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BIOLOGICAL ACTIVITY OF NOVEL MACROCYCLIC ALKALOIDS (BUDMUNCHIAMINES) FROM *ALBIZIA AMARA* DETECTED ON THE BASIS OF INTERACTION WITH DNA[1]

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ABSTRACT.—Extracts derived from *Albizia amara* were found to demonstrate activity in a recently developed hplc system designed to detect compounds capable of interacting with DNA. Further investigation led to the procurement of four sets of alkaloid isolates X_1–X_4 that were found to be macrocyclic pithecolobine alkaloids. All four isolates interacted with calf thymus DNA and were generally cytotoxic with a battery of cultured mammalian cells. As determined with *Salmonella typhimurium* strain TM677, isolates X_1 and X_3 were bactericidal, but not mutagenic. Isolate X_1 was found to inhibit the catalytic activity of DNA polymerase, RNA polymerase, and HIV-1 reverse transcriptase. With DNA polymerase, the reaction was shown to be inhibited in a manner that was competitive with respect to DNA. In addition, isolate X_1 inhibited each of the following: platelet aggregation, human lymphocyte transformation, phorbol-ester-induced chemiluminescence with human granulocytes, and cyclooxygenase activity. Detection of these alkaloids on the basis of their interaction with DNA exemplifies the validity of this approach.

As described in the preceding paper (1), we have devised an hplc-based method for detecting substances that interact with nucleic acids such as DNA. The method was characterized with a number of compounds known to interact with nucleic acids and then arbitrarily applied to a number of test materials. As a result, an extract derived from the seeds of *Albizia amara* Bolv. (Leguminosae) was found to demonstrate activity in the hplc detection system. *A. amara* has previously been studied phytochemically, and a variety of compounds have been reported; examples include triterpenes (2) flavonoids (2–4), rare amino acids (5), lipids (6), and steroids (7). However, none of these known constituents would be anticipated to interact with nucleic acids in the manner contemplated on the basis of demonstrating a positive response in this hplc-binding assay. Therefore, the extract was utilized for the isolation of active principles based on interaction with DNA. As described herein, these procedures resulted in the isolation of structurally unique macrocyclic alkaloids. Isolate X_1 has been identified as a mixture of budmunchiamines A–C in the ratio 4:1:1 (Figure 1).

Budmunchiamines belong to the class of pithecolobine alkaloids reported by Wiesner and co-workers (8–10). In determining the structures of budmunchiamines A–C (Figure 1), we have recently delineated all of the ^{1}H- and ^{13}C-nmr spectral parameters associated with the compounds (11). Three additional alkaloid preparations derived from this plant (designated as isolates X_2, X_3, and X_4) have recently been shown to contain alkaloids of this structural class (12). Because we initially became interested in elucidating the structures of these compounds due to their interaction with DNA, the ramifications of said interaction are of interest. This observation was also considered rel-

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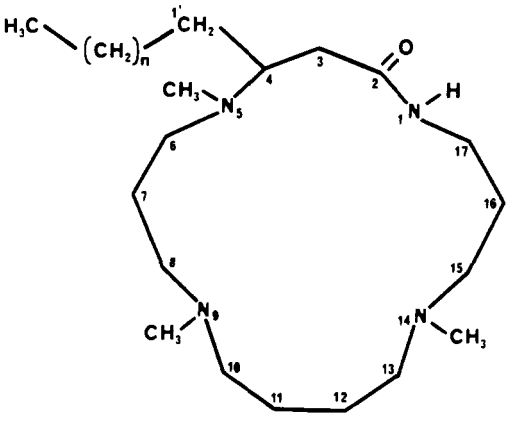

Budmunchiamine A n=9
Budmunchiamine B n=7
Budmunchiamine C n=11

FIGURE 1. Structures of budmunchiamines A–C (isolate X_1).

evant, for no reports have previously appeared in which the biological activity of pithecolobine alkaloids has been described. Thus, in addition to the procedures of isolation, we currently report the biologic potential of these compounds in a broad array of in vitro test systems.

EXPERIMENTAL

PLANT MATERIAL.—The seeds of *A. amara* were collected in India in 1974 by staff members of the Economic Botany Laboratory, Agriculture Research Service, BARC-East, USDA, Beltsville, Maryland. Voucher specimens are on deposit at the herbarium of the National Arboretum, Washington, D.C. The seeds were milled, defatted with petroleum ether, and extracted with MeOH three times overnight. This extract was evaluated for potential to bind to DNA using an hplc detection method (1) and found to result in complete diminution of the DNA peak observed by this technique. Based on this activity, the plant was selected for phytochemical analysis.

ISOLATION OF MARKER QUANTITIES OF DNA-BINDING COMPOUNDS USING DNA-CELLULOSE COLUMN CHROMATOGRAPHY.—DNA-cellulose (Sigma) (0.5 g) was suspended in 10 mM Tris-HCl buffer (pH 7.4) (10 ml) and allowed to settle to remove fine particles. After repeating this procedure several times, the resin was resuspended and loaded into a glass column (5 × 100 mm). The MeOH seed extract was evaporated to dryness and dissolved in DMSO (30 mg/ml). A small quantity (0.2 ml) was applied to the DNA-cellulose resin, and the column was eluted with 10 mM Tris-HCl buffer (pH 7.4) (20 ml). The resin was then removed from the column and washed with 10 mM Tris-HCl buffer (pH 7.4) in a batchwise manner (5 ml × 3). This procedure removed particulate manner that was not bound to the DNA-cellulose and facilitated subsequent elution procedures. The washed resin was then repacked into a glass column and eluted with additional 10 mM Tris-HCl buffer (pH 7.4) (ca. 50 ml) until no compounds could be detected in the eluate. For this procedure, fractions (10 ml) were collected and evaporated, and the residue was dissolved in MeOH (0.1 ml). Aliquots of the solutions were applied to Si gel tlc plates and developed with a variety of standard solvent systems. Attempts were then made to visualize the eluted compounds under uv light and after treatment with 50% aqueous H_2SO_4, vanillin-H_2SO_4 reagent, or Dragendorff's reagent. After eluting the column with approximately 50 ml of buffer, the eluate was free of compounds capable of being visualized by these procedures.

