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AZATOXIN DERIVATIVES WITH POTENT AND SELECTIVE ACTION ON TOPOISOMERASE II

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Abstract—Azatoxin was rationally designed as a DNA topoisomerase II (top2) inhibitor [Leteurtre *et al.*, *Cancer Res* **52**: 4478–4483, 1992] and was also found to inhibit tubulin polymerization. Its cytotoxicity is due to action on tubulin at lower concentrations and on top2 at higher concentrations. At intermediate concentrations, the combination of the two mechanisms appears antagonistic [Solary *et al.*, *Biochem Pharmacol* **45**: 2449–2456, 1993]. The aim of this study was to design azatoxin derivatives that would act only on tubulin or on top2. Selective targeting of top2 or tubulin was tested using top2-mediated DNA cleavage assays, and tubulin polymerization and tubulin proteolysis assays, as well as COMPARE analyses of cytotoxicity assays in the National Cancer Institute *in vitro* Drug Screening Program. Selective inhibitors of top2 and tubulin polymerization have been obtained. Top2 inhibition, abolished by methylation at position 4′, was enhanced by the addition of a bulky group at position 11. Bulky substitution at position 11 determined different patterns of top2 cleavage sites and suppressed the action on tubulin. Selective inhibition of tubulin was obtained with 4′-methylazatoxin that was found to bind to the colchicine site. These results are consistent with those obtained in the podophyllotoxin family to which azatoxin is structurally related. Some azatoxin derivatives are under consideration for further preclinical development.

Key words: topoisomerase II; tubulin; podophyllotoxin; azatoxin

Azatoxin is a synthetic molecule that was rationally designed as a top2¶ inhibitor, using molecular modeling of pre-existing top2 inhibitors [1]. Cytotoxicity data and COMPARE analyses from the NCI drug screen suggested anti-tubulin activity in addition to top2 inhibition [2]. Biochemical analyses confirmed that azatoxin inhibits tubulin polymerization at lower concentrations and top2 at higher concentrations. Cytotoxicity analyses suggested that the two actions were antagonist at intermediate concentrations [2].

The present study was performed with selected azatoxin derivatives (Fig. 1) to identify the drug structural determinants that are responsible for tubulin and top2 inhibition. Substitutions at two positions, 4′ and 11, were found to discriminate the activities against top2 and tubulin. 4′-Methylation abolished the anti-top2 activity and increased the anti-tubulin activity. Substitution at position 11 with anilino groups eliminated the anti-tubulin activity, dramatically potentiated the anti-top2 activity, and influenced the base sequence selectivity of top2 inhibition.

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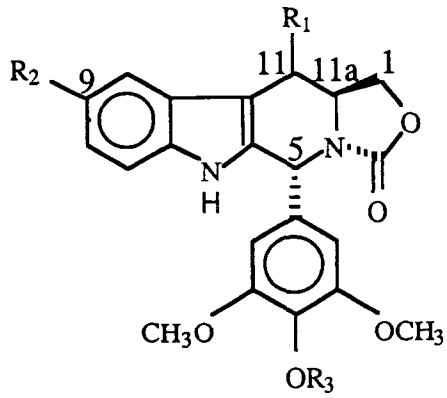
¶ Abbreviations: NCI drug screen, National Cancer Institute *in vitro* Drug Screening Program; PCR, polymerase chain reaction; top2, DNA topoisomerase II; VBL, vinblastine; and VP-16, etoposide.

MATERIALS AND METHODS

Materials, chemicals, and enzymes. The synthesis of azatoxin and azatoxin derivatives (Fig. 1) was done according to our previous procedures [1, 2]. VP-16 was obtained from the Bristol-Myers Co. (Wallingford, CT). Drug stock solutions were made in DMSO at 10 mM, and further dilutions were made in distilled water immediately before use.

SV40 DNA, human c-*myc* DNA, restriction enzymes, T4 polynucleotide kinase, Taq DNA polymerase, and polyacrylamide/bis-acrylamide were from GIBCO-BRL (Gaithersburg, MD), from the American Type Culture Collection (Rockville, MD), from Perkin Elmer Cetus (Norwalk, CT), or from New England Biolaboratories (Beverly, MA). [γ-^{32}P]ATP and [α^{32}P]dATP were purchased from New England Nuclear Research Products (Boston, MA). Top2 was purified from mouse leukemia L1210 cell nuclei as described previously, and was stored at −70° in 40% (v/v) glycerol, 0.35 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 0.2 mM dithiothreitol and 0.1 mM phenylmethanesulfonyl fluoride, pH 6.4. The purified enzyme yielded a single 170-kDa band after silver staining of SDS–polyacrylamide gels [3]. Primer oligonucleotides for PCR were prepared using a 392 DNA synthesizer and purified using oligonucleotide purification cartridges from Applied Biosystems (ABI, Foster City, CA).

