Solid Lipid Nanoparticles Containing Nimesulide: Preparation, Characterization and Cytotoxicity Studies

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Abstract: The prospect of improved cancer therapy using Solid Lipid Nanoparticles (SLNs) as drug delivery system is promising. Several obstacles frequently encountered with anticancer compounds, such as poor drug solubility, are overcome by delivering them using SLN.

Moreover, the intravenous administration of drugs into SLNs can potentially enhance drug blood circulation time and improve drug performance by inducing accumulation into tumours by enhanced permeability and retention (EPR) effect. This paper deals with the development of SLN containing nimesulide, a nonsteroidal anti-inflammatory drug with antitumour effect and low solubility in water.

Here, SLNs carrying nimesulide were prepared and characterized, and the antiproliferative effect of drug-loaded SLN versus free drug on HT-29 and SW-480 cell lines was here evaluated.

All the obtained systems possess colloidal size, ranging from 85 to 132 nm and negative zeta potential values. Moreover these systems show good loading capacity and drug release profile, and an in vitro antitumour activity comparable to free drug.

Keywords: Solid lipid nanoparticles; Nimesulide; Drug release; Human colon adenocarcinoma cells; Solid tumours.

1. INTRODUCTION

Except for a few cancer types (e.g. breast cancer), for which hormonal therapy or immunotherapy is used, cytotoxic drugs remain the major form of chemotherapy, being toxic to those cells that are rapidly growing and dividing, but presenting unique problems such as poor specificity and high toxicity [1]. Solid tumours present much more favourable conditions for preferential accumulation of colloidal sized drug delivery systems such as liposomes, polymeric and solid lipid nanoparticles (SLNs) [2-3]. In fact, the increased vascular permeability coupled with the impaired lymphatic drainage in tumours allows an enhanced permeability and retention (EPR) effect of these nanosystems in the tumour [4-5]. Other factors which may influence tumour accumulation by the EPR effect include the degree of tumour vascularization/angiogenesis, the gaps (600-800 nm) of the tumour vasculature endothelium and the size of the colloidal delivery system. Several colloidal systems have been successfully used to target anticancer drugs to tumour tissue in a passive manner. New delivery systems which could be used as carriers of anticancer drugs include liposomes, polymeric nanoparticles and macromolecule conjugates; they offer numerous advantages, e.g. improved efficacy and reduced toxicity, compared to conventional dosage forms [6-7]. Moreover, the colloidal carriers offer a great number of potential advantages as drug delivery systems, such as an improvement of bioavailability of poorly water-soluble drugs [8]. SLNs represent an attractive alternative to polymeric nanoparticles both for hydrophobic and hydrophilic drugs [9]. Basically, SLNs can be used for all parenteral applications ranging from intrarticular to intravenous administration [10]. For the latter, a limited uptake by mononuclear macrophages (MPS), in order to obtain a larger accumulation of SLNs in other tissues such as brain, can be achieved by the presence of polyethylene glycol (PEG-s) on nanoparticles surface, that reduces the hydrophobic surface exposed to blood components [11]. The oral administration of SLNs is possible as aqueous dispersion or alternatively after transformation into a traditional dosage form, i.e. tablets, capsules or powders in sachets [9,10,12].

The aim of this work is to prepare SLNs loaded with nimesulide, a non-steroidal anti-inflammatory drug (NSAID), which are among the few agents that inhibit colorectal carcinogenesis due to their action on cyclo-oxygenase enzymes (COX-1 and COX-2) [13]. Recent evidences indicate that COX-2 is an important molecular target for anticancer therapies [14-24]. Nimesulide, being a sulfonanilide class COX-2 inhibitor, possesses much less adverse effects on the gastrointestinal tract than non-specific NSAID, and shows antiproliferative activity against colon, lung, urinary bladder, breast, tongue, and liver carcinogenesis [25-31]. Considering the high hydrophobicity of nimesulide (water solubility is less than 10 μg x ml⁻¹), physical entrapment into SLNs can improve bioavailability, thus reducing long term side effects [32]. Moreover, intravenous administration of nimesulide into SLNs, in comparison with oral therapy, can potentially enhance drug blood circulation time and improve drug performance by inducing tumour accumulation by EPR effect. In this paper, nimesulide-loaded SLNs were prepared. Physico-chemical properties of empty and drug-loaded nanoparticles, such as size and zeta potential have been measured by photon correlation spectroscopy (PCS). Evaluation of drug release profile from the nimesulide-loaded SLNs in media mimicking biological fluids (buffer solution at pH 7.4) was also carried out. Finally, in vitro anti-proliferative activity studies on HT-29 and SW-480 cells (human colorectal cancer cells present in solid tumours) were carried out to verify the effect of drug entrapment into SLNs on cytotoxicity.

