Antitumor Efficacy, Pharmacokinetics, and Biodistribution of NX 211: A Low-Clearance Liposomal Formulation of Lurtotecan

INTRODUCTION

The camptothecin-based topoisomerase I inhibitors are an important emerging class of oncology drugs that inhibit the ability of DNA topoisomerase I to relax torsionally strained DNA (1–5). The relaxation reaction involves single-strand cleavage and the formation of a short-lived catalytic intermediate referred to as a “cleavable complex.” The intact DNA strand passes through the nicked strand, thereby relaxing torsion in the helix. The nick is then religated by the topoisomerase I enzyme, allowing completion of replication, transcription, and other DNA functions. Camptothecin and many of its analogues bind to and stabilize the cleavable complex, thereby preventing religation of the DNA strands and converting an essential nuclear enzyme into a cellular poison. This leads to inhibition of active replication forks and generates an accumulation of single- and double-strand breaks in the replicating DNA, resulting in cell cycle arrest and, in many cell types, apoptotic cell death (5–8).

Two camptothecins are clinically useful and commercially available [irinotecan (Camptosar) and topotecan (Hycamptin)], whereas many more camptothecin analogues are under intense study as oncolytic agents (1–4). Lurtotecan is a water-soluble analogue of camptothecin. It inhibits mammalian DNA topoisomerase I in vivo with greater potency than topotecan and demonstrates potent antitumor activity in multiple xenograft models (9–11). Phase I and II clinical development trails for lurtotecan were conducted in the United States and Europe by Glaxo-Wellcome Inc from 1994 to 1998 (12–25). Multicenter Phase II studies have shown that lurtotecan is active as a second-line agent in small cell lung cancer and ovarian cancer. Preclinical and clinical data suggest that prolonged exposure may enhance the cytotoxicity of camptothecins. We have formulated lurtotecan in low-clearance, small unilamellar liposomes (NX 211), with the expectation that liposomal encapsulation will prolong the plasma half-life of lurtotecan in human subjects, resulting in prolonged tumor exposure, enhanced efficacy, and an improved therapeutic index. The preclinical data indicate that: (a) liposomal encapsulation markedly increases the plasma residence time of lurtotecan in animals; (b) NX 211 is more potent than free lurtotecan in vitro and in vivo; and (c) NX 211 accumulates significantly better than free lurtotecan in xenografted tumors. Based on these data, it is believed that liposomal encapsulation may indeed improve the efficacy and therapeutic index of lurtotecan.

MATERIALS AND METHODS

Cells and Reagents. The ES-2 tumor cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in McCoy’s 5a media + 10% FCS. The U251 tumor cell line was obtained from the National Cancer Institute Tumor Repository (Frederick, MD) and cultured in RPMI 1640 + 10% FCS. The KB tumor cell line and the multidrug-resistant (MDR1) variant KBV were obtained from the laboratory of Dr. Igor Roninson (University of Illinois, Chicago, IL) and main-
tained in DMEM + 10% FCS ± 1 μg/ml vincristine (Sigma). Cells were subcultured in the usual fashion with mild trypsinization and maintained in the appropriate growth media at 37°C in a 5% CO₂ incubator. Lurtotecan, topotecan, and radiolabeled [14C]lurtotecan were obtained from Glaxo-Wellcome (Research Triangle Park, NC) and used in the preparation of [14C]NX 211. The specific activity of [14C]NX 211 was 9.75 μCi [14C]lurtotecan/ml, with a concentration of 0.4 mg lurtotecan/ml. Sterile D5W3 (McGaw, Inc., Irvine, CA) was used as a diluent. A stable liposome formulation of lurtotecan (NX 211) was formulated under 100 nM with a 20:1 lipid:drug ratio and a lipid composition (26, 27). This methodology yields small unilamellar liposomes.

The specific activity of [14C]NX 211 was 9.75 cpm divided by the weight of tissue in grams. The chemical counts in the tissue. The data were reported as the true counts.

### Statistical Analysis

All analysis was performed to compare for a drug effect for each of the 10 tissues measured. Wilcoxon rank-sum tests were performed to test for pairwise differences between drugs for each tumor type at each time point. The minimum achievable P was 0.05 for a one-sided test or 0.10 for a two-sided test. Tests that achieved this P were noted as significant on the summary tables. In addition, rank ANOVA was performed to test for differences between tumor types and drugs simultaneously. If the tumor types were able to be pooled, P > 0.05, and a significant difference between drugs was observed, P > 0.05, a significant difference was noted on the summary table for the pooled tumor types. However, if the tumor types were not able to be pooled, P ≤ 0.05, no statistical test results for the comparison of drugs were reported for the pooled tumor types.

