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Research paper

Cytotoxicity of anticancer drugs incorporated in solid lipid nanoparticles on HT-29 colorectal cancer cell line

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Abstract

Solid lipid nanoparticles (SLN) carrying cholesteryl butyrate (chol-but), doxorubicin and paclitaxel had previously been developed, and the antiproliferative effect of SLN formulations versus conventional drug formulations was here evaluated on HT-29 cells. The 50% inhibitory concentration (IC_{50}) values were interpolated from growth curves obtained by trypan blue exclusion assay. In vitro cytotoxicity of SLN carrying chol-but ($IC_{50\ 72\ h}$ 0.3 ± 0.03 mM vs >0.6 mM) and doxorubicin ($IC_{50\ 72\ h}$ 81.87 ± 4.11 vs 126.57 ± 0.72 nM) was higher than that of conventional drug formulations. Intracellular doxorubicin was double after 24 h exposure to loaded SLN versus the conventional drug formulation, at the highest concentration evaluated by flow cytometry. In vitro cytotoxicities of paclitaxel-loaded SLN and conventional drug formulation ($IC_{50\ 72\ h}$ 37.36 ± 6.41 vs 33.43 ± 1.17 nM) were similar. Moreover, the combination of low concentrations of chol-but SLN (0.1–0.2 mM) and doxorubicin (1.72 nM) or paclitaxel (1.17 nM) exerted a greater-than-additive antiproliferative effect at 24 h exposure, while the combination of Na-but and doxorubicin or paclitaxel did not. These preliminary in vitro results suggest that SLN could be proposed as alternative drug delivery system.

Keywords: Solid lipid nanoparticles; Sodium butyrate; Cholesteryl butyrate; Doxorubicin; Paclitaxel

1. Introduction

Targeting an anticancer drug to the disease location is a distinctive feature of most studies, the aim being to convey a sufficient dose of drug to the tumor. New delivery systems for use as carriers of anticancer drugs include liposomes, polymeric microspheres and macromolecule conjugates; they offer numerous advantages, eg improved efficacy and reduced toxicity, compared to conventional dosage forms [1]. Solid lipid nanoparticles (SLN) have been proposed as alternative drug carriers [2]. SLN are obtained using biocompatible components and are washed by diafiltration, leaving no toxic residues from the preparation process. SLN are in the colloidal size range and can be loaded with both hydrophilic and lipophilic drugs, depending on the preparation method [3,4]. The composition of the warm microemulsions from which SLN are prepared is flexible, and can be varied to suit the type of drug and administration route [5].

The short-chain fatty acid, butyric acid, a natural component of the colon milieu, is known to influence several kinds of pathophysiological process, producing changes in chromosome structure, inhibiting cell growth and inducing differentiation as well as apoptosis [6]. Butyrate has been shown to inhibit the growth of colon carcinoma cells, both in vivo, preventing and decreasing growth of chemically induced colon cancers in the rat [7], and in vitro in many colorectal cancer cell lines [8]. Although the molecular mechanisms by which butyrate mediates its effects are not well understood, it is known to induce a variety of changes within the nucleus, including histone hyperacetylation, which is the most extensively studied of its effects [9]. Furthermore, the effects of butyrate

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are enhanced additively or synergistically by combination with different types of agents such as phorbol ester, IFN-α, soluble Fas ligand and doxorubicin [10]. However, in vivo butyric acid exhibits low potency, most likely owing to its rapid metabolism [11].

The anthracycline antibiotic doxorubicin is one of the most useful antineoplastic agents, displaying a broad range of clinical activities against several solid tumors as well as hematological malignancies [12]. However, doxorubicin is not so active against colorectal cancer [13]. Furthermore, many colloidal carriers of doxorubicin, such as liposomes and polymeric nanoparticles, have been studied with the aim of reducing cardiac toxicity and improving therapeutic efficacy [14].

The diterpenoid derivative paclitaxel has broad antineoplastic activity, including against colorectal cancer cells, and a unique mechanism of action promoting the polymerization and stabilization of tubulin to microtubules [15–17]. One of the major clinical problems of using paclitaxel is its very low solubility in water, due to its extremely hydrophobic nature. In order to enhance paclitaxel's solubility, a mixture of 50:50 Cremophor EL (CrEL, a polyoxyethylated castor oil) and ethanol is used in the current clinical formulation. This determines the administration of a significant amounts of CrEL, with serious side effects for 25–30% of treated patients [16]. To minimize the incidence and severity of these reactions, premedication with histamine 1 and 2 blockers, as well as with glucocorticoids (usually dexamethasone) has become standard practice [18]. To circumvent these problems, a great deal of effort has been directed to developing new systemic paclitaxel formulations with enhanced circulation time and CrEL-free [19].

We previously developed two different types of SLN formulations: SLN constituted of chol-but as pro-drug to deliver butyric acid and SLN loaded with doxorubicin and paclitaxel [20–22].

We showed in animal studies that incorporation of anticancer agents in SLN can change their pharmacokinetic profiles, with increased half life, area under concentration-time curve (AUC), peak plasma concentration and concomitant decreased clearance [23,24]. We also showed chol-but SLN, and doxorubicin and paclitaxel-loaded SLN, to be significantly more potent than the conventional drug formulations in the in vitro inhibition of cancer cells proliferation [20,25,26].

