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XB596, a promising bis-naphthalimide anti-cancer agent

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We have synthesized a promising class of bis-naphthalimide anti-tumor agents. A representative compound in this series, XB596, exhibits potent *in vitro* growth inhibitory activity against several human and murine leukemic and solid tumor lines in culture, with IC_{50} values ranging from 7.2 to 147.5 nM. XB596 was almost as equally growth inhibitory against three doxorubicin-resistant cell lines compared with their parental lines. Using a human tumor colony-forming assay, XB596 demonstrated cytocidal activity against fresh human tumors taken directly from patients, with 23 of 25 evaluable tumors responding to a continuous exposure of 1 μg/ml of XB596. When L1210 cells were incubated with XB596 for 1 h, the incorporation of uridine and thymidine into RNA and DNA, respectively, was inhibited with IC_{50} values of 0.14 μM. DNA single-strand breaks, but not double-strand breaks, were detected in XB596-treated L1210 cells. XB596 bound to DNA with guanine–cytosine sequence selectivity as shown by an indirect ethidium bromide displacement assay. XB596 was showns to interact with DNA by a spectrophotometric titration assay, with an estimated binding constant of $4.7 \pm 2.2 \pm 10^6\ M^{-1}$. XB596 unwound supercoiled DNA as measured by agarose gel electrophoresis. These data are consistent with XB596 being a DNA intercalator. *In vivo*, XB596 demonstrated good anti-tumor activity against two human solid tumors (DLD-2 colon adenocarcinoma and MX-1 mammary carcinoma) xeonografted in nude mice, but has not demonstrated anti-leukemic activity. In summary, XB596 is a pre-clinical anti-cancer agent which interacts with DNA and demonstrates good *in vivo* anti-tumor activity against human solid tumor xenografts.

Key words: Anti-tumor agents, bis-naphthalimide compounds, DNA, XB596.

Introduction

DNA intercalating agents are one of the most widely used classes of cancer chemotherapeutic agents currently used for the management of human cancers. Doxorubicin, a DNA intercalator, is commonly used for the treatment of a variety of human hematological and solid tumors.[2] Following the successful introduction of doxorubicin, many synthetic or semi-synthetic DNA intercalators entered in clinical trials as anti-cancer agents. Several intercalators, including amsacrine,[3] mitoxantrone[4] and the anthrapyrazole DuP 941[5] have shown good clinical anti-tumor activity.

We have synthesized a series of bis-naphthalimide compounds as potential anti-cancer agents. The compounds are characterized by having two identical naphthalimide rings joined by an appropriate linker. A representative compound in this series, XB596, (4-[3-(3-nitronaphthalene-1,8-dicarboximido) propylamino]-1-(3-nitronaphthalene-1,8-dicarboximido)butane hydromethanesulfonate), which has two 3-nitro substituted naphthalimide rings joined by a spermidine linker is shown in Figure 1. Similar compounds have been independently discovered.[6] XB596 was designed as an analog in a bis-naphthalenesulfonamide series, which we had previously shown were protein kinase C (PKC) antagonists.[7] Although designed as a PKC antagonist, XB596 itself did not inhibit PKC activity and the subsequent biological activities of XB596 were distinct from kinase inhibition.

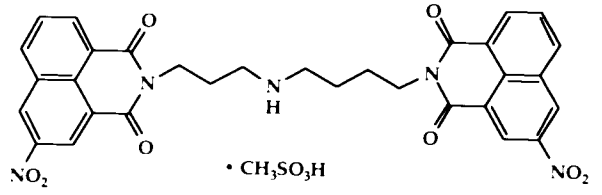

Figure 1. Chemical structure of XB596: 4-[3-(3-nitronaphthalene-1,8-dicarboximido)propylamino]-1-(3-nitronaphthalene-1,8-dicarboximido)butane hydromethanesulfonate.

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As shown below, the activity of XB596 is quite different from that of monomeric naphthalimides such as amonafide[8]. In this report, the *in vitro* growth inhibitory profile, *in vivo* anti-tumor activity and a possible mode of action of XB596 are described.

Materials and methods

Materials and chemicals

Tissue culture reagents were puchased from Gibco, Grand Island, NY. [5-^{3}H]Uridine (specific activity, 28.9 Ci/mmol), [methyl-1′,2′-^{3}H]thymidine (specific activity, 109 Ci/mmol) and [3,4,5-^{3}H(N)] leucine (specific activity, 156 Ci/mmol) were purchased from Du Pont-New England Nuclear Research Products (Boston, MA). The bacterial phage lambda DNA was purchased from New England Biolabs (Beverly, MA). Synthetic oliogomers poly(dG–dC)·poly(dG–dC) and poly(dA–dT)·poly(dA–dT) and pBR322 were purchased from Pharmacia (Piscataway, NJ). All other chemicals were purchased from Sigma (St Louis, MO) or Aldrich (Milwaukee, WI).

