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Anticancer exffect of 9-nitrocamptothecin liposome aerosol on human cancer xenografts in nude mice

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Abstract *Purpose*: To test the anticancer properties of the water-insoluble derivative of camptothecin, 9-nitrocamptothecin (9-NC) against human breast, colon and lung cancer xenografts in nude mice when administered in liposome aerosol. *Methods*: The drug was formulated with dilauroylphosphatidylcholine and nebulized in a particle size of 1.6 μm ± 2.0 mass median diameter to deliver doses of usually less than 200 μg/kg daily, 5 days per week. 9-NC liposome aerosols were generated with a Aerotech II nebulizer (CIS-USA) flowing at 10 l/min from a compressed air source and delivered to mice in sealed plastic cages or in a nose-only exposure chamber. *Results*: Tumor growth was greatly reduced or tumors were undetectable after several weeks of treatment. Colon tumor was least responsive. 9-NC was better than the parent compound, camptothecin, also water-insoluble, tested by aerosol in a similar liposomal preparation. Equivalent doses of 9-NC liposome preparations administered by mouth were substantially without effect while there was some effect, but limited, of the liposome preparation given intramuscularly. *Conclusions*: 9-NC liposome aerosol was strikingly effective in the treatment of three human cancer xenografts growing subcutaneously over the thorax in nude mice at doses much smaller than those traditionally used in mice administered by other routes.

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Introduction

Camptothecin (CPT) is a plant alkaloid first isolated from *Camptotheca acuminata* in 1966. As a topoisomerase I inhibitor, it has powerful anticancer properties [5, 16, 33] and has been used clinically in the treatment of a variety of cancers. It possesses significant toxicity, especially involving the bone marrow and gastrointestinal tract that has limited its use. Pantazis has recently reviewed this subject [27].

Derivatives of 20-(*S*)-CPT have been made to increase the aqueous solubility of these compounds and/or to modify the A-ring to increase membrane association. The 9-nitrocamptothecin (9-NC) derivative used in the present study is insoluble in water, but has demonstrated potent antitumor effects against human ovarian and malignant melanoma cells in the human xenograft-nude mouse model when administered intramuscularly at doses in the range 1–4 mg/kg per day [17, 24]. Following studies of oral administration of 9-NC as a dietary supplement [16], the same workers have also found that direct injection of cotton seed oil suspensions of 9-NC through the abdominal wall into the stomach at a dosage of 1.0 mg/kg per day 5 days per week for several weeks, is effective against several human tumor xenografts [25, 26]. A lower dose, 0.75 mg/kg per day given as above, is effective against some more sensitive tumors. In humans, oral treatment with 9-NC at a dosage of 1.0 mg/m^2 per day can be given safely for extended periods, but dose-limiting toxicities occur, especially myelosuppression [22].

Previous work in this laboratory has shown that certain drugs delivered to the respiratory tract in a liposome formulation may have advantages of noninvasive administration, including high pulmonary concentrations, often rapid entry into the systemic circulation, reduced toxicity and reduced dosage requirement compared to oral and parenteral adminis-

tration [11, 12, 29]. Burke et al. [3, 4] prepared liposomal formulations of CPT, 9-NC and other water-insoluble derivatives and Daoud et al. [6] described a potent antitumor effect of these preparations in malignant xenografts in nude mice. With other drug-liposome formulations, we have demonstrated the effectiveness of aerosol administration for the treatment of pulmonary disease as well as systemic disease [13–15]. We have also evaluated the safety and tolerability of the phospholipid used in the preparation of the 9-NC liposomes, dilauroylphosphatidylcholine (DLPC). Rats exposed to 1 h of continuous aerosol for 28 consecutive days show no effect of the phospholipid [10]. Phase I/II studies in humans with DLPC aerosol have also demonstrated its safety and tolerability [1, 31].

In this report we describe the beneficial effect of a liposome aerosol containing 9-NC on xenografts of human breast, colon and lung cancer in nude mice at doses that are considerably smaller than those in the studies described above.

Materials and methods

CPT, 9-NC and other chemicals

20-(*S*)-(CPT) and its derivative 9-NC were gifts from Dr. Beppino C. Giovanella of the Stehlin Foundation for Research, Houston, Tx. CPT was highly purified according to FDA regulations [22]. 9-NC was synthesized from CPT by Dr. Giovanella and was >99% pure as determined by HPLC analysis and has been given orally to patients [22]. DLPC was purchased from Avanti Polar Lipids, Alabaster, Ala., tertiary butanol (*t*-butanol) from Fisher Scientific, Houston, Tx, and sterile, pyrogen-free water for injection from Baxter Healthcare Corporation, Deerfield, Ill.

Nude mice

Swiss immunodeficient nude mice of the NIH-1 high-fertility strain, bred and housed at the Stehlin Foundation for Research, were used for the experiments [16, 23]. Tumors were implanted at the Stehlin Foundation for Research and the mice were housed at Baylor College of Medicine for treatments.

Human cancer xenografts

Human breast cancer (CLO; infiltrating duct carcinoma), human colon cancer (SQU; moderately differentiated adenocarcinoma) and human lung cancer (SPA; adenocarcinoma) cells were stored, grown and implanted into nude mice as previously reported [16, 18, 23]. Approximately 50 mg (wet weight) of finely minced tumor in 0.5 ml Eagle's minimum essential medium was injected under the skin over the right dorsal chest region. The animals were started on treatment with the experimental drug about 1–4 weeks after implantation of tumors. The sizes of breast tumors in one study were measured in two dimensions (area) with calipers. The sizes of all other tumors were measured in three dimensions (volume). When identifiable, individual tumor masses were measured separately and total area or volume recorded.

Preparation of liposomes containing CPT and 9-NC

CPT (10 mg/ml) and 9-NC (100 mg/ml) were first dissolved in DMSO and heated to 70 °C and 40–50 °C, respectively, to completely solubilize the drugs prior to addition to the phospholipid. DLPC (100 mg/ml) was dissolved in warmed *t*-butanol. Drug and phospholipid preparations, held at 40–50 °C, were mixed at a ratio of 1:50 (w/w). The volume of DMSO in the total organic mixture did not exceed 3–5%. Mixing was performed at room temperature. The material was distributed into 30-ml Wheaton vials (Fisher Scientific, Houston, Tx.), frozen in liquid nitrogen and lyophilized overnight or until thoroughly dried. After sealing the vials under vacuum, the material was stored frozen at −20 °C. For use, sterile, pyrogen-free water for injection was added to the vials to provide the desired concentration of drug. The suspension was gently vortexed until a homogeneous suspension was produced and then transferred to the reservoir of an Aerotech II nebulizer (CIS-USA, Bedford Mass.). The aqueous liposome aerosol suspension, 5–10 ml as needed, was added to the reservoir of the nebulizer. It is necessary to initiate nebulization of the liposomal formulation immediately after dispersing the liposomes in distilled water in order to ensure maximal output of 9-NC in the aerosol. Material left standing at room temperature shows reduced output of 9-NC apparently related to alterations in drug-liposome interactions.

