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Cellular uptake and cytotoxicity of solid lipid nanospheres (SLN) incorporating doxorubicin or paclitaxel

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Abstract

Solid Lipid Nanospheres (SLN) are colloidal therapeutic systems proposed for several administration routes and obtained by dispersing warm microemulsions in cold water. SLN as carriers of doxorubicin and paclitaxel have been previously studied. In this study, the cellular uptake of SLN and the cytotoxicity of doxorubicin and paclitaxel incorporated into SLN were investigated on two cell-lines, human promyelocytic leukemia (HL60) and human breast carcinoma (MCF-7). Cellular uptake of SLN was determined by incorporating 6-coumarin as fluorescent marker. The cellular uptake of fluorescent SLN was clearly evidenced by fluorescence microscopy. The cytotoxicity of doxorubicin incorporated in SLN was higher compared to the conventional doxorubicin solution, even at the lower concentrations. Paclitaxel in SLN was about 100-fold more effective than free paclitaxel on MCF-7 cells, while on HL60 cells a lower sensitivity was achieved with paclitaxel in SLN. Unloaded SLN had no cytotoxic effect on HL60 and MCF-7 cells.

Keywords: Doxorubicin-loaded solid lipid nanospheres; Paclitaxel-loaded solid lipid nanospheres; Cellular uptake; Cytotoxicity

1. Introduction

Solid Lipid Nanoparticles (SLN) have been prepared as colloidal therapeutic carriers by many researchers (Schwartz et al., 1992; Westesen et al., 1993; Domb, 1995; Yang et al., 1999); we prepared SLN by dispersing warm oil-in-water (o/w) microemulsions in cold water. This preparation method is simple and allows fairly high drug incorporation depending on the molecule employed; many lipophilic and hydrophilic drugs (including some peptides) have been incorporated into SLN using different approaches (Gasco, 1997).

In previous research (Cavalli et al., 1993, 2000) two different anticancer agents, doxorubicin (an anthracycline antibiotic) and paclitaxel (a diterpenoid derivative) were introduced into two different SLN formulations. The paclitaxel-loaded SLN were constituted mainly of tripalmitin and

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phosphatidylcholine and incorporated about 3% w/w of drug. The doxorubicin-loaded SLN were constituted mainly of stearic acid and phosphatidylcholine and had a higher percentage of drug incorporated as ion-pair, (about 7% w/w). Doxorubicin-loaded SLN were administered intravenously to rats (Zara et al., 1999; Fundarò et al., 2000) in two different animal models.

The activity of doxorubicin and paclitaxel against a variety of tumour types is well known and their efficacy has been measured in several human tumour cell-lines. Doxorubicin is an intercalating drug that stacks between paired bases in DNA. Cellular resistence mechanisms reduce the amount of DNA bound drugs; they include membrane transport mechanisms such as the P170 glycoprotein or others (Ling, 1992) as well as intracellular vescicular trapping.

Paclitaxel has a different mechanism of action. It promotes polymerization of tubulin dimers to form microtubules and stabilizes microtubules by preventing depolymerization (Kumar, 1981).

In the present study the behaviour of the two types of SLN, carrying doxorubicin or paclitaxel, was evaluated using two different tumour cell-lines: human breast carcinoma cells (MCF-7) and human promyelocitic leukemia cells (HL60). The main aim was to investigate the cytotoxic effect of the drug incorporated in SLN compared to free drug solutions. The cytotoxicity of the two types of SLN not carrying any drug was also evaluated on the two cell-lines. The cellular uptake of the two SLN was studied using fluorescent SLN containing 6-coumarin as fluorescent marker.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride was a kind gift from Farmitalia (Milan, I), Paclitaxel was a kind gift from Indena (Milan, I). Stearic acid, butanol and tripalmitin were from Fluka (Buchs, CH); cholesteryl hemisuccinate and phosphate buffer saline tablets were from Sigma Chem. Co. (Milan, I); Epikuron 200 (soya phosphatidylcholine 95%) was a kind gift from Lucas Meyer (Hamburg, D); taurocholate sodium salt was a kind gift from PCA (Basaluzzo, I). 6-coumarin was from Acros (Carlo Erba, Milan, I). RPMI 1640 medium, DMEM, streptomycin and foetal bovine serum (FBS) were from Gibco, Life Technologies. Sodium hexadecylphosphate was prepared as indicated by Brown (Brown et al., 1955). The other chemicals were of analytical grade.