2. MATERIALS AND METHODS

2.1. Materials

Nimesulide was purchased from Cayman Chemical Company (Ann Arbor, MI). Compritol 888 ATO (mixture of approximately mono-, di- and triglycerides of behenic acid, at 15, 50 and 35% w/w...
respectively) was a gift of Gattefossé, Saint-Priest, France. Palmitic acid and stearic acid were purchased from Fluka (Milan, Italy). Miglyol 812 (mixture of caprylic and capric triglycerides) was supplied by Sasol GmbH (Germany). Soy phosphatidylcholine 95% (Epikuron 200) was donated by Degussa Texturant Systems, Germany. Taurocholic acid sodium salt was a kind gift from PCA (Basiluzzo, Italy). Human colon adenocarcinoma (HT-29 and SW-480) cell lines were obtained from the American type culture collection (Rockville, MD, USA).

The other chemicals were obtained from Sigma and were of analytical grade.

2.2. Preparation and Purification of Empty and Drug-loaded Nanoparticles

SLNs were prepared through the precipitation technique. Epikuron 200 (0.14 mmol) was solubilized in ethanol (2 ml) and added, under stirring, to melted lipid (0.7 mmol) at 85°C. For obtaining drug-loaded nanoparticles, nimesulide was added, under mechanical stirring, to the melted lipid before the addition of Epikuron’s solution. SLNs were obtained by dispersing the warm solutions in cold twice-distilled water (100 ml at 2-3°C) containing taurocholate sodium salt (0.69 mmol), by using an Ultra Turrax T25 (IKA, D-Staufen). The obtained SLNs dispersions were purified by ultracentrifugation, by using a Centrifuge XL-90 Beckmann, equipped with a 70.1 rotor. The ultracentrifugation was carried out at 35,000 rpm, at 4°C for 30 min, washing several times the obtained nanoparticles with twice-distilled water.

After ultracentrifugation, the aqueous SLN dispersions were freeze-dried by using a Modulyo freeze-dryer (Labconco Corporation, Missouri, U.S.A.) and stored at room temperature for further characterizations. Ethanol is completely removed from the aqueous dispersions during ultracentrifugation and freeze-drying steps.

2.3. Particle Size and Zeta Potential Measurements

The mean diameter, width of distribution (polydispersity index, PDI) and zeta potential measurements of the obtained empty and drug-loaded nanoparticles in aqueous suspension, were determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instrument, Herrenberg, Germany), which utilizes Non-Invasive Back-Scatter (NIBS) technique. Each sample was appropriately diluted with filtered water (0.2 µm) and the reading was carried out at 25°C and at a 173º angle in respect to the incident beam. Each suspension was kept in a cuvette and analyzed in triplicate. The deconvolution of the measured correlation curve to an intensity size distribution was accomplished by using a non-negative least squares algorithm.

Zeta potential values were measured on dispersion of each lyophilised nanoparticle batch by using as dispersing media an aqueous solution of filtered double-distilled water (through 0.2µm filter) and using a Zetasizer Nano ZS. Each sample was analysed in triplicate.

2.4. Loading Capacity

An adequate HPLC method was developed to study the Loading Capacity (LC %) of drug-loaded SLNs. The HPLC analysis was performed at room temperature using a Shimatzu Instrument equipped with a reversed-phase C18 column (µBondapak, 3 µm, 150 x 4.6 mm i.d., Supelco) as a stationary phase and a mixture of CH3CN and an aqueous solution of ammonium phosphate (0.05 M) (50:50 v/v) as mobile phase, with a flow rate of 0.5 ml min⁻¹. The HPLC column system was connected with a UV-Vis detector (Shimatzu). The amount of nimesulide loaded into SLNs was measured and monitored at the wavelength of 234 nm.

For evaluating the amount of nimesulide entrapped into nanoparticles, 5 mg of each batch of freeze-dried drug-loaded NLC were dissolved in 5 ml of chloroform; then the solution was filtered through 0.45 µm (PTFE membrane) filters and analyzed by HPLC.

Results were expressed as the percentage of the drug amount contained in 100 mg of dried material.

2.5. Cell Culture and Viability Assays

Human colon adenocarcinoma cell lines HT-29 and SW-480 were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) at 37°C in 5% CO2 humidified atmosphere. These two cell lines have been extensively used for studying the effect of anti-inflammatory drugs on cell viability.