Percoll gradient analysis of efficiency of incorporation 9-NC and CPT into liposomes

9-NC-DLPC liposomes (100–200 μl of liposome suspension) dispersed in water were carefully layered on top of 2 ml Percoll (1.130 g/ml; Sigma, St. Louis, Mo.) and centrifuged at 2000 rpm for 15 min [23]. Liposomes with incorporated drug collected at the top of the Percoll interface while unincorporated drug was deposited at the bottom of the centrifuge tube. The supernatant fraction was carefully removed from the tube and the pellet was resuspended in 200–500 μl acetonitrile. The concentration of 9-NC in an aliquot of the pellet fraction (10 μl) was determined by HPLC analysis and the incorporation efficiency (IE) was calculated as follows: IE (%) = $[(A_T - A_P)/A_T] \times 100$, where A_T is the amount of drug in the original suspension and A_P is the amount of drug in the pellet after centrifugation.

Aerosol treatment

Aerosol was administered to groups of mice in clear, sealed plastic cages (11 × 7 × 5 in). Aerosol was supplied from the Aerotech II nebulizer flowing at 10 l/min via a 1-cm (i.d.) accordion tubing connected to an opening in one end of the cage; aerosol was discharged from an opening in the opposite end of the cage. In one experiment, a nose-only exposure chamber was used (Small Animal Exposure Chamber System, In-Tox Products, Albuquerque, N.M.). The nose-only exposure chamber was used to prevent ingestion of drug by licking or grooming activities by the mice.

Calculation of aerosol dosage of CPT derivatives to mice

Based on estimates of the minute volume [28], mice will exchange 1 l-min/kg of body weight of air (*ca.* 30 ml/min for the nude mice used in these studies) and it is estimated that mice will deposit about 30% of the inhaled particles [8]. It is estimated that one-half to two-thirds of the inhaled particles will deposit in the nose and head of the mouse. These particles and those deposited in the upper conducting airway will be carried promptly by mucociliary action to the orifice of the esophagus and swallowed. Depending on the drug, some fraction may be absorbed through mucus membranes in the nose, head and upper airway. Some 10–15% of the inhaled particles will deposit in the peripheral lungs. The estimated deposited dose (μg/kg) of 9-NC in mice was calculated by multiplying the concentration of 9-NC in the aerosol (μg/l) by the minute volume (l-min/kg), duration of treatment (min) and the estimated deposited fraction. A range of dosage calculations for 9-NC is shown in Table 1.

Table 1 9-NC-DLPC liposome aerosol characteristics and estimated dosage in mice. Mass median aerodynamic diameter (*MMAD*) and aerosol concentrations were determined using the Andersen cascade impactor. Dosage Calculations were based on a 30-g mouse with a minute volume of 1 l-min/kg of body weight [28], an average aerosol retention factor of 30% [8] and the aerosol concentration as indicated

MMAD (μm)	9-NC in		Treatment (min/day)	Dosage		
	Reservoir (μg/ml)	Aerosol (μg/l)		μg/kg/min	μg/kg/day	μg/day
0.8	100	1.8	15	0.54	8.1	0.24
0.8	100	1.8	30	0.54	16.1	0.48
1.6	200	3.6	30	1.08	32.4	0.97
1.2	500	8.5	15	2.56	38.3	1.15
1.2	500	8.5	30	2.56	76.7	2.30
1.2	500	8.5	60	2.56	153.4	4.60
1.2	500	8.5	120	2.56	306.7	9.20
1.5	1000	15.9	15	4.76	71.4	2.14
1.5	1000	15.9	30	4.76	143.1	4.29

Drug-DLPC aerosol and liposome particle size determinations

The particle sizes of aerosols containing CPT-DLPC or 9-NC-DLPC liposomes were measured with an Andersen/ACFM nonviable ambient particle sizing sampler (Andersen Instruments, Atlanta, Ga.) [32]. The concentrations of CPT or 9-NC in the aerosol generated with the Aerotech II nebulizer flowing at 10 l/min was also measured. Samples were collected over a 5-min period of operation. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated using KaleidaGraph 2.0 software (Synergy Software, Reading, Pa.). Liposome particle size was determined from samples in aqueous suspension with a Submicron Particle Sizer Model 370 (NICOMP Particle Sizing Systems, Santa Barbara, Calif.). Data were collected until the percent error was < 1.5.

Sampling of aerosol with the all-glass impinger (AGI)

Aerosol was drawn by vacuum through a calibrated glass tube with the tip 4 mm above a 10-ml volume of water in an AGI at a flow rate of 12.5 l/min. (Ace Glass, Vineland, N.J.). Collected fluids were used for analytical purposes.

Assay of CPTs by high-performance liquid chromatography

A Waters 710B WISP automatic injector and Waters Nova-Pak Cl8 column (3.9×150 mm; Waters, Milford, Mass.) at room temperature were used to quantitate CPT and 9-NC. The mobile phase was composed of 30% acetonitrile and 70% water containing 0.1% glacial acetic acid, and flowed at 1.2 ml/min. CPT was detected using a Waters 470 scanning fluorescence detector set to an excitation wavelength of 370 nm and an emission wavelength of 440 nm. 9-NC was detected using the Waters 440 absorbance detector with monitoring at 254 nm. The data were analyzed with Waters Millenium software.

Assay of DLPC

DLPC was measured by HPLC using a 717 WISP autosampler and a Nova-Pak silica column (3.9×150 mm; Waters). The mobile phase consisted of acetonitrile/methanol/10 m*M* ammonium trifluoroacetic acid, pH 4.8 (64:28:8 v/v/v). Peaks were detected with a SEDEX 55 mass evaporator detector (Sedere, Alfordville, France). Samples were dissolved in methanol or ethanol [30].