Chemical synthesis of XB596

A mixture of 3-nitro-1,8-naphthalic anhydride (32.14 g, 132.2 mmol) and spermidine (9.6 g, 66.1 mmol) in 700 ml of anhydrous ethanol was heated to reflux overnight. The product was collected on a filter, washed with ethanol and dried to give the XB596 free base (36.79 g, 93.5% yield) as a purple solid: m.p. 198–199°C. A portion of this free base (8.0 g, 13.4 mmol) was suspended in 75 ml of anhydrous ethanol and methane sulfonic acid (0.87 ml, 1.29 g, 13.4 mmol) was added to the reaction mixture. The reaction mixture was heated on a steam bath for 30 min. The resulting methane sulfonate salt was collected on a filter, washed with ethanol and dried to give XB596 (8.2 g, 88.5 yield) as a light tan solid. A portion of this product (4.0 g, 5.8 mmol) was heated in 200 ml of anhydrous ethanol on a steam bath for 2 h, filtered, dried *in vacuo* at 78°C to afford XB596 (3.0 g, 75% yield) as a solid: m.p. 185°C (dec); IR (KBr) 1706, 1665 (C=O) cm^{-1}; NMR (TFA-d1) δ 9.61 (d, 1H, J = 2.2 Hz), 9.59 (d, 1H, J = 2.2 Hz), 9.53 (d, 1H, J = ~2.2 Hz superimposed upon d, 1H, J = ~2.2 Hz), 9.11 (d, 2H, J = 7.6 Hz), 8.83 (d, 2H, J = 8.1 Hz), 8.27 (t, 1H, J = ~8.0 Hz superimposed upon t, 1H, J = ~8.0 Hz), 7.46 (broad, 2H), 4.72 (t, 2H, J = 6.6 Hz), 4.64 (t, 2H, J = 6.4 Hz), 3.64 (m, 4H), 3.30 (s, 3H), 2.67 (m, 2H), 2.31 (m, 4H); MS (m/e) 596 (M^+ + H-CH_3SO_3H).

Cell culture

Doxorubicin-sensitive and resistant murine P388 leukemia, B16 melanoma and M16c mammary carcinoma cell lines were kindly provided by Dr Robert C Jackson (Agouron Pharmaceutical Company, San Diego, CA). Clone A human colon cancer cells were isolated from the heterogeneous DLD-1 colon tumor line. Clone A, B16, B16/ADR$^+$, M16c and M16c/ADR$^+$ cells were grown in RPMI-C medium as reported.[10] Human melanoma RPMI 7272 cells were grown in RPMI 1640 supplemented with 10 mM Tricine (pH 7.8), 10 mM HEPES (pH 7.3), 0.075% sodium bicarbonate and 10% (v/v) heat inactivated (56°C, 30 min) fetal bovine serum. RPMI 7272 cells were obtained from Dr Daniel B Rifkin (New York University Medical Center, New York, NY). Murine leukemia L1210, P388 and P388/ADR$^+$ cells were maintained in RPMI-L medium as described.[11] All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air.

In vitro growth inhibitory activity

Exponentially growing cells (1 × 10^3 cells, unless specified otherwise) in 0.1 ml medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test analogs were added in hextuplet to the cell plates; standard derivations among replicates were less than 10%. After incubation at 37°C in a humidified incubator for 3 days, the plates were centrifuged briefly and 100 μl of the growth medium was removed. Cell cultures were incubated with 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide [MTT, 1 mg/ml in Dulbecco's phosphate buffered saline (PBS)] for 4 h at 37°C.[12] The resulting purple formazan precipitate was solubilized with 200 μl of 0.04 N HCl in isopropyl alcohol. Absorbance was monitored in a Titertek Multiskan MCC scanning well spectrophotometer (Flow Laboratoties, McClean, VA) at a test wavelength of 570 nm and a reference wavelength of 630 nm. The absorbances were stored on a floppy disk on a IBM-XT and uploaded on to a VAX computer. The IC_{50} values

were determined by a computer program, based on the enzyme analysis software EZ-FIT[13] that fit all of the data to the following four-parameter logistic equation:

$$Y = ((A_m - A_0)/(1 + (X/IC_{50})^n)) + A_0$$

where A_m is the absorbance of control cells, A_0 is the absorbance of cells in the presence of highest agent concentration, Y is the observed absorbance, X is the agent concentration and IC_{50} is the dose of agent that inhibits the number of population doublings of cells to one half that of the number of population doublings of the control cells and n is the slope of the curve.

Exponentially growing RPMI-7272 cells (3×10^5) in 2 ml of medium with 10% serum were grown in 35 mm sterile tissue culture dishes for 24 h. On day 1, the medium was removed and graded concentrations of test compounds or fresh media (control) were added to the cell plates in 2 ml volumes. After incubation at 37°C in a humidified incubator for 72 h, the cells were washed twice with 2 ml of Dulbecco's PBS and were then trypsinized with 2 ml of trypsin–EDTA (0.05% trypsin, 0.02% EDTA). A fraction of the cells was counted in filtered Isoton using a Coulter counter (Coulter Electronics, Hialeah, FL). Each experiment was performed in duplicate. The mean of the three measurements was used to calculate the IC_{50} which was determined by computer analysis. Experiments were only valid if control population doublings were >3 within 72 h.

