Preclinical Evaluation of LU 79553: A Novel Bis-naphthalimide with Potent Antitumor Activity

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ABSTRACT

LU 79553 is a novel bis-naphthalimide which is highly cytotoxic in vitro with EC_{50} (concentration required for 50% inhibition of growth) ranging from $2 \times 10^{-6}$ to $5 \times 10^{-10}$ M. A number of studies were conducted to examine its antitumor activity in human xenograft models. In addition, we wanted to explore the possible schedule dependency of LU 79553 cytotoxicity in these xenograft models. Complete regression of MX-1 (mammary carcinoma) xenografts was observed when LU 79553 was administered i.v. daily for 5 doses at 20 mg/kg (2 cycles starting on Days 6 and 20) or every 3 days for 2 doses at 55 mg/kg (2 cycles starting on Days 6 and 13) or every 7 days for 4 doses. Complete regression was also seen in the MX-1 model when tumors were staged at 1-2 g prior to the initiation of treatment. Regressions (complete or partial) were observed in the LX-1 (lung), CX-1 (colon), DLD (colon), and LOX (melanoma) xenograft models. A significant increase in the median survival time of OVCA-3- (ovarian carcinoma) bearing mice was noted in LU 79553-treated animals (treated/control = 195%). The excellent activity of this compound in such a wide variety of tumor types suggests LU 79553 merits further investigation in clinical trials.

INTRODUCTION

Naphthalimides are a new class of DNA intercalating agents synthesized in our laboratories which have demonstrated significant preclinical antitumor activity (1-3). Two members of this class, amonafide and mitonafide (Fig. 1) have been entered into clinical trials (4, 5). Since bis-intercalating agents have demonstrated a higher binding affinity for DNA (6), we synthesized and evaluated a number of bis-intercalating naphthalimides (bis-naphthalimides; Ref. 7). The structure of one of these compounds, LU 79553, is shown in Fig. 1. The bis-naphthalimides appear to bind DNA by intercalation of the chromophore group (3). While our research group (7) and others (8,9) synthesized in our laboratories which have demonstrated significant preclinical antitumor activity (1—3). Two members of this class, amonafide and mitonafide (Fig. 1) have been entered into clinical trials (4, 5). Since bis-intercalating agents have demonstrated a higher binding affinity for DNA (6), we synthesized and evaluated a number of bis-intercalating naphthalimides (bis-naphthalimides; Ref. 7). The structure of one of these compounds, LU 79553, is shown in Fig. 1. The bis-naphthalimides appear to bind DNA by intercalation of the chromophore group (3). While our research group (7) and others (8, 9) found that nitro substituents in the chromophore rings were, as in the mononaphthalimides (2), necessary for the antitumor activity of bis-naphthalimides, LU 79553 demonstrates potent activity yet lacks aromatic substituents. The purpose of these studies was to evaluate LU 79553 for efficacy against a panel of xenograft tumors.

MATERIALS AND METHODS

Animals. Specific pathogen-free female athymic Ncr-nude mice were used for all human tumor xenograft experiments, obtained from either Taconic Farms (Germantown, NY) or Charles River Breeding Laboratories (Wilmington, MA). Mice weighing between 18 and 25 g were used for all experiments (generally between 6 and 10 weeks old). The mice were housed in a pathogen-free barrier facility where ambient light was automatically controlled to produce 12-h light and dark cycles. Animals were maintained in an American Association for Accreditation of Laboratory Animal Care accredited facility, according to the NIH Guide for the Care and Use of Animals. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Tumors. MX-1 human mammary carcinoma, LX-1 human lung carcinoma, CX-1 and DLD-1 human colon carcinomas, and LOX human amelanotic melanoma were all obtained from the tumor repository of the National Cancer Institute (Bethesda, MD). OVCAR-3 human ovarian carcinoma cells were obtained from American Type Culture Collection. Solid tumors (MX-1, CX-1, LX-1, and DLD-1) were passaged in vivo by s.c. implant of approximately a 50-mg piece of a tumor dissected from the non-necrotic portion of a donor tumor. OVCAR-3 cells were grown i.p. as ascites, harvested around Day 30, and injected into passage or experimental mice at a ratio of 1 donor:5 recipients (approximately 1-4 x 10^7 cells/mouse). Cell lines were routinely tested to demonstrate that they remained free of Mycoplasma using the Gen-Probe rapid detection system (San Diego, CA). All tumors were negative for pathogenic murine viruses (AnMed Biosafe, Rockville, MD).