### Xenograft Studies

All antitumor efficacy studies were performed with s.c. established xenograft tumors with initial tumor size of 200 ± 50 mg in all treatment groups. All drug substances were prepared fresh each day in either D5W or D5W containing empty liposomes just before administration. Body weight and tumor size were determined twice weekly. In repeat-dose studies, the drug was administered weekly for either 2 or 3 consecutive weeks. Tumor growth was monitored until a maximum size was achieved (10% of animal body weight) or, in the case of cures, 60 days. Both the percentage of tumor volume and the percentage of body weight change were plotted for each experiment. Tumor measurements were used for determining the percentage TGI [%TGI = (Wc − Wt)/Wc where Wc is the mean tumor weight of control group, and Wt is the mean tumor weight of treated group] and the LCK [LCK = (T − C)/3.32 × (Tt) where T is the time in days for the treated group mean tumor volume to reach a final tumor volume, C is the time in days for the control treatment group to reach the defined final tumor volume, and Tt is the tumor doubling time of control tumors]. Any cures were excluded from the LCK calculations.

### PK Studies

Naïve, non-tumor-bearing female Nu/Nu mice (20–25 g) were divided into two treatment groups (28 mice/group) and given a 1 mg/kg i.v. bolus dose of lurtotecan (uncorrected for salt and water) or 1 mg/kg NX 211 (as free base GI47211) in D5W via the tail vein. Blood samples were obtained from four mice/group at predetermined time points by cardiac puncture using heparinized syringes. Samples were kept on wet ice until centrifuged to obtain plasma. Plasma samples were stored at −20°C until analyzed. The time points for blood collection after lurtotecan administration were 5, 15, and 30 min and 1, 2, 4, and 6 h. Blood was collected from NX 211-treated mice at 10 min and 2, 4, 8, 24, 32, and 48 h after the dose. Samples were prepared for HPLC analysis using a modification of a previously published method (28). Briefly, lurtotecan samples (50 μl) were precipitated and acidified with a 2:1 mixture of 10% perchloric acid:acetoni trile containing 6,7-dimethoxy-4-methyl-coumarin (Aldrich Chemical Co., St. Louis, MO) as an internal standard. After centrifugation, the supernatant was injected directly into the HPLC column. The mobile phase consisted of 84:15:1:0.1 sodium phosphate [0.1 m (pH 2.2)]; acetoni trile:tetrahydrofuran:acetic acid. The flow rate was 0.35 ml/min, and the column temperature was 45°C. The injection identity of the radioactive material was not identified in these studies.
volume was 25 μl. Detection was achieved using an excitation wavelength of 390 nm and emission at 425 nm. HPLC analyses were performed using a Model 600S Controller, a Model 717 Plus Autosampler, a Model 626 Pump, and a Model 474 Scanning Fluorescence Detector (Waters Corp., Milford, MA). A 3 mm × 25 cm Zorbax Rx C-18 HPLC column fitted with a 12.5 mm × 4.6 mm Zorbax Rx C-18 guard column was used. Chromatographic data were acquired and analyzed using a commercial computer system (Millennium 32; Waters Corp.). Samples containing less than 50 ng/ml drug were analyzed by preparation (as described above) using 100 μl of plasma, an increased injection volume of 70 μl, and incorporation of post-column photodegradation to enhance the fluorescence signal. A photodegradation cell (Beam Boost; Advanced Separation Technologies, Whippany, NJ) equipped with a 254 nm lamp and a 3 mm × 10 m reaction coil was used in this determination. Detection was achieved using an excitation wavelength of 378 nm and emission at 420 nm. The GH14721 calibration standards were prepared in control mouse plasma. Samples were quantitated against calibration curves obtained from the 1/c²-weighted linear regression of the peak height ratios of the drug to 6,7-dimethoxy-4-methyl-coumarin in the calibration standards. The precision and accuracy of the method were determined by the analysis of samples prepared by adding known amounts of NX 211 to control mouse plasma. Use of the liposomal material in this experiment assured that the method was capable of fully disrupting the liposomes. The method was demonstrated to give relative SDs of less than 15% and had a relative accuracy (percentage of deviation from nominal) of −2 to +16% over concentrations ranging from 0.5 to 200,000 ng/ml. The limit of quantitation was set at 1.4 ng/ml due to a potential interference observed in control mouse plasma. The mean plasma concentrations for each group at each time point were analyzed by noncompartmental analysis using commercial software (WinNonlin version 1.5; Scientific Consulting, Inc., Carey, NC).