NCI drug screen assays. The methodology employed by the National Cancer Institute *in vitro*



Compound	NSC	R_1	R_2	R_3
(1) Azatoxin	640737	H	H	H
(2) Methylazatoxin	662306	H	H	CH_3
3	659547	H	F	H
4	NA	H	OH	H
5	NA	O⌒OH	H	H
6	NA	NH⌒N(CH3)2	H	H
7	662304	NH–⬡–F	H	H
8	662305	NH–⬡–CN	H	H
9	668380	NH–⬡–NO_2	H	H

3: 9-Fluroazatoxin.
4: 9-Hydroxylazatoxin.
5: 11β (Hydroxyl ethyl) ether azatoxin.
6: 11β (2″-*N*,*N*-dimethylaminoethyl)-amino azatoxin.
7: 11β (4″-Fluoroanilino) azatoxin.
8: 11β (4″-Cyanoanilino) azatoxin.
9: 11β (4″-Nitroanilino) azatoxin.
NA: not assigned.

Fig. 1. Structure of azatoxin derivatives used in this study.

drug screening program has been discussed in detail previously [2, 4, 5].

Preparation of end-labeled DNA fragments. SV40 DNA was labeled at both termini of *Bcl*I restriction site using 1 unit of Taq DNA polymerase and [α-^{32}P]dATP. A human c-*myc* DNA fragment was prepared by PCR for top2 cleavage site sequencing. This 403 base pair DNA fragment encompassing the junction between the first intron and the first exon was amplified between positions 2671 and 3073, with numbers referring to GenBank genomic positions, using oligonucleotides: 5′-TGCCGCATCCACG-AAACTTTGC-3′ as sense primer and 5′-CTTGAC-AAGTCACTTTACCCCG-3′ as antisense primer. Single-end-labeling of the DNA fragment was obtained by 5′-end-labeling of the sense strand primer oligonucleotide [4]. Approximately 0.1 μg of the c-*myc* DNA that had been restricted by *Xho*I and *Xba*I was used as template for PCR [4].

Topoisomerase II-induced DNA cleavage reactions. DNA fragments were equilibrated with or without drug in 10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP and 15 μg/mL bovine serum albumin for 5 min before addition of purified top2 (40–70 ng) or HL60 nuclear extracts (2 μL corresponding to approximately the extract from 10^6 nuclei) in 20 μL final reaction volume [1]. Reactions were performed at 37° for 30 min, and then stopped by adding SDS to a final concentration of 1% and proteinase K to 0.4 mg/mL, followed by incubation for 1 hr at 42°.

Tubulin polymerization inhibition assays. Assays were essentially performed as described previously [2]. Briefly, autopolymerization of tubulin was

induced in the presence of GTP by a rapid shift in temperature from 0° to 30°; depolymerization occurred when the temperature was returned to 0°. Changes in tubulin polymerization status were followed by turbidimetry at 350 nm.

Tubulin proteolysis assays. Assays were performed as described previously [6]. Briefly, a 10 μM concentration of purified tubulin preincubated with or without an excess of drug was digested on ice with chymotrypsin or trypsin. Enzyme action was halted by addition of 0.2 mM phenylmethanesulfonyl fluoride (chymotrypsin) or 0.01 mM leupeptin (trypsin). Samples were separated by SDS–PAGE. Gels were stained with Coomassie Blue.

Electrophoresis and data analysis. For agarose gel analysis, 3 μL (10x) loading buffer (0.3% bromophenol blue, 16% Ficoll, 10 nM Na_2HPO_4) was added to each sample, which was then heated at 65° for 1–2 min before loading into an agarose gel made in (1x) TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). Agarose gel electrophoresis was at 2 V/cm overnight. The quantification of drug-induced DNA double-strand breaks in the presence of HL60 nuclear extracts was done as follows. Radioactive gels were scanned using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). For each lane, the radioactivity was measured for the DNA cleavage products (C), and the total DNA present in the lane (T). Drug-induced cleavage was expressed as:

$$\text{Percent DNA cleaved} = 100 \times (C/T - C_o/T_o)/(1 - C_o/T_o)$$

where C_o and T_o are the counts for cleaved and total DNA, in the presence of nuclear extracts without drug, respectively.

For DNA sequence analysis, samples were precipitated with ethanol and resuspended in 2.5 μL loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). Samples were heated to 90° and immediately loaded into DNA sequencing gels (7% polyacrylamide; 19:1 acrylamide:bis-acrylamide) containing 7 M urea in (1x) TBE buffer. Electrophoresis was at 2500 V (60 W) for 4 hr. Gels were dried on 3MM paper sheets and autoradiographed with Kodak XAR-2 film.

RESULTS

Sequencing of top2 cleavages induced by azatoxin derivatives

Top2 inhibitors have different spectra of activity in the clinics. This could be related to DNA sequence selectivity of top2 cleavage sites, which is different for each class of drug [7]. Top2 cleavage sites were mapped using purified murine leukemia top2 in the human c-*myc* proto-oncogene at the junction between the first intron and exon, a region important for transcription regulation [8]. Figure 2 shows cleavages induced by azatoxin derivatives at 100 μM. The cleavage patterns were different among derivatives. The same differences were observed over a wide concentration range (data not shown). The nature of the substituent at position 9 altered the cleavage pattern, as seen by comparison of azatoxin in lane D, with the 9-fluoro derivative (compound **3**) in lane H, and the 9-hydroxyl derivative (compound **4**) in lane I. The nature of the substituent also affected potency. The 9-hydroxyl derivative was less potent than azatoxin as more of the full size DNA was retained near the top of the gel. Modifications at position 11 also changed the cleavage pattern (Figs. 2 and 3, and data not shown). Important alterations of pattern were produced by the addition of an anilino group, as seen by the comparison of azatoxin in lane D with compound **6**