Statistics

Analysis of variance (ANOVA) was performed using the True Epistat statistical package from Epistat Services, Richardson, Tx. Input for analysis consisted of tumor size (surface area, one experiment, or volume measured by calipers), time of measurement and experimental group. *P*-values are based on the data using fixed factors and unweighted means. Multiple comparisons of the effect of day from start of treatment and/or treatment regimen on tumor size were made. Comparison of mean tumor sizes was performed using Student's *t*-test, two-tailed, which is a part of the statistical software of Microsoft Excel, v. 5.0.

Results

Aerosol characteristics and treatment

The aerosol particle size of 9-NC-DLPC liposomes was determined for 9-NC for drug concentrations ranging from 100 to 1000 μg/ml in the reservoir. As measured with the Andersen cascade impactor, the MMAD and GSD ranged from 0.8 to 1.6 μm and from 1.8 to 2.6, respectively. Aerosol particles with these MMAD characteristics are well suited for pulmonary deposition throughout the respiratory tract [24, 27, 28]. The concentration of DLPC in the aerosol was determined to be approximately 50 times the 9-NC concentration. Over this range of drug, the concentration of 9-NC in the aerosol was directly proportional to the concentration in the reservoir ($R^2 = 0.995$) and ranged from 1.8 to 15.9 μg/l aerosol (see Table 1).

Efficiency of incorporation of 9-NC and CPT into DLPC liposomes

Figure 1 shows the efficiency of incorporation of 9-NC into DLPC liposomes as determined by Percoll gradient analysis. The incorporation efficiency of 9-NC at a concentration of 0.1 or 0.2 mg/ml into DLPC liposomes in a range of drug to lipid ratios of 1:20 to 1:70 (w/w) was 100%. Incorporation of 0.5 mg/ml 9-NC was 78% at a 9-NC:DLPC ratio of 1:50 and was 100% at 1:60. Incorporation of 1.0 mg/ml 9-NC was lower but was above 90% at a 9-NC:DLPC ratio of 1:70. Microscopy with polarized light show a few crystals of 9-NC in all preparations, but many more where incorporation was low. This was quantitated by Percoll gradient analysis (Table 2). CPT was less efficiently incorporated but at a CPT to lipid ratio of 1:50 (w/w), concentrations of CPT of 0.1 to 0.5 mg/ml showed 80% or more incorporation (data not shown).

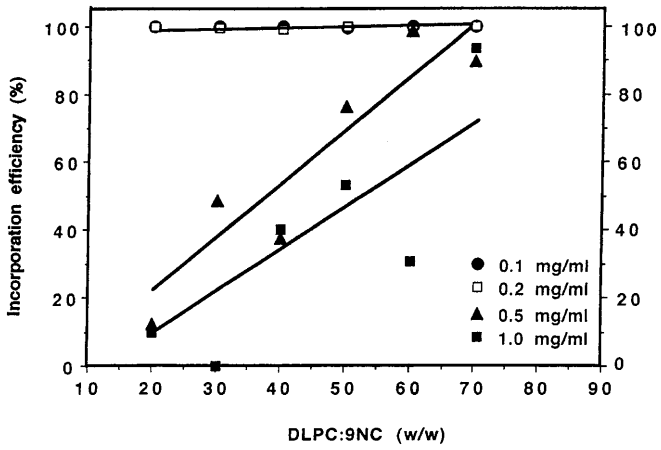

Fig. 1 Efficiency of incorporation of 9-NC into liposomes composed of DLPC as a function of drug: lipid ratio and concentration in water as determined by Percoll gradient analysis

Table 2 Percoll gradient analysis of 9-NC-DLPC liposome aerosol for presence of 9-NC Crystal

9-NC (mg/ml)	Ratio 9-NC:lipid	Crystals % ± SD
0.2	1:55	9.9 ± 5.0
0.2	1:60	16.4 ± 6.0
0.5	1:50	7.9 ± 6.4
0.5	1:60	7.9 ± 3.0

Material recovered from the Andersen sampler after nebulizing the 0.5 mg/ml 9-NC and 25 mg/ml DLPC liposome preparation showed a close correspondence of these constituents on the eight stages of the sampler ($R^2 = 0.831$). Similar values were found for lower concentration liposome preparations. The possibility of nebulization of 9-NC crystals not in a liposome formulation was explored by collecting AGI samples when 5 mg/ml 9-NC dispersed in 10 ml water was nebulized for 30 min. For comparison, the liposomal formulation with the same amount of 9-NC was nebulized similarly. Less than 1 μg of 9-NC was recovered from the drug-alone preparation, but 1270 μg was recovered from the liposome formulation.

Effect of nebulization and AGI sampling on liposome stability

9-NC-DLPC liposomes, 0.2 mg/ml 9-NC at 1:50 and 1:60 drug/lipid ratios, and 0.5 mg/ml 9-NC at the same drug/lipid ratios were each nebulized for 15 min in the Aerotech II nebulizer, 10 l/min. The total output of aerosol was collected for the various preparations in the AGI and the suspensions were then analyzed for the presence of crystals by Percoll gradient. Each preparation was analyzed in triplicate. The mean and standard deviations of the four preparations are shown in Table 2.

The small percentage of crystals from the liposomes was probably related to the shear forces associated with nebulization and from collection in the AGI. Both procedures are associated with cooling to 16–17 °C, which may have also contributed to the release of crystals. It is unlikely that free crystals were nebulized as noted above.

Electron microscopy

Aerosol containing 9-NC-DLPC liposomes was collected in an AGI containing distilled water and submitted for electron microscopic examination (Dr. Thomas Giddings, University of Colorado, Boulder, Colo.). The results of 1% uranyl acetate staining of the material are shown in Fig. 2. The 100-nm scale bar on Fig. 2 indicates that most of the multilamellar structures revealed were 100 nm or more in diameter. Much smaller particles, probably representing pieces of bilayer, were seen throughout the field.

Measurement of the size of liposomes

Samples were obtained from the reservoir of the Aerotech II nebulizer at the start, and at 17 and 30 min. Figure 3 shows the mean diameter of the liposomes at these time-points. The mean size at the beginning of nebulization was 2539 ± 91 nm, while at 17 and 30 min the diameters were reduced in size to <400 nm as a result of the shear effect of continuous cycling of the liposome suspension through the nebulizer which occurs within minutes.