Tumor Studies. The day tumors were implanted or injected was designated as Day 0. Tumors were allowed to grow for 5 to 10 days or until tumors measured 70—110 mg. LU 79553 was dissolved in sterile, pyrogen-free water and injected i.v. via the lateral tail vein at various doses and schedules (see figure legends 1—9 for details). Tumor growth was monitored by caliper measurements two to three times per week and converted to volume by the formula, $V = (W^2 \times L)/2$. Median tumor size was plotted. Regressions were defined as partial (PR, 2 tumor shrinkage, but always measurable) or complete (CR, no palpable tumor). An animal was considered cured if a CR was maintained past Day 70.

In Vivo Cytotoxicity. Inhibitory activity was measured using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (7). Cell lines were obtained from American Type Culture Collection and cultured in the recommended media. Drug-resistant sublines were removed from the selecting agent for two passages prior to testing. All assays were performed with exponentially growing cells. Cells were plated at 2500/well in 96-well plates. Test compounds were added 24 h later and the assay was ended after 72 h of drug exposure. The concentration of drug required to inhibit 50% of the cell growth (EC_{50}) was calculated using an in-house program (7).

Topoisomerase Inhibition. Inhibition of the decatenation activity of topoisomerase II by LU 79553 was measured according to the method of Sahai and Kaplan (10). Briefly, 450 mg C. fasiculata kDNA was incubated with 4 units of purified human topoisomerase II (TopoGen Inc., Columbus, OH) in 20 µl reaction buffer (0.05 M Tris-HCl, pH 8, 0.12 M KCl, 0.01 M MgCl_2, 0.5 mM ATP, 0.5 mM DTT) for 20 min at 37°C in the absence or presence of various concentrations of LU 79553 (range, 4—500 µM). The reaction mixture was stopped with 4 µl stop buffer (5% Sarkosyl, 0.0025% bromophenol blue, 25% glycerol) and further incubated with 50 µg/ml proteinase K at 37°C for 1 h. The reaction mixture was then loaded on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. After electrophoresis for 12 h at 1.7 V/cm in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA-Na_2, pH 8.5), the gel was destained and photographed under UV illumination. To determine the quantities of decatenated DNA produced in each reaction mixture, the minicircle bands on the negative were scanned with a densitometer. Peak area of the minicircle band for drug-treated (P_{control}) and control (P_{control}) reactions was measured. The percentage of inhibition of... 

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2 The abbreviations used are: PR, partial regression; CR, complete regression; EC_{50}, concentration required for 50% inhibition of growth; MTD, maximum tolerated dose; QID x 5, daily for 5 doses; QOD x 3, every 7 days for 3 doses; QD x 3, every 2 days for 3 doses; QID x 2, every 3 days for 2 doses; kDNA, kinetoplast DNA.
topoisomerase II activity by bis-naphthalimides at each concentration was calculated by the following equation:

\[
\% \text{ Inhibition} = \left[1 - \left(\frac{P_{\text{treated}}}{P_{\text{control}}}\right)\right] \times 100 \tag{1}
\]

The percentage of inhibition data were then used to determine the IC_{50}, the concentration at which 50% of enzyme activity was inhibited.

RESULTS

In Vitro Cytotoxicity. LU 79553 was tested in a cytotoxicity assay against a panel of human and murine cell lines. The EC_{50} values are summarized in Table 1. LU 79553 inhibited the growth of all cell lines tested, with EC_{50} values ranging from 2 \times 10^{-7} to 5 \times 10^{-10} M. Even cell lines selected for resistance to other chemotherapeutic drugs were sensitive to killing by LU 79553, although higher concentrations of drug were generally required. Due to this in vitro activity and solubility in aqueous solution, LU 79553 was chosen for extensive in vivo testing.

