Acta Pharm. 56 (2006) 143–155

Original research paper

Enhanced delivery of etoposide to Dalton's lymphoma in mice through polysorbate 20 micelles

LAKKIREDDY HARIVARDHAN REDDY[1]
RAKESH KUMAR SHARMA[2]
RAYASA RAMACHANDRA MURTHY[1]*

[1]*Drug Delivery Research Laboratory Center of Relevance and Excellence in NDDS, Pharmacy Department, G.H. Patel Building, Donor's Plaza, Fatehgunj Baroda-390002, Gujarat, India*

[2]*Division of Radiopharmaceuticals and Radiation Biology, Institute of Nuclear Medicine and Allied Sciences, Brig. S.K. Mazumdar Road, Delhi 110 054, India*

The study evaluates the possibility of enhancing uptake of etoposide (topoisomerase II inhibitor) by tumor when delivered through polysorbate 20 micelles. The micelle formation was ascertained by determining the critical micellar concentration (CMC) with a du Nouy ring tensiometer and by size measurement using dynamic light scattering. Addition of 5% ethanol decreased the CMC of Polysorbate 20 (from 5.0 x 10^{-5} to 4.54 x 10^{-5} mol L^{-1}). Etoposide (ET) and etoposide loaded polysorbate 20 micelles (EPM) were radiolabeled with ^{99m}Tc by the reduction method using stannous chloride. Labeling parameters were optimized to obtain high labeling efficiency. The diethylenetriaminepentaacetic acid and cysteine challenge tests showed very low transchelation of ^{99m}Tc-ET and ^{99m}Tc-EPM complexes indicating their *in vitro* stability. The complexes also exhibited serum stability assessed by ascending thin layer chromatography. Subcutaneous injection of EPM resulted in significantly higher tumor uptake (~ 100 folds compared to ET 6 h post injection) ($p < 0.001$) and prolonged tumor retention. Tumor uptake was also confirmed by gamma imaging studies. EPM exhibited relatively high brain concentrations (~ 7 fold 24 h post injection) compared to ET, suggesting the potential use of EPM in the treatment of brain malignancies.

Keywords: etoposide, polysorbate micelles, drug delivery, radiolabeling, biodistribution, tumor transport

Accepted November 11, 2005

Delivery of drugs to the target sites by the use of carrier systems has recently been the major area of drug delivery research. Incorporation of drugs into delivery carriers prevents drugs from degradation, targets the drug to the site of action and reduces toxicity or side effects by modifying their *in vivo* distribution (1). Delivery of anticancer agents using carrier systems with an objective of enhancing their tumor concentrations has been widely attempted (2–5). Over the years, surfactants have been of pharmaceutical interest either as drug carriers or, more recently, as targeting systems. From the toxicological point of view, non-ionic surfactants are generally regarded as safe and most suit-

* Correspondence, e-mail: m_rsr@rediffmail.com

able for pharmaceutical formulations (6). The very small size of micelles (< 100 nm) prevents uptake by the reticuloendothelial system and facilitates their extravasation at leaky sites of capillaries, leading to passive accumulation in tumor tissues and penetration into cells (6). The use of polysorbates (non-ionic surfactants) in drug delivery by parenteral route is well documented (7–9).

Major determinants of drug delivery to tumors are the tissue structure, tissue composition and tumor cell density (10). Both systemic and regional chemotherapy are used in the management of cancer. Following intravenous (*i.v.*) administration, the drug delivery to tumor involves various processes such as distribution through vascular space, transport across microvessel walls, and diffusion through interstitial space in the tumor tissue. In local chemotherapy such as after subcutaneous administration, the drug transport into tumors is mainly by diffusion through the interstitial space (10). Effective antitumor activity of subcutaneously administered 1,3-bis(2-chloroethyl)-1-nitrosourea and paclitaxel implants in human brain tumor xenografts developed after subcutaneous inoculation in nude mice has been reported by Vogelhuber *et al*. (11).

Radiolabeling of drugs and their carriers to study their *in vivo* distribution has been widely attempted. Our recent reports showed successful formation of stable ^{99m}Tc labeled drug and liposome/nanoparticle complexes and their use in biodistribution studies (12, 13).

Etoposide (ET) is an anticancer agent used in the treatment of a variety of malignancies and has been demonstrated to be effective in the treatment of small cell lung carcinoma, brain stem gliomas, malignant lymphoma and ovarian cancer. It acts by inhibition of topoisomerase II and activation of oxidation/reduction reactions to produce derivatives that bind directly to DNA and cause DNA damage (14, 15). The present study describes our attempt to enhance the delivery of etoposide to Dalton's lymphoma solid tumor in mice by incorporating etoposide into polysorbate 20 micelles. Polysorbate 20 micelles loaded with etoposide (EPM) were prepared, characterized and radiolabeled with ^{99m}Tc. The *in vitro* stability of radiolabeled complexes was examined by the diethylenetriaminepentaacetic acid (DTPA) and cysteine challenge tests; their stability in serum was established as well. These radiolabeled complexes were subcutaneously injected below the tumor region and the biodistribution and tumor transport in Dalton's lymphoma tumor bearing mice were studied.