The column was then eluted with 2 M NaCl (50 ml). The eluate was evaporated to dryness and extracted with absolute MeOH (2×). The supernatant fractions were combined, evaporated to dryness, and again extracted with absolute MeOH. This procedure was repeated until the concentration of NaCl was sufficiently reduced to analyze the residue by tlc. Thus, the final MeOH extracts were evaporated to dryness, redissolved in MeOH (0.1 ml), and analyzed by tlc. A variety of solvent systems was examined, but development with $CHCl_3$-diethylamine (10:1) and/or cyclohexane-diethylamine (5:1) followed by detection with Dragendorff's reagent proved most valuable and revealed the presence of four spots (alkaloids).

These alkaloids were collected and used as markers to direct the isolation of larger quantities of the same substances from the total MeOH extract using conventional procedures.

LARGE-SCALE ISOLATION OF DNA-BINDING ALKALOIDS.—The MeOH extract from *A. amara* seeds (100 g) was dissolved in 2% aqueous HOAc (500 ml) and partitioned with $CHCl_3$ (500 ml). The aqueous acidic fraction was saved, and the $CHCl_3$ fraction was extracted with additional 2% HOAc (500 ml). The aqueous fractions were combined, adjusted to a pH of 9.0 with NH_4OH, and extracted with $CHCl_3$ (1 liter). The $CHCl_3$ fraction was recovered and concentrated to dryness. This crude alkaloid fraction was separated into three fractions (A, B, and C) using Si gel cc with $CHCl_3$-diethylamine (150:1) as the solvent. Fraction A was further separated into isolates X_1 and X_2 with cyclohexane-diethylamine (50:1), and fraction B was further separated into isolates X_3 and X_4 with cyclohexane-diethylamine (25:1). As above, the eluates were evaluated by tlc using $CHCl_3$-diethylamine (10:1) and/or cyclohexane-diethylamine (5:1) as the solvent system and Dragendorff's reagent for detection. Isolates X_1–X_4 appeared to be identical to those isolated on the basis of DNA affinity using DNA-cellulose resin. Isolate X_1 has been structurally characterized and found to be a mixture of homologues, budmunchiamines A, B, and C (Figure 1), occurring in a ratio of approximately 4:1:1, respectively (11). The other three substances X_2–X_4 have recently been structurally characterized and found to belong to the same class of macrocyclic alkaloids (12).

CELL LINES AND EVALUATION OF CYTOTOXIC POTENTIAL.—Human breast cancer (UISO-BCA-1), colon cancer (UISO-COL-2), lung cancer (UISO-LUC-1), and melanoma (UISO-MEL-2) cell lines were established from primary human tumors in the Division of Surgical Oncology, University of Illinois College of Medicine at Chicago. These cell lines have been demonstrated to express human isozymes and are tumorigenic in athymic mice (J.M. Pezzuto, C.W. Beattie, K. Lowell, W. Mar, S.M. Swanson, V. Reutrakul, A.D. Kinghorn, and N.R. Farnsworth, unpublished observations). Human fibrosarcoma (HT-1080) and P-388 cells were purchased from the American Type Culture Collection (Rockville, MD). KB-3 and a multidrug-resistant cell line, KB-V1, which was established by treating KB-3 cells with a chronic sublethal dose of vinblastine (13), were supplied by Dr. I. Roninson, Department of Genetics, University of Illinois College of Medicine at Chicago, Chicago, Illinois.

The cytotoxic potential of the isolates derived from *A. amara* seeds was determined essentially by published procedures (14–17). P388 (5×10^4 cells/2.985 ml of medium, in logarithmic growth phase), UISO-BCA-1, UISO-COL-2, UISO-LUC-1, UISO-MEL-2 (15×10^4 cells/2.985 ml of medium, in logarithmic growth phase), KB-3, and KB-V1 (7.5×10^4 cells/2.985 ml of medium, in logarithmic growth phase) were incubated with various concentrations of the test compounds (initially dissolved in 15 μl of DMSO) for a period of 48 h (P388 and UISO-LUC-1) or 72 h (UISO-BCA-1, UISO-MEL-2, and UISO-COL-2, KB-3, KB-V1) (37°, 5% CO_2, 100% humidity). At the end of the incubation period, P-388 cells were counted, and the quantity of the other cell types surviving the treatment was determined by analysis of protein content (18). All determinations were performed in duplicate, and the averaged data were used for the construction of semi-logarithmic dose-response curves. Negative controls (treated with DMSO only) were used to establish 100% growth and at least four different concentrations of the compounds were used. The ED_{50} values (the concentration required to inhibit growth by 50%) were determined by linear regression analysis.