Efficacy in Human Tumor Xenografts. Initial dose-response studies were conducted in the MX-1 mammary carcinoma model (11). As shown in Fig. 2, when LU 79553 was administered to MX-1 tumor-bearing mice as two cycles of five daily injections (Q1D X 5; Days 6 and 20), regressions occurred in a dose-dependent fashion. At a daily dose of 5 mg/kg, three PRs were seen. When a daily dose of 10 mg/kg was given, two PRs and three CRs were noted. Doses of 20, 25, or 30 mg/kg/day were curative. One animal which received 30 mg/kg/day died at Day 42 with no signs of tumor progression (gross necropsy indicated no remarkable findings or cause of death). In these experiments, tumor regression begins at the end of the first treatment cycle and complete tumor disappearance occurs between Days 24 and 27. In control experiments, cures of MX-1 xenografts were also seen with cyclophosphamide (75 mg/kg/day i.v. twice/week for 5 doses) and partial and CRs were seen with taxol (20 mg/kg/day i.v. i.Q7D X 3; data not shown). We have treated 130 MX-1 tumor-bearing mice with the Q1D X 5 (2 cycles) schedule at doses between 20 and 30 mg/kg/day and have seen cures in 118 (91%) of the animals (data not shown).

To further elucidate the level of activity of LU 79553 in the MX-1 model, s.c. implanted tumors were allowed to grow for 20 days prior to treatment. When treatment was initiated, the tumor sizes of 1-2 g represented 5-10% of total body weight. However, even with this tremendous tumor burden, administration of LU 79553 at 20 mg/kg/day (Q1D X 5; Days 6 and 20) 5 of 5 animals had regressions of 5 animals (1 CR/cure, 4 PR). If LU 79553 was given at 40 mg/kg/day (Q2D X 3; Days 6 and 13) 5 of 5 animals had regressions (1 CR, 4 PR). At a dose of 55 mg/kg/day (Q3D X 2; Days 6 and 13) 5 of 5 animals also showed regressions (2 CR, 3 PR). Although not curative in this model, treatment with LU 79553 caused regressions (partial and complete) which resulted in tumor growth delay indicative of significant tumor killing.

LU 79553 also demonstrated excellent activity against the less sensitive LX-1 xenograft model (Ref. 11; Fig. 4). When administered at 20 mg/kg/day (Q1D X 5; Days 6 and 20) regressions occurred in 5 of 5 animals (1 CR/cure, 4 PR). If LU 79553 was given at 40 mg/kg/day (Q2D X 3; Days 6 and 13) 5 of 5 animals had regressions (1 CR, 4 PR). At a dose of 55 mg/kg/day (Q3D X 2; Days 6 and 13) 5 of 5 animals also showed regressions (2 CR, 3 PR). Although not curative in this model, treatment with LU 79553 caused regressions (partial and complete) which resulted in tumor growth delay indicative of significant tumor killing.

Figs. 5 and 6 show the results of LU 79553 treatment of mice with human colon carcinoma xenografts (CX-1 or DLD-1; Ref. 11). In the DLD model, treatment at 20 mg/kg/day (Q1D X 5; Days 5 and 19) resulted in 3 of 5 PRs while a dose of 55 mg/kg/day (Q3D X 2; Days 5, 12, and 33) gave 4 of 4 PRs. These effects translated into a significant tumor growth delay. Against CX-1 xenografts, LU 79553 caused PRs in 50-80% of treated animals. Using the Q1D X 5 (Days...
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The 03D x 2 (Days 6 and 13) schedule, CRs were seen with 55 (5/5) or 65 (6/6) mg/kg/day. Even with a less frequent Q7D x 3 schedule a dose of 70 mg/kg/day yielded 6 of 6 CRs. The Q7D x 4 schedule was also curative in the MX-1 model (data not shown).

The OVCAR-3 human ovarian carcinoma xenograft model was utilized to demonstrate activity of LU 79553 administered i.v. against an established i.p. multi-foci tumor (12). In untreated mice receiving i.p. injections of OVCAR-3, ascites formation becomes visible at 25—30 days and the median survival time is only 40—50 days. As shown in Fig. 8 when OVCAR-bearing mice are treated with LU 79553 at 20 mg/kg/day (Q1D x 5; Days 7 and 21), the median survival time is increased to 87 days (treated/control = 198%). One animal in the untreated group did not develop tumor.

To assess the activity of LU 79553 against a very rapidly growing tumor, the LOX human melanoma model was used. Nude mice developed measurable (100 mg) and rapidly progressing tumors 3 days after s.c. inoculation with 1 X 10⁶ LOX cells. When LU 79553 was administered to LOX tumor-bearing mice, CRs occurred (Fig. 7). Untreated LOX tumors grew rapidly and animals had to be sacrificed on Day 12, when median tumor size was almost 2 g. In the treated groups, tumors began to shrink by Day 7 and became nonpalpable beginning as early as Day 18. Treatment at 20 or 30 mg/kg/day (Q1D x 5; Days 6 and 13) resulted in 3 of 6 CRs and 55 mg/kg/day gave 3 of 6 PR. On the Q3D x 2 (Days 6 and 13) schedule, 55 mg/kg/day gave 3 of 6 PR and 65 mg/kg/day caused 4 of 5 PR. When LU 79553 was given at 40 mg/kg/day (Q1D x 5; Days 6 and 13), 3 of 5 PR occurred. No CRs or cures were seen in these models.

