequence of the steric hindrance exerted by the active compound. Contrarily, the surface charges of the nanosystems were not affected by the temperature, confirming the fundamental role of SD in their stabilization.

Table 2

| PTX concentration (µg/ml) | Mean Sizes (nm) | Polydispersity Index | Zeta Potential (mV) |
|--------------------------|----------------|----------------------|---------------------|
| 100                      | 110 ± 2        | 0.11 ± 0.01          | -32 ± 0.5           |
| 200                      | 131 ± 6**      | 0.18 ± 0.08          | -28 ± 2.2           |
| 300                      | 158 ± 5**      | 0.21 ± 0.04          | -31 ± 2.0           |

* p < 0.05, ** p < 0.01 (with respect to the empty formulation).
* Sodium azide (0.05% w/v) was added to the formulations as preservative.

Table 3

| PTX concentration (µg/ml) | Mean Sizes (nm) | Polydispersity Index | Zeta Potential (mV) |
|--------------------------|----------------|----------------------|---------------------|
| 100                      | 110 ± 2        | 0.13 ± 0.01          | 18 ± 2              |
| 200                      | 161 ± 9**      | 0.34 ± 0.09          | 20 ± 4              |
| 300                      | 265 ± 12**     | 0.45 ± 0.1           | 16 ± 5              |

* p < 0.05, ** p < 0.001 (with respect to the empty formulation).
The zein nanoparticles containing PTX were also incubated in 70% FBS at various pH values with the aim of fully investigating their stability features. As can be seen in Fig. 3A the sizes of the nanosystems showed only a slight increase in the mean diameter with respect to the empty

Fig. 1. TSI of zein nanoparticles prepared with various amounts of PTX as a function of time (t0: panels a and b; after 12 months: panels c and d) and temperature (20 °C: panels a and c; 37 °C: panels b and d).

Fig. 2. ΔT and BS profiles of empty SD-zein nanoparticles (A) and of nanosystems prepared with various amounts of PTX (B: 100 μg/ml; C: 200 μg/ml; D: 300 μg/ml) using Turbiscan Lab. The result was a representative experiment of three independent experiments.
In Fig. 3.

Table 5
TSI values of SD-zein nanoparticles prepared with various amounts of PTX as a function of time and temperature.

| Time (days) | Temperature | Empty PTX | PTX 100 μg/ml | PTX 200 μg/ml | PTX 300 μg/ml |
|-------------|-------------|-----------|---------------|---------------|---------------|
|             | 20 °C       | µg/ ml    | µg/ ml        | µg/ ml        | µg/ ml        |
|             | 37 °C       | ml        | ml            | ml            | ml            |
| 0           | 0.05        | 3.42      | 3.85          | 4.85          | 4.92          |
| 1           | 0.05        | 3.39      | 4.00          | 4.81          | 4.99          |
| 5           | 0.05        | 3.51      | 3.92          | 4.77          | 5.10          |
| 10          | 0.05        | 3.45      | 3.93          | 4.91          | 5.02          |
| 15          | 0.05        | 3.47      | 3.91          | 4.95          | 4.98          |
| 20          | 0.05        | 3.54      | 3.84          | 4.92          | 5.03          |
| 25          | 0.05        | 3.55      | 3.84          | 4.87          | 5.05          |
| 30          | 0.05        | 3.44      | 3.91          | 4.90          | 5.00          |

*p < 0.05 (with respect to the formulation at 30 °C).

Table 6
Effect of heating on the physico-chemical parameters of zein nanoparticles containing various amounts of PTX.

Table 6
Entrainment efficiency (EE%) and loading capacity (LC%) of various amounts of PTX in SD-stabilized zein nanoparticles prepared using a protein concentration of 2 mg/mL and 1.25% w/v of SD as a function of the drug concentration initially used.