Treatment of mice with xenografts of human breast cancer

In the initial experiment, six mice were started on treatment 25 days after xenograft implantation, while five implanted mice served as controls (Fig. 4, Table 3). Treatment consisted of 15-min inhalations of 9-NC-DLPC liposome aerosol (containing 1.8 μg/l 9-NC generated producing an estimated deposited dose of 8.1 μg/kg per day) for 5 days per week. ANOVA indicated a statistically significant difference between the two groups ($P < 0.0001$). There was an immediately discernible difference in the percent increase in tumor size compared to day 0 of treatment in the two groups of mice (initial mean ± SD tumor size, 90.7 ± 97.2 mm^2), with a rapid increase in mean tumor size in control mice (27.2% per day) and a sevenfold slower increase in the mean size in the treated mice (3.8% per day). The difference was statistically significant ($P < 0.05$; Student's *t*-test, two-tailed) by day 8 and continued through day 31 when the experiment was stopped because of the large size of the tumors and necrotic lesions in the untreated animals.

At that time the one control mouse and the two treated mice were saved for further study (Fig. 5). No treatment was given from day 32 to day 47. At that time,

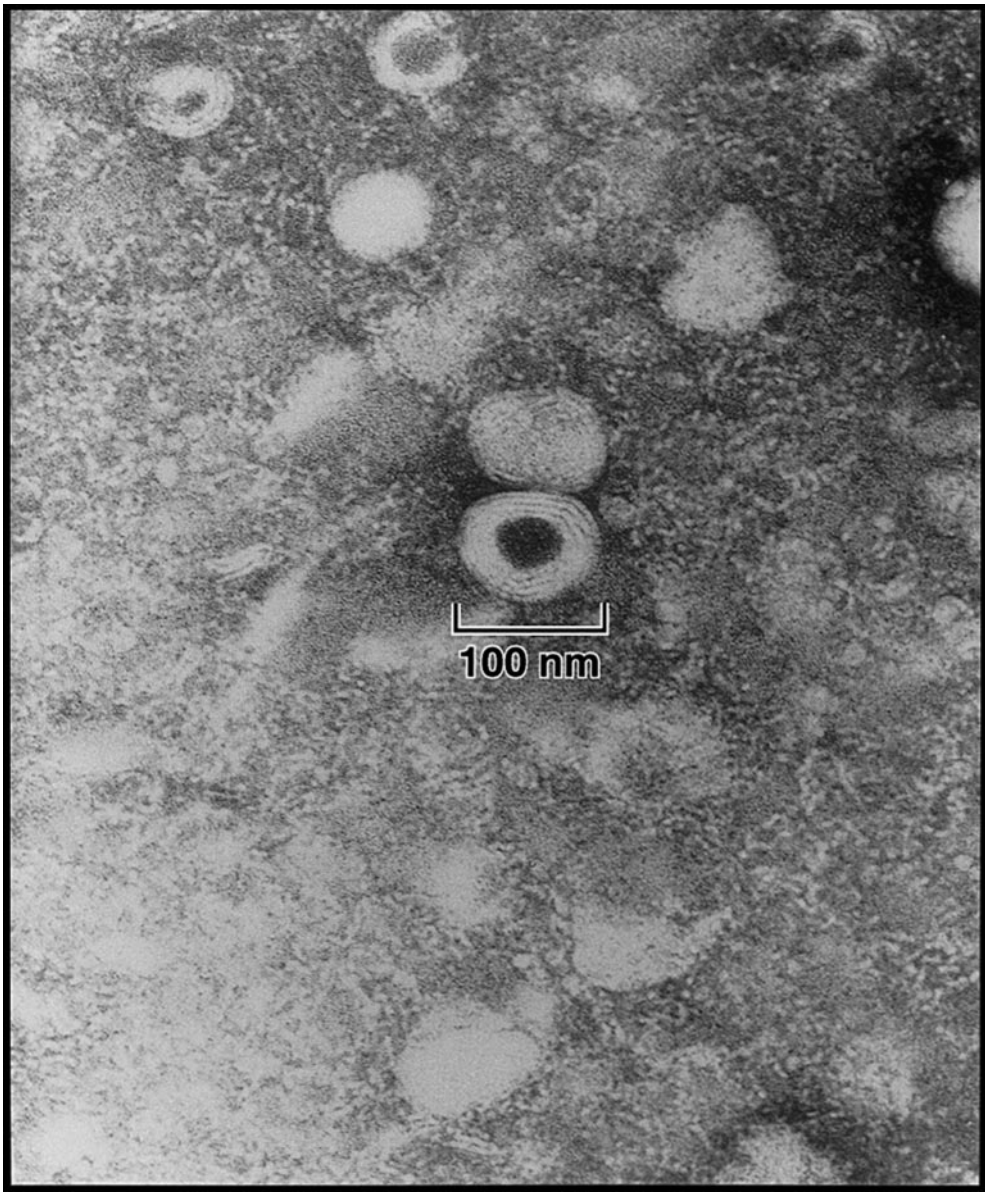

Fig. 2 Electron micrograph of material collected from an aerosol generated by an Aerotech II nebulizer containing 0.5 mg/ml 9-NC and 25 mg/ml DLPC over a 10-min period of operation. Uranyl acetate negative stain (1%) was used (performed by Thomas Giddings, Ph.D., Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colo.)

the two previously treated mice were again started on treatment, this time receiving 38.3 μg/kg 9-NC per day in a 15-min treatment period for 5 days per week. This treatment was continued to day 83 when treatment was stopped. During this later treatment, tumors disappeared in the two mice. These mice were followed for 75 days further. One mouse remained free of detectable tumor but a small tumor reappeared in the other.

Treatment of mice with xenografts of human colon cancer

Eight mice were started on treatment 12 days after implantation of tumor grafts with 9-NC-DLPC liposome aerosol (containing 8.5 μg/l 9-NC generated producing an estimated deposited dose of 76.7 μg/kg 9-NC per day) administered over a single 30-min period for 5 days per week (Fig. 6, Table 3). A second treatment group of seven mice received a similar dosage for 5 days per week for the first 23 days when the total daily dosage was increased to 153.4 μg/kg 9-NC administered as two daily 30-min treatments (a.m. and p.m.) for 5 days per week during the next 19-day period. At this time the dosage was further increased to 306.7 μg/kg 9-NC per day administered as two daily 60-min treatments (a.m. and p.m.) for 5 days per week for an additional 22-day period. Two other groups of ten mice each with xenografts received either DLPC-only liposome aerosol at the same dosage as that contained in the 9-NC aerosols initially used, or no treatment.

