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Preclinical paper

Makaluvamines vary in ability to induce dose-dependent DNA cleavage via topoisomerase II interaction

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The makaluvamines are marine natural products that were originally isolated because of their cytotoxicity in a cell-based mechanism screen. They have significant anti-cancer activity in animal models. There is, however, disagreement in the literature as to whether these compounds target topoisomerase II via a clinically relevant mechanism. This work shows that the makaluvamines can induce dose-dependent DNA cleavage via topoisomerase II. For most of the makaluvamines the levels of cleavage are significantly below those achieved by equimolar concentrations of etoposide. To some extent these results might explain the discrepancies present in the literature. [© 1999 Lippincott Williams & Wilkins.]

Key words: Cleavable complex formation, DNA cleavage, makaluvamine, topoisomerase II.

Introduction

The makaluvamines were discovered in 1993 as cytotoxic metabolites of the sponges *Zyzzya fuliginosa* (Figure 1).[1,2] They constituted a new class of marine pyrroloiminoquinone. Since then additional members of the class have been found.[3,4] These compounds possess promising anti-tumor activity in mouse xenograft models (Figure 2) and so the mechanism by which they exert their cytotoxicity is of interest. The original isolation/purification of these compounds was guided by their enhanced cytotoxicity to a DNA repair-deficient cell line, xrs-6, when compared to the repair-competent parental line.[5] This mutant CHO line is characterized by a mutated Ku86 protein and is deficient in DNA double-strand break repair.[6,7] The xrs-6 line has been shown to be especially sensitive to agents that either cleave DNA directly or induce topoisomerase II to cleave the DNA via 'cleavable complexes'.[8] Makaluvamines were also shown to inhibit the catalytic activity of topoisomerase II and it was therefore hypothesized early that their cytotoxicity might arise via topoisomerase II poisoning ('cleavable complex' formation).[9] While initial reports supported this hypothesis,[5] subsequent reports using other assays have not confirmed the activity.[2,3] The objective of this study was to assess the relative topoisomerase II cleavable complex formation of some makaluvamines and to compare them to clinically used topoisomerase II poisons.

Materials and methods

Reagents

Isolation and chemical characterization of the makaluvamines has been described.[1,4] Compound purity was assessed by chromatographic and NMR analysis. Drug standards were purchased from Sigma (St Louis, MO) or were supplied to us by Dr RA Kramer (Wyeth-Ayerst Research). pUC 19 DNA, Sequenase 2.0 and all sequencing supplies were purchased from US Biochemical (Cleveland, OH). Radioactive thymidine and deoxyadenosine triphosphate were purchased from New England Nuclear (Beverly, MA). Sequencing primers were synthesized by the University of Utah, Utah Huntsman Cancer Center DNA, Peptide Synthesizing and Sequencing core facility. All other chemicals

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	R^1	R^2	R^3	R^4
A	CH_3	H	H	H
C	H	CH_3	H	H
D	H	H	i	H
E	CH_3	H	ii	H
H	CH_3	CH_3	H	H
I	H	H	H	H
N	H	H	H	Br
V	H	CH_3	iii	H

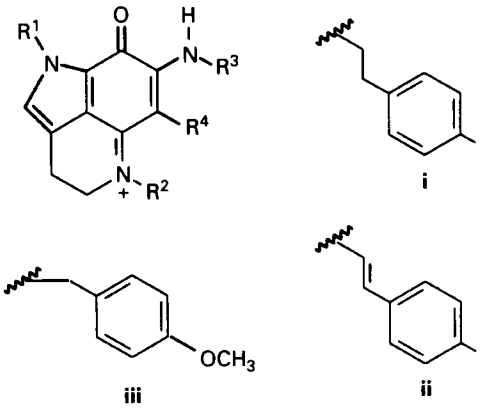

Figure 1. Structures of makaluvamines.