EXPERIMENTAL

Chemicals

Etoposide was a gift sample obtained from Dabur Research Center (India) and Cipla Limited (India). Polysorbate 20 was purchased from Merck Limited (India). Diethylenetriaminepentaacetic acid and cysteine were obtained from Sigma Chemical Co. (USA). Stannous chloride was purchased from Qualigens (India). Sodium pertechnetate eluted from molybdenum-99 by solvent extraction was procured from the Regional Center for Radiopharmaceutical Division (Northern region), Board of Radiation and Isotope Technology (BRIT), India. All other chemicals used in the study were of analytical grade.

Etoposide-loaded polysorbate 20 micelles

Etoposide-loaded polysorbate 20 micelles were prepared by dissolving etoposide (2 mg mL^{-1}) in 10% aqueous polysorbate 20 solution containing 5% ethanol. The mixture was slightly warmed to clarify the solution.

Characterization of micelles

The micelles were characterized by critical micellar concentration (CMC) measurements and size determination.

Determination of CMC of polysorbate 20

Determination of CMC of polysorbate 20 micelles was performed by the surface tension measurement by the ring method (16) using a du Nouy ring tensiometer (S. C. Dey & Co., India) at 30 ± 1 °C. The temperature was maintained by circulating thermostated water through a jacketed vessel containing the solution. The concentration was varied by adding aliquots of the stock surfactant solution of known concentration to a known volume of solution in the vessel using a Hamilton microsyringe. The ring was cleaned by heating in alcohol flame. The measured surface tension values were plotted as a function of the logarithm of surfactant concentration and the CMC was estimated from the break-point in the resultant curve. Reproducibility of the surface tension *vs.* concentration curve was checked by triplicate runs.

Determination of the size of micelles

The micellar size was determined in a particle size analyzer (Malvern Zetasizer $3000HS_A$ Particle size analyzer, Malvern Instruments, UK) by the injection method. The micellar preparation was injected into the injection port of the particle size analyzer and the average mean diameter was estimated.

Radiolabeling of etoposide and polysorbate 20 micelles

ET and EPM were labeled with technetium (^{99m}Tc) after reduction with stannous chloride similarly to the previously reported methods (17, 18). Briefly, 2 mCi pertechnetate (TcO_4^-) was reduced with 20 μg $SnCl_2 \times 2\ H_2O$ for both ET and EPM (in 10% acetic acid) and pH was adjusted to 6.5 with 0.5 mol L^{-1} sodium bicarbonate. The volume of the reaction medium was 0.5 mL. ET (2 mg mL^{-1}) and/or EPM (equivalent to 2 mg mL^{-1} of ET) was added and incubated at room temperature for 10 min. Quality control was performed as for the method described earlier (17). The labeling efficiency of ET and EPM was determined by ascending thin layer chromatography using silica gel coated fiber sheets (Gelman Sciences Inc., USA). Ascending thin layer chromatography was performed by spotting 2–3 μL on an instant thin layer chromatography (ITLC) strip and developed using acetone as the mobile phase. The strip was cut into two halves and the radioactivity in each half was determined with a well type gamma ray spectrometer (Type GRS23C, Electronics Corporation of India Limited, India). Free pertechnetate (R_f = 0.9–1.0) migrates to the top portion of the ITLC strip, leaving the reduced/hydrolyzed

^{99m}Tc along with the labeled complex at the bottom. Incorporation of excess of stannous chloride for reduction of ^{99m}Tc may lead to the formation of undesirable radiocolloids. Colloids formation was determined in pyridine/acetic acid/water (3:5:1.5). In the case of acetone as the mobile phase (developed for determining the labeling efficiency), only free pertechnetate moves with the solvent front leaving the labeled complex (plus radiocolloids) at the bottom. The mobile phase pyridine/acetic acid/water (3:5:1.5) separates radiocolloids from the labeled complex and free pertechnetate, and allows the labeled complex to move with the solvent front, with radiocolloids remaining at the bottom. The net amount of ^{99m}Tc-ET or ^{99m}Tc-EPM was calculated by subtracting the migration with the solvent front using acetone from that using pyridine/acetic acid/water mixture.

Stability of the ^{99m}Tc labeled complexes

Stability of the ^{99m}Tc labeled complexes of ET and EPM was determined *in vitro* in freshly collected human serum by the ascending TLC technique. The labeled complex (0.1 mL) was incubated with human serum (0.4 mL) at 37 °C. The stability test was performed by determining the changes in labeling efficiency by subjecting the samples to ITLC at regular intervals up to 24 h and analyzing the chromatograms in a gamma ray spectrometer.

DTPA and cysteine challenge

In vitro stability studies of ^{99m}Tc labeled complexes were performed with DTPA and cysteine as previously reported (19). Briefly, fresh solutions of DTPA and cysteine (10, 30 and 50 mmol L^{-1}) were prepared in 0.9% saline. 500 μL of the labeled preparation was incubated with different concentrations of DTPA and cysteine for 1 h at 37 °C. 500 μL of 0.9% saline served as control. The effect of DTPA and cysteine on the labeling efficiency of complexes was measured by ITLC-silica gel strips using acetone as the mobile phase. In this system, ^{99m}Tc–ET and ^{99m}Tc–EPM remain at the start (R_f = 0.0), while pertechnetate (R_f = 0.9–1.0) and all known chemical forms of ^{99m}Tc-DTPA and ^{99m}Tc–cysteine complexes migrate (R_f = 0.7–1.0). After developing, each paper was cut into two halves and each half was counted for radioactivity in the gamma ray spectrometer.