Human tumor cloning assay

The effect of XB596 on the formation of human tumor colony-forming units (TCFUs) was determined by utilizing fresh human tumors taken directly from patients and cultured in soft agar. Cell suspensions of solid tumor specimens were prepared within 4 h after excision from patients with neoplasia of varying histology. Preparation of tumor cells and the method for *in vitro* exposure of tumor cells to XB596 followed by soft agar clonogenic assay has been described previously.[14,15]

Inhibition of precursor incorporation into macromolecules

Incorporation of tritium-labeled precursors (uridine, thymidine and leucine) into macromolecules (RNA, DNA and protein) was carried out in a 96-well microtiter plate. Exponentially growing murine leukemia L1210 cells (1×10^5 cell in leucine-free RPMI 1640 medium) were pre-incubated at 37°C for 30 min with varying concentrations of test agents in a total volume of 80 μl. Leucine-free RPMI 1640 medium (20 μl) with [5-^{3}H]uridine, [methyl-1′,2′-^{3}H]thymidine or [3,4,5-^{3}H(N)]leucine was added to the appropriate wells and the cells were incubated at 37°C for an additional 30 min. The final radioactivity was 1 μCi/well. The incorporation reaction was stopped by adding 40 μl of 100% ethanol. The samples were harvested onto Filtermats (Skatron Inc., Sterling, VA) using a Skatron cell harvester. The unincorporated precursors were removed by washing filters with water. After drying, the radioactivity retained on the filters was measured using Econoflour (DuPont NEN, Boston, MA) and a Packard Tricarb scintillation counter (Packard, Downersgrove, IL).

Indirect binding of XB596 to DNA

The binding of XB596 to DNA and to synthetic polynucleotide duplexes was determined by an ethidium bromide displacement assay, which measures the reduction of fluorescence when ethidium bromide is displaced from DNA.[16] The fluorescence intensity of a 3 ml solution containing 9.3 mM NaCl, 2 mM Tris–HCl (pH 7.0), 100 μM EDTA, 1 μM DNA (in nucleotides) and 1.26 μM ethidium bromide in a quartz cuvette was measured at the excitation (546 nm) and emission (600 nm) wavelengths. Graded concentrations of agent were then added and the fluorescence intensity was measured using a Gilford Fluoro IV spectrofluorometer (Oberlin, OH). The concentration of compound required to reduce the fluorescence intensity by 50% of that of control (IC_{50}) was determined.

Direct binding of XB596 to DNA

A spectrophotometric titration assay was used to determine the binding constant of XB596 to DNA. Measurements were made in a 3 ml, 1 cm path length quartz cuvette using a Perkin-Elmer Lambda 2 UV vis spectrometer (Uberlingen, Germany). XB596 was dissolved in 25 mM 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid buffer (pH 7.3), 100 mM NaCl, 1 mM EDTA and 25% ethanol at 10 μm, and the UV and visible absorption spectrum (250–450 nm) was determined for XB596 in the presence of varying concentrations of human

placental DNA. Small aliquots of DNA were titrated into the compound solution and the extinction coefficient was calculated after each addition at 340 nm. The extinction coefficient of the bound compound was determined by extrapolation to infinite DNA concentration by the method of Bontemps and Fredericq.[17] The fraction of compound bound to DNA (c) after each addition during the titration was calculated from the equation

$$c = (E_f - E)/(E_f - E_b)$$

where E_f is the molar extinction coefficient of free compound, E_b is the molar extinction coefficient of the bound compound and E is the observed extinction coefficient. From the value of c, the variables C_f (molar concentration of free compound) and r, the binding ratio (compound bound per base pair) were calculated. The binding parameters were estimated using the model of McGhee and von Hippel.[18] The data were fit to the equation

$$r/C_f = K(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1}$$

where K is the intrinsic association constant and n is the binding site size or the number of base pairs occluded by one bound XB596.

DNA intercalation assay

Supercoiled pBR322 plasmid DNA (0.26 μg, 20 μM base pairs) was incubated in the presence of varying concentrations of XB596 for 30 min at 25°C in a total volume of 20 μl of 10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA and 5 mM NaCl. Loading buffer (4 μl of 60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanole FF and 10 mM Tris–HCl, pH 7.9) was added and products were subjected to electrophoresis at 10 V/cm in 1.0% agarose gels in 40 mM Tris–acetate, pH 8.3 and 2 mM EDTA. Following electrophoresis, gels were stained in an aqueous solution of ethidium bromide (0.8 μg/ml). DNA bands were visualized by transillumination with UV light (300 nm) and photographed through a Kodak 24A filter with Polaroid Type 55 positive/negative film.

Induction of DNA single- and double-strand breaks by XB596

DNA single- and double-strand breaks in XB596-treated cultured L1210 cells were measured by the filter elution assay described by Kohn *et al.*[19] Briefly, cellular DNA was radioactively labeled in exponentially growing cells by incubation of cells with [2-^{14}C]thymidine (0.02 μCi/ml) for 24 h at 37°C. Cells were then treated with various concentrations of compound for 1 h before being deposited on polycarbonate filters (2 μm pore diameter, Nucleopore Corp., Pleasanton, CA). Cells were lysed with a 2% sodium dodecyl sulfate (SDS)–0.025 M EDTA solution, pH 9.7. Two milliliters of Proteinase K (0.5 mg/ml) diluted in the above lysis solution was then added. The eluting solution was tetrapropyl ammonium hydroxide with 0.02 M EDTA containing 0.1% SDS, pH 12.1, for assaying single-strand breaks and 0.2% SDS, pH 9.6, for assaying double-strand breaks. An increase in the elution rate of the drug treated versus the control signifies DNA strand breaks. Calculation of rad-equivalents was as described by Kohn *et al.*[19]

In vivo human tumor models

The DLD-2 tumor was originally obtained from primary colon carcinoma surgically removed from a male patient.[9,20] The MX-1 human breast carcinoma xenograft and LX-1 human lung carinoma xenograft were both established by Dr B. Gionevella of the Stehlin Foundation of Houston, Texas, both tumor models were utilized for many years in the NCl screening panel of experimental tumors.[21] These three solid tumors were propagated in athymic nude mice.