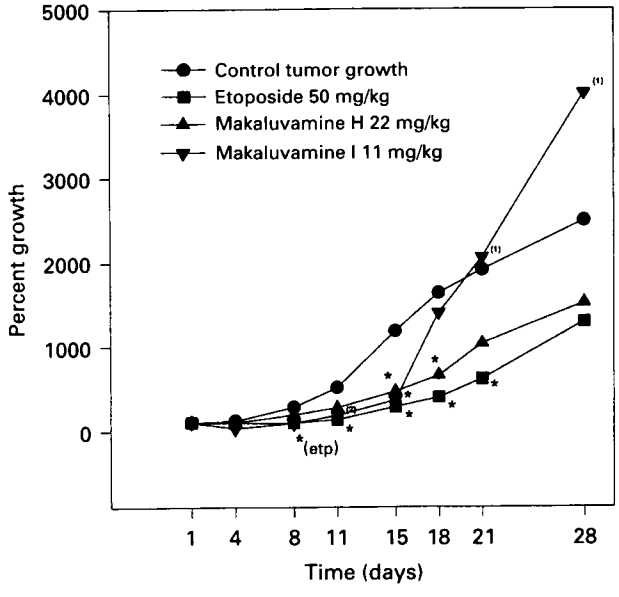

Figure 2. *In vivo* antitumor activity of makaluvamines H and I and the positive control etoposide. Athymic, nude mice were injected on the shoulder with KB nasopharyngeal-epidermoid carcinoma cells. Once tumors were established and tumor volumes measured to be at least 50 mm^3, drug treatment was initiated. Animals were treated with drug i.p. on days 1, 4 and 8 (after staging), n=5 except for makaluvamine I indicated in parentheses. Exceptional activity was observed for makaluvamine H at a dose of 22 mg/kg with a maximal difference in T/C tumor volume of 38% on both days 15 and 18. The lower dose of 11 mg/kg also showed significant effects. Makaluvamine I was much more toxic *in vivo*. However, a significant anti-tumor effect was seen in the two survivors receiving only one injection (the next lower dose of makaluvamine I, 5 mg/kg, showed no effect). The positive control etoposide data is shown at an MTD dose of 50 mg/kg.

were purchased from Sigma (Pittsburgh, PA). Radiolabeled (4.4×10^3 c.p.m./μg) ^{3}H replicative form (r.f.) of M13 mp 19 was isolated by the alkaline lysis method as described.[10]

In vivo anti-tumor activity

Athymic, nude mice were fed *ad libitum* and housed in a 12 h light:12 h dark, temperature controlled, sterile environment. All mice were marked by ear punches, weighed and injected on the shoulder with $1\text{-}3 \times 10^6$ KB nasopharyngeal-epidermoid carcinoma cells. Tumor size was measured with calipers and calculated using the equation tumor volume=width^2 × length. Once tumor volume was measured to be at least 50 mm^3, drug treatment was initiated. Animals were treated with drug on days 1, 4 and 8 i.p., after staging. Each treatment group consisted of five mice. Tumor size and body weight were measured twice weekly throughout the duration of the experiment. Mice with tumors greater than 15% of body weight were euthanized. All remaining mice were euthanized on day 28 of the study.

Yeast strains and plasmids

Yeast strain BCY 123, a protease-deficient, *ura3* strain, was kindly supplied by Dr J Lindsley (University of Utah). Plasmid YEpWOb6, which contains the human topoisomerase II gene under control of a galactose promoter, was kindly supplied by Dr J Wang (Harvard University). Plasmid YEpWOb6 was transfected into

yeast strain BCY 123 by the lithium acetate method.[11] Transformants were selected for by plating on minimal plates containing glucose (2%) and lacking uracil.