Animals

Balb/c mice of either sex weighing about 25–30 g were used in the study. The mice were housed in cages and were provided with water and standard mouse food (Liptin, India) *ad libitum*. All animal experiments in this study were approved by the Social Justice & Empowerment Committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India, New Delhi.

Tumor implantation in mice

Dalton's lymphoma tumor cells maintained in the ascites form in the peritoneum of Balb/c mice by serial weekly passage were subcutaneously inoculated (5×10^6 cells per mouse) into the right hind leg of Balb/c mice (7–8 weeks old). After 8–10 days, a palpable tumor in the volume range of 0.9 ± 0.1 cm^3 was observed and used for further studies.

Biodistribution study

^{99m}Tc-ET and ^{99m}Tc-EPM were subcutaneously injected into tumor bearing Balb/c mice. Two groups containing nine tumor bearing mice each, one for the ^{99m}Tc-ET and the other for ^{99m}Tc-EPM were used in the study. The biodistribution study was performed 1, 6 and 24 h post injection. At these time intervals, blood was collected by cardiac puncture, animals were sacrificed and their organs were isolated. The organs were then weighed and measured for radioactivity using a gamma ray spectrometer. The radioactivity was interpreted as % injected dose (% ID) per gram of organ/tissue. To correct for physical decay of radiolabel and to calculate radiopharmaceutical uptake in each organ as a fraction of the injected dose, aliquots of the injectate containing 2% of the injected dose were counted simultaneously at each time point.

Tumor imaging by gamma scintigraphy

100 μCi of the ^{99m}Tc-EPM was administered subcutaneously below the tumor region on the right hind leg. 24 h after injection, the mice were fixed on an animal fixing tray board and imaging was performed with a Single Photon Emission Computed Tomography (SPECT, LC 75-005, Diacam, Siemens, USA) gamma camera.

Statistical analysis

Statistical comparisons of the experimental results were performed by Student's *t* test at 0.05 and 0.001 significance levels.

RESULTS AND DISCUSSION

Polyoxyethylene surfactants possess a hydrophobic core, which can incorporate hydrophobic drugs (20). ET being a hydrophobic drug gets incorporated into the inner hydrophobic portion of micelles. Polysorbate 20 spontaneously forms micelles above its CMC. At very low concentrations, the surfactant molecules remain as single chains. But, as the concentration reaches a critical value (*i.e.*, critical micellar concentration), the polymer chains associate to form micelles such that the hydrophilic portion projects into the aqueous phase and hydrophobic portion moves towards the inner core. At CMC, the micelle core incorporates more solvent and they appear as loose aggregates with a size larger than the micelles formed at high surfactant concentrations (21). At concentrations above the CMC, the equilibrium favors the formation of micelles with a smaller size as they adopt low energy configuration (22). Fig. 1 represents the determination of CMC of polysorbate 20 in both pure water and water containing 5% ethanol. The CMC of polysorbate 20 in pure water at 30 °C is 5.0×10^{-5} mol L^{-1}, which is in good agreement with the earlier reports (23). Reduced CMC of polysorbate 20 was found (4.54×10^{-5} mol L^{-1}) in water containing 5% ethanol, indicating the effect of ethanol on CMC of polysorbate 20. The size of the micelles was determined by dynamic light scattering, and was found to be (mean ± SD, $n = 3$) 40.0 nm ± 13.2 nm (range 28–55 nm). Polar organic compounds such as alcohols were reported to affect the CMC by being incorporated into the micelles

(24). This is probably due to the adsorption of ethanol mainly in the outer portion of the micelle, close to the water-micelle interface (24). Ethanol forms hydrogen bonds with water, and hydrogen bonding helps counterbalance the lateral pressure tending to push the additive (ethanol) into the interior of the micelle resulting in a decrease in CMC. The concentration of polysorbate 20 used in the formulation of drug loaded micelles is about 100 times higher than the CMC, indicating its existence in the form of micelles. Etoposide being a hydrophobic drug gets incorporated into the inner hydrophobic portion of micelles. The EPM remained clear upon storage, and the absence of drug precipitate and a minor increase in size even after one week of storage (43.0 ± 24.8 nm, range 33–62 nm, $n = 3$) indicate the stability of drug loaded micelles.

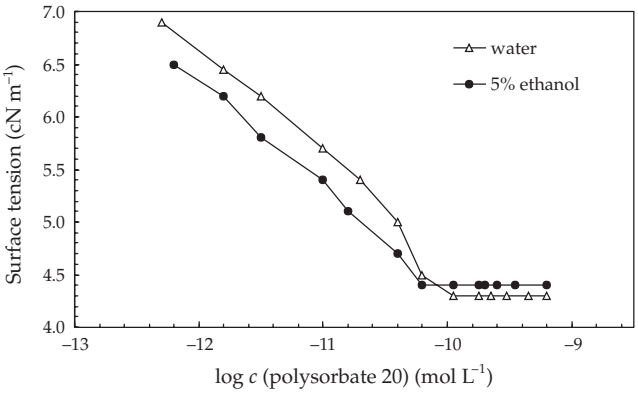

Fig. 1. Plot of surface tension (γ) *vs.* log *c* of polysorbate 20 in pure water and water containing 5% ethanol (mean of 3 determinations).