2.2. Preparation of doxorubicin-loaded and paclitaxel-loaded solid lipid nanospheres

To obtain doxorubicin-loaded SLN, a warm oil-in-water (o/w) microemulsion was prepared using stearic acid (0.70 mmol) as internal phase, Epikuron 200 (0.20 mmol) as surfactant, sodium taurocholate (0.68 mmol) as cosurfactant and filtered water (111.11 mmol) 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 (2.48 mmol) as internal phase, Epikuron 200 (2.46 mmol) as surfactant, cholesteryl hemisuccinate (2.14 mmol), butanol (3.50 mmol) and taurocholate (1.14 mmol) as cosurfactants, and distilled filtered water (111.11 mmol) as continuous phase.

Paclitaxel was added to the melted tripalmitin; Epikuron 200, cosurfactants and filtered water were then added obtaining a clear microemulsion at about 70°C.

SLN, either with doxorubicin or paclitaxel, were obtained by dispersing the warm o/w microemulsion (about 70°C) in cold water (about 2°C) under mechanical stirring at 1:10 ratio (microemulsion:water, v/v).

The SLN dispersions were washed three times with distilled water by diafiltration (TCF2-Amicon, Danvers, USA) using a Diaflo YM100 membrane (cut off 100 000 Dalton).

The SLN dispersions were then diluted with filtered water to obtain the selected drug concentration (1 or 10 μM).

To determine the amount of the incorporated drug, the SLN dispersions were freeze-dried using a Modulyo freeze-dryer (Edwards, Crawley, UK).

Unloaded SLN for each formulation, i.e. SLN not carrying either drug, were also prepared as described above.

2.3. Preparation of fluorescent solid lipid nanospheres

Fluorescent SLN were prepared adding 3.4×10^{-3} mmol of 6-coumarin (0.04% calculated on the whole microemulsion) to the internal phase of the two microemulsions and maintaining the other components of the formulations fixed. The fluorescent SLN were obtained as described above for drug-loaded SLN.

2.4. Characterization of solid lipid nanospheres

2.4.1. Determination of the sizes of solid lipid nanospheres

All SLN were characterised by photon correlation spectroscopy (PCS) using a 90 PLUS instrument (Brookhaven Instrument Corporation, USA) at a fixed angle of 90°C and at a temperature of 25°C. The SLN water dispersions were diluted with filtered water before analysis. Each value reported is the average of six measurements. The average diameter and the polydispersity index of SLN were determined. The polydispersity index measures the size distribution of the nanosphere population (Koppel, 1972).

2.4.2. Determination of the zeta potential of solid lipid nanospheres

The electrophoretic mobility and the zeta potential were measured using a 90 PLUS instrument (Brookhaven Instrument Corporation, USA).

To determine the zeta potential, SLN samples were diluted with distilled water and placed in the electrophoretic cell, where an electric field of 15.24 V/cm was applied. Each sample was analyzed in triplicate.

2.4.3. Determination of the amount of drug or fluorescent agent incorporated in SLN

The percentage of doxorubicin and paclitaxel in the SLN were determined by reverse phase HPLC methods (Rose et al., 1988; Sharma et al., 1994). The percentage of 6-coumarin in the SLN was determined spectrofluorimetrically.

The amount of paclitaxel or doxorubicin or 6-coumarin incorporated into SLN was determined on a weighed amount of freeze-dried SLN dissolved in methanol.