Induction of human topoisomerase II in yeast

Yeast strain BCY 123, containing plasmid YEpWOb6, was grown overnight at 30°C in 1 ml of rich media containing 20 mg/ml of glucose as a carbon source. The overnight culture was then added to 1000 ml of minimal media lacking uracil but containing 3% glycerol, 2% lactic acid (pH 5.5–6.0), 1.5 mg/ml yeast nitrogen base without amino acids and ammonium sulfate (Difco, Detroit, MI), 5 ml ammonium sulfate and amino acids. When the optical density of the culture at 600 nm was between 0.6 and 0.8, galactose (100 ml of 20 mg/ml) was added to induce expression of the human topoisomerase II gene. After 24 h the yeast were harvested by centrifugation at 2000 *g* for 20 min, and resuspended in 30 ml of 50 mM Tris-HCl (pH 7.5), 10% glycerol, 0.3 M NaCl, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM dithiothreitol (DTT). The cells were lysed by the addition of 30 g of glass beads (425–600 μM, Sigma) and vortex mixing for 30 s intervals alternating with cooling in an ice water bath. Adequate cell breakage was determined under light microscopy. Cell debris and glass beads were pelleted by centrifugation at 5000 *g* for 10 min. The supernatant, referred to as the crude extract, contained abundant topoisomerase II enzymatic activity (determined by assaying for decatenation of kinetoplast DNA).[10] When an aliquot of the crude extract was subjected to denaturing SDS gel electrophoresis, a major protein species at 170 kDa was observed which was confirmed to be human topoisomerase II by Western blot analysis.[12]

Isolation of human topoisomerase II

All procedures were performed at 4°C unless otherwise noted. To the yeast crude extract an equal volume of 50 mM Tris-HCl (pH 7.5), 2 M NaCl, 1 mM PMSF and 2 mM DTT was added, and the mixture stirred for 15 min. Following this, 1/3 volume of 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 18% (w/v) polyethylene glycol, 1 mM PMSF and 2 mM DTT was added with additional stirring for 15 min. Precipitated DNA and cell debris were removed by centrifugation at 2000 *g* for 30 min and the supernatant was removed. This clarified supernatant was fractionated over a 10 ml hydroxylapatite column and the topoisomerase II eluted with a 0.2–0.7 M KCl gradient as described previously.[10]

Active fractions from hydroxylapatite chromatography were pooled and diluted 1:5 with 10% glycerol, 0.1 mM EDTA, 1 mM PMSF and 2 mM DTT, and applied to a FPLC mono Q column equilibrated with 20 mM Tris-HCl (pH 7.5), 10% glycerol, 100 mM NaCl, 0.1 mM EDTA, 1 mM PMSF and 2 mM DTT. After washing with the equilibration buffer, topoisomerase II was eluted with a 20 ml NaCl gradient (0.1–1.0 M) in 20 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1 M EDTA, 1 mM PMSF and 2 mM DTT. Fraction size was 0.5 ml. The topoisomerase elutes about 0.4 M NaCl. Fractions containing the 170 kDa species of topoisomerase were identified by denaturing PAGE analysis. The diluted enzyme was then applied to a FPLC mono S column equilibrated with 50 mM HEPES (pH 7.5), 10% glycerol, 100 mM NaCl, 0.1 mM EDTA, 1 mM PMSF and 2 mM DTT. After washing with the equilibration buffer, topoisomerase IIα was eluted from the mono S column with a 10 ml NaCl gradient (0.1–1.0 M) in 50 mM HEPES (pH 7.5), 10% glycerol, 100 mM NaCl, 0.1 mM EDTA, 1 mM PMSF and 2 mM DTT. Fraction size was 0.25 ml. Topoisomerase II elutes from the mono S column at about 0.6 M NaCl. Fractions containing the 170 kDa species of topoisomerase were identified by denaturing PAGE analysis.[13]

Radiolabeling DNA for cleavage reactions

DNA (pUC 19) was radiolabeled for topoisomerase cleavage using Sequenase 2.0 obtained from US Biochemical and the following approach.[14] This strategy was adapted to enable quantitative comparison between topoisomerase-DNA cleavage reactions containing varying concentrations of drug. To radiolabel enough DNA for 10 subsequent cleavage reactions, 10 μl pUC 19 DNA (1.5 μg) was aliquoted into a 0.5 ml microfuge tube and mixed with 10 μl H_2O and 5 μl 1 M NaOH. To this, 2.5 μl of primer (5 pmol) was added with 10 min incubation at 37°C, the mixture was then placed on ice. Then 1 M HCl (5 μl) was added along with 5 μl of labeling reaction buffer (400 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$ and 250 mM NaCl) and the mixture is incubated again at 37°C for 10 min, and again placed on ice. DTT (2.5 μl of a 0.1 M stock) was added with 5 μl of labeling mix (1.5 μM dCTP, dGTP and dTTP) and 1.25 μl [^{32}P]dATP (10 mCi/ml) and 2.5 μl diluted Sequenase 2.0 (1 μl of Sequenase, 13 U, plus 1 μl of pyrophosphatase, 5 U, and 6 μl of glycerol enzyme dilution buffer, 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA and 50%