Radiolabeling of etoposide and EPM

ET and EPM were radiolabeled with ^{99m}Tc with high labeling efficiency. The pertechnetate existing in its seventh oxidative state was reduced to its lower valence (fourth) state by stannous chloride and the pH adjusted to 6.5 before addition of ET or EPM. The amount of stannous chloride played an important role in the labeling process. The influence of stannous chloride on labeling efficiency and formation of radiocolloids is shown in Table I. The optimum amount of stannous chloride resulted in high labeling efficiency and a small amount of radiocolloids. The volume of the reaction mixture was kept constant for both ET and EPM. Smaller amount of $SnCl_2$, led to poor labeling efficiency and its larger amount, led to greater formation of undesirable radiocolloids. These radiocolloids distribute extensively to the organs of the reticuloendothelial system due to their macrophage uptake.

In vitro *stability of labeled complexes*

The *in vitro* stability of radiolabeled complexes needs to be confirmed before their use for biodistribution studies. Determination of the stability of radiolabeled complexes

Table I. Influence of the amount of stannous chloride on the labeling efficiency of etoposide and etoposide loaded polysorbate 20 micelles[a]

$SnCl_2 \times 2H_2O$ (μg)[b]	ET[c]			EPM[c]		
	Labeled (%)	Colloids (%)	Free (%)	Labeled (%)	Colloids (%)	Free (%)
5	80.24 ± 1.20	0.45 ± 0.34	19.31 ± 1.85	84.61 ± 2.15	0.45 ± 0.56	14.94 ± 1.80
10	88.76 ± 1.34	0.48 ± 0.54	1.76 ± 0.12	92.17 ± 1.45	0.56 ± 0.75	8.39 ± 1.25
20	99.28 ± 2.05	0.68 ± 0.31	0.04 ± 0.42	98.78 ± 1.78	0.83 ± 1.05	0.39 ± 1.12
25	98.25 ± 1.54	1.18 ± 0.64	0.57 ± 0.34	96.82 ± 2.34	2.38 ± 0.88	0.80 ± 1.56
50	96.78 ± 1.42	3.01 ± 1.15	0.21 ± 0.86	94.16 ± 1.64	4.98 ± 1.25	0.86 ± 0.98

[a] Each value is the mean of three experiments ± SD.
[b] $c(Sn^{2+}) = 2.4 \times 10^{-8} - 2.4 \times 10^{-7}$ mol L^{-1}.
[c] ET – etoposide, PM – etoposide loaded polysorbate 20 micelles.

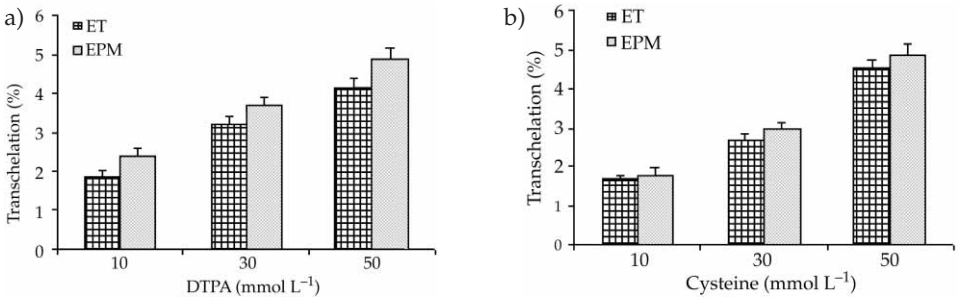

Fig. 2. *In vitro* stability of ^{99m}Tc labeled complexes of etoposide and etoposide loaded polysorbate 20 micelles by: a) DTPA challenge test; b) cysteine challenge test. Each data point is the mean ± SD of 3 experiments. ET – etoposide, EPM – etoposide loaded polysorbate 20 micelles.

Table II. Stability of ^{99m}Tc labeled complexes of etoposide and etoposide loaded polysorbate 20 micelles in human serum[a]

Time (h)	Radiolabeling (%)	
	ET[b]	EPM[b]
	99.28 ± 1.58	98.78 ± 1.68
0.25	99.20 ± 1.98	98.80 ± 2.15
0.5	99.22 ± 1.58	98.71 ± 1.42
1	99.10 ± 1.87	98.60 ± 2.25
2	99.01 ± 1.25	98.42 ± 1.38
4	98.94 ± 2.15	98.12 ± 1.63
8	98.78 ± 1.75	97.96 ± 1.97
24	97.79 ± 2.23	97.22 ± 2.53

[a] Each value is the mean ± SD of three experiments.
[b] ET – etoposide, EPM – etoposide loaded polysorbate 20 micelles.

in serum *in vitro* is an important parameter because the serum contains proteins which may chelate and bind to ^{99m}Tc (termed transchelation), disturbing the stability of ^{99m}Tc--labeled complexes. The stability of ^{99m}Tc-labeled complexes in serum also supports their stability in a biological environment upon administration to the body. ^{99m}Tc-labeled complexes of ET and EPM were challenged with 10, 30 and 50 mmol L^{-1} of DTPA and cysteine to test the strength of binding and stability. Challenge studies demonstrated that the labeling efficiency of the complexes did not alter much in the presence of DTPA (Fig. 2a) and cysteine (Fig. 2b). Even at 50 mmol L^{-1} concentration of DTPA and cysteine, the transchelation was found to be less than 5% indicating a high degree of stability of the radiolabeled complexes. The data (Table II) demonstrated the stability of ^{99m}Tc-ET and ^{99m}Tc-EPM in serum determined up to 24 h, indicating their use as markers for biodistribution studies.