Outbred Swiss mice bearing the nude (*nu/nu*) gene[22,23] weighing 18–22 g were inoculated s.c. with 0.2 ml of a mince prepared from DLD-2, MX-1 or LX-1 tumors excised from tumors propagated in nude mice. Palpable tumors appeared within 1 week and had estimated weights of ~50 mg at that time. The mice were pair-matched in groups of 10 each and compounds or vehicles were administered intravenously (i.v.) on a daily ×9 schedule. On the final day of the test, the body weights were recorded, and the tumors were excised and weighed. The percentage of tumor growth inhibition (%TGI) was calculated according to the formula:

$$\%TGI = 100\% - \frac{\text{mean tumor weight treated (T)}}{\text{mean tumor weight control (C)}} \times 100\%$$

In vivo murine tumor models

The methods of evluating the efficacy of XB596 against murine L1210 and P388 leukemia were as previously described.[24] Efficacy is expressed as a percentage of the mean survival time of the control group:

$$\%T/C = \frac{\text{mean survival time of treated}}{\text{mean survival time of control}} \times 100\%$$

Results

In vitro growth inhibitory activity

The growth inhibitory activities of XB596 and selected anti-cancer agents against several cultured murine and human tumor cell lines were examined (Table 1). XB596 potently inhibited the growth of mouse and human tumor cell lines, with IC_{50} values ranging from 7.2 to 147.5 nM. Except for the B16 melanoma line, XB596 was approximately equipotent in inhibiting the growth of all cell lines tested. The potency of growth inhibitory activity of XB596 was similar to that of doxorubicin and mitoxantrone. It is of interest to note that XB596 was at least 1–2 orders of magnitude more potent than a mono-naphthalimide, amonafide.[8]

The growth inhibitory activities of XB596 against three doxorubicin-resistant cell lines (P388/ADR$^+$, B16/ADR$^+$ and M16c/ADR$^+$) and their corresponding parental lines are shown in Table 2. The murine leukemia P388 doxorubicin-resistant line (P388/ADR$^+$) was 85.7- and 41.8-fold resistant to doxorubicin and mitoxantrone, respectively, but was only 4.5-fold resistant to XB596, compared with the parental P388 line. The murine B16 melanoma (B16/ADR$^+$) line exhibited 9.7-fold resistance to doxorubicin but was equisensitive to XB596. The murine mammary carcinoma M16c/ADR$^+$ line was resistant to both doxorubicin (14.3-fold) and mitoxantrone (11.2-fold), but was significantly less resistant to XB596 (2.6-fold), compared with the M16c-sensitive line. All three doxorubicin-resistant lines exhibited 2.8- to 4.0-fold resistance to amonafide, whereas two lines (B16, M16c) were less resistant to XB596. These data suggest that XB596 might be an effective tumoricidal agent against doxorubicin-resistant tumors.

Effect of XB596 against human TCFUs

Human primary tumor samples of varying histologic types were treated continuously with 0.01, 0.1 and 1 μg/ml of XB596 using the human tumor colony forming assay.[14,15] Dose-dependent responses of 8, 44 and 92% were obtained when tumors were exposed to 0.01, 0.1 and 1 μg/ml of XB596, respectively (Table 3). XB596 demonstrated broad spectrum anti-tumor activity, since it was equally active against all of the tumors studied at the effective concentration of 1 μg/ml.

Table 1. Growth inhibitory activity of XB596 and known anti-cancer agents against murine and human tumor cell lines

Cell line	Species	Tumor type	50% growth inhibitory concentration IC_{50} (nM)			
			XB596	doxorubicin	mitoxantrone	amonafide
P388[a]	mouse	leukemia	8.8	12.6	1.1	203
L1210[a]	mouse	leukemia	7.2	36.2	2.2	188
B16[a]	mouse	melanoma	147.5	25.5	743	1170
M16c[a]	mouse	mammary	11.9	6.9	1.5	268
Clone A[a]	human	colon carcinoma	18.8	32.8	113	2512
RPMI-7272	human	melanoma	26.0	17.0	3.2	2540

The 50% growth inhibitory concentration (IC_{50}) of XB596 and selected anticancer agents against several murine and human tumor cell lines were determined by the MTT assay as described in Materials and methods.
[a]Exponentially growing L1210 cells (1×10^3), P388 cells (1×10^3), B16 ($2–3 \times 10^3$), M16c (3×10^3) or Clone A cells (3×10^3) in 0.1 ml medium were seeded in a 96-well microtiter plate. The RPMI-7272 cells were plated at 3×10^5 cells per 35 mm plate and growth inhibition was measured as described in Materials and methods. The dose of compound that inhibits the number of population doublings of cells to half that of the number of populaton doublings of the control cells (IC_{50}) was determined as described in Materials and methods. The data are averaged from at least two independent experiments.