glycerol). This mixture was incubated for 5 min at room temperature. The reaction was chased with 25 μl chase mix (1.7 mM of each deoxynucleotide triphosphate in H_2O) and 7.5 μl of additional diluted Sequenase 2.0 for 6 min at 37°C.

The reaction was stopped by the addition of 40 μl each of phenol and chloroform:isoamyl alcohol (24:1) with mixing. The reaction was then centrifuged for 15 min at 15000 r.p.m. Then 65 μl of the aqueous phase (80% of the volume) was transferred to a second microfuge tube to which 130 μl of ice-cold ethanol and 2.5 μl of 5 M NaCl and 1 μl 1 M $MgCl_2$ was added and mixed. Radiolabeled DNA was precipitated by freezing on solid CO_2 for 20 min and centrifugation at 40°C, 15000 r.p.m. for 20 min. Ethanol was aspirated, 130 μl of ice-cold 70% ethanol was added with mixing, freezing on solid CO_2 for 5 min and centrifugation at 40°C, 15000 r.p.m. for 5 min. Ethanol was again aspirated and the labeled DNA was allowed to dry in air.

Topoisomerase II cleavage of radiolabeled DNA

Radioactive DNA was dissolved in 115 μl TE (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). Each cleavage reaction (sufficient DNA for three to four lanes upon electrophoresis) utilizes 11.5 μl of the radiolabeled DNA solution. To each 11.5 μl, 1.5 μl of 10 × topoisomerase I reaction buffer was added (1 × buffer contains 50 mM Tris-HCl, pH 8.0, 20 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 0.5 mM EDTA and 30 μg BSA/ml) followed by 1 μl of DMSO or drug dissolved in DMSO and 1 μl pure topoisomerase II (50 ng protein) with mixing. The reaction was incubated for 30 min at 37°C, and stopped with 0.8 μl 10% SDS and 1 μl of proteinase K (4 mg/ml), and further incubated for 1 h at 37°C. Loading solution (5 μl) was added (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and the samples were stored at −20°C until electrophoresis. Topoisomerase II-drug stabilized complexes were attached to the DNA via 5′ hydroxy links. Thus the radiolabeled DNA fragments generated by this procedure terminate at the nucleotide indicated on the sequencing gel.

DNA sequencing

DNA sequencing and PAGE were performed as described in the Sequenase 'Quick-Denature' plasmid sequencing kit using either the commercially supplied or custom-synthesized primer.

Quantitation of cleavage complex formation

DNA cleavage assays were performed in 20 μl volumes containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.2 mg/ml BSA, 2 mM DTT, 500 ng of radiolabeled and supercoiled r.f. M13 mp 19 DNA and 20–40 ng purified topoisomerase. After incubation at 30°C for 30 min, the reaction was stopped by the addition of 2 μl of 1.5 mg/ml proteinase K in 0.5% SDS and incubated at 37°C for 60 min. The DNAs were fractionated by electrophoresis in 0.8% agarose to separate nicked, cut and supercoiled DNA. After staining with ethidium bromide the DNA was visualized under UV light and sliced out of the gel. Gel slices were placed in scintillation vials with 1 ml water, melted in a microwave and mixed with 10 ml Opti-Fluor (Packer, Meriden, CT) while molten and radioactivity determined by standard liquid scintillation counting. The fraction of the total radioactivity found in the linear DNA band from the reaction incubated with topoisomerase and 100 μM etoposide was set to 100%. Cleavage induced by other test compounds was compared to this.