RESULTS

Xenograft Studies. The therapeutic index of lurtotecan, topotecan, and NX 211 was determined in two separate xenograft models. This experiment was initiated to compare the antitumor efficacy of NX 211 with that of free drug and with topotecan, an approved topoisomerase I inhibitor with clinical activity against a variety of solid tumors and hematological malignancies. A broad range of dosages was evaluated for each agent in an attempt to determine a therapeutic index as well as the antitumor efficacy at an optimum dosage for each compound. Two experimental controls were included, untreated and empty liposomes. All treatments were administered as single bolus i.v. injections, and each group contained 10 mice. Topotecan was administered at 6, 9, 12, 16, 20, 30, and 40 mg/kg; lurtotecan was administered at 6, 9, 12, 16, 20, and 30 mg/kg; and NX 211 was administered at 3, 6, 9, 12, 16, 20, 30, and 40 mg/kg. The therapeutic index for each experiment was determined by extrapolating the LD₅₀, ED₆₀, and ED₈₀ values from the dose versus mortality curves and the dose versus %TGI graphs. The results of the KB tumor study demonstrated that the 3 mg/kg dose of NX 211 produced significantly greater tumor growth delay than that produced by either 6 mg/kg lurtotecan or 12 mg/kg topotecan (P = 0.00004). The growth delays produced by lurtotecan and topotecan were not significantly different from each other. In the ES-2 experiment, slightly different MTDs were determined. However, the overall pattern of differences remained the same. The growth delay produced by 9 mg/kg NX 211 was significantly greater than that produced by either 12 mg/kg lurtotecan (P = 0.0006) or 16 mg/kg topotecan (P = 0.00004). The overall results demonstrate that NX 211 is more potent and has a consistent increase in the therapeutic index ranging from 3–14-fold over that of lurtotecan (Table 1).

Additional antitumor efficacy studies with repetitive administration of NX 211 and lurtotecan were performed in several other xenografts. These studies compared free drug and liposomal drug at equitoxic doses and schedules in the ES-2 ovarian tumor and the KB and KBV epidermoid tumors. Drugs were administered i.v. once weekly for 3 consecutive weeks for the KB and ES-2 xenografts and administered once every 2 weeks for the KBV xenograft. The MTD for each drug tested in these models was determined on the basis of body weight loss and toxic deaths. For NX 211, the MTD was determined to be 9 mg/kg/week, whereas the MTD for lurtotecan and topotecan were 14 and 16 mg/kg/week, respectively. The repeat-dose efficacy results for the ES-2 xenograft study are presented in Table 2 and Fig. 1. In this study, both lurtotecan and topotecan were compared with NX 211 at equitoxic doses, and all three drug groups had a significant effect on tumor growth compared with the vehicle control group. However, among the different treatment groups, there were significant differences in the magnitude of response and the duration of response, with NX 211 demonstrating a significant advantage. The amount of body weight loss and the number of toxic deaths that occurred in this experiment demonstrated that all three groups were dosed at their maximum tolerated levels. In the NX 211-treated group, three durable cures were generated with an overall LCK of 4.08, whereas no durable cure occurred in the free lurtotecan group, which had a LCK of 2.14. The topotecan group failed to generate any cures, and the LCK for this group was 0.58. The difference in TGI between the NX 211 and lurtotecan groups in the KB xenograft model was less dramatic, but NX 211 still showed greater activity. In this experiment, two different groups were tested at an identical dose of 14 mg/kg lurtotecan. In one group, D5W was used as the diluent, whereas in the second group, a suspension of empty liposomes was used as the diluent to control for any additional effects the lipid may have had on tumor growth. The results shown in Table 3 demonstrate that the empty liposomes had no effect on the observed efficacy because the two lurtotecan-treated groups

### Table 1  Determination of the therapeutic index for topotecan, lurtotecan, and NX 211 in the KB and ES-2 xenograft models

<table>
<thead>
<tr>
<th>Drug</th>
<th>KB tumor (LD₅₀/ED₆₀)</th>
<th>ES-2 tumor (LD₅₀/ED₆₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topotecan</td>
<td>0.5</td>
<td>ND²</td>
</tr>
<tr>
<td>Lurtotecan</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>NX 211</td>
<td>2.9</td>
<td>14.4</td>
</tr>
</tbody>
</table>