The main aim of the present study was to evaluate the cytotoxic effect of these drugs incorporated, versus free drugs, on the human colorectal cancer cell line, HT-29. Moreover, as it has been suggested that a combination of cytotoxic agents and histone deacetylase inhibitors (HDIs) such as butyric acid could enhance inhibition of cancer cell proliferation [27], we examined the cytotoxic effects of the combination of chol-but SLN and free paclitaxel or doxorubicin, again on HT-29 cells.

2. Materials and methods

2.1. Drugs and chemicals

Doxorubicin hydrochloride was a kind gift from Farmitalia (Milan, Italy) and paclitaxel was a kind gift from Indena (Milan, Italy). For the in vitro tests the commercial products Adriamycin® (Pharmacia, Sweden) and Taxol® (Bristol–Myers Squibb, United Kingdom) were used as free drugs.

Chol-but, stearic acid, butanol and tripalmitin were purchased from Fluka (Buchs, Switzerland). Cholesteryl hemisuccinate, phosphate buffer saline tablets, RPMI 1640 medium, sera and antibiotics for cell culture were purchased from Sigma Chem. Co. (Milan, Italy). Epikuron 200® (containing about 95% of soy phosphatidylcholine) was a kind gift from Lucas Meyer (Hamburg, Germany); taurocholate sodium salt was a kind gift from PCA (Basaluzzo, Italy). Sodium hexadecylphosphate was prepared as indicated by Brown [28]. The other chemicals were of analytical grade.

2.2. Preparation of solid lipid nanoparticles

To obtain chol-but SLN, Epikuron 200® and chol-but, used as a lipid matrix, were melted at 85 °C and a warm water solution of biliar salt and butanol was added to obtain a clear system. The microemulsion was immediately dispersed in cold water and successively washed twice, filtered by diafiltration (TCF 2A-Amicon-Grace, Danvers, MA, USA; membrane Diaflo YM 100). The dispersions were then sterilized by autoclaving (15 min at 121 °C, 2 bar).

Chol-but concentration was determined by high pressure liquid chromatography ultraviolet equipment, following Duncan [29].

To obtain doxorubicin-loaded SLN, a warm oil-in-water (o/w) microemulsion was prepared using stearic acid as internal phase, Epikuron 200® as surfactant, sodium taurocholate as cosurfactant and ultrapure water as continuous phase. Doxorubicin hydrochloride and hexadecylphosphate were added to the internal phase. Hexadecylphosphate was used as doxorubicin counter ion in a 1:2 molar ratio (doxorubicin:hexadecylphosphate). Paclitaxel-loaded SLN were prepared from a warm oil-in-water (o/w) microemulsion containing tripalmitin as internal phase, Epikuron 200® as surfactant, cholesteryl hemisuccinate, butanol and taurocholate as cosurfactants, and ultrapure water as continuous phase. Paclitaxel was added to the melted tripalmitin; Epikuron 200®, cosurfactants and ultrapure water were then added obtaining a clear microemulsion at about 70 °C. SLN, either with doxorubicin or paclitaxel, were obtained as reported elsewhere [21,22]. Unloaded SLN for each formulation, i.e. SLN not carrying any drug, were also prepared. Both paclitaxel and doxorubicin-loaded SLN were sterilized as described for

Table 1
Composition of the microemulsions

	Chol-but SLN	Doxorubicin-loaded SLN	Paclitaxel-loaded SLN
Lipid matrix	Cholesteryl butyrate	Stearic acid	Tripalmitin
External phase	Water	Water	Water
Surfactant	Epikuron 200	Epikuron 200	Epikuron 200
Cosurfactants	Na-taurocholate, Butanol	Na-taurocholate	Na-taurocholate, Butanol, Cholesteryl hemisuccinate

chol-but SLN. The amount of drug incorporated was determined as reported elsewhere [26]. Table 1 shows the composition.

2.3. Characterization of solid lipid nanoparticles

All SLN were characterized by photon correlation spectroscopy (PCS) using a 90 PLUS instrument (Brookhaven Instrument Corporation, Holtsville, NY, USA) at a fixed angle of 90° and at a temperature of 25 °C to determine the average diameter and the polydispersity index. The polydispersity index measures the size distribution of the SLN population [30]. SLN water dispersions were diluted with ultrapure water before analysis. Each value reported is the average of six measurements.

The electrophoretic mobility and zeta potential were measured using a 90 PLUS instrument (Brookhaven Instrument Corporation, Holtsville, NY USA). To determine the zeta potential, samples of doxorubicin or paclitaxel-loaded SLN were diluted with KCl 1×10^{-4} M and placed in the electrophoretic cell, where an electric field of 15.24 V/cm was applied. Each sample was analysed in triplicate. The zeta potential was calculated as described by Helmholtz–Smoluchowski. The SLN aqueous dispersions were stable for more than 12 months stored at 4 °C.