Table 2. Growth inhibitory activity of XB596 and selected anti-cancer agents on doxorubicin-resistant tumor cell lines

Cell line	50% growth inhibitory concentration IC_{50} (nM)			
	XB596	doxorubicin	mitoxantrone	amonafide
P388	8.8	12.6	1.1	203
P388/ADR$^+$	39.4 (4.5)[a]	1080 (85.7)	47.3 (41.8)	585 (2.8)
B16	147	25.5	743	1170
B16/ADR$^+$	127 (1.1)	247 (9.7)	2770 (3.7)	4660 (4.0)
M16c	11.9	6.9	1.5	268
M16c/ADR$^+$	31.4 (2.6)	98.9 (14.3)	16.9 (11.2)	954 (3.6)

The 50% growth inhibitory concentration (IC_{50}) of XB596 and selected anti-cancer agents against doxorubicin-sensitive and reistant P388, B16 and M16c lines was determined as described in Materials and methods. The data for XB596 are the mean of at least two separate experiments.

[a]Numbers in parentheses are the ratios of the IC_{50} value of the ADR$^+$ line/the IC_{50} value of the parental line. A ratio of 1 indicates that the agent is equally cytotoxic to the parental and doxorubicin-resistant (ADR$^+$) cell line.

Biochemical pharmacology of XB596

To assess the mechanism of tumoricidal activity of XB596, the incorporation of tritium-labeled uridine, thymidine or leucine into RNA, DNA or protein was measured. Both the incorporation of uridine and thymidine into RNA and DNA was inhibited equally with an IC_{50} value of 0.14 μM (Table 4). Protein biosynthesis was also inhibited by XB596, but at a concentration 80-fold higher than that required to inhibit DNA and RNA synthesis. XB596 was considerably more potent than doxorubicin, mitoxantrone and amonafide at inhibiting uridine and thymidine incorporation (Table 4).

The ability of XB596 to bind to DNA and to synthetic oligonucleotide duplexes was measured using an ethidium bromide displacement assay.[16] Ethidium bromide, a known DNA intercalator, has no fluorescence in the absence of DNA; however, fluorescence intensity increases when ethidium bromide binds to DNA. The ethidium bromide displacement assay measures the decrease in fluorescence intensity caused by displacing DNA-bound ethidium bromide with DNA interacting agents. Figure 2 shows a dose-dependent decrease in DNA fluorescence intensity when graded concentrations of XB596 were added. XB596

Table 3. Effect of XB596 against human tumor colony-forming units

Tumor type	No. response/no. evaluable[a] for different concentrations of XB596 (μg/ml)		
	0.01	0.1	1.0
Bladder	0/2	0/2	2/2
Breast	1/6	2/6	5/6
Colon	0/1	0/1	1/1
Kidney	0/1	1/1	1/1
Lung (non-small cell)	0/2	0/2	1/2
Ovary	0/10	6/10	10/10
Prostate	1/1	1/1	1/1
Stomach	0/2	1/2	2/2
Overall summary	2/25 (8%)	11/25 (44%)	23/25 (92%)

Cell suspensions of human solid tumors with varying histologic types were treated continuously with indicated concentrations of XB596 as previously described.[14,15] A positive response is defined as $\leq$50% survival in XB596 treated cultures as compared to untreated control cells.

[a]An evaluable test is one having a mean of 20 or more colonies present on day 14 in untreated control cultures and $\leq$30% survival in a positive control (orthosodium vanadate, non-specific cell poison) culture when compared to untreated controls. A response is defined as a reduction to $\leq$50% in colony formation, compared to untreated control cells.

Table 4. Effect of XB596 and selected anti-cancer agents on precursor incorporation into macromolecules in L1210 cells

Compound	[^{3}H]Uridine	[^{3}H]Thymidine	[^{3}H]Leucine
XB596	0.14	0.14	11
Doxorubicin	13.2	16.6	>36
Mitoxantrone	14.6	45.0	>50
Amonafide	14.0	37.1	>70

The effect of XB596 and selected anti-cancer agents on inhibiting the incorporation of tritium-labeled uridine, thymidine and leucine into RNA, DNA and protein was measured as described in Materials and methods. The concentrations of XB596 and selected anti-cancer agents necessary to inhibit precursor incorporation by 50% (IC_{50}) were determined. The data are averaged from at least two independent experiments.

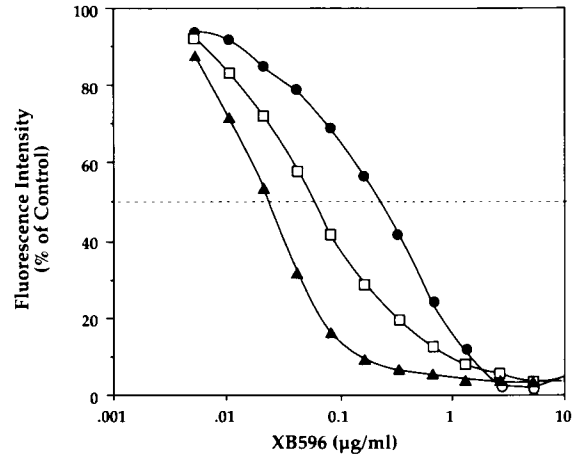

Figure 2. Displacement of DNA-bound ethidium bromide by XB596. The fluorescence intensity of a 3 ml solution containing 9.3 mM NaCl, 2 mM Tris–HCl (pH 7.0), 100 μM EDTA, 1 μM (in nucleotides) of poly(dA–dT)·poly(dA–dT) (●), phage λDNA (□), poly(dG–dC)·poly(dG–dC) (▲) and 1.26 μM ethidium bromide in a quartz cuvette was measured at the excitation wavelength (546 nm) and emission wavelength (600 nm). The fluorescence intensity of the solution was monitored following addition of graded concentrations of XB596 as described in Materials and methods.

bound tightly to synthetic poly(dG–dC)·poly(dG–dC) and lambda DNA, with IC_{50} values of 30.4 and 81 nM, respectively. In contrast, XB596 had a weaker affinity for poly(dA–dT)·poly(dA–dT), with an IC_{50} value of 278 nM.