Biodistribution study

Dalton's lymphoma is a lymphoma of T-cell type. In the case of Dalton's lymphoma, the regional lymph nodes are affected by cancer. According to Muranishi and coworkers (25) and Takahashi (26), tumor cells that have detached from the tissue or have invaded a lymphatic vessel would be trapped in the meshwork of a lymph node. Earlier studies showed that the optimal size range of colloidal species (such as ^{99m}Tc-sulphur colloid, ^{198}Au colloid, *etc*.) for better lymphatic drainage was 10–50 nm (27). The biodistribution of subcutaneously injected ^{99m}Tc labeled complexes of ET and EPM was determined in Balb/c mice 1, 6 and 24 h post injection (Table III) below the tumor region in the right hind limb of mice. EPM uptake was low initially (1 h post injection) in the organs such as liver, lung and spleen, and increased slightly 6 h post injection. This suggests a slower clearance of micelles from the site of injection and also after being concentrated in the tumor. The relatively low concentration of micelles in the liver throughout the study indicates reduced phagocytosis. The EPM concentration in spleen and lung 24 h post injection was significantly higher than ET ($p < 0.001$). Concentrations of both ET and EPM in the kidney decreased with time. However, the EPM concentration in the kidney was significantly lower than ET. The uptake of EPM by bones was higher than ET throughout the experiment. However, the concentrations were significantly higher only 6 h post injection. The brain uptake of EPM increased 6 h post injection and remained higher even 24 h post injection (7 fold higher than ET). This is in good agreement with earlier reports on high brain uptake of polysorbate 80 coated systems (28–30). The possible mechanisms suggested for enhanced brain penetration of the polysorbate coated systems are endocytosis, opening of the tight endothelial junctions between the brain endothelial cells, and the third possibility is the inhibition of P-glycoprotein efflux pump responsible for multi-drug resistance, which is a major obstacle to cancer chemotherapy. This observation could be extended to confirm the higher brain transport of EPM after intravenous administration and its possible use in the treatment of brain malignancies (14). EPM showed greater muscle concentrations 24 h post injection. The tumor uptake of EPM was significantly higher than that of ET (2.06% ID of ET, 10.3% ID of EPM after 1 h ($p < 0.001$) and 0.08% ID of ET, 4.18% ID of EPM remained in tumor 24 h post injection ($p < 0.001$)) (Fig. 3) indicating rapid diffusion of EPM into tumor and prolonged tumor retention. EPM showed a 102-fold increase in tumor concentration compared to ET 6 h post injection. This may be attributed to the smaller size of micelles aiding

their infiltration into tumor and resulting in high tumor transfer. Increase in membrane permeability by EPM could also be a contributing factor for high tumor uptake. The hydrophilic surface of EPM might have contributed to the high tumor uptake as well. An earlier report on extensive localization of hydrophilic polymer Poloxamine 904 coated

Table III. Biodistribution of ^{99m}Tc labeled complexes of ET and EPM after subcutaneous injection in Dalton's lymphoma tumor bearing mice

Organ/tissue	Injected dose per gram of organ/tissue (%)[a,b]					
	1 h		6 h		24 h	
	ET	EPM	ET	EPM	ET	EPM
Blood	0.13 ± 0.01	0.13 ± 0.01	0.19 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.22 ± 0.01[d]
Heart	0.14 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.30 ± 0.01
Liver	0.30 ± 0.05	0.09 ± 0.01[d]	0.21 ± 0.05	0.10 ± 0.02[d]	0.28 ± 0.03	0.19 ± 0.02[d]
Lung	0.18 ± 0.03	0.08 ± 0.01[d]	0.14 ± 0.03	0.10 ± 0.02[d]	0.08 ± 0.01	0.36 ± 0.05[d]
Stomach	0.28 ± 0.03	0.36 ± 0.05	0.25 ± 0.03	0.16 ± 0.02	0.16 ± 0.02	0.26 ± 0.04
Intestine	0.24 ± 0.04	0.08 ± 0.03	0.38 ± 0.05	0.23 ± 0.03	0.59 ± 0.03	0.06 ± 0.01
Spleen	0.05 ± 0.01	0.03 ± 0.01[e]	0.04 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.71 ± 0.06[d]
Kidney	1.40 ± 0.09	0.66 ± 0.06[d]	0.91 ± 0.08	0.55 ± 0.03[d]	0.68 ± 0.04	0.42 ± 0.05[d]
Muscle[c]	0.05 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.12 ± 0.01[d]
Bone	0.09 ± 0.01	0.11 ± 0.03	0.08 ± 0.01	0.26 ± 0.05[d]	0.18 ± 0.01	0.36 ± 0.04[d]
Brain	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.02[d]	0.01 ± 0.01	0.07 ± 0.03[d]

[a] The animals were subcutaneously injected with 100 μCi of the ^{99m}Tc labeled complexes of etoposide and etoposide loaded polysorbate 20 micellar formulation, and were sacrificed at 1, 6 and 24 h post injection. Radioactivity was counted in each organ and expressed as % injected dose per gram of organ/tissue.
[b] Each value is the mean ± SEM of 3 animals.
[c] The muscle contralateral to the tumor inoculated thigh was collected for the study.
Significant difference between ET and EPM: [d] $p < 0.001$, [e] $p < 0.05$.
ET – etoposide, EPM – etoposide loaded polysorbate 20 micelles.