2.4. Cell culture and cytotoxicity

The human colorectal adenocarcinoma cell line, HT-29, was obtained from the American Type Culture Collection (Rockville, MD, USA). HT-29 cells were grown as a monolayer culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/l L-glutamine and penicillin/streptomycin (100 units/ml), at 37 °C in 5% CO_2 humidified atmosphere, and passaged weekly. At the beginning of the experiments, cells in exponential growth phase were removed from the flasks with 0.05% trypsin-0.02% EDTA solution. Cells were seeded in 24 wells/plate (25,000 cells/well) in RPMI 1640 medium with 10% FCS. The cells were allowed to attach for 72 h, and seeding medium was removed and replaced by experimental medium. Cells were maintained for 3 days in medium supplemented with increasing concentrations of chol-but SLN and the sodium salt of butyric acid (Na-but), doxorubicin (free or loaded SLN) and paclitaxel (free or loaded SLN). The concentrations of chol-but SLN and Na-but varied from 0.05 to 0.6 mM and the concentrations of doxorubicin and paclitaxel (free or loaded SLN) varied from 1.72 to 172 nM and from 1.17 to 117 nM, respectively. HT-29 cells were also cultured in the presence of two different concentrations of free doxorubicin (1.72 and 34.4 nM) or free paclitaxel (1.17 and 23.44 nM) in combination with different concentrations of chol-but SLN or Na-but (0.05, 0.1 and 0.2 mM).

Since chol-but is equimolarly composed of cholesterol and butyrate, we also evaluated the effect of 0.1 mM free cholesterol on cell growth. We were unable to assay a higher cholesterol concentration because of the cytotoxicity of the corresponding concentration of ethanol (above 0.1%) used to solubilize the organic compound. Unloaded SLN were also evaluated on cell line growth at the highest SLN concentration corresponding to 1 mg of SLN/ml dispersion for doxorubicin formulation and 2.5 mg of SLN/ml dispersion for paclitaxel formulation. All experiments were done three times, each condition being performed in triplicate. Cell viability was assessed by trypan blue exclusion assay. Cytotoxicity was expressed as percentage of control cells. The inhibition concentration 50% (IC_{50}), defined as the dose of compound that inhibited 50% of cell growth, was interpolated from the growth curves thus obtained.

2.5. Determination of doxorubicin cellular uptake by flow cytometry

Taking advantage of the natural fluorescent property of anthracycline, the amount of intracellular doxorubicin (free or loaded SLN) was determined by flow cytometry in HT-29 cells. Cells were seeded in six well/plate (150,000 cells/well) in RPMI 1640 medium with 10% fetal calf serum. They were allowed to attach for 72 h, after which seeding medium was removed and replaced by experimental medium. After 0.08, 0.25, 1 and 24 h of exposure to different concentrations (86 and 516 nM) of the above compounds, cells were washed, trypsinized and resuspended in PBS. Cells were kept in the dark until flow cytometry analysis. Fluorescence profiles of doxorubicin stained cell suspensions were generated on the FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). Results are expressed as mean fluorescence intensity (MFI). Laser excitation was at 488 nm and the doxorubicin fluorescence was recorded through a 575 nm wavelength. Typically, 10,000 events were assayed for each sample. Experiment was carried out in triplicate.

2.6. Statistical analyses

Data were expressed as means of three separate experiments, and were compared by analysis of variance (ANOVA). A p-value <0.05 was considered statistically significant in all cases.

3. Results

3.1. Characterization of solid lipid nanoparticles

The average diameter of chol-but SLN was about 130 nm and the polydispersity index was 0.2.

The average diameter of doxorubicin-loaded SLN was about 80 nm, the polydispersity index was 0.2 and the zeta potential −35 mV, while the average diameter of paclitaxel-loaded SLN was about 160 nm, the polydispersity index was 0.2 and the zeta potential −31 mV.

3.2. Cytotoxicity of solid lipid nanoparticles

Fig. 1 shows the percentage survival of HT-29 cells after exposure to butyrate, either as sodium salt or in SLN. None of the concentrations of sodium butyrate (Na-but) that were used caused 50% inhibition of cell growth at 72 h exposure. Indeed, HT-29 cells were more sensitive to chol-but SLN than to Na-but, 50% inhibition of cell growth being achieved with 0.3 mM of chol-but SLN at 24 h exposure; the cytotoxicity did not increase with exposure time (Table 2)

Cholesterol, equimolarly present in chol-but, did not affect cell growth at the concentration tested in all cell lines (data not shown).

The amount of doxorubicin required to achieve the IC_{50} value was lower with loaded SLN than with free drug and the cytotoxicity was time dependent (Table 2). The IC_{50} of free doxorubicin and SLN-borne doxorubicin were respectively 126.75 ± 0.72 nM and 81.87 ± 4.11 nM at 72 h exposure (Fig. 2).

Fig. 3 reports the survival curves of HT-29 cells after 72 h exposure to paclitaxel, free and SLN-borne. The two formulations of paclitaxel tested showed a similar dose-dependent inhibitory activity, which caused a strong decrease of cell growth after 24 h exposure. In Table 2 the IC_{50} of free paclitaxel and paclitaxel-loaded SLN in HT-29 cells are reported.

The different lipid matrices of doxorubicin and paclitaxel-loaded SLN, namely stearic acid and tripalmitin, were both well tolerated; no cytotoxicity of the unloaded SLN being observed on HT-29 cell line at the highest SLN concentration used, as cell viability remained constant.