² ND, not determined because an ED₆₀ value was never reached.
were essentially identical, with TGI values of 90% and 92% and a LCK of 1.66. The group treated with 9 mg/kg NX 211 had a 98% TGI and a LCK index of 2.7. Thus, the NX 211-treated group demonstrated a greater tumor cell kill effect than the lurtotecan-treated groups. In the multidrug-resistant tumor model, KBV, the NX 211-treated groups demonstrated a dose-response inhibition of tumor growth, whereas the lurtotecan-treated group did not (Fig. 2). In fact, the lowest dose of NX 211, 4 mg/kg, was as effective as the high dose of lurtotecan, 16 mg/kg. Overall, the effectiveness of lurtotecan in this tumor model was minimal compared with the control, with a LCK index of 0.59 for the group treated with 16 mg/kg lurtotecan (Table 4). In contrast, NX 211 produced a LCK index of 1.63 at the 9 mg/kg dose level, demonstrating that NX 211 is nearly three times as effective in cell kill. These results are consistent with those seen in previous studies of equal dose comparison and single-dose therapeutic index determinations where NX 211 consistently demonstrated superior efficacy compared with lurtotecan and topotecan.

**PK Studies.** In the PK studies, NX 211 and lurtotecan were administered at 1 mg/kg on an “as is” basis. This dose

<table>
<thead>
<tr>
<th>Drug (dose)</th>
<th>Maximum body weight loss</th>
<th>No. of toxic deaths</th>
<th>% TGI$^a$</th>
<th>LCK$^b$</th>
<th>T-C$^c$</th>
<th>No. of durable cures</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX 211 (9 mg/kg)</td>
<td>19%</td>
<td>0/8</td>
<td>99.5%</td>
<td>4.08</td>
<td>51.4</td>
<td>3/8</td>
</tr>
<tr>
<td>Lurtotecan (14 mg/kg)</td>
<td>9%</td>
<td>3/8</td>
<td>95.0%</td>
<td>2.14</td>
<td>27.0</td>
<td>0/8</td>
</tr>
<tr>
<td>Topotecan (16 mg/kg)</td>
<td>16%</td>
<td>3/8</td>
<td>57.0%</td>
<td>0.58</td>
<td>7.4</td>
<td>0/8</td>
</tr>
</tbody>
</table>

$^a$ TGI = 100 (1 – Wt/Wc); Wt, mean tumor volume of treated group at day 15; Wc, mean tumor volume of control group at day 15.

$^b$ LCK = T-C/(3.32) (Td) where Td = tumor doubling time of control tumors (days).

$^c$ T-C, difference in days for treated and control groups to reach 400% tumor volume increase.

**Fig. 1** Tumor growth curve and body weight graph of the ES-2 ovarian tumor xenograft experiment comparing lurtotecan, NX 211, and topotecan at an equitoxic dose. All three compounds were administered i.v. weekly via tail vein. Data shown are the mean ± SE from eight mice at each time point, except where noted. Arrows indicate dose days.
translated to 1 mg/kg GI147211 as the free base for the NX 211-treated group and 0.78 mg/kg GI147211 for the lurtotecan-treated group (corrected for the dihydrochloride salt and water content). The results, shown in Fig. 3 and Table 5, demonstrated that NX 211 (liposomal encapsulated lurtotecan) has significantly increased plasma $C_{\text{max}}$, AUC, mean residence time (MRT), and half-life. Plasma clearance (Cl) for NX 211 was decreased almost 1500-fold, and the extensive volume of distribution ($V_{ss}$) observed for lurtotecan (9 liters/kg) was reduced essentially to that of the plasma compartment (35 ml/kg). These results are consistent with a model of a stable liposomal drug whose circulation is restricted primarily to the plasma compartment.

**Biodistribution Studies.** These studies were carried out in several different xenograft models and revealed that consistently greater amounts of radioactive material accumulated in
The tumors of the NX 211-treated mice than in the tumors of lurtotecan-treated mice. Fig. 4 compares the results of four different tumor xenografts and shows the cpm/g tumor tissue, 24 h after injection with either [14C]NX 211 or [14C]lurtotecan. Tumors from animals treated with [14C]NX 211 contained 10,255 (ES-2), 7,817 (KB), 9,474 (KBV), and 1,981 (U251) cpm/g tumor tissue compared with 256 (ES-2), 118 (KB), 162 (KBV), and 0/8 (U251) cpm/g tumor tissue in mice treated with [14C]lurtotecan. These data reflect a 9–67-fold increase in the amount of radioactive material in the tumors of [14C]NX 211-treated mice, whereas the liver was the major site of localization of radioactive material for the [14C]lurtotecan-treated animals. The spleen was the major site of localization at all time points examined, which is consistent with the known uptake of liposomes by the reticuloendothelial system. The KB tumor showed an apparent peak of radioactive material of over 2-fold by 6 h, compared with the amount at 1 h. The spleen and intestines also had a peak in radioactive material by 6 h. The other tissues examined had a peak in radioactive material at 1 h, followed by a gradual elimination of radioactive material until 48 h. Thus, NX 211 showed a greater propensity for tumor localization, compared to free lurtotecan, which varied with different tumor types.