EVALUATION OF BACTERICIDAL AND MUTAGENIC POTENTIAL WITH *SALMONELLA TYPHIMURIUM* STRAIN TM677.—The procedures for assessing forward mutation to 8-azaguanine resistance and bactericidal activity have been described previously (19–21). In brief, various concentrations of test compounds (initially dissolved in 20 μl of DMSO) were added to an incubation mixture (0.98 ml) containing $NADP^+$ (1.0 mg), glucose 6-phosphate (1.0 mg), glucose 6-phosphate dehydrogenase (0.8 units) $MgCl_2$ (0.67 mg), rat liver S-9 (the 9000 × g supernatant fraction derived from the liver of Aroclor 1254-pretreated rats; 1.4 mg protein), and *S. typhimurium* strain TM677 (carrying the 'R-factor' plasmid pKM101; approximately 7×10^6 cells) that had previously been cultured for 1 h at 37° in minimal essential medium ($MgSO_4 \cdot 7H_2O$, 0.2 mg/ml; citric acid, 2.0 mg/ml; K_2HPO_4, 10.0 mg/ml; $NaNH_4HPO \cdot 4H_2O$, 3.5 mg/ml; glucose, 20 mg/ml; biotin, 0.012 mg/ml). After a 2 h incubation period at 37°, the reaction was quenched by the addition of phosphate-buffered saline (4 ml). The bacteria were then recovered by centrifugation, resuspended in phosphate-buffered saline, and plated (in triplicate) in the presence and absence of 8-azaguanine. The plates were incubated for 48 h at 37° and colonies were scored. The mutagenic potential was expressed as the number of 8-azaguanine-resistant bacteria divided by the total number of surviving bacteria in the absence of 8-azaguanine, after correcting for dilution factors. Bactericidal activity was determined by the number of bacteria capable of growing on 8-azaguanine-free plates, relative to negative controls in which the bacteria were treated with an equivalent amount of DMSO. All determinations were performed in duplicate, and 2-aminofluorene (Sigma) was used as a positive control mutagen.

INTERACTION WITH CALF THYMUS DNA.—The potential of the isolates to interact with DNA, as judged by modulation of absorption spectra, was determined essentially as described previously (22,23). Incubation mixtures (1 ml) were prepared consisting of 20 mM sodium phosphate buffer (pH 7.4), 100 mM NaCl, various amounts of calf thymus DNA (0–480 μM), and 88.5 μM of the test compounds (initially dissolved in 50 μl of MeOH). Difference spectra were recorded from 190 to 300 nm, using mixtures containing the same concentrations of DNA (without test compounds) as reference solutions.

SCATCHARD ANALYSIS OF THE INTERACTION OF ALKALOIDS AND CALF THYMUS DNA.—Binding parameters were determined spectrophotometrically with test compounds and calf thymus DNA. Incubation mixtures (1 ml) were prepared containing 20 mM sodium phosphate buffer (pH 7.4), 100 mM NaCl, 100 μM calf thymus DNA (on the basis of phosphate), and various amounts of test compounds (50–170 μM for isolates X_1 and X_3, 25–250 μM for isolates X_2 and X_4). As described previously (24), absorbance data (at the maximum of 202 nm) were used to calculate concentrations required for the construction of Scatchard plots. The following equation was used: $r/m = K_{app}(B_{app} - r)$, where r is a molar ratio (bound compound per DNA phosphate), m is the molar concentration of free compound, K_{app} is the apparent binding constant, and B_{app} is the apparent number of binding sites per DNA phosphate.

EFFECT OF ISOLATE X_1 ON THE CATALYTIC ACTIVITY OF MAMMALIAN DNA POLYMERASE.—*Preparation of DNA polymerase.*—The procedure used for the purification of DNA polymerase was similar to that described by Sedwick *et al.* (25), with some modifications (26). Cultured UISO-BCA-1 cells were suspended in 1.0 M sucrose buffer (1.0 M sucrose, 2.5 mM KCl, 25 mM 2-mercaptoethanol, 1.0 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.5) (1×10^8 cells/ml), homogenized and centrifuged ($1,000 \times g$, 15 min, 4°). The resulting pellet was resuspended in 10 volumes (v/w) of 0.25 M sucrose buffer (0.25 M sucrose, 2.5 mM KCl, 25 mM 2-mercaptoethanol, 1.0 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.5) and centrifuged ($800 \times g$, 5 min, 4°). This washing procedure was repeated, and the pellet was suspended in 9 volumes (v/w) of a detergent-containing buffer (0.3% Triton-X100, 1.0 mM 2-mercaptoethanol, 1.0 mM $MgCl_2$, 1.0 mM K_2HPO_4 buffer, pH 6.5) and centrifuged ($800 \times g$, 5 min, 4°). The nuclei were suspended in 9 volumes (v/w) of 0.2 M sucrose buffer (0.2 M sucrose, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.5). EDTA (1 mM final concentration) and NaCl (4 M final concentration) were added. The mixture was stirred (4°, 4 h) and dialyzed (12,000–14,000 mol wt cut-off) against buffer (0.025 M K_2HPO_4, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, pH 7.5) for a period of 24 h at 4°. The buffer solution was changed three times during the dialysis period. The resulting precipitate was removed by centrifugation, protein concentration was determined by the method of Lowry *et al.* (27), and the preparation was stored as small aliquots at −80°.

Inhibition of DNA polymerase mediated by isolate X_1.—The assay for DNA polymerase activity was based on that described by Miller and Chinault (28), as modified previously (26). Activated calf thymus was prepared as substrate by the method of Schlabach *et al.* (29). Briefly, calf thymus DNA (100 μM on the basis of phosphate) was dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and pancreatic deoxyribonuclease (40 ng of DNase/mg of DNA). The reaction mixture was incubated at 37° for 35 min, and DNase was heat-inactivated (10 min, 60°). The mixture was finally cooled on ice and stored as small aliquots at −80°.