Table 2
IC_{50} values obtained in the HT-29 cell line depending on the drug formulation used. IC_{50} values were evaluated after 24, 48 and 72 h exposure of cells to butyric acid (sodium salt and chol-but SLN), doxorubicin (free and loaded SLN) and paclitaxel (free and loaded SLN). The results are mean values ± SD of three independent experiments performed in triplicate

Drug formulation	IC_{50} (mean ± SD)		
	24 h	48 h	72 h
Na-but	>0.6 mM	>0.6 mM	>0.6 mM
Chol-but SLN	0.29 ± 0.03* mM	0.30 ± 0.04* mM	0.30 ± 0.03* mM
Doxorubicin	>172 nM	>172 nM	126.57 ± 0.72 nM
Doxorubicin-loaded SLN	130.10 ± 16.98° nM	101.57 ± 9.01° nM	81.87 ± 4.11° nM
Paclitaxel	71.40 ± 1.37 nM	41.00 ± 5.50 nM	33.43 ± 1.66 nM
Paclitaxel-loaded SLN	81.46 ± 0.26 nM	47.16 ± 1.87 nM	37.36 ± 6.41 nM

*$P < 0.05$ (chol-but SLN vs Na-but), °$P < 0.05$ (doxorubicin-loaded SLN vs doxorubicin).

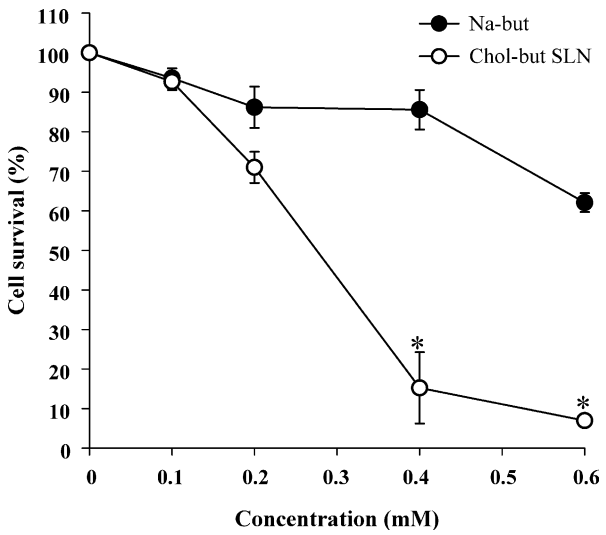

Fig. 1. Cytotoxicity of Na-but (●) and chol-but SLN (○) in HT-29 cells after 72 h exposure. The results are mean values ± SD of three independent experiments performed in triplicate. *$P < 0.05$ (chol-but SLN vs Na-but).

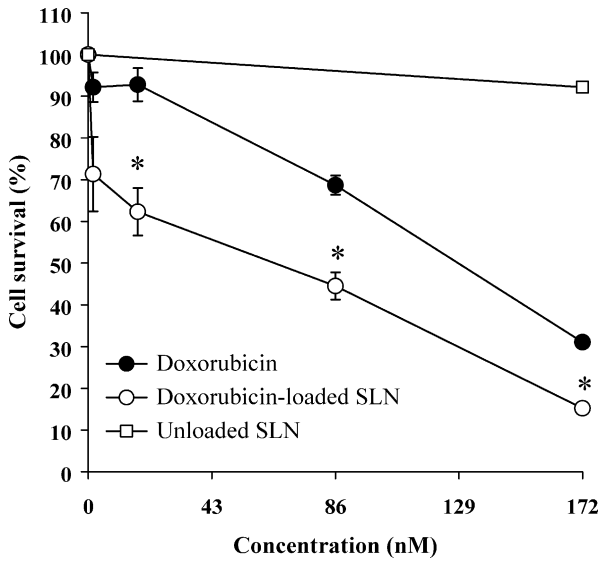

Fig. 2. Cytotoxicity of doxorubicin (●) doxorubicin-loaded SLN (○) and unloaded SLN (□) in HT-29 cells after 72 h exposure. The results are mean values ± SD of three independent experiments performed in triplicate. *$P < 0.05$ (doxorubicin-loaded SLN vs doxorubicin).

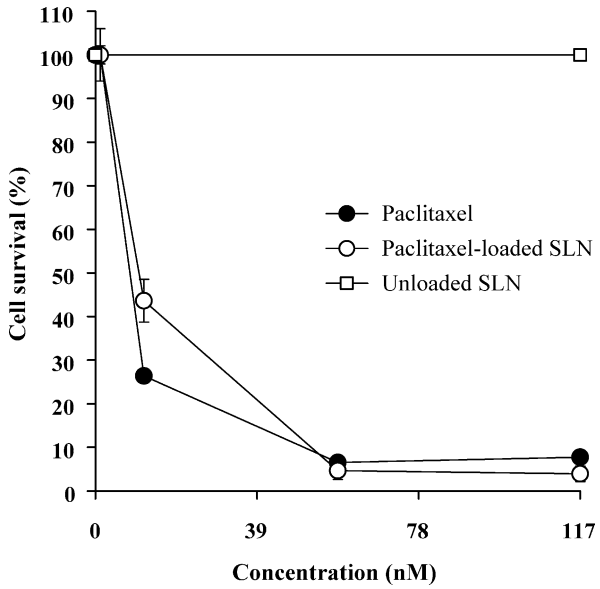

Fig. 3. Cytotoxicity of paclitaxel (●), paclitaxel-loaded SLN (○) and unloaded SLN (□) in HT-29 cells after 72 h exposure. The results are mean values ± SD of three independent experiments performed in triplicate. *$P < 0.05$ (paclitaxel-loaded SLN vs paclitaxel).