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DISCUSSION

LU 79553 is a new bis-naphthalimide selected for clinical Phase I studies in cancer patients. In preclinical efficacy studies this compound has demonstrated excellent activity in a variety of human tumor xenografts grown in nude mice. These tumor types (lung, breast, ovarian, and colon) represent some of the most challenging neoplasms in the clinic today. Whereas the mononaphthalimide amonafide demonstrated little activity against solid tumor xenografts, LU 79553 treatment has resulted in not only tumor growth inhibition, but tumor regression and long term tumor-free survival in several of these models. Cured animals, submitted for histopathological analysis, show no signs of tumor at the implant site. In the MX-1 human mammary carcinoma model, LU 79553 was curative even when the initial tumor burden represented 5–10% of an animal’s body weight. Equally impressive was the demonstration that LU 79553 could cause CRs in the more refractory LX-1 human lung carcinoma model. The ability of LU 79553 to demonstrate this outstanding activity at doses below the MTD using a variety of schedules indicates a potentially exploitable therapeutic window. Few experimental compounds have displayed such high-level activity over such a broad spectrum of tumor types.

There does not seem to be a strong schedule dependence for LU 79553 in the models tested. Against most xenograft tumors, the efficacy of daily dosing (Q1D X 5) is equivalent to higher doses given once or twice per week. In preliminary experiments in the MX-1 model, high single doses resulted in partial but not CRs (data not shown). These studies were done at levels below the MTD and further

Topoisomerase II Inhibition. Previous studies (13) have demonstrated that the mononaphthalimide amonafide could inhibit topoisomerase II activity. In order to test if the bis-naphthalimides retained this inhibitory activity, we conducted enzyme assays using purified human topoisomerase II and C. fasciculata kDNA to measure the effects of LU 79553 on this enzyme. As shown in Fig. 9, exposure of topoisomerase II to LU 79553 (Lanes 5–8) could suppress the ATP-dependent decatenation activity of this enzyme. The inhibitory effects of LU 79553 started at 1 μM of drug concentration and reached a plateau at 100 μM, with an IC50 of 18 μM. These experiments also demonstrated that bis-naphthalimides are much more potent topoisomerase II inhibitors than the mononaphthalimide amonafide, which had an IC50 of 220 μM in this assay (data not shown).

![Fig.7: LOX human melanoma was serially passaged as ascites. Cells were collected, washed, and suspended in HBSS at a concentration of 5 × 10⁶ cells/ml. Cells (1 × 10⁶) were injected s.c. per mouse on Day 0. Treatment was initiated on Day 3 (median tumor size above 100 mg) and consisted of i.v. (lateral tail vein) injections of LU 79553 (dissolved in sterile, nonpyrogenic water) at 0 ( ), 20 ( ), 30 ( ), and 55 ( ) mg/kg/day Q1D X 5, Days 3, 17, 30 ( ), and 65 ( ) mg/kg/day Q3D X 2, Days 3, 10; or 70 ( ) mg/kg/day i.v. Q7D X 3, Day 3.

![Fig.8: OVCAR-3 human ovarian carcinoma was serially passaged as ascites (approximately 3 × 10⁶ cells/mouse) on Day 0. Treatment was initiated on Day 7 and consisted of 5 daily i.v. (lateral tail vein) injections of LU 79553 (dissolved in sterile, nonpyrogenic water) at 0 ( ) or 20 ( ) mg/kg/day Q1D X 5, Days 3, 7, 11, 21. Animals were monitored daily for survival. The percentage of animals surviving in each group is plotted (n = 5).