For the determination of enzymatic activity, reaction mixtures were prepared in 50 mM Tris-HCl buffer, pH 7.5, containing 8 mM $MgCl_2$, 4 mM KCl, 0.5 mM dithiothreitol, 12.5% glycerol, 20 μM ATP, 5 μM dATP, 5 μM dGTP, 5 μM dCTP, 0.2 μM [methyl-^{3}H]TTP (1.22 μCi, 61 Ci/mmol, ICN Radiochemicals, Irvine, CA), 5 μM activated calf thymus DNA, and various concentrations of test compound (0–180 μM; 0–80 μg/ml) (initially dissolved in 10 μl of DMSO). The reactions were started by the addition of partially purified DNA polymerase (15 μg based on protein) (final volume, 0.1 ml), and the mixtures were incubated at 37° for 30 min. The reactions were then stopped by the addition of 10 μl of 0.25 M EDTA and placed on ice. Aliquots (100 μl) of the reaction mixtures were spotted onto circular discs (2.5 cm) of Whatman DE81 ion exchange paper and washed with 5% aqueous Na_2HPO_4 (3×) and distilled H_2O. The discs were dried, and radioactivity was determined by liquid scintillation counting. Activity was expressed as a percentage relative to the solvent-treated control, and the IC_{50} value was determined using linear regression analysis with a semi-logarithmic response curve. All determinations were performed in triplicate.

Kinetic analysis of the inhibition of DNA polymerase by isolate X_1.—The assay system was similar to that described above except that various concentrations of [methyl-^{3}H]TTP were used (0.04–0.2 μM, 61 Ci/mmol) with a fixed concentration of DNA (5 μM), or various concentrations of DNA (3.33–20 μM) were used with a fixed concentration of [methyl-^{3}H]TTP (0.2 μM, 61 Ci/mmole). Five concentrations of the test compound (0–20 μM) were used, and the reaction mixtures were incubated for 8 min at 37°. All assays were performed in triplicate. K_m and V_{max} values were determined from Lineweaver-Burk reciprocal plots, and K_i values was determined from Dixon plots.

EFFECT OF ISOLATE X_1 ON THE CATALYTIC ACTIVITY OF MAMMALIAN RNA POLYMERASE.—*Preparation of partially purified RNA polymerase.*—Nuclei were prepared from cultured UISO-BCA-1 cells as described for the preparation of DNA polymerase, and partially purified RNA polymerase was isolated using a modification (26) of the procedure of Rose *et al.* (30). The nuclei were suspended in 4 volumes (v/w) of buffer (50 mM Tris-HCl, pH 8.9, 50 mM KCl, 1.0 mM $MgCl_2$, 0.5 mM phenylmethylsulfonylfluoride, 0.1 mM EDTA, 20% glycerol), and $(NH_4)_2SO_4$ was added to a final concentration of 0.42 g/ml. The suspension was incubated at 35° for 10 min and then stirred for 3 h at 4°. The mixture was dialyzed (mol wt cut-off 12,000–14,000) against a buffer solution (50 mM Tris-HCl, pH 7.9, 2.0 mM dithiothreitol, 0.1 mM EDTA, 25% glycerol, 1.0 mM $MgCl_2$, and 50 mM KCl) (24 h, 4°, with two changes of the buffer). The resulting precipitate was removed by centrifugation, glycerol was added to a final concentration of 50%, and the extract was stored at −20° as small aliquots. The protein concentration was determined by the method of Lowry *et al.* (27).

Activity of partially purified RNA polymerase.—The assay was conducted by a modification of the procedure described by Rose *et al.* (31). Reaction mixtures were prepared containing calf thymus DNA (5.0 μM), [5,6-^{3}H]-UTP (1.28 μM, 5 μCi, 39 Ci/mmol), spermidine (3 mM), $MnCl_2$ (1.67 mM), ATP, CTP, and GTP (0.32 mM each), ribonuclease (RNase) inhibitor (3 units, Sigma), NaF (3.3 mM), KCl (8 mM), $(NH_4)_2SO_4$ (60 mM), $MgCl_2$ (1.5 mM), Tris-HCl buffer, pH 8.0 (60 mM) and various concentrations of the test compounds (0–75 μM, initially dissolved in 5 μl of DMSO). The reaction was initiated by the addition of partially purified RNA polymerase (15 μg protein) (final volume 100 μl) and incubated at 37° for a period of 30 min. The reactions were stopped by the addition of 0.25 M EDTA (10 μl) and placing the reaction tubes on ice. Aliquots (100 μl) were then transferred to 2.5-cm circular discs of Whatman DE81 ion exchange paper, and the discs were processed as described above for the DNA polymerase reaction.

EFFECT OF ISOLATE X_1 ON THE CATALYTIC ACTIVITY OF HIV-1 REVERSE TRANSCRIPTASE.—These analyses were performed as described previously (32). In brief, assay mixtures (final volume 100 μl) contained the following: 50 mM Tris·HCl buffer (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM ethylene glyco-bis-(β-amino-ethylether) *N,N'*-tetraacetic acid (EGTA), 5 mM dithiothreitol, 0.3 mM glutathione, 2.5 μg/ml bovine serum albumin, 5 μM poly A [ϵ_{260} (mM) = 7.8], 1.2 μM oligo dT [ϵ_{265} (mM) = 5.6], 0.05% Triton X-100, 20 μM TTP, and 0.5 μCi of [^{3}H]TTP. Alternatively, activated calf thymus DNA (5 μM) was used in place of poly A·oligo dT. The reaction was initiated by the addition of HIV-1 reverse transcriptase (0.1 μg protein), and the mixture was permitted to incubate at 37° for 1 h. Reactions were terminated by the addition of 25 μl of 0.1 M EGTA followed by chilling in ice. Aliquots of each reaction mixture (100 μl) were then spotted uniformly onto circular 2.5-cm DE-81 (Whatman) filters, kept at ambient temperature for 15 min, and washed four times with 5% $Na_2HPO_4 \cdot 7H_2O$. This was followed by additional washing with distilled H_2O. Finally, the filters were thoroughly dried and subjected to scintillation counting in a toluene-based scintillation fluid. For testing enzyme inhibition, various concentrations of isolate X_1 (5–200 μM) were added to the reaction mixtures prior to the addition of enzyme.