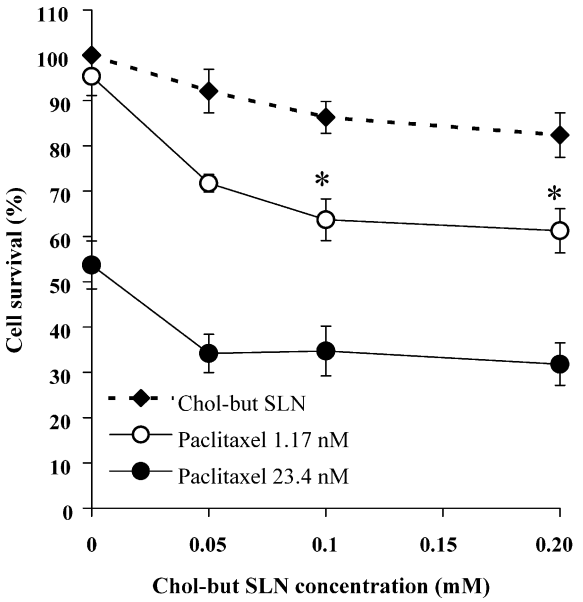

Fig. 4. Cytotoxicity of chol-but SLN (◆), doxorubicin 1.72 nM (○) and 34.4 nM (●) in HT-29 cells after 24 h exposure to the drugs and their combination. The results are mean values ± SD of three independent experiments performed in triplicate. *$P < 0.05$ (chol-but SLN and doxorubicin combination vs doxorubicin).

3.3. Combination of cholesteryl butyrate solid lipid nanoparticles with free doxorubicin and free paclitaxel

HT-29 cells were cultured with different concentrations of chol-but SLN (0.05, 0.1 and 0.2 mM) in combination with different concentrations of doxorubicin (1.72 and 34.4 nM). The effect was greater than additive when chol-but SLN 0.1 and 0.2 mM were used with 1.72 nM doxorubicin at 24 and 48 h of exposure. The best combination in terms of low drug concentrations and greater-than-additive effect was chol-but SLN at 0.1 mM and doxorubicin at 1.72 nM at 24 h exposure (Fig. 4). The same treatment schedule was applied with Na-but and doxorubicin, but it did not determine a greater-than-additive effect (data not shown).

HT-29 cells were also cultured with different concentrations of chol-but SLN (0.05, 0.1 and 0.2 mM) in combination with different concentrations of paclitaxel (1.17 and 23.4 nM). The effect was greater than additive when chol-but SLN 0.05, 0.1 and 0.2 mM were used with 1.17 nM paclitaxel at 24, 48 and 72 h exposure, and with 23.4 nM paclitaxel at 24 h exposure. The best combination in terms of low drug concentrations and greater-than-additive effect was chol-but SLN 0.05 mM and paclitaxel at 1.17 nM at 24 h exposure (Fig. 5). The same treatment schedule was applied with Na-but and paclitaxel but did not determine a greater-than-additive effect (data not shown).

3.4. Cellular uptake of free doxorubicin and doxorubicin-loaded solid lipid nanoparticles

Doxorubicin accumulation, as measured by flow cytometry, was used to compare the cellular uptake of free doxorubicin and doxorubicin-loaded SLN in HT-29 cells.

Fig. 6 shows the MFI of doxorubicin stained HT-29 cells after incubation with two different concentrations of free doxorubicin and doxorubicin-loaded SLN at 0.08, 0.25, 1 and 24 h exposure. HT-29 cells incubated with free doxorubicin and doxorubicin-loaded SLN accumulated the cytotoxic drug, and a single predominant population was present, suggesting that HT-29 cells are a functionally homogeneous cell population with regard to doxorubicin uptake. HT-29 cells exposed to the lowest concentration tested (86 nM) accumulated the drug after 24 h exposure, and the difference in uptake between commercial

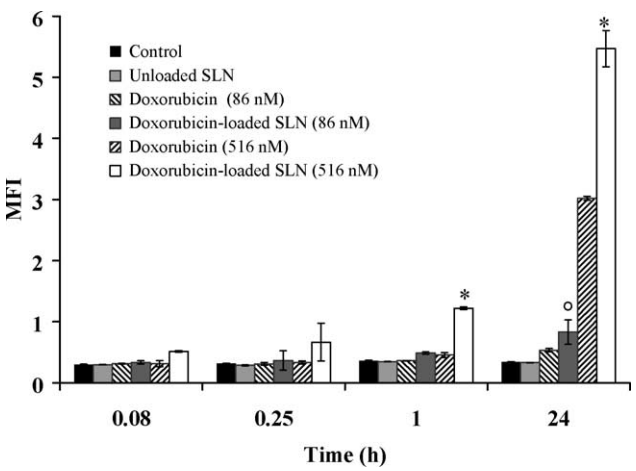

Fig. 5. Cytotoxicity of chol-but SLN (◆), paclitaxel 1.17 nM (○) or 23.4 nM (●) in HT-29 cells after 24 h exposure to the drugs and their combination. The results are mean values ± SD of three independent experiments performed in triplicate. *$P < 0.05$ (chol-but SLN and paclitaxel combination vs paclitaxel).