| Amount of drug used (μg/ml) | EE (%) | LC (%) | Amount of entrapped drug (μg/ml) |
|---------------------------|--------|--------|-------------------------------|
| 100                       | 29 ± 3 | 0.9 ± 0.03 | 29 ± 1.2                       |
| 200                       | 32 ± 3 | 2.12 ± 0.03 | 64.5 ± 2.3                     |
| 300                       | 40 ± 4 | 3.84 ± 0.12 | 120 ± 3.9                      |
evidenced during our research concerning the encapsulation of other lipophilic compounds (ATRA and Red Oil) in SD-stabilized zein nanoparticles (Luo et al., 2012; Gagliardi et al., 2018). This is probably due to the peculiar chemical structure of PTX, which renders it able to be perfectly integrated into the polymeric structure of the nanosystems. A validation of this hypothesis was provided by the evaluation of the drug release profiles from the zein nanoparticles; namely, the drug leakage was constant and prolonged (Fig. 4) and it was modulated by the amount of the entrapped active compound. In fact, the samples prepared using 100 and 200 μg/ml of PTX showed full release of the drug after 48 and 120 h, respectively, while the formulation containing the highest amount of the active compound was characterized by a release of PTX of ~70% after 5 days of analysis (Fig. 4).

3.4. Freeze-drying of PTX-loaded zein nanoparticles

The freeze-drying features of an innovative nanosystem are also critical aspects to be evaluated in order to provide long-term storage and stability of the formulation and its potential practical application (Chang et al., 2017). As previously described, sodium deoxycholate (SD) was proposed as a promising stabilizer for use in developing freeze-dried zein nanoparticles characterized by a high level of stability (Gagliardi et al., 2018). For this reason, additional lyophilization studies were performed on the nanoformulation prepared with 0.3 mg/ml of PTX in order to evaluate the influence of various cryoprotectants on the physico-chemical properties of the colloidal systems. As shown in Table 7, no significant differences in particle size, polydispersity index or zeta potential were observed when glucose (5% w/v), sucrose, mannitol and trehalose were used, suggesting the high stability and potential long-term storability of these formulations. Only when mannose and 10% w/v glucose was used did problems of redispersion of the samples and a discrete degree of aggregation occur, confirming the unsuitability of the saccharide in the development of acceptable freeze-dried zein nanoparticles (Table 7).

3.5. Cytotoxicity of PTX-loaded zein nanoparticles

The evaluation of the cytotoxicity of a colloidal system is another fundamental parameter to be evaluated in the phases of preformulation (Gupta et al., 2017). The SD-stabilized zein nanosystems manifested a favorable safety profile towards various cell lines up to 50 μg/ml of protein (Gagliardi et al., 2018). For this reason, the pharmacological efficacy of the PTX-loaded zein nanosystems, obtained using 300 μg/ml of drug during the preparation procedure, was evaluated on K562 and MCF-7 cells, as models of human cancer cells sensitive to the lipophilic drug, with respect to the free form of the active compound (Fig. 5). The choice of this formulation was due to the favorable physico-chemical and technological features obtained during the characterization of the nanosystems. The cytotoxic effects were evaluated as a function of both the incubation times (24, 48, or 72 h) and the drug concentrations (0.1–1 μM) in order to define the exposition time and the dose-response effects, respectively.

In detail, the nanoencapsulation of the anticancer agent promoted a greater decrease in cell viability as compared to the free form of PTX at all the concentrations used (Fig. 5); the best anticancer effects were exerted by the colloidal formulation containing the lipophilic drug on the MCF-7 cells because noteworthy pharmacological efficacy was shown early on during incubation. Contrarily, the increased toxicity of nanoencapsulated PTX on K562 cells was more evident only after 72 h (Fig. 5). The IC50 evaluation of the drug encapsulated within the colloidal systems confirmed the difference in pharmacological efficacy on the aforementioned cell lines (Table 8). The uptake of the nanosystems containing PTX could be a plausible explanation for the increased cytotoxic effect of the bioactive, as has been true in the case of many other polymeric carriers and it could favor a significant decrease of the efficacious concentration of the drug (Mura et al., 2019).

In fact, the evaluation of the cell interaction rate of titrated zein-nanoparticles confirmed a time-dependent cell uptake of the nanosystems that occurred after only 1 h incubation (Fig. 6). In addition, it was interesting to note that the interaction between the K562 cells and