As shown in Fig. 6, there was a pronounced reduction in the rate of increase of tumor size (initial mean ± SD tumor volume, 95.3 ± 42.0 mm^3) in the two treated groups that was highly statistically significant ($P < 0.0001$, ANOVA). The mean maximum rate

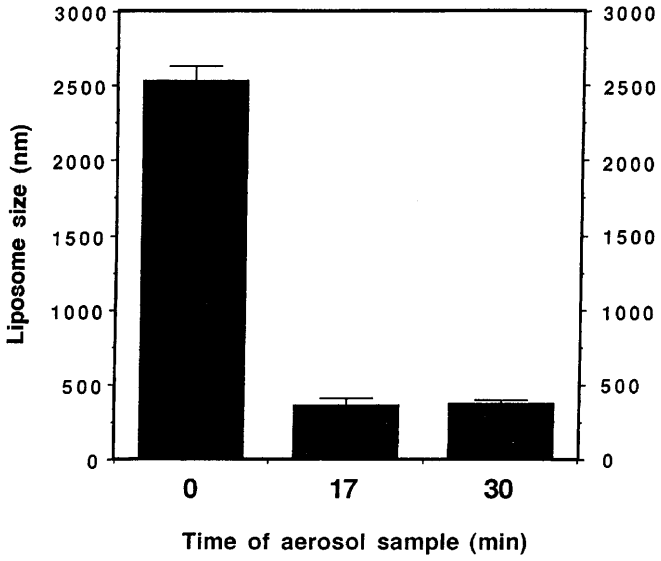

Fig. 3 Particle size of 9-NC-DLPC (1:50, w/w) liposomes. Particle size was determined from samples taken at 0, 17 and 30 min of nebulization from the reservoir of an Aerotech II nebulizer flowing at 10 l/min and containing 0.2 mg/ml 9-NC

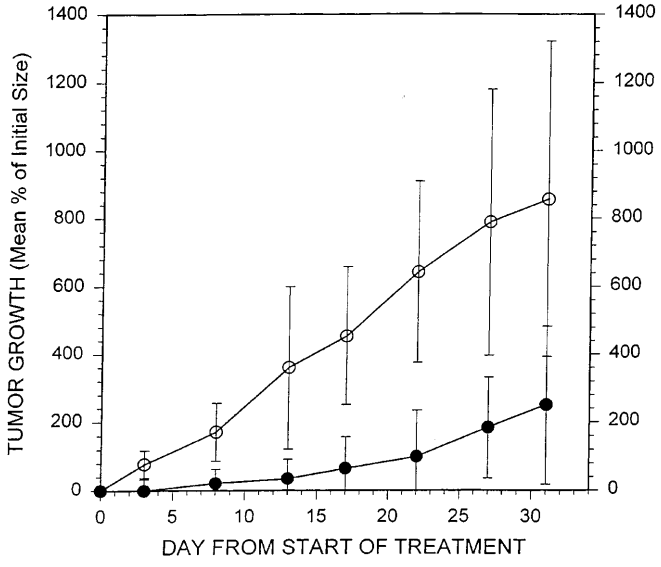

Fig. 4 Treatment of human breast cancer (CLO) xenografts in nude mice with 9-NC-DLPC liposome aerosol. Aerosol was administered for 15 min daily for 5 consecutive days per week for a period of 31 days. The calculated dose was 8.1 μg/kg of 9-NC per day. Values are means ± SD. The mean (±SD) size of tumors at the start of treatment was 90.7 ± 97.2 mm^2 (○ untreated, $n = 5$; ● 9-NC-DLPC liposomes, $n = 6$)

of tumor growth in the two treated groups over the first 42 days of treatment (70.4–113.6 mm^3/day) was seven- to tenfold slower than either the untreated (575 mm^3/day) or the DLPC-only liposome-treated (768 mm^3/day) control groups. The rate of tumor growth in mice receiving the constant low dose of 9-NC (38.3 μg/kg per day) remained at about the same level until day 56 when the size of their tumors increased slightly. However, in the treated group that received the increasing doses, the rate of tumor growth after day 42 decreased from 93.4 mm^3/day to 22.3 mm^3/day for the remainder of the experiment. This decrease in growth rate was statistically significant ($P = 0.012$; Student's t-test, two-tailed).

Treatment of mice with xenografts of human lung cancer

Starting 13 days after implantation, groups of 11 mice with xenografts were treated by aerosol with each liposomal drug regimen (aerosols containing 8.5 μg/l 9-NC generated producing an estimated deposited dose of 76.7 μg/kg 9-NC per day) for 5 days per week, while 11 other mice received no treatment (Fig. 7, Table 3). Initial mean ± SD tumor size at the start of treatment was 457 ± 76 mm^3 and the subsequent mean maximum rate of tumor growth in the untreated mice was 351 mm^3/day. Mean tumor growth was significantly reduced in both treatment groups ($P < 0.0001$, ANOVA), but the effect was greatest in animals receiving 9-NC. The difference in mean tumor volume between the untreated and 9-NC-treated mice was statistically significant ($P < 0.05$; Student's t-test, two-tailed) on day 7 while it required 14 days for the CPT-treated group to achieve significance. At all time-points, the mean size of tumors in the 9-NC-treated group was statistically significantly less than in the CPT group. Five animals in the 9-NC treatment group and six in the CPT treatment group died during the experiment. Most of the animals in the treatment groups developed a skin lesion over their dorsal skin which on histologic examination was found to be a pyoderma with micro-abscesses and many gram-positive cocci resembling staphylococci. Most deaths, either by sacrifice or spontaneous, occurred during the period from day 14 through day 24 of treatment. Later, as the tumor size reduced, the skin lesions disappeared and the animals gained weight and became more active. The increase in mean tumor volume of the CPT-DLPC-treated mice on day 35 compared to that on day 21 was not statistically significant ($P = 0.129$; Student's t-test, two-tailed).