2.5. Cell cultures and viability assay

HL60 cells were grown as a suspension culture in RPMI 1640 medium; MCF-7 cells were grown in monolayer in Dulbecco's modified Eagle's medium (DMEM). Both cells lines were cultured in media supplemented with 10% heat-inactivated foetal bovine serum and antibiotics at 37°C in a humidified atmosphere containing 5% CO_2.

Exponentially growing cells were seeded in 12-well plates and treated for 72 h with various concentration of doxorubicin (in solution or SLN) and for 48 h with various concentrations of paclitaxel (in solution or SLN). The concentrations of paclitaxel and doxorubicin varied from 1 to 100 ng/ml respectively for SLN and solution.

Unloaded SLN were also evaluated on the two cell-lines 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 the experiments were performed in triplicate.

Cell viability was assessed by trypan blue dye exclusion assay. Cytotoxicity was expressed as percentage of control (unloaded SLN). The IC_{50} value was defined as the drug concentration required to inhibit growth by 50% relative to controls.

2.6. Fluorescence microscopy

The cellular uptake of fluorescent SLN was studied on MCF-7 cells using fluorescence microscopy. MCF-7 cells were grown on coverslips for 24 h in a wet box at 37°C and further incubated for 1 or 24 h with the two types of fluores-

cent SLN at a concentration of 2 mg/ml employed in the viability experiments. After rinsing with 0.01 M phosphate-buffered saline (PBS), stained coverslips were mounted on a glass slide and photographed using a microscope equipped with epifluorescence optics.

3. Results and discussion

3.1. Cytotoxicity of unloaded SLN

No cytotoxicity of the two unloaded SLN was observed on either the cell-lines studied even at the highest SLN concentration (corresponding to 1 and 2.5 mg/ml respectively) as the cell viability did not decrease. The two different lipid matrices of SLN were both well tolerated. Phosphatidylcholine, a natural bioacceptable surfactant, is principally located on the SLN surface.

Previously Müller and coworkers (Müller et al., 1997; Müller and Olbrich, 1999) have studied the cytotoxicity of unloaded SLN (constituted of triglycerides and containing soya lecithin, poloxamers and poloxamine as surfactants) in comparison to polymeric (polyalkylcyanoacrylate and polylactic/polyglycolic acid) nanoparticles using HL60 cell line differentiated to granulocytes. They observed that the cytotoxicity of SLN was lower compared to polymeric nanoparticles; in particular SLN containing lecithin had no cytotoxic effect, allowing their use as colloidal drug carriers in vivo. They also showed that the nature of the lipid matrix had no effect on cell viability.

3.2. Cellular uptake of fluorescent SLN

Fluorescent SLN had an average diameter of about 70 nm, a polydispersity index of 0.2 and a zeta potential of -32 mV.

Fig. 1 shows the internalization of the two types of fluorescent SLN in MCF-7 cells following 24 h treatment.

The cellular uptake of fluorescent SLN was clearly evidenced by fluorescence microscopy.

The fluorescence inside the cells was already marked after 1 h of incubation; nanosphere accumulation was time-dependent since fluorescence increased in the cells after 24 h of exposure. No difference was shown between the two fluorescent SLN formulations.

The rapid internalization of fluorescent SLN could be related to their size and composition; they are chiefly constituted of bioacceptable and biodegradable substances, solid lipids and phosphatidylcholine.

3.3. Cytotoxicity of doxorubicin-loaded SLN

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

Fig. 2 shows the survival curves of HL60 cells after exposure to doxorubicin, either in solution or in SLN. None of the concentrations of doxorubicin in solution that were used caused 50% inhibition of cell growth after 72 h exposure, whereas doxorubicin in SLN strongly enhanced cytotoxic-