Cells in complete medium were seeded on 96 well plates (100 µl for well) at a density of 10,000 per well and allowed to adhere overnight. At time 0, the medium was replaced with fresh complete medium, and various concentrations (12.5, 25, 50, 100 and 200 µM) of free nimesulide (dissolved in dimethyl sulphoxide) and nimesulide-loaded SLNs were diluted with one volume of 2X concentrated RPMI complete medium and added to the cells. Empty SLNs were also evaluated on the two cell lines growth at the highest concentrations corresponding to that of 200 µM of nimesulide-loaded SLNs. All SLNs samples were dispersed using a water bath sonication for 2 x 10 min (Transsonic 310, Elma, Germany) before use. The cells were cultured for 72 hours in atmosphere enriched of 5% CO2. Cells incubated with the medium were used as negative control. After the incubation time with various concentrations of the inhibitors, viability assay was performed using the CellTiter Aqueous OneSolution kit (Promega Corporation, Madison, WI, USA). Briefly, 15 µl of a commercial solution (obtained from Promega Corporation, Madison, WI, USA) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazoliun (MTS) and phenazine ethosulfate were added to each well. Further the plates were incubated for 1 h in a humidified atmosphere at 37°C in 5% CO2. The bioreduction of the MTS dye was assessed by measuring the absorbance of each well at 490 nm. Cytotoxicity was expressed as a percentage of the absorbance measured in the control cells. Values were expressed as means ± SD of three separate experiments, each of which was performed in triplicate.

2.6. Drug Release at pH 7.4

Drug release was assayed at eleven prefixed time intervals. For this purpose, eleven suspensions, each containing 5 mg of nimesulide-loaded SLNs were dispersed in 100 ml of PBS at pH 7.4 and kept at 37°C ± 1°C by a water bath under mechanical stirring. At suitable time intervals, samples dispersed in PBS were centrifuged at 18,000 rpm for 15 min at 4°C, filtered through 0.45 µm nylon membrane filters (Whatmann) and analyzed by HPLC, by using the method described above. Each experiment was carried out in triplicate.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of Empty and Drug-loaded SLNs

In this paper, the preparation, the physical-chemical characterization and in vitro biological studies of SLNs are described. Moreover, the potential of these SLNs as drug delivery system was investigated by evaluating the ability of nanoparticles to physically entrap nimesulide, to release it in a physiological medium, and the in vitro cytotoxicity on cancer cell line. Nimesulide, 4'-nitro-2'phenoxy methane sulfonalide, is a non-steroidal anti-inflammatory drug (NSAID) and it was selected in this study as a model drug because of its lipophilic nature and a very low aqueous
That nonsteroidal anti-inflammatory drugs (NSAIDs), particularly the highly selective cyclooxygenase (COX)-2 inhibitors, have promise as anticancer agents. NSAIDs have been shown experimentally to stimulate apoptosis and to inhibit angiogenesis, two mechanisms that help to suppress malignant transformation and tumour growth [14-15].

In particular, four different samples of empty and drug-loaded SLNs were prepared by using as lipid matrix palmitic acid (sample A), stearic acid (sample B), Compritol 888 ATO (sample C) and a mixture between Compritol 888 ATO and Miglyol at 20 w/w % on the total lipid weight (sample D).

The complete process of empty or drug-loaded SLNs preparation is schematically depicted (Fig. (1)).

Epikuron 200 and taurocholate sodium salt were chosen as surfactant and cosurfactant, respectively, because they are acceptable components in parenteral administration. A solution of the Epikuron 200 in warm ethanol was added to the melted lipid at 85 °C. Empty SLNs (empty A, B, C and D samples) were obtained by dispersing the warm solution in cold water containing taurocholate sodium salt, under mechanical stirring. For obtaining drug loaded samples (A, B, C and D samples), nimesulide was added to the melted lipid before the addiction of Epikuron’s ethanolic solution.

Since some physical-chemical properties (size, surface charge, polydispersity index) are quite critical for biopharmaceutical behavior of SLNs, all the obtained samples were characterized in terms of particle size, PDI and zeta potential. In Table 1 the physical-chemical characteristics of empty and nimesulide-loaded SLNs are showed.

As can be seen, all the obtained samples, empty or drug-loaded showed colloidal size ranging between 85 and 132 nm for empty SLNs and between 93 and 170 nm for drug-loaded SLNs, respectively. This result is probably due to a higher amount of total lipid phase, containing also the drug, precipitated in the aqueous cold phase for obtaining drug-loaded nanoparticles, as reported by other authors [33-35]. Moreover, all the samples resulted to be homogeneous being the PDI values quite small.

These systems, thanks to their small size, could minimize the uptake of macrophages of MPS [36], giving long circulating systems in the blood stream, that could potentially accumulated in tumour masses as a consequence of EPR effect.