Table 7

| Cryoprotectant | Cryoprotectant concentration (% w/v) | Mean size (nm) | Polydispersity Index | Zeta Potential (mV) |
|---------------|-------------------------------------|----------------|----------------------|---------------------|
| Before lyophilization | - | 110 ± 1 | 0.11 ± 0.01 | -34 ± 2.9 |
| - | - | >1000** | 0.9 ± 0.05 | -25 ± 1.8 |
| Glucose | 5% | 158 ± 6** | 0.21 ± 0.03 | -39 ± 3.1 |
| Glucose | 10% | 450 ± 20** | 0.89 ± 0.04 | -19 ± 0.1 |
| Mannose | 5% | >1000** | 0.9 ± 0.08 | -21 ± 7.4 |
| Mannose | 10% | 500 ± 31** | 0.66 ± 0.03 | -19 ± 8.2 |
| Trehalose | 5% | 150 ± 2** | 0.19 ± 0.01 | -35 ± 2.7 |
| Trehalose | 10% | 166 ± 2** | 0.25 ± 0.05 | -36 ± 2.6 |
| Sucrose | 5% | 175 ± 8** | 0.27 ± 0.07 | -38 ± 5.2 |
| Sucrose | 10% | 190 ± 9** | 0.29 ± 0.09 | -37 ± 3.3 |
| Mannitol | 5% | 160 ± 5** | 0.24 ± 0.06 | -38 ± 2.1 |
| Mannitol | 10% | 180 ± 3** | 0.19 ± 0.04 | -38 ± 3.9 |

* *p < 0.001 (with respect to the formulation before the freeze-drying procedure).
zein nanoparticles was less than that obtained in the case of MCF-7 cells, probably as a consequence of the physical deposition of the polymeric systems on the plate which promoted their accumulation in the adherent cells. It is well known that the cell uptake of polymeric nanoparticles having a mean diameter <200 nm is characterized by an endocytotic mechanism (Conner and Schmid, 2003). It has been demonstrated that various zein nanoparticles are characterized by an energy-dependent cell uptake but the specific mechanisms governing the intracellular localization of the SD-zein nanosystems have not yet been investigated (Luo et al., 2013).

Moreover, the influence of various surfactants on the inhibition of P-glycoprotein (P-gp) efflux pumps was described in recent experimental investigations (Han et al., 2018; Al-Ali et al., 2019); sodium deoxycholate could compromise the efficacy of P-gp promoting the pharmacological effects of PTX following its nanocapsulation in SD-zein nanoparticles (Sandhu et al., 2015; Rocheblave et al., 2016).

The results demonstrate that zein nanoparticles preclude the use of organic solvents to solubilize the lipophilic compound, which would...
Table 8

| Incubation | IC50 PTX | IC50 PTX-loaded zein nanoparticles |
|------------|---------|----------------------------------|
| Cell lines | time    | (µM)                             | (µM) |
| MCF-7      | 24 h    | >1                               | 1    |
|           | 48 h    | >1                               | 0.49 |
|           | 72 h    | 0.55                             | 0.05 |
| K562      | 24 h    | >1                               | >1   |
|           | 48 h    | >1                               | 1    |
|           | 72 h    | 0.72                             | 0.19 |

The values have been obtained fitting the MTT-test curves to the following Four Parameter Logistic Equation:

\[ y = \frac{\text{max} - \text{min}}{1 + \left( \frac{x}{\text{IC50}} \right)^{\text{HillSlop}}} + \text{min} \]

mean a potential dramatic decrease in the side effects related to their use in human beings.

4. Conclusions

The use of biomaterials to deliver traditional anticancer compounds is a widely-recognized approach for developing innovative nanomedicines characterized by a high level of pharmacological efficacy and decreased side effects. Moreover, the encapsulation of lipophilic compounds in colloidal systems promotes the modulation of their biopharmaceutical properties avoiding the use of organic solvents. The physical entrapment of PTX in zein nanoparticles demonstrated its potentiality for use as a novel anticancer formulation due to its properties of high stability and in vitro anticancer efficacy. Obviously, there are several sticking points in this reason, additional studies concerning the nature of the plasmatic proteins adsorbed on the colloidal surface after serum incubation/in vivo injection, and the biodistribution and pharmacokinetic profiles of the described formulation are in progress. Moreover, the investigation of the anticancer properties of PTX-loaded-SD-stabilized zein nanoparticles on murine tumor xenograft models will be evaluated in order to demonstrate the real efficacy of this nanomedicine in preclinical and clinical applications.

Declarations

Author contribution statement

Donato Cosco: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Agnese Gagliardi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sonia Bonacci: Performed the experiments; Wrote the paper.

Donatella Paolino, Christian Celia: Performed the experiments. Antonio Procopio: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Massimo Fresta: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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