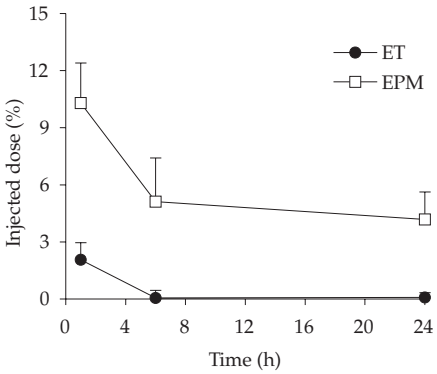

Fig. 3. Tumor concentrations of ^{99m}Tc labeled complexes of etoposide and etoposide loaded polysorbate 20 micelles. Each data point is the mean ± SD of 3 experiments. ET – etoposide, EPM – etoposide loaded polysorbate 20 micelles.

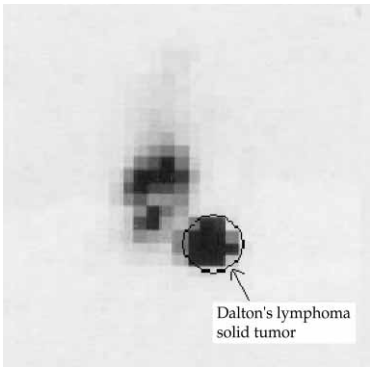

Fig. 4. Gamma scintigraphic image of Dalton's lymphoma tumor bearing mice 24 h post subcutaneous injection of ^{99m}Tc-etoposide loaded micellar formulation. The circle portion indicates high tumor uptake and prolonged tumor retention of etoposide loaded polysorbate 20 micelles in Dalton's lymphoma solid tumor.

polystyrene microspheres (60 nm) in the regional lymph nodes after local administration in the foot pad in rats supports our finding (31). The gamma scintigraphic image of mice taken 24 h post injection of EPM clearly demonstrated prolonged tumor retention (Fig. 4). A previous report on the subcutaneously administered liposomal bupivacaine in the local region resulting in wound infiltration with increased duration of analgesia and slow release plasma profiles compared to the standard aqueous formulation supports our observation of significantly higher tumor uptake after subcutaneous (*s.c.*) injection below the tumor region (32). Distribution of EPM to femur bone in the tumor region was relatively higher than that of ET after 1 h and significantly higher after 6 h (14.5 folds) and 24 h (1.8 folds) post injection (Fig. 5). The ^{99m}Tc separated from the labeled complex inside the body concentrate mainly in the stomach, intestine and the thyroid. A very low recovery of ^{99m}TC from stomach and intestine indicates the intactness of the labeled complexes inside the body and their *in vivo* stability. From the biodistribution data it is evident that the subcutaneous injection of EPM below the tumor region resulted in long lasting tumor concentrations, which would lead to prolonged exposure of tumor tissue to etoposide, resulting in a stronger antitumor effect and tumor regression.

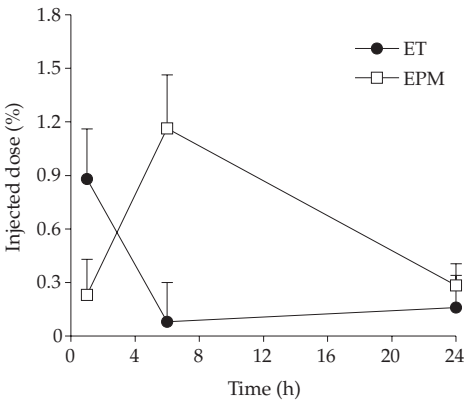

Fig. 5. Concentrations of ^{99m}Tc labeled complexes of etoposide and etoposide loaded polysorbate 20 micelles in femur bone in the tumor region. Each data point is the mean ± SD of 3 experiments. ET – etoposide, EPM – etoposide loaded polysorbate 20 micelles.

CONCLUSIONS

Administration of the etoposide loaded polysorbate 20 micellar formulation by subcutaneous injection significantly enhanced the tumor uptake. The micellar encapsulated etoposide also exhibited prolonged tumor retention. The relatively high brain concentrations of EPM compared to free etoposide indicate its potential use in the treatment of brain malignancies. The study points to the advantage of polysorbate 20 micelles in delivering etoposide to tumors at high concentrations for a prolonged time, which is expected to lead to enhanced antitumor activity and tumor regression. Though the micellar formulation of etoposide showed good tumor uptake, its detailed toxicity need to be evaluated before clinical trials on human patients are initiated.

Acknowledgements. – Financial support from the University Grants Commission (F.10-32/2000 (SA-II)), New Delhi, India, is gratefully acknowledged. Lt. Gen. T. Ravindranath, AVSM, VSM, Director, Institute of Nuclear Medicine and Allied Sciences, Delhi, India, is acknowledged for providing the necessary facilities to carry out the radiolabeling and biodistribution studies.