![Fig.9: Inhibition of catalytic activity of topoisomerase II by LU 79553. The topoisomerase II inhibition assay was done, except where noted otherwise, using 450 ng kDNA and 4 units of purified human topoisomerase II in a total volume of 20 μl reaction buffer (0.05 M Tris-HCl, pH 8, 0.12 M KCl, 0.01 M MgCl₂, 0.5 mM ATP, 0.5 mM DTT). The reaction mixture was incubated for 20 min at 37°C with various concentrations of LU 79553 (range, 4–500 μM) and was stopped subsequently with 4 μl stop buffer (5% Sarkosyl, 0.0025% bromophenol blue, 25% glycerol). After further incubation with 50 μg/ml Protease K at 37°C for 1 h, the reaction mixture was analyzed on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Lane 1, kDNA control; Lane 2, kDNA plus 4 units of topoisomerase II minus AlP control; Lanes 4-8, kDNA plus enzyme plus AlP in the presence of 0, 4, 20, 50, 100, 200, or 500 μM LU 79553, respectively.]

10000

C

1000

N

100

10

0

Time [Days]

Animals Surviving [%]

Time [Days]

1 2 3 4 5 6 7 8

Fig. 7. LOX human melanoma was serially passaged as ascites. Cells were collected, washed, and suspended in HBSS at a concentration of 5 × 10⁶ cells/ml. Cells (1 × 10⁶) were injected s.c. per mouse on Day 0. Treatment was initiated on Day 3 (median tumor size above 100 mg) and consisted of i.v. (lateral tail vein) injections of LU 79553 (dissolved in sterile, nonpyrogenic water) at 0 ( ), 20 ( ), 30 ( ), and 55 ( ) mg/kg/day Q1D X 5, Days 3, 17, 30 ( ), and 65 ( ) mg/kg/day Q3D X 2, Days 3, 10; or 70 ( ) mg/kg/day i.v. Q7D X 3, Day 3.

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studies will be needed to elucidate the effectiveness of single-dose therapy.

Interestingly, LU 79553 showed only slight activity against the murine leukemia P388 (treated/control = 120%). A similar lack of activity against murine tumors has been reported for other members of this class and it has been suggested that these compounds demonstrate a human tumor Selectivity (14). DMP-840 (DuPont-Merck Pharmaceuticals) is a bis-naphthalimide which has recently been described (7). It has a similar antitumor profile to LU 79553 but, as with most of the very active bisintercalating agents, a nitro substitution on the chromophore is required for activity. In the mononaphthalimide series, a basic terminal nitrogen was also required for activity (4) but nitro substitutions were associated with unacceptable toxicities (irreversible memory loss leading to disorientation and confusion; Ref. 5). The lack of any chromophore substitution on LU 79553 may reduce the frequency of these unwanted side effects.

Toxicity based on loss of body weight was seen at the higher doses of LU 79553. At the MTD for the schedules tested, 15–20% weight loss was noted. At lower (but equally efficacious) doses, weight loss ranged from 0 to 10% of total body weight (data not shown). Specific organ toxicity has not been determined yet. Local irritation has been noted at the injection site and appears to be concentration dependent. The lesions heal rapidly once treatment has been completed.

One of the most difficult aspects of treating patients with cancer is the ability of many tumors to develop resistance to chemotherapeutic drugs. Many mechanisms of resistance have been identified including the increased expression of P-glycoprotein which acts as an efflux pump to reduce the intracellular concentration of a number of different classes of chemotherapeutic agents (antracyclines, Vinca alkaloids, podophyllotoxins, taxanes). This multidrug resistance may be a common reason for the failure of cancer therapy. LU 79553 has been shown to be effective against a variety of multidrug resistance-positive sublines in vitro (Table 1). Although a slightly higher drug concentration was generally required to effect the same degree of killing, EC$_{50}$ values for resistant lines were still in the 10$^{-7}$ to 10$^{-8}$ molar range. Pharmacokinetic studies have determined that this concentration is easily achievable in vivo (data not shown). In vivo models of multidrug resistance, LU 79553 also demonstrated activity (data not shown). This ability to circumvent clinically relevant mechanisms of drug resistance may be an important aspect of the therapeutic potential of LU 79553.

Although the critical biochemical target of the bis-naphthalimides has not been identified, several lines of evidence suggest that these compounds are DNA intercalators which cause DNA damage in treated cells. In addition to DNA intercalation activity, the monomeric compounds seem to be warranted.

In these experiments, LU 79553 has demonstrated dramatic antitumor activity in a variety of xenograft models. Treatment of tumor-bearing mice over a range of doses below the MTD has resulted in CRs and PRs as well as long-term tumor-free survivors in many of the models tested. This degree of activity over such a broad spectrum of tumor types is not often seen with experimental therapeutics and make LU 79553 an excellent candidate for clinical trials.

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