Comparison of oral and aerosol treatment with 9-NC liposomes

A second experimental treatment of nude mice with the same human lung carcinoma was performed with 9-NC (Fig. 8, Table 3). It consisted of 11 mice treated with 9-NC-DLPC liposome aerosol and 11 mice given orally the same liposome preparation. The dose by aerosol was 76.6 μg/kg 9-NC per day for 5 days per week and the oral dose was 100 μg/kg 9-NC per day on the same schedule. Treatment was started on day 15 after xenograft implantation. By this time the tumor sizes were larger than in previous experiments (initial mean ± SD tumor volume, 760 ± 527 mm^3). Because of little apparent effect on the initial mean maximum rate of tumor growth (246 mm^3/day), the doses of both aerosol and

Table 3 Summary of the survival of nude mice during treatment with 9-NC and CPT

Experimental group	Liposome treatment	No. of mice		Duration of study (days)	No. of mice died or sacrificed (day)	Explanation[b]
		Start of experiment	End of experiment			
Breast cancer (CLO)	9-NC aerosol	6	5	31	1 (24)	Sacrificed for histology
	No treatment	5	3		2 (17)	Histology (1); died (1)
Continuation of above experiment:[a]						
Breast cancer (CLO)	9-NC aerosol	2	2	83	0	Large tumors
	No treatment	1	1		1 (51)	
Colon cancer (SQU)	9-NC aerosol, constant dose	8	6	63	2 (14, 37)	Large tumors
	9-NC aerosol, variable dose	7	7		0	
	DLPC only aerosol	10	0		3 (23), 4 (28), 3 (33)	Large tumors
	No treatment	10	0		1 (14), 2 (23), 4 (28), 3 (33)	Large tumors
Lung cancer (SPA)	9-NC aerosol	11	5	36	1 (11), 2 (14), 2 (17), 1 (24)	Emaciation, sacrificed or spontaneous death[c]
	CPT aerosol	11	6		2 (14), 2 (17), 1 (35)	As above
	No treatment	11	0		1 (24), 8 (28), 2 (32)	Large tumors
Lung cancer (SPA)	9-NC aerosol	11	9	49	1 (30), 1 (33)	Eye injury, emaciation
	9-NC oral	11	0		11 (33)	Large tumors
	No treatment	11	0		1 (19), 10 (33)	Large tumors
Lung cancer (SPA)	9-NC aerosol (nose-only)	8	7	36	1 (1)	Accident
	9-NC intramuscularly	8	8		0	
	No treatment	8	8		0	

[a] One of the three surviving untreated, and two of the five surviving 9-NC aerosol-treated mice were followed further at an increased dosage. The other mice were sacrificed for toxicity studies
[b] Animals were sacrificed due to large and/or necrotic tumors
[c] Several of these animals had a gram-positive coccal pyoderma which cleared as tumor size diminished

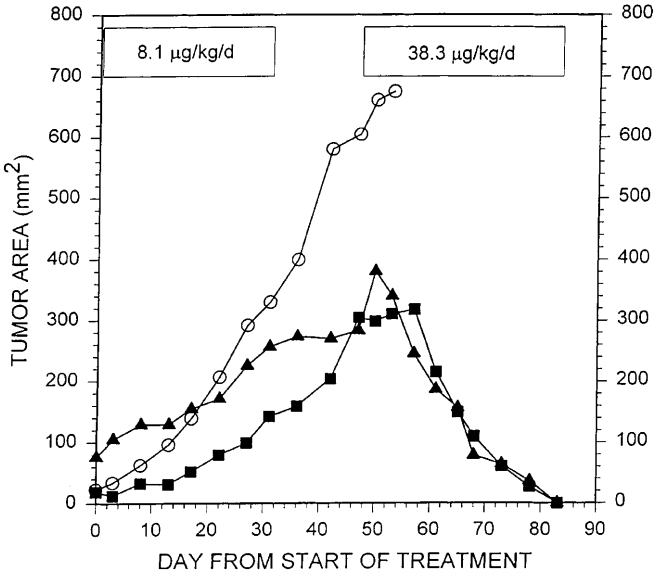

Fig. 5 Treatment of human breast cancer (CLO) xenografts in two nude mice from the experiment described in Fig. 4, followed by no treatment from day 32 to day 47, then treatment with 38.3 µg/kg 9-NC-DLPC per day for 5 consecutive days per week from day 47 to day 83 (○ untreated mouse; ■ mouse #3, ▲ mouse #5)

oral drug were doubled on day 13. Aerosol was administered twice daily for 30 min (a.m. and p.m.). By day 19 the tumors of the aerosol-treated animals were smaller in size than those in the untreated mice ($P = 0.004$; Student's t-test, two-tailed) and the mean rate of growth was decreasing (-138 mm^3/day). This decrease in tumor volume was statistically significant for days 19 through 33 ($P \geq 0.004$; Student's t-test, two-tailed). The tumors animals receiving oral dosage continued to increase in size in a closely comparable manner to those of the untreated group. By day 40, tumors in the aerosol-treated animals had regressed in size nearly to that at the start of treatment. The difference in mean tumor size between aerosol-treated animals and the other two groups of animals was highly significant ($P < 0.0001$, ANOVA). Of the nine 9-NC-DLPC-treated mice surviving on day 49, four did not have any measurable tumor mass. The increase in mean tumor volume in the 9-NC-DLPC aerosol-treated mice which occurred after treatment was stopped was not statistically significant ($P = 0.480$; Student's t-test, two-tailed). This increase was due to the fact that two of the mice had large tumors which had not decreased in size as rapidly as those in the other animals, suggesting that longer treatment might have been more effective.

Nose-Only 9-NC liposome aerosol treatment of mice with xenografts of human lung cancer

A third experimental treatment of nude mice with human lung carcinoma was conducted to evaluate the