REFERENCES

1. J. Kreuter, *Nanoparticles*, in *Colloidal Drug Delivery Systems* (Ed. J. Kreuter), Marcel Dekker, New York 1994, pp. 219–342.
2. G. S. Kwon, S. Suwa, M. Yokoyama, T. Okano, Y. Sakurai and K. Kataoka, Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxide-aspartate) block copolymer-Adriamycin conjugate, *J. Control. Release* **29** (1994) 17–23.
3. K. Kataoka, T. Matsumoto, M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto and G. S. Kwon, Doxorubicin-loaded poly(ethylene glycol)-poly(ß-benzyl-L-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance, *J. Control. Release* **64** (2000) 143–153.
4. J. S. Chawla and M. M. Amiji, Biodegradable poly(ε-caprolactone) nanoparticles for tumor targeted delivery of tamoxifen, *Int. J. Pharm.* **249** (2002) 127–138.
5. T. Nakanishi, S. Fukushima, K. Okamoto, M. Suzuki, Y. Matsumura, M. Yokoyama, T. Okano, Y. Sakurai and K. Kataoka, Development of the polymer micelle carrier system for doxorubicin, *J. Control. Release* **74** (2001) 295–302.
6. M. J. Lawrence, Surfactant systems: microemulsions and vesicles as vehicles for drug delivery, *Eur. J. Drug Metab. Pharmacokinet.* **19** (1994) 257–269.
7. A. E. Gulyaev, S. E. Gelperina, I. N. Skidan, A. S. Antropov, G. Y. Kivman and J. Kreuter, Significant transport of doxorubicin into the brain with polysorbate 80-coated nanoparticles, *Pharm. Res.* **16** (1999) 1564–1569.
8. S. E. Gelperina, A. S. Khalansky, I. N. Skidan, Z. S. Smirnova, A. I. Bobruskin, S. E. Severin, B. Turowski, F. E. Zanella and J. Kreuter, Toxicological studies of doxorubicin bound to polysorbate-80 coated poly(butyl cyanoacrylate) nanoparticles in healthy rats and rats with intracranial glioblastoma, *Toxicol. Lett.* **126** (2002) 131–141.
9. D. Le Garre, M. Ranger and J.-C. Leroux, Micelles in anticancer drug delivery, *Am. J. Drug Del.* **2** (2004) 15–42.
10. J. L.-S. Au, S. H. Jang, J. Zheng, C.-T. Chen, S. Song, L. Hu and M. G. Wientjes, Determinants of drug delivery and transport to solid tumors, *J. Control. Release* **74** (2001) 31–46.

11. W. Vogelhuber, T. Spruß, G. Bernhardt, A. Buschauer and A. Gopferich, Efficacy of BCNU and paclitaxel loaded subcutaneous implants in the interstitial chemotherapy of U-87 MG human glioblastoma xenografts, *Int. J. Pharm.* **238** (2002) 111–121.
12. L. H. Reddy, R. K. Sharma and R. S. R. Murthy, Enhanced tumor uptake of doxorubicin loaded poly(butyl cyanoacrylate) nanoparticles in mice bearing Dalton's lymphoma tumor, *J. Drug Target.* **12** (2004) 443–451.
13. N. Arulsudar, N. Subramanian, P. Mishra, K. Chuttani, R. K. Sharma and R. S. R. Murthy, Preparation, characterization and biodistribution study of Technetium-99m-labeled leuprolide acetate-loaded liposomes in Ehrlich Ascites tumor-bearing mice, *AAPS Journal* **6** (2004) 1–13 (http://www.aapspharmsci.org).
14. M. Chamberlain, Recurrent brainstem gliomas treated with oral VP-16, *J. Neuro-Oncol.* **15** (1993) 133–139.
15. D. Ashley, L. Meier, T. Kerby, F. Zlduondo, H. Friedman, A. Gajjar, L. Kun, P. Duffner, S. Smith and D. Longee, Response of recurrent medulloblastoma to low-dose oral etoposide, *J. Clin. Oncol.* **14** (1996) 1922–1927.
16. K. S. Sharma, C. Rodgers, R. M. Palepu and A. K. Rakshit, Studies of mixed surfactant solutions of cationic dimeric (Gemini) surfactant with nonionic surfactant $C_{12}E_6$ in aqueous medium, *J. Colloid Interface Sci.* **268** (2003) 482–488.
17. V. J. Richardson, K. Jeyasingh and R. F. Jewkes, Properties of [^{99m}Tc] technetium labeled liposomes in normal and tumor bearing rats, *Biochem. Soc. Trans.* **5** (1977) 290–291.
18. N. Arulsudar, N. Subramanian, P. Mishra, R. K. Sharma and R. S. R. Murthy, Preparation, characterization and biodistribution of ^{99m}Tc-labeled liposome encapsulated cyclosporine, *J. Drug Target*. **11** (2003) 187–196.
19. A. K. Mishra, N. Iznaga-Escobar, R. Figueredo, V. K. Jain, B. S. Dwarakanath, R. Perez-Rodriguez, R. K. Sharma and T. Lazar Mathew, Preparation and comparative evaluation of ^{99m}Tc-labeled 2-iminothiolane modified antibodies and CITC-DTPA immunoconjugates of anti-EGF-receptor antibodies, *Methods Find. Exp. Clin. Pharmacol.* **24** (2002) 653–660.
20. K. T. Oh, T. K. Bronich and A. T. Kabanov, Micellar formulations for drug delivery based on mixtures of hydrophobic and hydrophilic Pluronic® block copolymers, *J. Control. Release* **94** (2004) 411–422.
21. Z. Gao and A. Eisenberg, Model of micellization for block copolymers in solutions, *Macromolecules* **26** (1993) 7353–7360.
22. M.-C. Jones and J.-C. Leroux, Polymeric micelles – A new generation of colloidal drug carriers, *Eur. J. Pharm. Biopharm.* **48** (1999) 101–111.
23. C. C. Ruiz, J. A. Molina-Bolivar, J. Aguiar, G. MacIsaac, S. Mooroze and R. Palepu, Effect of ethylene glycol on the thermodynamic and micellar properties of Tween 20, *Colloid Polym. Sci.* **281** (2003) 531–541.
24. M. J. Rosen, *Surfactants and Interfacial Phenomena,* Wiley, New York 1989, pp. 108–168.
25. S. Muranishi, T. Fuijita, M. Murakami and A. Yamamoto, Potential for lymphatic targeting of peptides, *J. Control. Release* **46** (1997) 157–164.
26. T. Takahashi, Emulsion and activated carbon in chemotherapy, *CRC Crit. Rev. Ther. Drug Carrier Syst.* **2** (1985) 245–274.
27. S. E. Strand and L. Bergqvist, Radiolabeled colloids and macromolecules in the lymphatic system, *Crit. Rev. Ther. Drug Carrier Syst.* **6** (1989) 211–238.
28. G. Borchard, K. L. Audus, F. Shi and J. Kreuter, Uptake of surfactant-coated poly(methylmethacrylate)-nanoparticles by bovine brain microvessel endothelial cell monolayers, *Int. J. Pharm.* **110** (1994) 29–35.