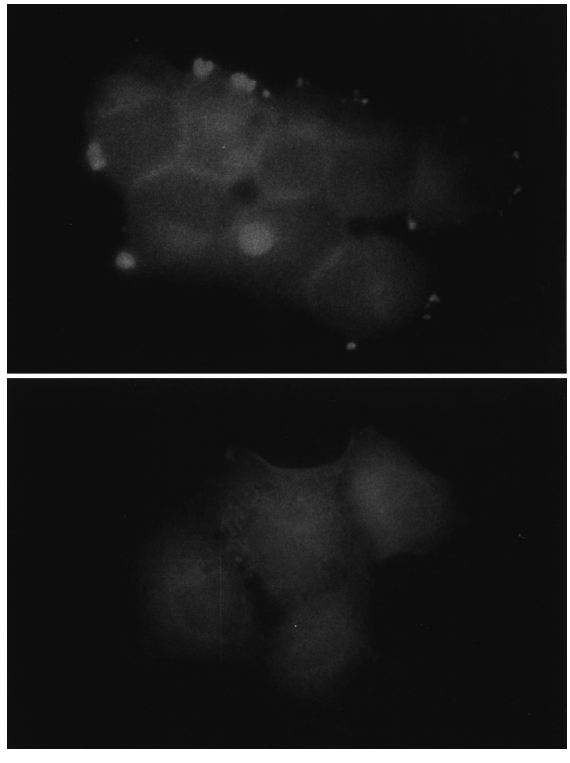

Fig. 1. MCF-7 cells 24 h after incubation with fluorescent SLN. Upper part: doxorubicin formulation. Lower part: paclitaxel formulation.

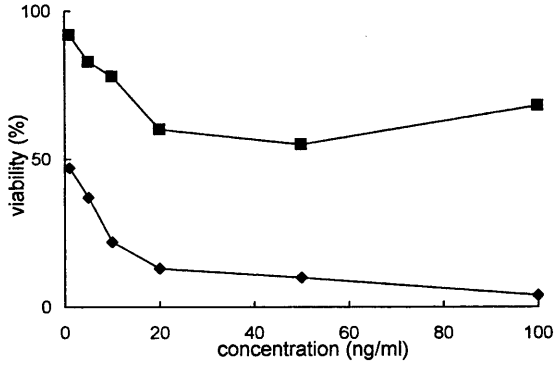

Fig. 2. Survival curves of (HL60) cells after exposure to doxorubicin in solution■ and doxorubicin in SLN◆.

ity, with values ranging from about 50% at 1 ng/ml to nearly 100% at 100 ng/ml. Thus, a strong sensitization was achieved with doxorubicin-loaded SLN, which appear to decrease cell resistance to the drug.

The activity of doxorubicin on leukemia cells is known and anthracyclines are widely used in hematological oncology as they are rapidly distributed in myeloid and lymphatic leukemia cells (Gieseler et al., 1998).

The MCF-7 cells were more sensitive than the HL60 cells to doxorubicin in solution; indeed, 50% inhibition of growth was achieved with about 10 ng/ml of doxorubicin, and the cytotoxicity increased on increasing the concentration, as shown in Fig. 3. MCF-7 cells were more sensitive to doxorubicin-loaded SLN than to doxorubicin solution and 50% growth inhibition was achieved

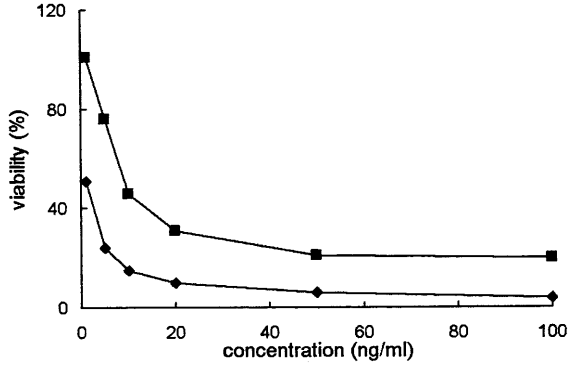

Fig. 3. Survival curves of MCF-7 cells after exposure to doxorubicin in solution■and doxorubicin in SLN◆.