The biodistribution of NX 211 over time is seen in Fig. 6, which plots the cpm/g tissue at selected times over a 48-h period from tumor-bearing mice treated with [14C]NX 211. The spleen was the major site of localization at all time points examined, which is consistent with the known uptake of liposomes by the reticuloendothelial system. The KB tumor showed an apparent peak of radioactive material of over 2-fold by 6 h, compared with the amount at 1 h. The spleen and intestines also had a peak in radioactive material by 6 h. The other tissues examined had a peak in radioactive material at 1 h, followed by a gradual elimination of radioactive material until 48 h. Thus, NX 211 showed a greater propensity for tumor localization, compared to free lurtotecan, which varied with different tumor types.

**DISCUSSION**

Liposomal encapsulation can significantly prolong the plasma residence time of drugs that would otherwise be rapidly distributed or cleared (28–32). Altered plasma pharmacokinetics is one mechanism by which liposomal drugs may demonstrate substantial changes in tissue distribution, efficacy, and toxicity from their nonencapsulated counterparts. This may be especially important with cytotoxic drugs such as camptothecins, where the major cytotoxic effects are manifested in a cell cycle-specific fashion (5). Thus, the increase in LCK and the greater overall antitumor efficacy seen in the present experiments may be due in large part to prolonged exposure as...
bloodstream prohibits the accurate assessment of fold). The rapid and extensive distribution of free drug from the volume of distribution after liposomal encapsulation (over 250-fold drug) is ascribed to the dramatic decrease in tumor sites due to leaky vasculature (38–40). The variation we see within different xenografts would also argue that the increased exposure is due in part to liposomal extravasation, which varies between different tumors.

The increased efficacy seen with NX 211 compared with lurtotecan was evident in both single-dose and repeat-dose xenograft experiments, where a consistent increase in the therapeutic index, LCK, and the number of durable cures generated is consistent with an improved formulation of this active agent. This improvement in antitumor efficacy is significant and is consistent with the increased plasma AUC and tumor exposure. The increased efficacy of NX 211 in the KBV xenograft is especially intriguing because the free drug demonstrated only slight activity in this model, and active efflux of camptothecin molecules by the PGP-170 protein is believed to be less important as a multidrug resistance factor against this class of cytotoxic drugs.

In the clinic, continuous infusion schedules have been used with topotecan and lurtotecan in an attempt to prolong tumor exposure while reducing toxicity by attenuation of peak plasma drug levels. Phase I studies have evaluated lurtotecan in several i.v. dosing schedules, including daily i.v. doses for 5 consecutive days (12–14), continuous infusion for 3 days (14–19), and continuous infusion for 7, 14, or 21 days (19). Tumor responses in these Phase I studies were observed in the 3- and 21-day infusion schedules, but not in the consecutive 5-day i.v. schedule. Although the number of patients was limited, these results suggest enhancement of the antitumor activity of lurtotecan by continuous infusion. Prolongation of the infusion caused more pronounced thrombocytopenia but did not increase the severity of neutropenia, suggesting that the toxicity profile might also be influenced by the schedule of administration. Clinical findings suggest that prolonged i.v. infusion of topotecan may also increase tumor response (41). Collectively, the data are consistent with the hypothesis that a low-clearance liposomal lurtotecan formulation such as NX 211 will have a superior efficacy and therapeutic index in the treatment of solid tumors. Human clinical studies to determine the safety, tolerability, and phar-
Fig. 5  Biodistribution of $^{14}\text{C}$-NX 211 and $^{14}\text{C}$-lurtotecan in eight different tissues from four different xenograft studies. Data shown are the mean ± SD from three mice at each time point. Tu, tumor; Li, liver; Ki, kidney; Sp, spleen; In, intestine; Lu, lung; He, heart; Br, brain.
The macokinetics of NX 211 treatment in patients with advanced solid tumors are currently under way.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 6 Time course biodistribution of [14C]NX 211 in six different tissues harvested from mice engrafted with the KB tumor. Data shown are the mean ± SD from three mice at each time point. Missing error bar from the 6 h spleen time point represents the mean of two mice.
2912 NX 211, Liposomal Lurtotecan


Antitumor Efficacy, Pharmacokinetics, and Biodistribution of NX 211: A Low-Clearance Liposomal Formulation of Lurutocan


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