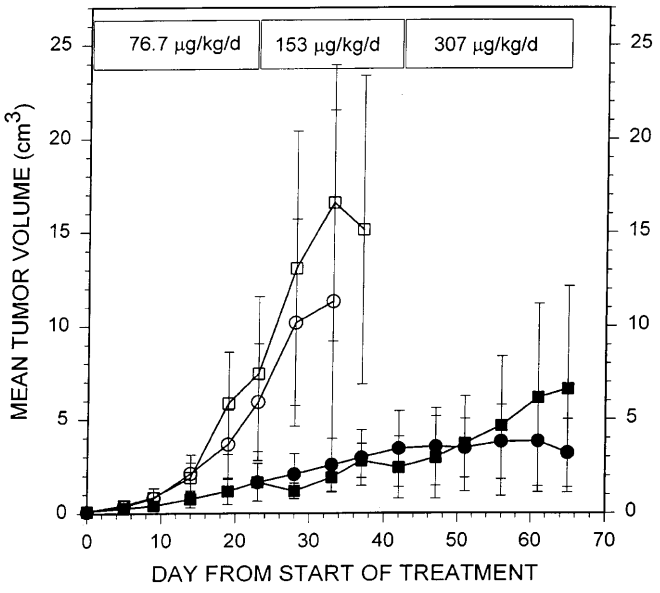

Fig. 6 Treatment of human colon cancer (SQU) xenografts in nude mice with 9-NC-DLPC liposome aerosol. The following dosing schedules were used: 76.7 μg/kg 9-NC per day for 5 consecutive days per week from day 0 to day 62 (constant dose): or 76.7 μg/kg 9-NC per day for 5 consecutive days per week from day 0 to day 23, 153.4 μg/kg per day twice daily for 5 consecutive days per week from day 24 to 42, and 306.7 μg/kg per day twice daily for 5 consecutive days per week from day 43 to day 65 (increasing dose). Values are means ± SD (○ untreated, $n = 10$; □ DLPC only liposomes, $n = 10$; ■ 9-NC-DLPC liposomes, constant dose, $n = 8$; ● 9-NC-DLPC liposomes, increasing dose, $n = 7$)

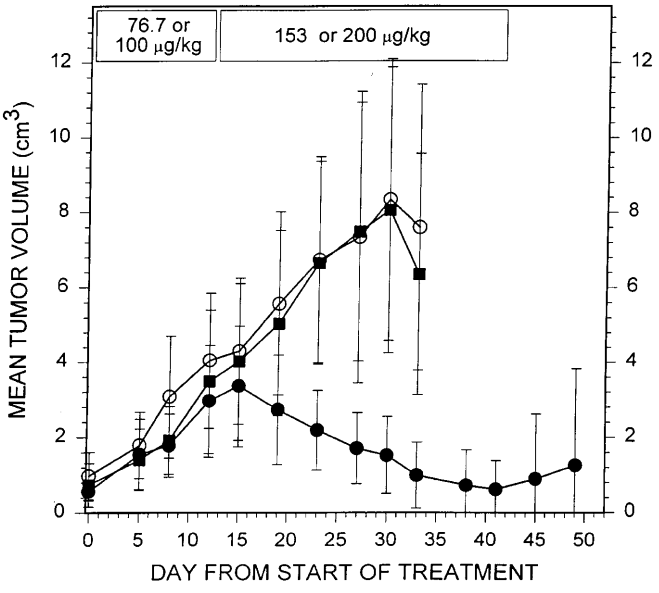

Fig. 8 Treatment of human lung cancer (SPA) xenografts in nude mice with 9-NC-DLPC liposome aerosol or oral administration. The aerosol dosage was 76.7 μg/kg 9-NC per day for 5 consecutive days per week from day 0 to day 12. The dose was increased to 153.4 μg/kg per day twice daily for 5 days per week from day 13 to day 41. The oral 9-NC-DLPC liposomes in aqueous suspension were administered at 100 and 200 μg/kg 9-NC per day following the same regimen as aerosol treatments. Values are means ± SD (○ untreated, $n = 11$; ■ oral 9-NC-DLPC liposomes, $n = 11$; ● aerosol 9-NC-DLPC liposomes, $n = 11$)

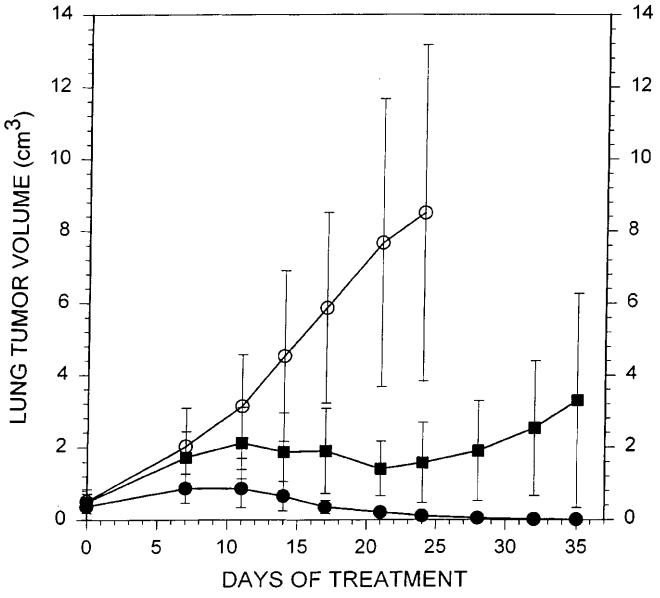

Fig. 7 Treatment of human lung cancer (SPA) xenografts in nude mice with 9-NC-DLPC or CPT-DLPC liposome aerosol. The liposome aerosols were administered in a dosage of 76.7 μg/kg 9-NC or CPT per day for 5 consecutive days per week from day 0 to day 35. Values are means ± SD (○ untreated, $n = 11$; ■ CPT-DLPC liposomes, $n = 11$; ● 9-NC-DLPC liposomes, $n = 11$)

effect of nose-only aerosol exposure or intramuscular injection of 9-NC-DLPC liposomes on tumor growth. Nose-only aerosol exposure was used to evaluate the possible effect of drug consumed orally as a consequence of animal grooming. Each group consisted of eight mice and were untreated, given a single intramuscular injection into the hind leg of 100 μg/kg 9-NC per day on days 0–23 followed by 200 μg/kg per day on days 24–36, or given 77 μg/kg 9-NC per day by nose-only aerosol exposure on days 0–23 followed by 153 μg/kg per day twice daily on days 24–36. Treatment was started 8 days after xenograft implantation and administered for 5 days per week. The mean initial tumor size was 96.8 ± 65.0 mm^3.

By day 19, mean tumor volumes were significantly different in the three groups (Fig. 9, Table 3; $P < 0.001$, ANOVA). While intramuscular injection was moderately effective compared to no treatment from day 19 on ($P < 0.02$; Student's t-test, two-tailed), this treatment was not as effective as the nose-only aerosol exposure ($P < 0.04$; Student's t-test, two-tailed). Following 12 days of aerosol treatment, tumor volume was statistically significantly less than in the untreated controls ($P \leq 0.014$; Student's t-test, two-tailed). Mean tumor growth rate in the aerosol treatment group was more than seven times slower than in the untreated controls from day 8 through 23 (57.9 mm^3/day vs 441 mm^3/day, respectively).