Table 1 shows the zeta potential values of all empty and drug-loaded SLN batches. The surface charge values are negative for all empty and drug-loaded samples. Moreover, these values are lower in drug-loaded samples than empty. The presence of drug causes a

Table 1. Mean size, PDI and Zeta Potential Values of empty and Nimesulide-Loaded SLNs in Twice-Distilled Water and Drug Loading Capacity (LC%) of Drug-Loaded SLNs. Values are Reported as Means ± S.D. The Average Size of Nanoparticles was Calculated in the Range Between 5 and 95 % for Each Sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lipid Matrix</th>
<th>Mean Size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>LC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty A</td>
<td>Palmitic acid</td>
<td>85.62 ± 2.67</td>
<td>0.148 ± 0.07</td>
<td>-35.43 ± 1.68</td>
<td>------</td>
</tr>
<tr>
<td>A</td>
<td>Palmitic acid</td>
<td>93.17 ± 3.21</td>
<td>0.243 ± 0.06</td>
<td>-32.42 ± 1.21</td>
<td>9.3%</td>
</tr>
<tr>
<td>Empty B</td>
<td>Stearic acid</td>
<td>130.02 ± 2.92</td>
<td>0.244 ± 0.07</td>
<td>-38.23 ± 2.54</td>
<td>------</td>
</tr>
<tr>
<td>B</td>
<td>Stearic acid</td>
<td>170.02 ± 4.12</td>
<td>0.261 ± 0.05</td>
<td>-32.36 ± 2.17</td>
<td>8.7%</td>
</tr>
<tr>
<td>Empty C</td>
<td>Compritol</td>
<td>101.00 ± 1.64</td>
<td>0.189 ± 0.05</td>
<td>-28.24 ± 3.15</td>
<td>------</td>
</tr>
<tr>
<td>C</td>
<td>Compritol</td>
<td>120.00 ± 1.56</td>
<td>0.227 ± 0.07</td>
<td>-26.18 ± 1.02</td>
<td>17.8%</td>
</tr>
<tr>
<td>Empty D</td>
<td>Compritol/Miglyol (20%)</td>
<td>111.07 ± 2.32</td>
<td>0.174 ± 0.03</td>
<td>-26.15 ± 2.85</td>
<td>------</td>
</tr>
<tr>
<td>D</td>
<td>Compritol/Miglyol (20%)</td>
<td>132.00 ± 5.87</td>
<td>0.303 ± 0.02</td>
<td>-20.79 ± 1.54</td>
<td>15.8%</td>
</tr>
</tbody>
</table>
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diminution of surface charge of all the investigated samples because probably a share of drug is situated onto nanoparticles surface.

Moreover all samples have different zeta potential values because the surface charge depends on lipid matrix used.

In Table 1, the LC% values (expressed as weight percent of drug amount in 100 mg of dried material) of drug-loaded SLNs (samples A, B, C and D) are also reported. The loading capacity (LC%) of nanoparticles, evaluated by dissolving the batch into chloroform and subsequent HPLC analysis of the solution, resulted to be 9.3, 8.7, 17.8 and 15.8 wt.-%, respectively.

As can be seen, the best results were obtained when Compritol 888 ATO was used as lipid matrix, and this batch was chosen for further characterization. This result is very important considering the high hydrophobicity of nimesulide, which physical entrapment into SLNs can improve bioavailability, and reduce long term side effects. Moreover, intravenous administration of nimesulide into SLNs, in comparison with oral therapy, can potentially enhance drug blood circulation time and improve drug performance by inducing tumour accumulation by EPR effect [2-3].

To evaluate the possibility of drug-loaded sample C to release nimesulide, drug release profile was investigated in PBS at pH 7.4, under experimental conditions mimicking extracellular fluids (Fig. 2).

As it can be seen, after 1 h, the amount of nimesulide released from sample C was equal to 38 %. An initial burst effect in the drug-release profile of sample C is evident and it can be probably ascribed to the presence of the unloaded drug. The drug delivery system releases 100 % of drug within 14 hours.

Fig. (3) shows the survival curves of HT-29 (Fig. 3A), and SW-480 (Fig. 3B) cells after exposure to nimesulide, either in DMSO solution or in sample C aqueous suspension.

To ensure that the cytotoxicity was caused by the drug itself and not by formulation components, cells were incubated with un-
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CONCLUSIONS

In this study the potential of SLNs dispersions as carriers for parenteral delivery of antitumour drugs was exploited. SLNs, constituted of bioacceptable lipids, were prepared by the precipitation technique as empty or nimesulide-loaded systems. Empty and drug-loaded SLNs showed average diameters in the colloidal size range, a good loading capacity and drug release. In vitro cytotoxicity assay showed that nimesulide-loaded SLNs maintain an antitumour activity comparable to free drug, demonstrating that nimesulide activity is not reduced in the presence of the nanoparticle carrier.

Considering that solid tumours present much more favourable conditions for preferential accumulation of colloidal sized drug delivery systems such as SLNs, these systems can be useful for application in cancer therapy.

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