Evidence for direct interaction of XB596 with DNA was further provided by a spectrophotometric titration assay. XB596 had a well-defined spectrum with an absorption maximum near 336 nm. When XB596 bound to DNA, the UV spectrum underwent a bathochromic and hypochromic shift, and an isosbestic point was evident at 374 nm (Figure 3A). The appearance of an isosbestic point suggests that the titration spectrum represents the sum of the contributions of two species of agent, bound and free. Scatchard plots of the titration data were constructed and the data were curve-fit to the McGhee and Von Hippel equation (Figure 3B). The values of K (association constant) of $4.7 \pm 2.2 \times 10^6\ M^{-1}$ and n (binding site size) of 2.2 ± 0.1 bp (mean ± SEM of three determinations) were obtained.

To confirm the intercalation of XB596 into DNA, supercoiled pBR322 plasmid DNA was incubated for 30 min in the presence of graded concentrations of XB596. Compounds that intercalate between nucleic acid bases will alter the density of covalently closed supercoiled DNA. With increasing amounts of intercalated agent, negatively supercoiled DNA is converted to a form which has a density more closely resembling that of a relaxed plasmid. Additional intercalation restores the density of the supercoiled plasmid as it is converted into a form that resembles positively supercoiled plasmid. In contrast, the density of nicked circular DNA (present as a contaminant in the supercoiled pBR322 preparation) will not dramatically change upon drug binding since this form of the plasmid is not topologically constrained. Therefore, by monitoring the relative electrophoretic mobility of the supercoiled form of the DNA with respect to the nicked component (which runs as the completely relaxed form of the

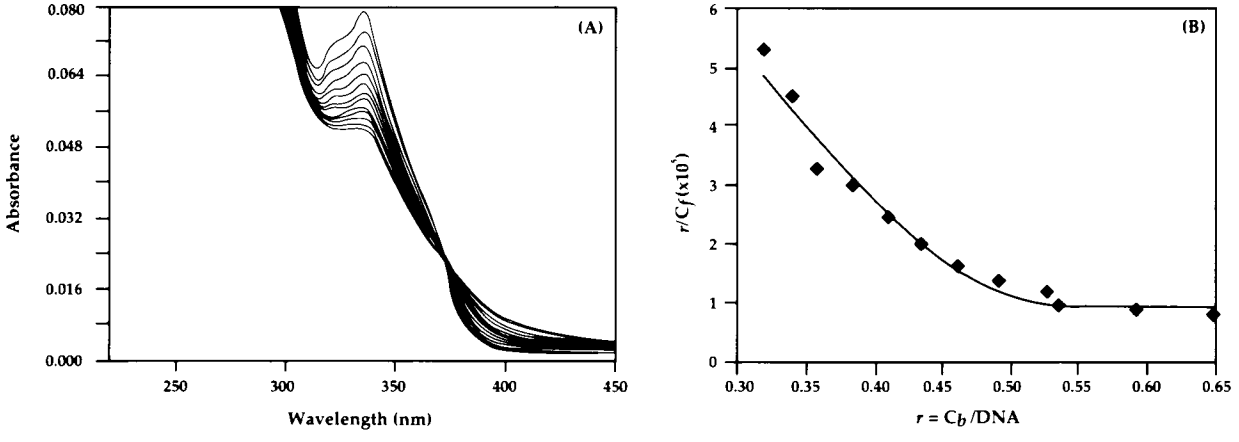

Figure 3. (A) The UV spectrum of a 10 μM XB596 solution was measured in 25 mM HEPES buffer (pH 7.3), 100 mM NaCl, 1 mM EDTA and 25% ethanol, and is shown as the uppermost spectrum. Graded concentrations of human placental DNA were added and after thoroughly mixing, the UV spectrum of the solution was recorded. The DNA (μM base pairs) to XB596 (μM) ratios from the highest absorbance maximum to the lowest, respectively, were 0, 0.27, 0.54, 0.80, 1.05, 1.30, 1.56, 1.80, 2.04, 2.27, 2.50, 2.73, 2.95 and 3.16. (B) The data points generated from spectrophotometric titration were fit based on the theoretical McGhee and von Hippel equation as described in Materials and methods.