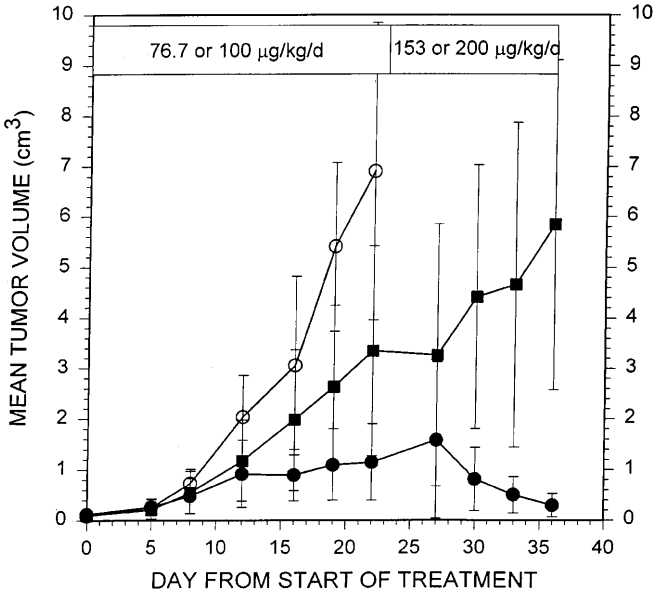

Fig. 9 Treatment of nude mice with human lung cancer (SPA) xenografts with 9-NC-DLPC liposomes administered by aerosol using a nose-only exposure device or by intramuscular injection. Values are means ± SD (○ untreated, $n = 8$; ● aerosol, 76.7 μg/kg 9-NC per day, 5 days per week from day 0 to day 23, then dosage doubled from day 24 to day 36, $n = 8$; ■ intramuscular injections into the hind legs, 100 μg/kg 9-NC per day, then 200 μg/kg per day on same schedule as aerosol treatment, $n = 8$)

Discussion

These studies showed a potent anticancer effect of 9-NC incorporated into DLPC liposomes and administered as an aerosol to nude mice implanted with three different human cancer xenografts (breast, colon and lung). The daily estimated doses associated with easily detectable suppression of tumor growth ranged from 8.1 μg/kg 9-NC per day in a 15-min inhalation to as high as 306.7 μg/kg per day in multiple inhalation periods. Most doses were 38.3 to 76.7 μg/kg per day in a single treatment period. Treatments ranged from 15 to 60 min in duration and were administered five times per week over periods of several weeks to several months. Since oral and intramuscular administration of the liposome formulations at comparable doses was only marginally effective at best, the substantial benefit of the liposome preparation administered by aerosol suggests a major role of this route in producing the favorable response.

We found that incorporation of 9-NC and CPT into DLPC liposomes was nearly complete when first formed. However, after nebulization, a small percentage of crystals was found in aerosols sampled using the AGI. There was no evidence of pulmonary toxicity in this study and thus there were apparently no untoward effects of the small proportion of crystals that may have been inhaled.

In our studies, treatment was continued long enough to establish a statistically significant difference in tumor size between treated and control groups. Since tumor size was receding in most treatment groups at the close of the experiments, further treatment would likely have resulted in greater regression in size of tumors. The least favorable results were with animals xenografted with human colon cancer. With this aggressive tumor (highest growth rate of those tested), longer treatments may be needed. This was suggested by the stabilization of growth in the group treated with an increased dose after day 42 (Fig. 6).

For comparison, investigators at the Stehlin Foundation [16, 24, 25] have used 9-NC by intramuscular injection of 1.5–2.0 mg/kg twice weekly for 3–4 weeks, producing complete remissions in colon, lung and breast cancer, malignant melanoma and ovarian carcinoma xenografts in nude mice. In some experiments partial remissions occurred. There were very few treatment failures. The authors report that the above dosage is about the largest reasonably well-tolerated dose in this model, and that lower doses are not effective. In comparison, the largest dose we used in these studies was only about 1/5 or less of the intramuscular dose on a weekly basis. The doses we used were also smaller than the generally effective dose of 1.0 mg/kg per day given 5 days per week for several weeks by direct injection into the stomach of mice with human cancer xenografts cited earlier in this report.

We examined lungs and other organs of a number of mice involved in this study, many after a month or more of treatment. None of the histological sections showed evidence of drug toxicity. Likewise, bone marrow from 16 treated mice was examined and no findings indicated myelosuppression (unpublished results).

As described previously, about 30% of aerosol inhaled by mice is deposits in the respiratory tract; the remaining 70% is exhaled [8]. At least one-half of the deposited aerosol is found on mucus membranes of the nose, head, trachea and upper bronchi. Particles deposited at these sites are promptly transported to the esophageal orifice and swallowed. Despite this distribution of inhaled particles, as shown above, only aerosol treatment was effective, implying that it was the pulmonary deposition that was essential. We found (unpublished observations) that drug deposited in the lungs by inhalation was transported in a few minutes to the systemic circulation and accumulated principally in the liver and to a lesser extent in the spleen and kidney. Drug was found also in the tumor tissue.

The use of 9-NC-DLPC liposome aerosol in humans has advantages over its use in mice. About 70% of inhaled aerosol will be deposited by nasal breathing in an adult human [20, 21]. More than one-half of this amount will be deposited in the nasopharynx. A large fraction of this deposition will be transported to the esophageal orifice and swallowed. In addition, some of the drug deposited in the trachea and upper bronchi will be transported upwards and swallowed. Alternatively, the aerosol can be administered by mouth breathing. Less than 5% given by this route will be deposited in the mouth and about 27% of the total inhaled amount will

be deposited in the lungs. As in rodents, some material deposited in the trachea and upper bronchi will be transported upward and swallowed, but the majority of the dose deposited in the lungs will remain there to diffuse into the systemic circulation. Thus, administration of aerosol particles to the lungs is more efficient in humans than in mice.

Other differences between the nude mouse-xenograft models of human cancer and the treatment of cancer in humans may be significant. Besides the benefit of pulmonary administration cited above, the greater inactivation of the CPTs by human serum albumin [2, 9], the differences in metabolic conversion of 9-NC to 9-amino-CPT [19], the greater myelosuppressive effects in humans [7, 22] and the use of immunodeficient mice raise important questions that can only be answered by studies in humans.

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