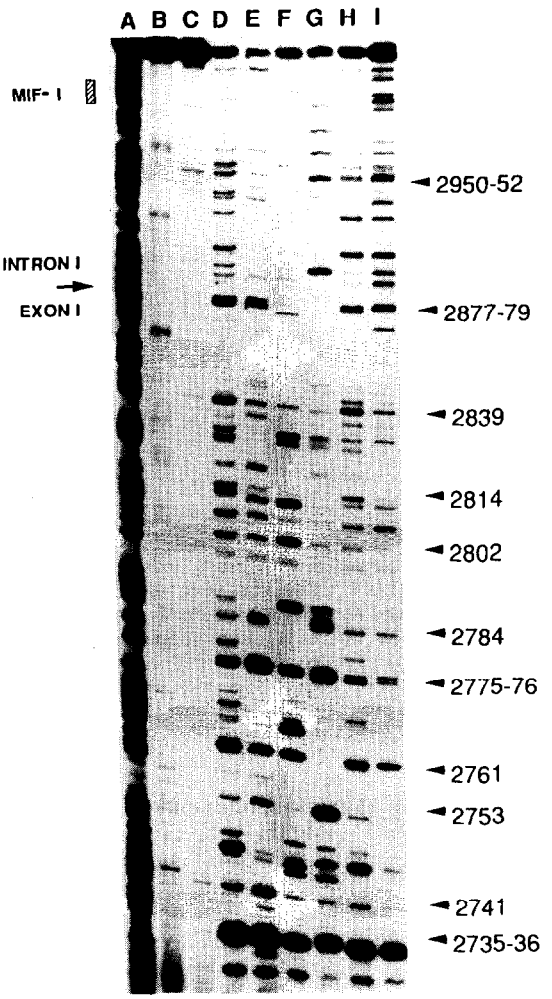


Fig. 2. Top2 cleavage pattern induced by azatoxin derivatives in the human c-*myc* proto-oncogene at the junction between the first intron and the first exon. The 5'-end-labeled sense DNA strand was reacted with drugs and purified top2 for 30 min at 37°. Reactions were stopped by adding SDS and proteinase K, and DNA fragments were separated in a 7% denaturing acrylamide gel. Lane A, purine lane; lane B, DNA control; lane C, top2 without drug; lanes D–I: + 100 μM drug; lane D, azatoxin (compound **1**); lane E, VP-16; lane F, compound **6**; lane G, compound **7**; lane H, compound **3**; and lane I, compound **4**. Numbers to the right of the autoradiogram correspond to the genomic position in GenBank of the nucleotide covalently linked to top2.

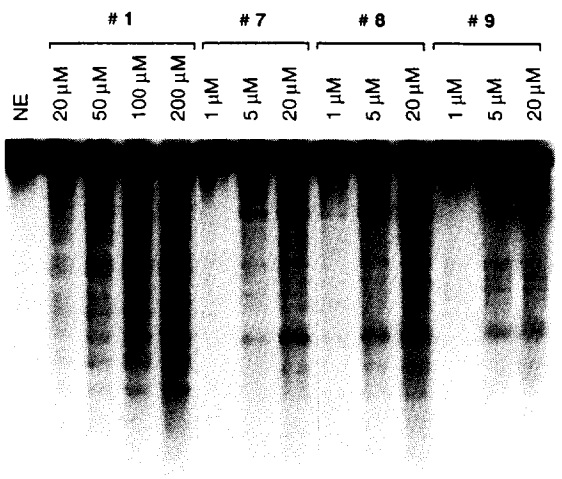


Fig. 3. Top2-mediated DNA double-strand breaks induced by the most active azatoxin derivatives. ^{32}P-End-labeled SV40 DNA was reacted with drugs and HL60 nuclear extract for 30 min at 37°. Reactions were stopped by adding SDS and proteinase K, and DNA fragments were separated in 1% agarose gels. A typical autoradiogram is presented. The potency of each derivative to induce top2-mediated DNA double-strand breaks was evaluated relative to azatoxin; results are reported in Table 1. NE = nuclear extract in the absence of drug.

(addition of an aliphatic substituent) in lane F, or with compound **7** (addition of an aromatic anilino substituent) in lane G. Additional data (not shown) demonstrated that the 11-substituted compounds (**7–9**) were more potent than azatoxin (see below). Therefore, each azatoxin derivative exhibited a specific cleavage pattern that was also distinct from that of the other top2 inhibitors [VP-16 in lane E, mitoxantrone or amsarine (*m*-AMSA), results not shown].