EFFECT OF ISOLATE X_1 ON PLATELET AGGREGATION.—The inhibitory effect of isolate X_1 on platelet aggregation was investigated by the method of Born and Cross (33). Human blood was collected (3.13% trisodium citrate) and centrifuged (150 × *g*, 10 min, 22°) to yield an upper layer of platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by recentrifugation of the lower portion (2000 × *g*, 10 min), and this was used to adjust the PRP to a density of 2.55×10^8 platelets/ml The extent of platelet aggregation, as reflected by optical density, was then measured using a dual-channel aggregometer (Labor, Ahrensburg, Germany). In brief, modified Tyrode solution (136.9 mM NaCl, 2.68 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.42 mM NaH_2PO_4, 5.55 mM glucose, 11.9 mM $NaHCO_3$) (45 μl) was added to PRP (200 μl), and the mixture was stirred (1000 rpm, 37°) in the aggregometer. Various concentrations of the test substance were then added (initially dissolved in 2.5 μl of MeOH), and aggregation was induced by adding solutions of platelet activating factor (PAF) (1.0 μM final concentration) or ADP (10 μM final concentration). The increase in light transmission (aggregation) was recorded using PPP as a reference, and the inhibitory effect of isolate X_1 was calculated as a percentage of the maximum aggregation induced by PAF or ADP with PRP treated with 2.5 μl of MeOH.

EFFECT OF ISOLATE X_1 ON THE PROLIFERATION OF HUMAN LYMPHOCYTES.—The procedure has been described previously by Wagner *et al.* (34). In brief, human lymphocytes were isolated from heparinized peripheral blood by Ficoll gradient centrifugation. The cells were washed three times with Hanks balanced salt solution and resuspended in RPMI-1640 medium containing Hepes buffer (20 mM), L-glutamine (4 mM), minimal essential medium vitamins, non-essential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (10%). Cells (10^5 in 0.2 ml of medium) were then incubated with various concentrations of the test substance (ranging from 1 μg/ml to 100 ng/ml) for a

period of 70 h (37°, 5% CO_2), in the presence or absence of concanavalin A (0.8 μg). The incubation mixtures were then supplemented with [^{3}H]thymidine (0.17 μCi; final volume, 0.25 ml), and the incubation was continued for an additional 18 h. At the end of the incubation period, the cells were collected on glass fiber filters, washed with H_2O, dried, and counted by liquid scintillation. Incubations were performed in 96-well plates, and all determinations were performed in triplicate.

EFFECT OF ISOLATE X_1 ON PHORBOL-ESTER-INDUCED CHEMILUMINESCENCE WITH HUMAN GRANULOCYTES.—The procedure has been described previously by Wagner *et al.* (35). Human granulocytes were isolated from peripheral blood by consecutive centrifugations through Dextran T500 (1.5%) and a Ficoll gradient. The cells were suspended in RPMI-1640 medium and counted. Incubation mixtures were then prepared containing granulocytes (10^6 cells/ml), buffer (Veronal, 10 mg/ml; $CaCl_2$, 0.2 mg/ml; $MgCl_2$, 0.10 mg/ml; NaCl, 10 mg/ml; glucose, 1 mg/ml; bovine serum albumin, 1 mg/ml; pH 7.0), luminol (0.02 mmol), and various concentrations of the test substance (dissolved in 10 μl of DMSO). After preincubation at 37° for 10 min, the reaction mixtures were supplemented with phorbol-12-myristate-13-acetate (PMA) (10 pmol) (final volume, 1.0 ml). The incubation was then continued (37°), and the chemiluminescence response was recorded for a period of 30 min (Berthold Biolumat LB 9500T luminometer linked to an Apple IIE computer with Berthold software).

EFFECT OF ISOLATE X_1 ON CYCLOOXYGENASE ACTIVITY.—For the evaluation of cyclooxygenase activity, sheep seminal vesicle microsomes were isolated by a modification of the method of Nutgeren and Hazelhof (36). In brief, sheep vesicular glands were cleaned and homogenized in three volumes (v/w) of buffer (0.1 M KH_2PO_4, pH 7.4, 1.0 mM EDTA) using an Ultra-Turax blender (0–4°). Coarse material was removed by centrifugation (4000 × *g*, 10 min), and the supernatant was then centrifuged at 105,000 × *g* for 1 h. The resulting pellet was suspended in 0.1 M KH_2PO_4 buffer, pH 7.4, and stored as small aliquots at −70°. Cyclooxygenase activity was determined essentially by the method of Kuhl *et al.* (37). Incubation mixtures were prepared containing 0.1 M Tris-HCl buffer, pH 8.0, 1.0 mM reduced glutathione, 1 mM epinephrine, 0.05 mM EDTA, microsomes (8.0 μg protein), and various concentrations of the test substance (in triplicate). After a preincubation period (5 min, 20°), the reactions were started by the addition of [^{14}C]arachidonic acid (1 μM, 54 mCi/mmol) (Amersham, Braunschweig). After an incubation period (20 min, 37°), the reaction was stopped by the addition of 1% HCO_2H (0.525 ml, 0–4°), and the mixture was extracted with EtOAc (4 ml, 2×). The organic phase was evaporated to dryness, the residue was dissolved in EtOH, and arachidonic acid metabolites were analyzed by reversed-phase hplc. Radioactivity was monitored (Berthold, HPLC radioactivity Monitor LB 506 C-1), and peak areas were determined by integration. The results were expressed as a percentage relative to control incubations and used to construct semi-logarithmic plots for the calculation of IC_{50} values.