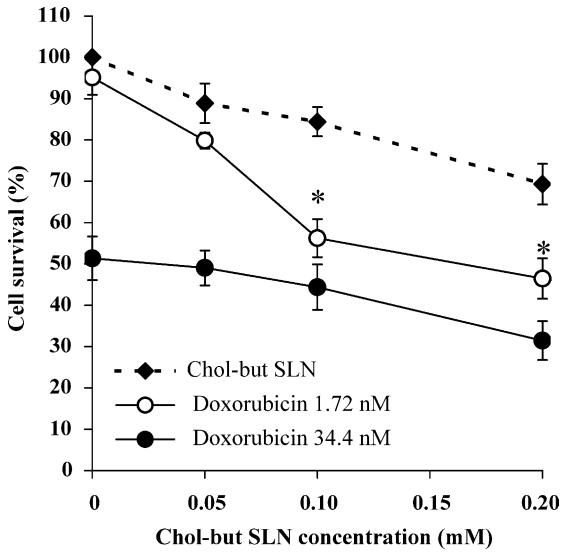

Fig. 6. Doxorubicin uptake of free and loaded SLN drug by HT-29 cells. The amount of drug taken up by the cells was measured by flow cytometry and expressed as mean fluorescence intensity (MFI). The results are mean values ± SD of an experiment performed in triplicate. °$P < 0.05$ (doxorubicin-loaded SLN 86 nM vs doxorubicin 86 nM); *$P < 0.05$ (doxorubicin-loaded SLN 172 nM vs doxorubicin 172 nM).

formulation and SLN-borne doxorubicin was significant. The fluorescence inside the cells was already marked after 1 h incubation with the highest concentration tested (516 nM), and doxorubicin accumulation from loaded SLN was twofold that from the commercial formulation.

4. Discussion

In most cases, chemotherapic treatment of tumors is limited by the low therapeutic index of the anticancer drugs in current use. A significant pharmacokinetic problem is to determine the reason for low anticancer activity and/or severe side effects. The drugs are not specific against tumor cells, thus they accumulate not only in tumors but also in healthy tissues. It is clear that new approaches that can deliver drugs more specifically and produce less toxicity are required [31]. Some studies have shown that polymeric nanoparticle-bound anti-tumor agents prolong drug retention in tumors, reduce tumor growth and increase survival of tumor-bearing animals [32–34]. In order to evaluate the anti-tumoral activity of chol-but as a butyric acid pro-drug, as well as of doxorubicin and paclitaxel, on colorectal cancer cell proliferation, we studied the inhibitory effects of SLN versus free drug on HT-29 cell growth. In previous work we showed that chol-but SLN penetrate quickly into cancer cells in vitro and proposed that butyric acid could be released from chol-but, and that its efficacy might realistically be higher than that of butyric acid, because it accumulates within cells [20]. HT-29 cells were more sensitive to chol-but SLN than Na-but, showing that chol-but SLN may be an alternative approach to delivering butyric acid to colorectal cancer cells (Table 2). Chol-but SLN efficacy has been demonstrated on different tumor cell lines (non-small-cell lung carcinoma and melanoma cells) [20,25]. Several in vivo studies have shown only partial anti-tumor activity for butyrate, which is probably due to the impossibility of achieving biologically relevant plasma drug concentrations and to the rapid clearance rate. For these reasons, many butyric acid derivatives and precursors have been prepared and screened [35–37].

Improved efficacy was also achieved with doxorubicin-loaded SLN, the amount of doxorubicin required to achieve 50% HT-29 cell growth inhibition at 72 h exposure was significantly lower with loaded SLN than with commercial formulation (Table 2). In previous research we showed that doxorubicin-loaded SLN increase the drug's cytotoxicity against the human breast carcinoma cell line, MCF-7, and against the human promyelocitic leukaemia cell line, HL-60 [26]. The increased cytotoxicity of doxorubicin when incorporated in SLN may be related to the fast internalization of doxorubicin-loaded SLN, followed by the drug's release from SLN inside the cells, enhancing its action. Our in vitro results show a time- and dose-dependent accumulation of both free drug and loaded SLN. Doxorubicin accumulation in HT-29 cells incubated with doxorubicin-loaded SLN was significantly higher than with free drug (Fig. 6). We also observed important effects of doxorubicin incorporated in SLN in vivo; we showed that doxorubicin-loaded SLN have a higher AUC and half-life and a lower clearance and volume of distribution, than the commercial formulation [23].

At each time of exposure the same dose-dependent inhibition of HT-29 cell growth was observed with the two different formulations of paclitaxel tested (Table 1). Data reported in the literature suggest that, in the case of the commercial formulation, a significant inhibitory effect is due to the diluent CrEL, whereas for paclitaxel-loaded SLN the cytotoxicity is due only to the amount of paclitaxel released (since SLN are non-toxic). In previous research we observed the same cytotoxic activity on HL-60 cells, whereas on MCF-7 cells the cytotoxic effect of paclitaxel-loaded SLN was significantly higher versus the commercial formulation [26].

Some in vitro and in vivo data have recently been published on polymeric and protein-stabilized nanoparticles as carriers of paclitaxel [32]. Nevertheless, to our knowledge the incorporation of paclitaxel into solid lipid nanoparticles, and the effect on in vitro HT-29 cell culture, have not been reported.