Induction of DNA double-strand breaks by azatoxin derivatives in the presence of HL60 nuclear extract. Most potent azatoxin derivatives to induce top2 cleavages, as judged from the above data, were selected to measure the drug-induced DNA double-strand breaks in SV40 DNA in the presence of HL60 nuclear extract. Top2-mediated double-strand breaks increased with drug concentration (Fig. 3). No inhibition of cleavage was observed at the highest concentration tested (500 μM, not shown), consistent with the absence of DNA intercalation of these derivatives [9, 10] as previously observed with azatoxin [1]. The potency of azatoxin derivatives was evaluated relative to azatoxin, and results are reported in Table 1. The presence of a hydroxyl at position 4′ was shown previously to be necessary for anti-top2 activity [1], and the structure–activity relationship can be extended by modifications at other positions. At position 11, the presence of an anilino group, compounds **7–9**, markedly increased the drug potency to induce DNA cleavage (Fig. 3 and Table 1) and changed the top2-mediated DNA cleavage pattern (Figs. 2 and 3). The nature of the substitution on the anilino group also played a role in drug potency, a cyano group being more effective than a nitro or a fluoro group (Table 1). In contrast, the addition of an aliphatic group at position 11 decreased potency (compound **5**, Table 1). At position 9, addition of a fluoro group (compound **3**) yields a top2 inhibitor. However, this modification altered the slope of the curve of top2 inhibition relative to drug concentration. Thus, azatoxin and **3** were equipotent at 20 μM, but azatoxin was eight times more potent than **3** at 200 μM (Table 1). These results indicate that potency of top2 inhibition by azatoxin derivatives is highly sensitive to structural modifications [2].

Activity of azatoxin derivatives on tubulin polymerization. Azatoxin was shown previously to interact with tubulin using tubulin polymerization assays [2]. The effect of azatoxin derivatives on tubulin polymerization was tested. The polyring system must be planar for tubulin interaction (results not shown). Methylazatoxin (compound **2**) was 2-fold more potent than azatoxin (Table 1). Substitutions at position 11 dramatically reduced tubulin polymerization inhibition. Addition of a fluoro group at position 9 (compound **3**) also decreased drug potency. Thus, substitutions at positions 9 and 11 reduced the ability of the drug to inhibit tubulin polymerization, while the presence of a 4′-methoxy increased the anti-tubulin activity.

Alteration of tubulin proteolysis in the presence of azatoxin derivatives. Colchicine has been shown to induce a structural change in β-tubulin, namely the unfolding of the carboxyl end of an amphipathic helix that ends around residue 391 [6]. The resulting unfolded loop can be cleaved by trypsin or chymotrypsin, but detection of unfolding is easier with chymotrypsin due to the complete lack of cleavage without drug. To determine if azatoxin can induce the same structural changes in tubulin, chymotryptic digestion of tubulin in the presence of azatoxin and analogs was performed, and the results are shown in Fig. 4. The induction of a new cleavage site by colchicine can clearly be seen by comparing lane 2 with the control in lane 1. Podophyllotoxin can also induce this cleavage, as shown in lane 3. Of the azatoxin analogs tested, only azatoxin itself and methylazatoxin induced colchicine-like cleavage. Both induced cleavage more weakly than podophyllotoxin or colchicine, and azatoxin was less potent than methylazatoxin. All other analogs failed to induce cleavage, consistent with their failure to inhibit polymerization. Similar results were obtained with trypsin (data not shown). These results show that azatoxin and methylazatoxin bind to tubulin at the colchicine site to induce an alteration of tubulin structure.

Cytotoxicity of azatoxin derivatives. A selection of azatoxins was tested in the NCI drug screen, based on their mechanisms of action in the biochemical systems described above. They were methylazatoxin (compound **2**), a tubulin inhibitor, compounds **7–9** with an anilino substituent at position 11 that provides selectivity for top2, and 9-fluoroazatoxin (compound **3**) that targets both top2 and tubulin (Table 1). The cytotoxic profiles in the NCI drug screen were compared with that of drugs with a known mechanism of action using the COMPARE software. This approach has been validated recently for several drugs such as benzamide

Table 1. Activity of azatoxin derivatives on topoisomerase II and tubulin

Compound	NSC	Purified systems: Top2-induced DNA cleavage relative to azatoxin*	Purified systems: Tubulin polymerization inhibition IC_{50}† (μM)	NCI cell screen: GI_{50}‡ (μM)	NCI cell screen: COMPARE coefficient with: VBL	NCI cell screen: COMPARE coefficient with: VP-16
(1) Azatoxin	640737	1	3.3 ± 0.4	0.16	0.55	0.30
(2) Methyl-azatoxin	662306	0	2.4 ± 0.5	0.11	0.65	0.04
3	659547	NQ§	11 ± 2	9.5	0.24	0.26
5	NT‖	<0.04¶	>100	NT	NT	NT
7	662304	4/3	>100	1.4	0.24	0.58
8	662305	8/10	>100	0.15	0.47	0.66
9	668380	5/8	>100	0.25	0.50	0.58

* Drug-induced DNA double-strand breaks produced by top2 were quantitated, and results were expressed relative to azatoxin. Values of independent experiments are provided.

† IC_{50}: drug concentration leading to 50% inhibition of tubulin polymerization. Values for compounds **1–3** are means ± SD, N = 3 or more.

‡ GI_{50}: drug concentration leading to 50% growth inhibition.

§ NQ: not quantitatable: the slope of the concentration–responsive curve differed from that of other azatoxins. Compound **3** relative potency to azatoxin was 1 at 20 μM, but only 1/8 (0.125) at 200 μM.

‖ NT: not tested.

¶ Top2-mediated double-strand breaks induced by compound **5** were barely detectable.