RESULTS AND DISCUSSION

In order to investigate the utility of a recently developed assay involving hplc for the detection of DNA-binding compounds in plant extracts (1), several plants, including *A. amara*, were selected on a random basis. An extract derived from this plant displayed a positive response with the hplc system, and it was also found to demonstrate cytotoxity when evaluated with cultured cells (Table 1). Based on these observations, the *Al-*

TABLE 1. Cytotoxic Activity of the MeOH Extract and Alkaloid Isolates X_1–X_4 Obtained from the Seeds of *Albizia amara*.

Cell line	MeOH extract	Alkaloid Isolate Mixtures[a]			
		X_1	X_2	X_3	X_4
P388	1.02	0.09	0.68	0.10	0.91
KB-3	2.01	0.27	1.36	0.29	1.90
colon	5.24	0.56	2.30	1.60	1.70
breast	10.10	0.77	4.72	1.42	4.65
lung	12.40	0.74	7.70	1.40	11.40
melanoma	6.40	0.58	2.85	0.75	2.80
sarcoma	2.02	0.17	1.40	0.29	1.31
KB-V1	ND[b]	0.56	ND	ND	ND

[a]The results are expressed as ED_{50} values (μg/ml).
[b]ND=not determined.

bizia extract was subjected to a fractionation scheme in order to isolate active principles. Four sets of alkaloid isolates were obtained; the composition of one of these (isolate X_1) is shown in Figure 1 (11). These materials, assigned the trivial names budmunchiamines A–C, belong to the pithecolobine class of alkaloids, previously isolated from *Pithecolobium saman* (syn. *Samania saman*) (8–10). [The name budmunchiamine (11) commemorates the collaboration between Budapest, Munich, and Chicago that led to the discovery of these alkaloids.] Alkaloids of this type have not previously been reported to interact with DNA, nor have they previously been reported as an isolate from plants other than *P. saman*, a close taxonomic relative of *A. amara* in the family Leguminosae. More recently, we have also elucidated the structures of isolates X_2–X_4 and found that they are closely rel3ated to isolate X_1 (12).

Due to the structural similarity of bundmunchiamines A–C, separation is not a trivial undertaking. For example, a variety of elution techniques employing C-18 hplc were not of value in facilitating separation (R. Bauer, unpublished observations). Nonetheless, it should not be anticipated that the structural variation among these homologues would affect observations of biological activity, and a number of evaluation procedures were therefore performed. As illustrated in Figure 2, addition of calf thymus

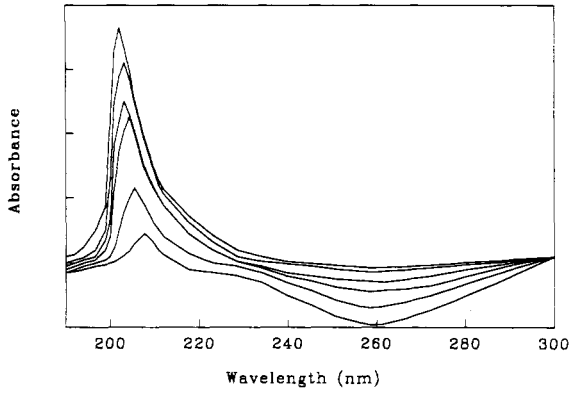

FIGURE 2. Difference spectra demonstrating the dose-dependent diminution in the absorbance of isolate X_1 (88.5 μM) that results from the addition of increasing quantities of calf thymus DNA (0, 60, 120, 240, 360, and 480 μM). Additional details are provided in Experimental.

DNA produced a dose-dependent diminution in the uv absorbance maxima of isolate X_1; this is indicative of DNA interaction. Similar spectral perturbations were produced when DNA was admixed with isolates X_2–X_4 (data not shown). Further, this spectrophotometric procedure could be used to evaluate the interaction quantitatively, and Scatchard analysis (Figure 3) revealed $K_{app} = 0.47 \times 10^5\ M^{-1}$ and $B_{app} = 1.03$. Thus, isolate X_1 binds to calf thymus DNA with relatively high affinity, at a maximal level of one molecule of alkaloid per nucleotide. The nature of this interaction is currently under investigation.

The cytotoxic activity of the alkaloids was then assessed with a number of cultured mammalian cell lines. As summarized in Table 1, the original extract and all four of the isolates demonstrated a general (nonspecific) cytotoxic response. The intensity of the response demonstrated by isolate X_1 was similar to that demonstrated by isolate X_3, and approximately 10-fold more intense than that demonstrated by the original extract. The responses demonstrated by isolates X_2 and X_4 were similar to each other, but less

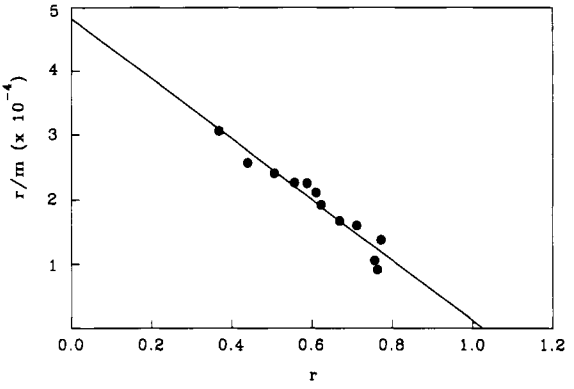

FIGURE 3. Scatchard plot illustrating the quantitative interaction of calf thymus DNA and compound isolate X_1 (see Experimental).

intense than those demonstrated by isolates X_1 and X_3. These results show that significant enrichment of activity was obtained using DNA-based isolation procedures. Also, it is of interest that multidrug-resistant KB-V1 cells were susceptible to the effect of isolate X_1.