Table 1
Inhibitory concentration of doxorubicin and paclitaxel producing 50% of cell inhibition or dead

Drug formulation[a]	IC_{50} (HL60 cells) (ng/ml)	IC_{50} (MCF-7 cells) (ng/ml)
Doxorubicin solution	45.5	10.9
Doxorubicin-loaded SLN	0.9	1.0
Paclitaxel solution	11.1	102.0
Paclitaxel-loaded SLN	16.7	1.16

[a] The two formulations of unloaded SLN did not decrease the viability of the two cell lines.

with a doxorubicin concentration of about 1 ng/ml.

Table 1 reports the growth inhibitory concentrations of doxorubicin-loaded SLN and of doxorubicin solution on the two cell-lines. The amount of doxorubicin required to achieve 50% of growth inhibition (IC_{50}) was much lower in SLN than in solution. The SLN enhanced the cytotoxicity of doxorubicin about 10-fold on MCF-7 cell and more than 40-fold on HL60 compared with the doxorubicin solution.

Many authors studied the incorporation of doxorubicin in colloidal carriers. The increase of doxorubicin cytotoxicity compared to the solution has already been observed with polymeric nanoparticles, micelles and liposomes carrying doxorubicin. Kabanov and coworkers reported the enhanced intracellular uptake and enhanced cytotoxicity of doxorubicin delivered in Pluronic copolymer micelles (Venne et al., 1996). Couvreur showed that doxorubicin-loaded polyalkylcyanoacrylate nanoparticle were more cytotoxic than doxorubicin solution against P388 leukemia cells and allowed to overcome multidrug resistence (MDR) (Couvreur and Vauthier, 1991). The effect of polymeric nanoparticles loaded with doxorubicin against resistant MCF-7 cell-lines, revealed a decrease of the cell viability (Couvreur and Vauthier, 1991). Liposomes encapsulating doxorubicin showed an increased cytotoxicity on HL60 cells and vincristine-resistant HL60 cells (Gokhale et al., 1996).

The increase cytotoxicity of doxorubicin incorporated in SLN may be related to the fast internalization of doxorubicin-loaded SLN and the successive drug release from SLN inside the cells, enhancing the action of doxorubicin.

3.4. Cytotoxicity of paclitaxel-loaded SLN

The average diameter of paclitaxel-loaded SLN was about 160 nm, the polydispersity index of 0.2 and the zeta potential −31 mv.

HL60 cells showed a different behaviour with paclitaxel than with doxorubicin on HL60 cells. At the lower paclitaxel concentrations they were less sensitive to paclitaxel incorporated in SLN than in solution. Cytotoxicity was enhanced when HL60 cells were exposed to more than 5 ng/ml of paclitaxel in SLN. Fig. 4 reports the viability curves of HL60 cells.

On MCF-7 cells, paclitaxel solution did not show a strong dose-dependent inhibitory activity up to a concentration of 100 ng/ml, which caused a strong decrease of cell growth after 2 days of exposure. Paclitaxel incorporated in SLN was more effective, since it was inhibitory already at nearly 1 ng/ml; a slight enhancement of cytotoxicity was observed by increasing the drug concentration. Fig. 5 reports the viability curves of MCF-7 cells.

This cell line was more sensitive to paclitaxel incorporated in SLN than to the solution; incorporation into SLN appeared to enhance the cytotoxic effect of this drug.

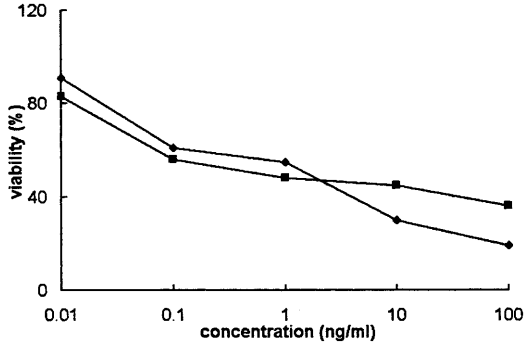

Fig. 4. Survival curves of HL60 cells after exposure to paclitaxel in solution■ and paclitaxel in SLN◆.