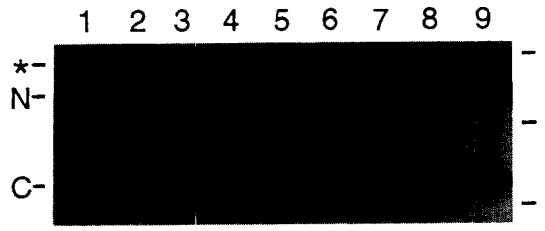


Fig. 4. Chymotryptic digestion of tubulin in the presence of azatoxin and analogs. Structural changes induced in tubulin by azatoxin and analogs were revealed by proteolysis with chymotrypsin. Purified rat brain tubulin (10 μM) was incubated with an excess (100 μM) of the tested compound for 30 minutes at 30°. Chymotrypsin was then added at a weight ratio of 1:50 to tubulin. Digestion on ice and SDS electrophoresis of samples were as described in Ref. 6. The portion of the gel containing digestion products is shown. The positions of the 31-kDa amino terminal and the 19-kDa carboxyl terminal chymotryptic fragments of β-tubulin are indicated on the left of the gel by "N" and "C," respectively. The asterisk indicates the position of the band produced by cleavage of β-tubulin at the position of colchicine-induced unfolding. Lane 1 shows the digestion in the absence of added drug. Lane 2 is colchicine; lane 3 is podophyllotoxin; lane 4 is azatoxin; lane 5 is methylazatoxin; lane 6 is compound **3**; lane 7 is compound **5**; lane 8 is compound **7**; and lane 9 is compound **8**. The unlabeled tics to the right show the positions of molecular weight marker proteins. Top to bottom (mass in kDa): ovalbumin (45), carbonic anhydrase (31.5), and soybean trypsin inhibitor (21.5).

riboside, an inhibitor of the IMP dehydrogenase [11], and DuP-941 and DuP-937, two top2 inhibitors of the anthrapyrazole family [4]. COMPARE analysis showed that azatoxin acts first on tubulin and, then, at higher concentrations on top2 [2]. Figure 5 compares the cytotoxicity of methylazatoxin (compound **2**) and fluoroanilinoazatoxin (compound **7**) with that of vinblastine and VP-16, tubulin and top2 inhibitors, respectively. The concentrations inhibiting 50% of cell growth (GI_{50}) expressed as the logarithm of the concentrations were plotted for each drug. Correlation coefficients of linear regression of reciprocal plots were used to assess the similarities between two drugs. This coefficient, r, is equal to 1 when the cytotoxic profiles are identical. Methylazatoxin, compound **2**, on the lower panel gave a good correlation coefficient with vinblastine ($r = 0.61$), but not with VP-16 ($r = 0.25$). Tubulin active agents such as **2** do not correlate well with alkylating agents, e.g. chlorambucil ($r = 0.15$), carboplatin ($r = 0.31$) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) ($r = 0.22$). Top2 agents such as **7** may sometimes correlate a little with alkylating agents, e.g. chlorambucil ($r = 0.50$), or in other cases hardly at all, e.g. CCNU ($r = 0.13$). Neither tubulin active compounds nor top2 inhibitors are likely to be mistaken for antimetabolites. The glutamine antagonist acivicin correlated weakly with **2**. In fact, the correlation of the tubulin active **2** with acivicin ($r = 0.31$) was about the same as the top2 inhibitor **7** with acivicin ($r = 0.29$). Compound **2** correlated insignificantly with the IMP dehydrogenase inhibitor tiazofurin ($r = 0.33$), and **7** was about the same ($r = 0.20$). Brequinar, a dihydroorotate dehydrogenase inhibitor, correlated better with **2**