In accord with these relative degrees of cytotoxic activity, bactericidal responses were also demonstrated with isolates X_1 and X_3, but not with isolates X_2 and X_4, when tested with *S. typhimurium* TM677 at concentrations as high as 200 μg/ml (Table 2). Because these alkaloids were established as having the potential to interact with DNA, it was speculated that they might mediate a mutagenic response. As shown in Table 2,

TABLE 2. Evaluation of the Bactericidal and Mutagenicity Potential of Alkaloid Isolate Mixtures X_1–X_4 Obtained from the Seeds of *Albizia amara* Utilizing *Salmonella typhimurium* Strain TM677 in the Presence of Rat Liver S-9.

Concentration tested (μg/ml)	Alkaloid Isolate Mixtures				
	X_1	X_2	X_3	X_4	2AF[b]
0	7.8 ± 1.6 (100)	7.8 ± 1.6 (100)	7.8 ± 1.6 (100)	7.8 ± 1.6 (100)	7.8 ± 1.6 (100)
6.25	10.3 ± 0.1 (90)	NT	10.2 ± 0.1 (103)	NT	NT
12.50	11.0 ± 0.7 (82)	9.8 ± 0.5 (92)	12.6 ± 11.5 (79)	11.5 ± 1.7 (95)	NT
25.00	toxic[c] (0.1)	9.1 ± 2.3 (107)	toxic (2.3)	11.1 ± 0.6 (93)	NT
50.00	toxic (<0.1)	12.3 ± 3.9 (97)	toxic (<0.1)	11.0 ± 1.5 (92)	NT
100.00	toxic (<0.1)	10.0 ± 0.1 (105)	toxic (<0.1)	11.0 ± 2.0 (94)	715 ± 96 (1)
200.00	toxic (<0.1)	10.0 ± 1.4 (109)	toxic (<0.1)	9.4 ± 0.4 (101)	NT

[a]The values are the average of duplicate determinations (plated in triplicate) and are expressed as a mutant fraction ($\times 10^5$) ± SD; the parenthetical values represent the percentage of bacteria that survived the treatment. NT = not tested.

[b]2-AF = 2-aminofluorene.

[c]Due to the extent of bactericidal activity, the mutant fraction could not be determined with these experimental conditions.

however, no significant mutagenicity was observed when the alkaloids were evaluated with *S. typhimurium* in the presence of a metabolic activating system. Since a relatively strong bactericidal response was observed with isolates X_1 and X_3, it was not possible to increase the concentration further. However, these results are reasonable since it is unlikely that these compounds interact with DNA in a covalent manner (based on the isolation procedure), and covalent interactions are generally considered important in the process of chemically-induced mutagenicity.

In order to explore further the biological ramifications of interaction with DNA, the effect of isolate X_1 on nucleic acid polymerization reactions was investigated. As shown in Figure 4, isolate X_1 inhibited the catalytic activity of DNA polymerase and

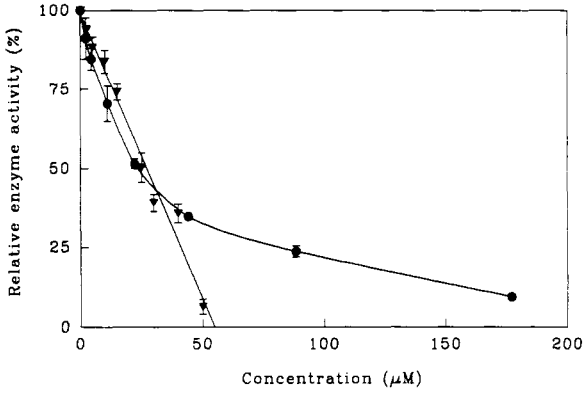

FIGURE 4. Inhibition of the catalytic activity of DNA polymerase (▼) and RNA polymerase (●) by isolate X_1. Reactions were performed in the presence of the indicated concentrations of isolate X_1 as described in Experimental.

RNA polymerase. The corresponding IC_{50} values were approximately 25 μM, but as illustrated in the figure, a biphasic inhibition response was observed with the reaction catalyzed by RNA polymerase. Inhibition of the reactions catalyzed by reverse transcriptase was also mediated by isolate X_1, yielding IC_{50} values of 467 and 335 μM, respectively, using poly A oligo dT and activated calf thymus DNA as substrates (data not shown). The nature of the inhibition was examined in greater detail using DNA polymerase. As summarized in Table 3, the K_i for this reaction was approximately 20 μM, which was similar to the K_m value for DNA as substrate. In addition, the inhibition was competitive with respect to DNA. These results are consistent with the previous Scatchard analysis (Figure 3), where it was demonstrated that isolate X_1 interacted with DNA with a maximum binding value of one molecule of alkaloid per DNA base, and provide evidence that this interaction is biologically relevant.

TABLE 3. Kinetics of DNA Polymerase Inhibition Mediated by Isolate X_1.[a]

Substrate	Km (μM)	V max (μM/min)	Ki (μM)	Type of inhibition
DNA	15.13	92.89	19.86	competitive
TTP	0.073	26.35	22.06	noncompetitive

[a]Reactions were performed as described in Experimental using various concentrations of TTP (0.04–0.2 μM), activated calf thymus DNA (3.33–20 μM), and isolate X_1 (0–20 μM). Kinetic constants were derived from Lineweaver-Burk and Dixon plots.