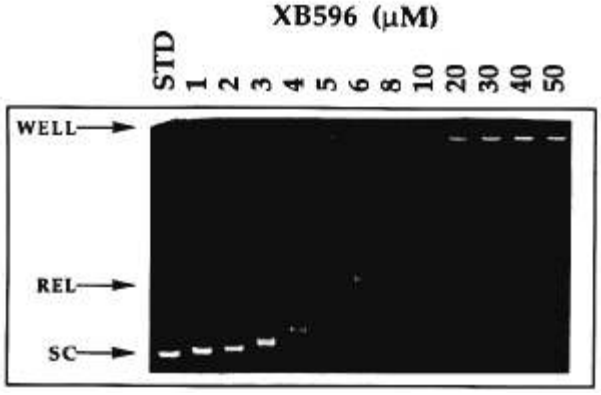

Figure 4. DNA unwiding studies. Supercoiled pBR322 DNA (20 μM in base pairs) was incubated with 0–40 μM of XB596 (lanes 1–9) for 30 min at 25°C. The DNA was separated in 1.0% agarose gels as described in Materials and methods.

plasmid) an apparent affinity for agent intercalation can be shown.

An ethidium bromide-stained gel showing a representative drug titration for XB596 intercalation into plasmid DNA is shown in Figure 4. A concentration of 6 μM XB596 converted the negatively supercoiled pBR322 into a form that resembles completely relaxed plasmid. For comparison, the concentrations of doxorubicin and amonafide that are required to observe the same effect were 4 μM and >50 μM, respectively (data not shown).

The ability of XB596 to induce DNA single-strand breaks in L1210 cells was measured by a filter elution technique.[19] A typical elution profile of DNA of L1210 cells treated with 0–14.4 μM XB596 for 1 h is shown in Figure 5(A). Elution rates increased in a dose-dependent fashion in the treated cells, indicating the production of single-strand breaks by XB596. At 1.2 and 3.6 μM, for example, 240 and 750 rad-equivalent single-strand breaks were produced by XB596. However, no measurable DNA double-strand breaks were detected when cells were treated with the same concentrations of XB596 for 1 h under elution conditions necessary for the identification of double-strand breaks (Figure 5B).

In vivo anti-tumor activity against human tumor xenografts

XB596 demonstrated good activity against human tumor xenografts in nude mice (Table 5). At the maximum tolerated dose, XB596 inhibited the growth of DLD-2 human colon adenocarcinoma and MX-1 human mammary carcinoma xenografts by 91 and 78%, respectively. In contrast, doxorubicin and amonafide were both inactive in the DLD-2 and MX-1 xenografts, where modest activity is defined as >58% TGI and excellent

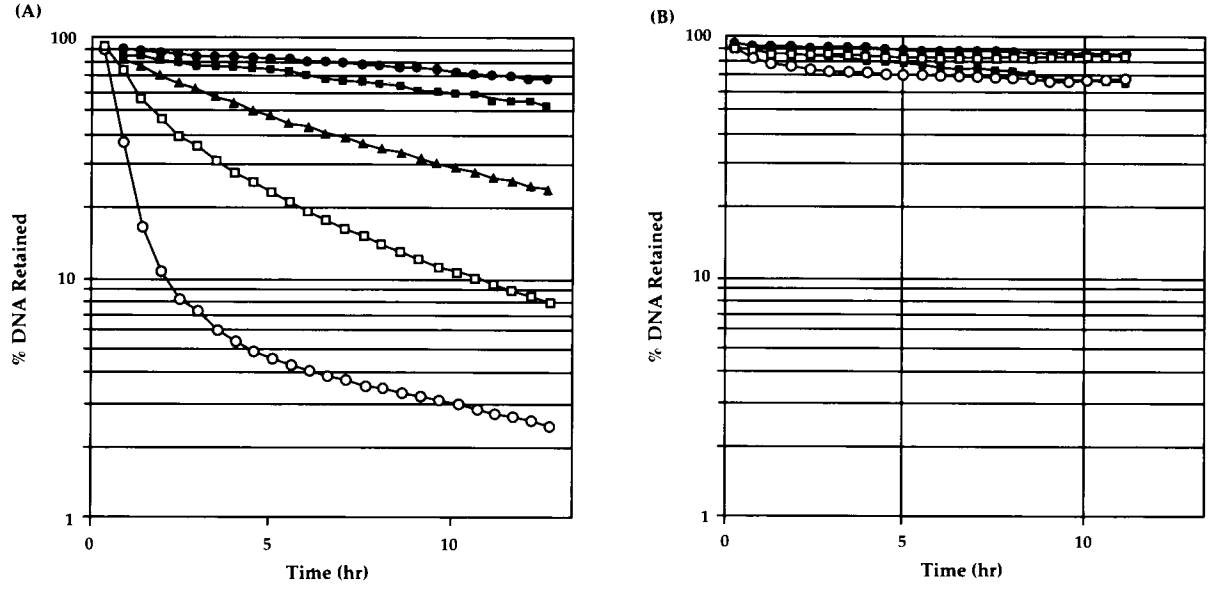

Figure 5. DNA single- and double-strand breaks induced by XB596. L1210 cells (3 × 10^5 cells/ml) were incubated with [2-^{14}C]thymidine (0.02 μCi/ml) for 24 h. The cells (2 × 10^6 cells) were harvested and incubated with 0 (●), 0.3 (■), 1 (▲), 3 (□) and 10 (○) μg/ml of XB596 at 37°C for 1 h. (A) DNA single-strand breaks were measured using Pr4NOH-EDTA elution solution (pH 12.1) and (B) DNA double-strand breaks were measured using Pr4NOH-EDTA elution solution (pH 9.6) as describe by Kohn *et al.*[19] The data is representative of one experiments, using the HCl salt of XB596.