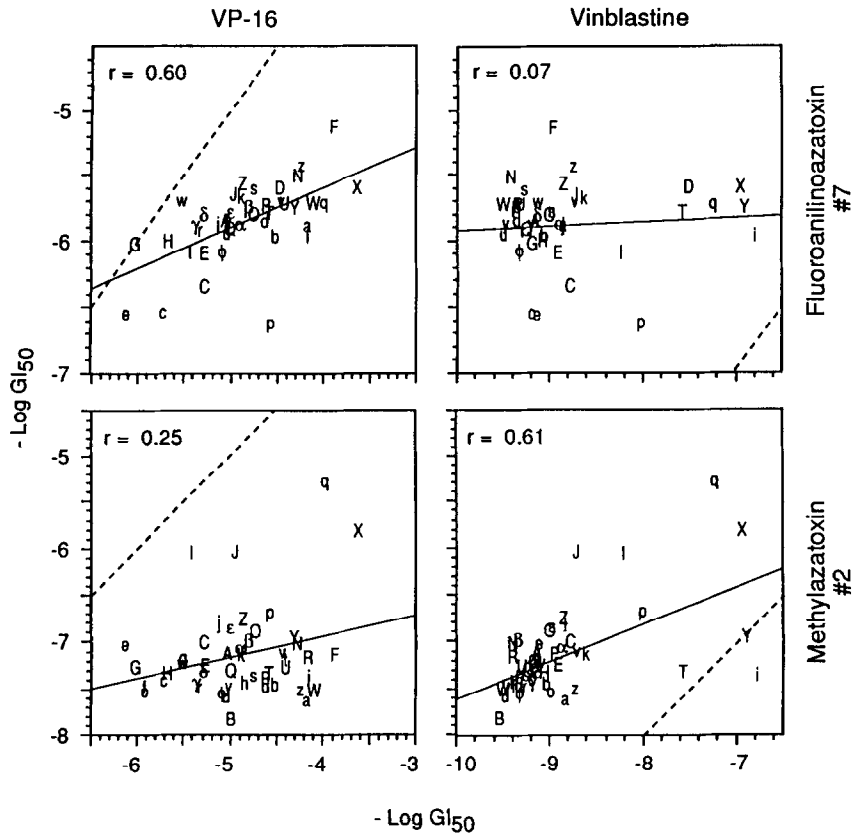


Fig. 5. Reciprocal plot of the 50% growth inhibition (GI_{50}) for a panel of cell lines from the NCI drug screen for compound **2** (bottom) and **7** (top) relative to a classical top2 inhibitor (VP-16), on the left and a classical tubulin inhibitor (vinblastine, on the right). In each panel, the unbroken line corresponds to the linear regression with r, its correlation coefficient; the broken line corresponds to the equipotency line, e.g. assuming equal GI_{50} for both drugs in all cell lines. Identification of each cell line was: *NSCLC*, A: NCI-H23, B: NCI-H522, C: A549/ATCC, D: EKVX, E: NCI-H226, F: NCI-H322M, G: NCI-H460, H: HOP-62, I: HOP-18, J: HOP-92, K: LXFL 529, L: DMS 114, M: DMS 273; *Colon*, N: HT29, O: HCC-2998, P: HCT-116, Q: SW-620, R: COLO 205, S: DLD-1, T: HCT-15, U: KM12, V: KM20L2; *Ovarian*, W: OVCAR-3, X: OVCAR-4, Y: OVCAR-5, Z: OVCAR-8, a: IGROV1, b: SK-OV-3; *Leukemia*, c: CCRF-CEM, d: K-562, e: MOLT-4, f: HL60(TB), g: RPMI-8226, h: SR; *Renal*, i: UO-31, j: SN12C, k: A498, l: CAKI-1, m: RXF-393, n: RXF-631, o: 786-0, p: ACHN, q: TK-10; *Melanoma*, r: LOX IMVI, s: MALME-3M, t: SK-MEL-2, u: SK-MEL-5, v: SK-MEL-28, w: M14, x: M19-MEL, y: UACC-62, z: UACC-257; *CNS*, **a**: SNB-19, **b**: SNB-75, **c**: SNB-78, **d**: U251, **e**: SF-268, **f**: SF-295, **g**: SF-539, η: XF 498.

($r = 0.46$) than with **7** ($r = 0.27$), but of course neither correlation was high enough to infer a mechanism. Together, these results suggest that methylazatoxin (**2**) targets tubulin in cells, in agreement with the results obtained in the biochemical systems. On the other hand, fluoro-anilinoazatoxin (**7**, upper panel) gave a good correlation coefficient with VP-16 ($r = 0.60$), but not with vinblastine ($r = 0.07$), suggesting a cytotoxicity involving top2 inhibition. 9-Fluoroazatoxin, compound **3**, showed low correlation coefficients with both vinblastine and VP-16 (Table 1). Therefore, mechanism of action can be deduced from COMPARE analysis with compound **3**, a combination of anti-top2 and anti-tubulin activities being likely.

The potency of azatoxins was evaluated using the mean concentration producing 50% growth inhibition (Table 1). Among the drugs acting on tubulin, methylazatoxin (compound **2**) was slightly more potent ($GI_{50} = 0.11\ \mu M$) than azatoxin ($GI_{50} = 0.16\ \mu M$), but still almost two orders of magnitude less potent than vinblastine ($GI_{50} = 0.0014\ \mu M$). Compound **3** showed a low potency ($GI_{50/13} = 9.5\ \mu M$) that was quite uniform in all the cell lines of the NCI drug screen (result not shown). Among the drugs acting on top2, the tested anilinoazatoxins were more potent than VP-16 ($GI_{50} = 14.2\ \mu M$) by one order of magnitude for compound **7** ($GI_{50} = 1.4\ \mu M$), and by two orders of magnitude for compound **8** ($GI_{50} = 0.15\ \mu M$). Compared with other top2 inhibitors, compound **8** was three times more potent than *m*-AMSA ($GI_{50} = 0.46\ \mu M$), almost as potent as doxorubicin ($GI_{50} = 0.11\ \mu M$) and the

anthrapyrazole DuP-941 (GI_{50} = 0.132 μM), but less potent that mitoxantrone (GI_{50} = 0.05 μM). Thus, compounds **2** and **7–9** are potent cytotoxic agents that display selective inhibition of either top2 or tubulin.

DISCUSSION

The present study was undertaken to obtain azatoxin derivatives with greater potency and selectivity. We had shown previously that azatoxin, a non-intercalator cytotoxic agent, is highly cytotoxic and acts on two targets, tubulin at lower doses and tubulin plus top2 at higher doses. At intermediate concentrations, cytotoxicity does not increase and can even decrease with the drug concentration, suggesting an antagonism between the anti-tubulin and anti-top2 activities [2]. Antagonism of these two activities was probably found for 9-fluoroazatoxin (compound **3**), since compared with azatoxin, a potency reduction against tubulin and an at least equipotency on top2 at low doses were found (Table 1), possibly bringing together the two activities in the same range of concentrations. This antagonism might explain the low potency of the drug. Also, 9-fluoroazatoxin was found to lack cytotoxic selectivity and, therefore, was not selected for further preclinical development. Thus, the presence of a unique target for azatoxin derivatives might be important for cytotoxic selectivity and anticancer activity.