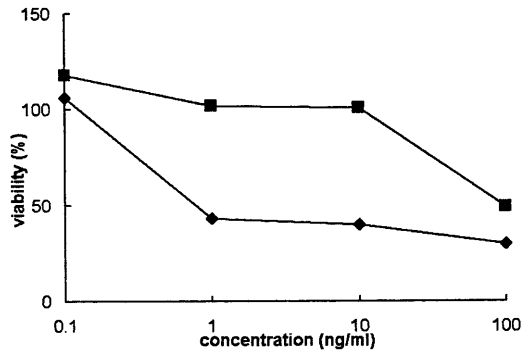

Fig. 5. Survival curves of MCF-7 cells after exposure to paclitaxel in solution■ and paclitaxel in SLN◆.

In Table 1 the IC_{50} of the paclitaxel-loaded SLN and paclitaxel in solution on the two cell-lines are reported. On MCF-7 cell line the concentration of paclitaxel useful to obtain a cytotoxic effect was lower with paclitaxel-loaded SLN than with the paclitaxel solution. The cytotoxic enhancement was about 100-fold.

An increased cell growth inhibition of paclitaxel was already shown using other colloidal carriers such as liposomes and micelles (Cabanes et al., 1998; Miwa et al., 1998).

Liposome-encapsulated paclitaxel has been shown to decrease toxicity (Cabanes et al., 1998) indicating that therapeutic efficacy of conventional paclitaxel can be improved.

Paclitaxel is poorly soluble in aqueous media and the vehicle (Cremophor EL) commonly used for its administration has toxic side effects. Therefore its incorporation into SLN might decrease its toxicity after in vivo administration as all the SLN components are biocompatible and biodegradable.

4. Conclusions

From the results obtained, some conclusions may be drawn:

1. Neither of the unloaded SLN formulations, obtained with different lipid matrices, are toxic to either of the cell-lines studied.
2. SLN are rapidly entrapped into both cell-lines, as shown with the fluorescent SLN. This be-

haviour confirms that SLN are easily internalized.

3. The cytotoxicity of doxorubicin and paclitaxel incorporated in SLN is consistently higher than that of the drug solutions on both cell-lines. This effect is particularly marked on HL60 with doxorubicin-loaded SLN and on MCF-7 with paclitaxel-loaded SLN.

The higher sensitivity of the cells to the drugs incorporated into SLN than to the drugs in solution may be related to the marked uptake and accumulation of drug-loaded SLN in the cells, where the loaded-SLN should release the drugs, so enhancing their action.

SLN may be proposed as alternative colloidal drug carriers for the administration of chemotherapeutic agents.

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References

Brown, D.A., Malkin, T., Maliphant, G.K., 1955. An X-ray examination of long-chain alkyldihydrogen phosphates and dialkylhydrogen phosphates and their sodium salts. J. Chem. Soc. 1584–1588.

Cabanes, A., Briggs, K.E., Gokhale, P.C., Treat, J.A., Rahman, A., 1998. Comparative in vivo studies with paclitaxel and liposome-encapsulated paclitaxel. Int. J. Oncol. 12, 1035–1040.

Cavalli, R., Caputo, O., Gasco, M.R., 1993. Solid lipospheres of doxorubicin and idarubicin. Int. J. Pharm. 89, R9–R12.

Cavalli, R., Caputo, O., Gasco, M.R., 2000. Preparation and characterization of Solid Lipid Nanoparticles incorporating paclitaxel. Eur. J. Pharm. Sci. 10, 305–309.

Couvreur, P., Vauthier, C., 1991. Polyalkylcyanoacrylate nanoparticles as drug carrier: present state and perspectives. J. Controlled Release 17, 187–198.