The combination of HDIs and conventional antineoplastic drugs has been suggested as a potential new approach in cancer treatment [27]. We therefore, assayed the cytotoxic effect of chol-but SLN in association with paclitaxel or doxorubicin on HT-29 cells. The cytotoxic effect increased in a greater-than-additive manner when 0.1 and 0.2 mM chol-but SLN were combined with the lowest concentration of doxorubicin tested (Fig. 4). Butyrate induction of histone hyperacetylation may facilitate DNA breakage by DNAse-I,

which is functionally and antigenically indistinguishable from the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis. Histone hyperacetylation loosens the chromatin structure, and this may facilitate the accessibility of intercalating agents such as doxorubicin to nucleosomal DNA, as has been proposed for the additive effect of Na-but and daunorubicin [38].

The association of low concentrations of chol-but and paclitaxel also determined a greater-than-additive effect (Fig. 5). The same treatment schedule was applied with Na-but and free doxorubicin or paclitaxel, but none of the associations determined a greater-than-additive effect.

HDIs can reactivate gene expression and inhibit growth and survival of tumor cells. The remarkable tumor-specificity of the compounds, and thus their potency in vitro and in vivo, underscores the potential of HDAC inhibitors as exciting new agents for cancer treatment.

In conclusion, the results of this study show that the SLN formulation of anticancer drugs can improve the cytotoxic activity of butyrate and doxorubicin in a colorectal cancer cell model, with more than additive effects being produced by combinations of chol-but SLN with doxorubicin and paclitaxel. Further studies are in progress to evaluate the possible toxicological effects of these new formulations in animal models.

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References

[1] I. Brigger, C. Dubernet, P. Couvreur, Nanoparticles in cancer therapy and diagnosis, Adv. Drug. Del. Rev. 54 (2002) 631–651.
[2] M.N.V. Ravi Kumar, Nano and microparticles as controlled drug delivery devices, J. Pharm. Pharmaceut. Sci. 3 (2) (2000) 234–258.
[3] M.R. Gasco, Solid lipid nanospheres from warm micro-emulsions, Pharm. Technol. Eur. 9 (11) (1997) 52–58.
[4] R. Cavalli, A. Bargoni, V. Podio, E. Muntoni, G.P. Zara, M.R. Gasco, Duodenal administration of solid lipid nanoparticles loaded with different percentages of tobramycin, J. Pharm. Sci. 92 (5) (2003) 1085–1094.
[5] M.R. Gasco, Solid lipid nanoparticles for drug delivery, Pharm. Technol. Eur. 13 (2001) 32–41.
[6] V. Santini, A. Gozzini, B. Scappini, A. Grossi, P. Rossi Ferrini, Searching for the magic bullet against cancer: the butyrate saga, Leuk. Lymph. 42 (3) (2001) 275–289.
[7] A. McIntyre, P.R. Gibson, G.P. Young, Butyrate production from dietary fibre and protection against large bowel cancer in a rat model, Gut 34 (3) (1993) 386–391.
[8] J.A. Barnard, G. Warwick, rapidly induces growth inhibition and differentiation in HT-29 cells, Cell. Growth Differ. 4 (6) (1993) 495–501.
[9] S.Y. Archer, S. Meng, A. Shei, R.A. Hodin, $p21^{WAF1}$ is required for butyrate mediated growth inhibition of human colon cancer cells, Proc. Natl Acad. Sci. 95 (1998) 6791–6796.
[10] H. Miyake, S. Hara, S. Arakawa, S. Kamidono, I. Hara, Overexpression of Bcl-2 regulates sodium butyrate- and/or docetaxel-induced apoptosis in human bladder cancer cells both in vitro and in vivo, Int. J. Cancer 93 (1) (2001) 26–32.
[11] P.R. Pouillart, Role of butyric acid and its derivatives in the treatment of colorectal cancer and hemoglobinopathies, Life Sci. 63 (20) (1998) 1739–1760.
[12] D.J. Booser, G.N. Hortobagyi, Anthracycline antibiotics in cancer therapy: focus on drug resistance, Drugs 47 (1994) 223–258.
[13] D. Nielsen, C. Maare, T. Skovsgaard, Cellular resistance to anthracyclines, Gen. Pharmacol. 27 (1996) 251–255.
[14] G. Batist, G. Ramakrishnan, C.S. Rao, A. Chandrasekharan, J. Gutheil, T. Guthrie, P. Shah, A. Khojasteh, M.K. Nair, K. Hoelzer, K. Tkaczuk, Y.C. Park, L.W. Lee, Cardiotoxicity and preserved antitumour efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer, J. Clin. Oncol. 19 (5) (2001) 1444–1454.
[15] C.M. Spencer, D. Faulds, Paclitaxel. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in the treatment of cancer, Drug 48 (1994) 794–847.
[16] A.K. Singla, A. Garg, D. Aggarwal, Paclitaxel and its formulations, Int. J. Pharm. 235 (1–2) (2002) 179–192.
[17] S. Horwitz, Mechanism of action of taxol, Trend Pharmachol. Sci. 13 (1992) 134–135.
[18] R.S. Finley, Patient care issue: the management of paclitaxel-related toxicities, Ann. Pharmacother. 28 (1994) S27–S30.
[19] X. Zhang, H.M. Burt, D. Von Hoff, D. Dexter, G. Mangold, D. Degen, A.M. Oktaba, W.L. Hunter, An investigation of the antitumour activity and biodistribution of polymeric micellar paclitaxel, Cancer Chemother. Pharmacol. 40 (1997) 81–86.
[20] C. Pellizzaro, D. Coradini, S. Morel, E. Ugazio, M.R. Gasco, M.G. Daidone, Cholesteryl butyrate in solid lipid nanoparticles as an alternative approach for butyric acid delivery, Anticancer Res. 19 (5B)) (1999) 3921–3926.
[21] R. Cavalli, O. Caputo, M.R. Gasco, Solid lipospheres of doxorubicin and idarubicin, Int. J. Pharm. 89 (1993) 9–12.
[22] R. Cavalli, O. Caputo, M.R. Gasco, Preparation and characterization of solid lipid nanoparticles incorporating paclitaxel, Eur. J. Pharm. Sci. 10 (2000) 305–309.
[23] A. Fundarò, R. Cavalli, A. Bargoni, D. Vighetto, G.P. Zara, M.R. Gasco, Stealth and non-stealth solid lipid nanoparticles (SLN) carrying doxorubicin: Pharmacokinetics and tissue distribution after I.V. administration to rats, Pharmacol. Res. 42 (2000) 337–343.
[24] G.P. Zara, R. Cavalli, A. Bargoni, A. Fundarò, D. Vighetto, M.R. Gasco, Intravenous administration to rabbits of non-stealth and stealth doxorubicin-loaded solid lipid nanoparticles at increasing concentration of stealth agent: pharmacokinetics and distribution in brain and other tissues, J. Drug. Target. 10 (2002) 327–335.
[25] B. Salomone, R. Ponti, M.R. Gasco, E. Ugazio, P. Quaglino, S. Osella-Abate, M.G. Bernengo, In vitro effects of cholesteryl butyrate solid lipid nanoparticles as a butyric acid pro-drug on melanoma cells: evaluation of antiproliferative activity and apoptosis induction, Clin. Exp. Metastasis 18 (8) (2001) 663–673.
[26] A. Miglietta, R. Cavalli, C. Bocca, L. Gabriel, M.R. Gasco, Cellular uptake and cytotoxicity of solid lipid nanospheres (SLN) incorporating doxorubicin or paclitaxel, Int. J. Pharm. 210 (2000) 61–67.
[27] R.W. Johnstone, Histone-deacetylase inhibitors: novel drugs for the treatment of cancer, Nat. Rev. 1 (2002) 287–299.
[28] D.A. Brown, T. Malkin, G.K. Maliphant, An X-ray examination of long-chain alkyldihydrogen phosphates and dialkylhydrogen phosphates and their sodium salts, J. Chem. Soc. (1955) 1584–1588.
[29] I.W. Duncan, P.H. Culbreth, C.A. Burtis, Determination of free, total and esterified cholesterol by high-performance liquid chromatography, J. Chromatogr. 162 (3) (1979) 281–292.
[30] D.E. Koppel, Analysis of macromolecular polydispersity in intensity correlation spectroscopy the method of cumulants, J. Chem. Phys. 57 (1972) 4814–4818.