Table 5. *In vivo* anti-tumor activity of XB596 and selected anti-cancer agents

Compound	Dose (mg/kg)	Percent tumor growth inhibition (% TGI)		
		DLD-2 (colon)	MX-1 (breast)	LX-1 (lung)
XB596	20	91 ± 3 (30)[a]	78 ± 4 (6)[b]	31
Doxorubicin	1	30	47	31
Amonafide	6	0	I[c]	I[c]

Tumors were innoculated subcutaneously in nude mice with 0.2 ml of a mince prepared from DLD-2, MX-1 or LX-1 tumors excised from tumors propagated in nude mice. Animals were pair-matched into control and treatment groups 7 days post-inoculation when palpable tumors were approximately 5 × 5 mm^2 (50 mg). Mice were treated i.v. with agents daily for 9 days at the maximum tolerated dose. Experiments were terminated on day 18, mice were sacrificed and tumors were excised and weighed. Tumor growth inhibition percentage (%TGI) was calculated as described in Materials and methods. At the termination of the experiment, DLD-2 control tumors weighed 4290 ± mg, MX-1 control tumors weighed 3150 ± mg and LX-1 tumors weighed 2790 ± mg.
[a]Mean ± of 30 different experiments.
[b]Mean ± of six different experiments.
[c]I = inactive, reported in Szez *et al.*[28]

activity is defined as >90% TGI.[24] None of the agents evaluated including XB596 was active versus the LX-1 human lung tumor xenograft.

In vivo anti-tumor activity against murine leukemia tumors

Although XB596 has shown potent growth inhibitory activity *in vitro*, it demonstrated marginal (if any) *in vivo* anti-tumor activity against murine leukemia L1210 and P388, with %T/C values of 138 and 106%, respectively, at the maximal tolerated dose of 20 mg/kg (data not shown).

Discussion

We have synthesized a series of bis-naphthalimide anti-tumor agents. A representative compound in this series, XB596, demonstrated good *in vivo* anti-tumor activity against the MX-1 human breast carinoma and the DLD-2 human colon adenocarcinoma.

In vitro, XB596 showed potent growth inhibitory activity against a broad spectrum of murine and human leukemia and solid tumor cell lines. Differential cytotoxicity was not observed against the growth of either the leukemic or the solid tumor cells based on an *in vitro* MTT assay. XB596 was also growth inhibitory against three doxorubicin-resistant (multidrug resistant phenotype) tumor cell lines (P388 ADR^-, B16 ADR^- and M16c ADR^-). The growth inhibitory activities of XB596 against these doxorubicin-resistant cells lines were almost comparable to those obtained with the corresponding parental lines. These results suggest that XB596 might be an effective tumoricidal agent against doxorubicin-resistant tumors.

The human tumor colony-forming assay has been used to predict clinical activity of chemotherapeutic agents against human solid tumors.[14,15] In this assay, XB596 demonstrated excellent activity against a variety of human solid tumors *in vitro*, suggesting that XB596 could be useful in treating human tumors if a plasma concentration of 1 8μg/ml were achieved.

XB596 inhibited uridine and thymidine incorporation into RNA and DNA at a concentration 80-fold lower than that required to inhibit leucine incorporation into protein. These data suggest that XB596 might exert its cytotoxic effect(s) at the level of DNA and/or RNA biosynthesis. XB596 binding to DNA was demonstrated by an ethidium bromide displacement assay and by a spectrophotometric titration assay. It is of interest to note that XB596 has a higher binding affinity toward guanine–cytosine than to adenine–thymine sequences. The relationship of the guanine–cytosine binding selectivity to the tumoricidal activity of XB596 remains to be clarified.

Evidence that XB596 intercalates into DNA was provided by an unwinding assay using supercoiled DNA. The negatively supercoiled pBR322 was converted to a form which has a density closely resembling that of a relaxed plasmid when appropriate concentrations of XB596 were added. Additional XB596 restored the density of the supercoiled plasmid as it was converted into a form that resembled positively supercoiled plasmid.

XB596 treatment produced DNA single-strand breaks in cultured L1210 leukemia cells. This finding was consistent with other evidence suggesting that XB596 intercalates into DNA. Many DNA intercalating agents have been shown to induce DNA single-[25] and double-strand breaks[26] in treated tumor cells. However, XB596 appears to be unique compared to other DNA intercalators such as doxorubicin and ellipticine[26] because it produced no detectable double-strand DNA breaks. The DNA intercalator amonafide, a mono-naphthalimide, was shown to inhibit topoisomerase II and to result in intercalator-stabilized-topoisomerase II–DNA cleavable complex formation;[27] however, XB596 does not share this property (preliminary results).

In summary, we have synthesized an interesting class of bis-naphthalimide anti-cancer agents. A representative compound, XB596, interacts with and/or intercalates into DNA, causes DNA single-strand breaks, and inhibits DNA and RNA biosynthesis in tumor cells. The elucidation of the precise mechanism of tumoricidal activity of bis-naphthalimides is currently in progress and includes investigating whether XB596 inhibits topoisomerase I or II activities in a novel manner. Our results with this series, as exemplified by these data on XB596, indicate that the bis-naphthalimides are promising anti-tumor agents. Further studies are being conducted on this series of compounds to further improve their water solubility and to optimize their efficacy against human solid tumor animal models.

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