Domb, A.J., 1995. Long-acting injectable oxytetracycline lipospheres formulation. Int. J. Pharm. 124, 271–275.

Fundarò, A., Cavalli, R., Bargoni, A., Vighetto, D., Zara, G.P., Gasco, M.R., 2000. Non-stealth and stealth solid lipid nanospheres carrying doxorubicin: pharmacokinetics and tissue distribution after iv administration to rats. Pharmacol. Res. 42, 337–343.

Gasco, M.R., 1997. Solid Lipid Nanoparticles from warm microemulsion. Pharm. Tech. Eur. 9, 52–59.

Gieseler, F., Nubler, V., Brieden, T., Kunze, J., Valsamas, S., 1998. Intracellular pharmacokinetics of anthracyclines in human leukemia cells: correlation of DNA-binding with apoptotic cell death. Int. J. Clin. Pharm. Therap. 30, 25–28.

Gokhale, P.C., Radhakrishnan, B., Husain, S.R., Abernethy, D.R., Sacher, R., Dritschilo, A., Rahman, A., 1996. An improved method of encapsulation of doxorubicin in liposomes: pharmacological, toxicological and therapeutic evaluation. Br. J. Cancer 34, 45–48.

Koppel, D.E., 1972. Analysis of macromolecular polydispersity in intensity correlation spectroscopy the method of cumulants. J. Chem. Phys. 57, 4814–4818.

Kumar, N., 1981. Taxol-induced polimerization of purified tubulin. Mechanism of action. J. Biol. Chem. 256, 10435–10441.

Ling, V., 1992. P-glycoprotein and resistance to anticancer drugs. Cancer 69, 2603–2609.

Miwa, A., Ishibe, A., Nakano, M., Yamashira, T., Itai, S., Jinno, S., Kawahara, H., 1998. Development of novel chitosan derivatives as micellar carriers of taxol. Pharm. Res. 15, 1844–1850.

Müller, R.H., Olbrich, C., 1999. Solid lipid Nanoparticles: Phagocytic uptake, in vitro cytotoxicity and in vitro biodegradation. Pharm. Ind. 61, 564–569.

Müller, R.H., Rühl, D., Runge, S., Schulze-Foster, K., Mehnert, W., 1997. Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant. Pharm. Res. 14, 458–462.

Rose, L.M., Tillery, K.F., El Dareer, S.M., Hill, D.L., 1988. High-performance liquid chromatographic determination of doxorubicin and its metabolites in plasma and tissue. J. Chromatog. 425, 419–423.

Schwartz, C., Mehenert, W., Lucks, J.S., Müller, R.H., 1992. Solid lipid nanoparticles (SLN) for controlled drug delivery. I Production, characterization and sterilization. Int. J. Pharm. 88, 53–58.

Sharma, A., Conway, W.D., Straubinger, R.M., 1994. Reversed-phase high performance liquid chromatografic determination of taxol in mouse plasma. J. Chromatog. B. 655, 315–319.

Venne, A., Li, S., Mandeville, R., Kabanov, A.V., Alakhov, V.J., 1996. Hypersensitizing effect of Pluronic L61 on cytotoxicity activity, transport and subcellular distribution of doxorubicin in multiple drug-resistant cells. Cancer Res. 56, 3626–3629.

Westesen, K., Siekmann, B., Koch, M.J.H., 1993. Investigation on physical state of lipid nanoparticles by synchroton radiation X-ray diffraction. Int. J. Pharm. 93, 199–203.

Yang, S., Zhu, J., Lu, Y., Liang, B., Yang, C., 1999. Body distribution of camptothecin Solid Lipid Nanoparticles after oral administration. Pharm. Res. 16, 751–757.

Zara, G.P., Cavalli, R., Fundarò, A., Bargoni, A., Caputo, O., Gasco, M.R., 1999. Pharmacokinetics of doxorubicin incorporated in solid lipid nanoparticles (SLN). Pharmacol. Res. 40, 281–286.