[31] U. Massing, S. Fuxius, Liposomal formulations of anticancer drugs. Selectivity and effectiveness,, Drug Resist. Updat. 3 (3) (2000) 171–177.
[32] L. Mu, S.S. Feng, A novel controlled release formulation for the anticancer drug paclitaxel (Taxol®): PGLA nanoparticles containing vitamin E TPGS, J. Controll. Rel. 86 (2003) 33–48.
[33] P. Beck, J. Kreuter, R. Reszka, I. Fichtner, Influence of polybutylcyanoacrylate nanoparticles and liposomes on the efficacy and toxicity of the anticancer drug mitoxantrone in murine tumour models, J. Microencapsul. 10 (1993) 101–114.
[34] C. Verdun, F. Brasseur, H. Vranokx, P. Couvreur, M. Roland, Tissue distribution of doxorubicin associated with polyisohexylcyanoacrylate nonoparticles, Cancer Chemother. Pharmacol. 26 (1990) 13–18.
[35] R. Feiman, O.K. Clarke, L.E. Harrison, Phenylbutyrate-induced apoptosis is associated with inactivation of NF-$_K$B in HT-29 colon cancer cells, Cancer Chemother. Pharmacol. 49 (1) (2002) 27–34.
[36] A. Patnaik, E.K. Rowinsky, M.A. Villalona, L.A. Hammond, C.D. Britten, L.L. Siu, A. Goetz, S.A. Felton, S. Burton, F.H. Valone, S.G. Eckhardt, A phase I study of pivaloyloxymethyl butyrate, a prodrug of the differentiating agent butyric acid, in patients with advanced solid malignancies, Clin. Cancer Res. 8 (7) (2002) 2142–2148.
[37] A. Batova, L.E. Shao, M.B. Diccianni, A.L. Yu, T. Tanaka, A. Rephaeli, A. Nudelman, J. Yu, The histone deacetylase inhibitor AN-9 has selective toxicity to acute leukemia and drug-resistant primary leukemia and cancer cell lines, Blood 100 (9) (2002) 3319–3324.
[38] E. Rabizadeh, O. Bairey, A. Aviram, I. Ben-Dror, M. Shaklai, Y. Zimra, Doxorubicin and a butyric acid derivative effectively reduce levels of Bcl-2 protein in the cells of chronic lymphocytic leukemia patient, Eur. J. Haematol. 66 (2001) 263–271.