Azatoxin determinants for action on top2 can be summarized as follows. We had shown previously that the polyring system must be planar, and that a hydroxyl group at position 4′ is required for anti-top2 activity [1], as in the podophyllotoxin family [12, 13]. Similarities between the azatoxin and the epipodophyllotoxin families are of special interest since the overall spatial structures of azatoxin and demethylpodophyllotoxin are comparable [1]. Substitutions at position 9 with a hydroxyl group seems a hindrance for interactions with top2 as revealed by the low potency of compound **4**. Similarly, the presence of a 9-fluoro group changed the interactions with top2, as the slope of the concentration–response curve of top2 cleavage activity was decreased. The 9-substituent might also be in close proximity with the DNA because the sequence selectivity of top2 cleavage changed depending upon the substituent. Substitutions at position 11 were also critical for top2 inhibition, and results were in agreement with substitutions in the podophyllotoxin family. Addition of a 2-hydroxyl-acivicin ethoxy group (compound **5**) reduced the potency, whereas addition of anilino substituents increased it, as in the podophyllotoxin family [14, 19]. Cyanoanilinoazatoxin (compound **8**) was especially potent, being one order of magnitude more potent than azatoxin against purified top2 and two orders of magnitude more cytotoxic. The sequence selectivity of top2 cleavages also dramatically depended upon the nature of the 11-substituent. Sequence selectivity differs for each class on top2 inhibitor examined thus far [7], except for bisantrene and amsacrine [20]. Within a drug family, minor alterations of the structure usually have minimal effects on the cleavage pattern [1, 4, 21]. The alteration of cleavage pattern observed with azatoxin and anilino-azatoxins was of unusual magnitude. Sequence selectivity of drug-induced top2 cleavage has been interpreted as specific contacts between drug and DNA bases [7]. The most recent arguments in favor of specific drug–DNA interactions come from the same base preference observed for top1 and top2 cleavages in the presence of saintopin, a dual inhibitor of topoisomerases I and II [22], and from specific interactions of the non-DNA intercalators camptothecin and VP-16 with DNA bases [23, 24] that are preferentially found at the cleavage sites [7]. Thus, we propose that bulky groups at position 11 can be accommodated inside the top2 DNA complexes and probably interact with the DNA.

Azatoxin determinants for action on tubulin can be summarized as follows. Azatoxin and methylazatoxin bind to tubulin at the colchicine site. As for top2 activity, the polyring system must be planar for inhibition of tubulin polymerization. In contrast to the podophyllotoxin family, methylation of the hydroxyl group on the pendant ring at position 4′ increased azatoxin interaction with tubulin [13, 25]. However, azatoxins and methylazatoxin were less potent than podophyllotoxin in their interactions with tubulin. Substitutions at positions 9 and 11 reduced the interaction with tubulin, regardless of the nature of the 11-substituent. These results are in agreement with those obtained in the podophyllotoxin family [19].

Azatoxin structure–activity relationship had allowed the specific targeting of tubulin or top2. Important similarities were found between the azatoxin and podophyllotoxin families. On the basis of drug cytotoxic profiles, several molecules have been selected for further preclinical development by the NCI. They are methylazatoxin (compound **2**) as a tubulin inhibitor, and the fluoroanilinoazatoxin (compound **7**) as a top2 inhibitor.

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REFERENCES

1. Leteurtre F, Madalengoitia J, Orr A, Guzi TJ, Lehnert E, Macdonald T and Pommier Y, Rational design and molecular effects of a new topoisomerase II inhibitor, azatoxin. *Cancer Res* **52**: 4478–4483, 1992.
2. Solary E, Leteurtre F, Paull K, Scudiero D, Hamel E and Pommier Y, Dual inhibition of topoisomerase II and tubulin polymerization by azatoxin, a novel cytotoxic agent. *Biochem Pharmacol* **45**: 2449–2456, 1993.
3. Minford J, Pommier Y, Filipski J, Kohn KW, Kerrigan D, Mattern M, Michaels S, Schwartz R and Zwelling LA, Isolation of intercalator-dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. *Biochemistry* **25**: 9–16, 1986.
4. Leteurtre F, Kohlhagen G, Paull KD and Pommier Y, Topoisomerase II inhibition and cytotoxicity of the anthrapyrazoles DuP 937 and DuP 941 (Losoxantrone) in the National Cancer Institute Preclinical Antitumor

Drug Discovery Screen. *J Natl Cancer Inst* **86**: 1239–1244, 1994.

5. Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J and Boyd M, Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human cell lines. *J Natl Cancer Inst* **83**: 757–766, 1991.
6. Sackett DL and Varma JK, Molecular mechanism of colchicine action: Induced local unfolding of β-tubulin. *Biochemistry* **32**: 13560–13565, 1993.
7. Pommier Y, Kohn KW, Capranico G and Jaxel C, Base sequence selectivity of topoisomerase inhibitors suggests a common model for drug action. In: *Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy* (Eds. Andoh T, Ikeda H and Oguro M), pp. 215–227. CRC Press, Boca Raton, FL, 1993.
8. Zajac-Kaye M, Yu B and Ben-Baruch N, Downstream regulatory elements in the *c-myc* gene. *Curr Top Microbiol Immunol* **166**: 279–284, 1990.
9. Liu LF, DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* **58**: 351–375, 1989.
10. Pommier Y and Kohn KW, Topoisomerase II inhibition by antitumor intercalators and demethylepipodophyllotoxins. In: *Developments in Cancer Chemotherapy* (Ed. Galazer RI), Vol. 2, pp. 175–196. CRC Press, Boca Raton, FL, 1989.
11. Jayaram H, Gharehbaghi K, Rieser J, Krohn K and Paull K, Cytotoxicity of a new IMP dehydrogenase inhibitor, benzamide riboside, to human myelogenous leukemia K562 cells. *Biochem Biophys Res Commun* **186**: 1600–1606, 1992.
12. Sinha B, Politi P, Eliot H, Kerrigan D and Pommier Y, Structure–activity relations, cytotoxicity and toposomerase II dependent cleavage induced by pendulum ring analogues of etoposide. *Eur J Cancer* **26**: 590–593, 1990.
13. Long B, Musial S and Brattain M, Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP16–213 and VM26: A quantitative structure–activity relationship. *Biochemistry* **23**: 1183–1188, 1984.
14. Liu S, Hwang B, Haruma M, Imakura Y, Lee K and Cheng Y, Podophyllotoxin analogs: Effects on topoisomerase II, tubulin polymerization, human tumor KB cells, and their VP-16 resistant variants. *Mol Pharmacol* **36**: 78–82, 1989.
15. Zhou X, Wang Z, Chang J, Chen H, Cheng Y and Lee K, Antitumors agents. 120. New 4-substituted benzylamine and benzyl ether derivatives of 4′-*O*-demethylepipodophyllotoxins as potent inhibitors of human topoisomerase II. *J Med Chem* **34**: 3346–3350, 1991.
16. Wang Z, Kuo Y, Schnur D, Bowen P, Liu S, Han F, Chang J, Cheng Y and Lee K, Antitumor agents. 113. New 4β-arylamino derivatives of 4′-*O*-demethylepipodophyllotoxin and related compounds as potent inhibitors of human DNA topoisomerase II. *J Med Chem* **33**: 2660–2666, 1990.
17. Wang Z, Shen Y, Chen H, Chang J, Guo X, Cheng Y and Lee K, Antitumor agents. 126. Novel 4 beta-substituted anilino derivatives of 3′,4′-*O*,*O*-demethylepipodophyllotoxin as potent inhibitors of human DNA topoisomerase II. *Pharm Res* **10**: 343–350, 1993.
18. Lee K, Beers S, Mori M, Wang Z, Kuo Y, Li L, Liu S, Chang J, Han F and Cheng Y, Antitumor agents. 111. New 4-hydroxylated and 4-halogenated anilino derivatives of 4′-demethylepipodophyllotoxin as potent inhibitors of human DNA topoisomerase II. *J Med Chem* **33**: 1364–1368, 1990.
19. Chang J-Y, Han F-S, Liu S, Wang Z-Q, Lee K-H and Cheng Y, Effect of 4β-arylamino derivatives of 4′-*O*-demethylepipodophyllotoxin on human DNA topoisomerase II, tubulin polymerization, KB cells, and their resistant variants. *Cancer Res* **51**: 1755–1759, 1991.
20. Capranico G, Palumbo M, Tinelli S, Mabilia M, Pozzan. A and Zunino F, Conformational drug determinants of the sequence specificity of drug-stimulated topoisomerase II DNA cleavage. *J Mol Biol* **235**: 1218–1230, 1994.
21. Capranico G, Zunino F, Kohn KW and Pommier Y, Sequence-selective topoisomerase II inhibition by anthracycline derivatives in SV40 DNA: Relationship with DNA affinity and cytotoxicity. *Biochemistry* **29**: 562–569, 1990.
22. Leteurtre F, Fujimori A, Tanizawa A, Chhabra A, Mazumder A, Kohlhagen G, Nakano H and Pommier Y, Saintopin, a dual inhibitor of DNA topoisomerases I and II, as a probe for drug–enzyme interactions. *J Biol Chem* **269**: 28702–28707, 1994.
23. Leteurtre F, Fesen M, Kohlhagen G, Kohn KW and Pommier Y, Specific interaction of camptothecin, a topoisomerase I inhibitor, with guanine residues of DNA detected by photoactivation at 365 nm. *Biochemistry* **32**: 8955–8962, 1993.
24. Yamashita A, Tawa R, Imakura Y, Lee K and Sakurai H, Site specific DNA cleavage by Cu(II) complexes of podophyllotoxin derivatives. *Biochem Pharmacol* **47**: 1920–1925, 1994.
25. Loike J, Brewer C, Sternlicht H, Gensler W and Horwitz S, Structure–activity study of the inhibition of microtubule assembly *in vitro* by podophyllotoxin and its congeners. *Cancer Res* **38**: 2688–2693, 1978.