29. R. N. Alyautdin, D. Gothier, V. Petrov, D. Kharkevich and J. Kreuter, Analgesic activity of hexapeptide dalargin adsorbed on the surface of polysorbate 80-coated poly(butylcyanoacrylate) nanoparticles, *Eur. J. Pharm. Biopharm.* **41** (1995) 44–48.
30. J. Kreuter, R. N. Alyautdin, D. Kharkevich and A. A. Ivanov, Passage of peptides through the blood-brain barrier with colloidal polymer particles (nanoparticles), *Brain Res.* **674** (1995) 171–174.
31. S. M. Moghimi, A. E. Hawley, N. M. Christy, T. Gray, L. Illum and S. S. Davis, Surface engineered nanospheres with enhanced drainage into lymphatics and uptake by macrophages of the regional lymph nodes, *FEBS Lett.* **344** (1994) 25–30.
32. D. B. Larsen, S. Joergensen, N. V. Olsen, S. H. Hansen and C. Larsen, In vivo release of bupivacaine from subcutaneously administered oily solution. Comparison with in vitro release, *J. Control. Release* **81** (2002) 145–154.

SAŽETAK

Povećana isporuka etopozida u Daltonov limfom u miševa pomoću micela s polisorbatom 20

LAKKIREDDY HARIVARDHAN REDDY, RAKESH KUMAR SHARMA i RAYASA RAMACHANDRA MURTHY

U radu je proučavan ulazak etopozida (inhibitora topoizomeraze II) u tumorsko tkivo iz micela s polisorbatom 20. Oblikovanje micela je potvrđeno određivanjem kritične micelarne koncentracije (CMC) pomoću du Nouy kružnog tenziometra i mjerenjem veličine čestica metodom rasapa svjetlosti. Dodatak 5% etanola smanjuje CMC polisorbata 20 (od 5,0 x 10^{-5} do 4,54 x 10^{-5} mol L^{-1}). Etoposid (ET) i micele etoposida s polisorbatom 20 (EPM) obilježene su radioizotopom ^{99m}Tc redukcijom pomoću kositrova klorida. Parameteri markiranja su optimirani. Testovi s dietilentriaminpentaoctenom kiselinom i cisteinom pokazali su vrlo nisko transkeliranje ^{99m}Tc-ET i ^{99m}Tc-EPM kompleksa, što ukazuje na njihovu stabilnost u uvjetima *in vitro*. Uzlaznom tankoslojnom kromatografijom dokazana je i stabilnost kompleksa u serumu. Nakon subkutane primjene EPM isporuka etopozida u tumorsko tkivo bila je značajno veća (~ 100 puta u odnosu na ET 6 h poslije injiciranja) ($p < 0{,}001$), a dokazano je i produljeno zadržavanje EPM u tumoru. Ulazak u tumor potvrđen je i gama analizom slike. EPM je postigao relativno visoku koncentraciju u mozgu u usporedbi s ET (~ 7 puta veću 24 h poslije injiciranja), zbog čega bi se potencijalno mogao upotrijebiti u terapiji malignih tumora mozga.

Ključne riječi: etopozid, micele s polisorbatom, isporuka lijeka, obilježavanje radioizotopom, biodistribucija, transport u tumor

Drug Delivery Research Laboratory, Center of Relevance and Excellence in NDDS, Pharmacy Department, G.H. Patel Building, Donor's Plaza, Fatehgunj, Baroda-390002, Gujarat, India

Division of Radiopharmaceuticals and Radiation Biology, Institute of Nuclear Medicine and Allied Sciences, Brig. S.K. Mazumdar Road, Delhi-110 054, India