Because the biological potential of pithecolobine alkaloids has never been reported, additional evaluation procedures were performed. Inasmuch as isolate X_1 has been structurally defined and shown to mediate one of the most intense responses in the systems described above, X_1 was used for the additional test procedures. As illustrated in Figure 5, isolate X_1 mediated a dose-dependent inhibition of platelet aggregation that was induced by PAF or ADP. The corresponding IC_{50} values were 18.8 and 24.3 μM, respectively. One of the most potent natural product agents previously tested in this assay system is ginkgolide B, which demonstrates an IC_{50} value of 10.5 μM in PAF-induced platelet aggregation (data not shown). Thus, isolate X_1 mediates a relatively strong inhibition of platelet aggregation, but unlike ginkgolide B, which is a specific PAF antagonist, isolate X_1 is nonspecific.

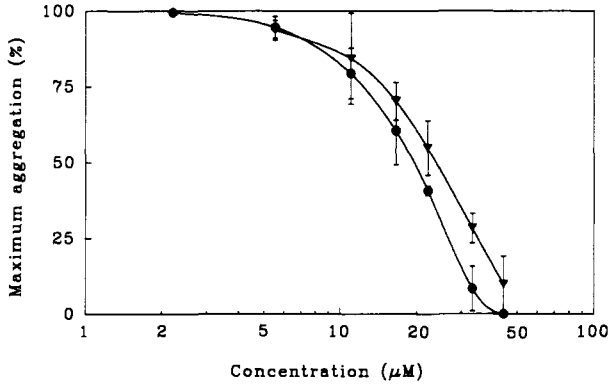

FIGURE 5. Inhibition of platelet aggregation by isolate X_1. Platelet aggregation was induced by treatment with PAF (●) or ADP (▼) in the presence of the indicated concentrations of isolate X_1, as described in Experimental.

Isolate X_1 was also evaluated for potential to modulate the proliferation of human lymphocytes. As reported previously, some well-known antitumor agents (e.g., vinblastine) inhibit lymphocyte proliferation at high doses, but they stimulate the cells, as judged by thymidine incorporation, when treated with doses in the pico- and femtogram range (34). When human lymphocytes were treated with isolate X_1, dose-dependent inhibition of thymidine incorporation was observed in the range of 0.01 to 1.0 μg/ml (data not shown). When tested at lower doses (10 ng–100 ag/ml), however, the compound had no significant effect on lymphocyte proliferation (data not shown). Similar effects were observed when lymphocytes were treated with isolate X_1 either in the presence or absence of concanavalin A. Therefore, isolate X_1 does not demonstrate a "reversal effect" (i.e., suppression at high doses and stimulation at low doses) in this assay, nor does the substance co-stimulate lymphocytes in the presence of concanavalin A. Rather, isolate X_1 only suppresses lymphocyte proliferation at higher doses, which is the response demonstrated by the majority of antitumor agents that have been tested in this system (38).

In addition, isolate X_1 was analyzed for potential to inhibit PMA-induced chemiluminescence with human granulocytes. As illustrated in Figure 6, significant inhibition was observed. The alkaloid demonstrated a sharp dose-response relationship in the range of 0.5 to 1.0 μg/ml, with 50% inhibition being facilitated at a concentration of approximately 0.5 μg/ml. This type of activity is consistent with inhibition of tumor promotion. For example, retinoids are active in this process; the concentration of retinyl acetate required to reduce the response by 50% is approximately 12 μg/ml

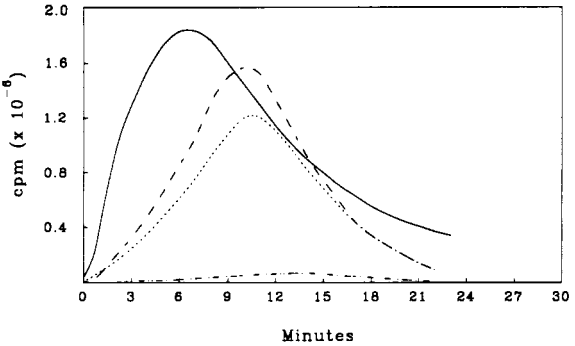

FIGURE 6. Inhibition of PMA-induced chemiluminescence by compound isolate X_1. The assay was performed in the presence of the following concentrations of isolate X_1: 0 (———); 0.25 μg/ml (— —); 0.50 μg/ml (– – –); 1.0 μg/ml (– · · –); 2.0 μg/ml (line not visible). Additional details are provided in Experimental.

(J.M. Pezzuto, unpublished observations). Activity in this test system is also consistent with anti-inflammatory activity (39).

Another response consistent with anti-inflammatory activity is inhibition of cyclooxygenase activity (39). Isolate X_1 was evaluated and found to demonstrate a dose-dependent inhibition of the enzyme, with an IC_{50} of 27 μg/ml (59.6 μM). This activity is considered in the moderate range, being comparable to allicin, a compound that inhibits prostaglandin biosynthesis by 67% at a concentration of 50 μM. One of the most potent agents in this test system is indomethacin, which demonstrates an IC_{50} of 1.2 μM. Studies are currently in progress to determine the effect of budmunchiamines on related activities such as the reaction catalyzed by protein kinase C.

To conclude, the budmunchiamines demonstrate a broad range of biological activities. While studies to characterize more fully the structural and biological activities of these alkaloids are currently in progress, the results thus far obtained are sufficient to support the value of DNA-binding as a screen. It should be of interest to continue investigations along similar lines with other plant species. Since *A. amara* was selected on a semi-empirical basis, it may be suggested that an unanticipated array of structurally diverse compounds will be discovered through utilization, in the future, of these DNA-based procedures, and it is likely that some of these isolates may be of applied interest.

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