Results

In vivo activity

Upon discovery of the improved *in vitro* topoisomerase II activity of the makaluvamines H and I (over A and C for instance, see Table 1), they were tested *in vivo* in a flank epidermoid cancer line KB xenograft model and compared to etoposide. Exceptional activity was observed for H at a dose of 22 mg/kg (the MTD was not reached in this experiment). A maximal difference in T/C tumor volume was ob-

Table 1. Topoisomerase II-mediated cleavage of plasmid DNA by makaluvamines as a percentage of etoposide cleavage

Drug (91 mM)	Cleavage (%)
Etoposide	100
Makaluvamine A	17
Makaluvamine C	16
Makaluvamine D	5
Makaluvamine E	22
Makaluvamine H	33
Makaluvamine I	61
Makaluvamine N	26
Makaluvamine V	2

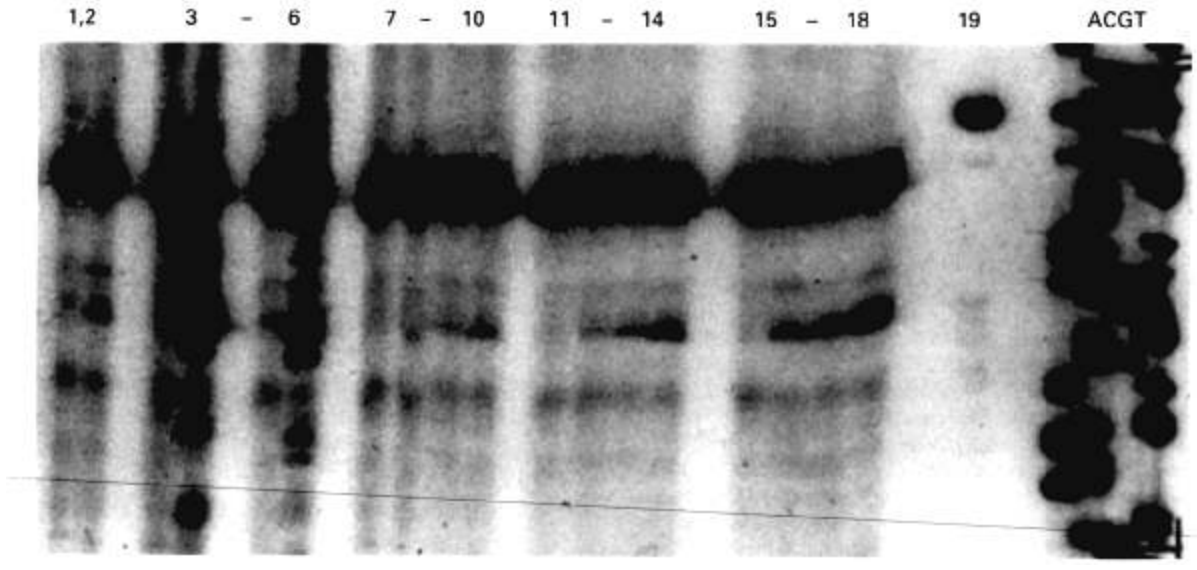

Figure 3. DNA cleavage assayed by PAGE. Lane 1, substrate DNA only. Lane 2, a reaction of substrate DNA and topoisomerase II. Lanes 3 and 4, reactions of DNA substrate and topoisomerase II without (lane 3) and with (lane 4) 6.7 μM mitoxantrone. Lanes 5 and 6, reactions of DNA substrate and topoisomerase II without (lane 5) and with (lane 6) 133 μM etoposide. Lanes 7, 11 and 15, reactions of substrate DNA and 466 μM of makaluvamines C, D and E, respectively. Lanes 8–10, 12–14 and 16–18, reactions of DNA substrate, topoisomerase II and makaluvamine C (lanes 8–10), makaluvamine D (lanes 12–14) or makaluvamine E (lanes 16–18). Each makaluvamine was tested at 33 μM (lanes 8, 12 and 16), 133 μM (lanes 9, 13 and 17) and 466 μM (lanes 10, 14 and 18). Lane 19, substrate DNA digested with the restriction enzyme *Pvu*II. Lanes A, C, G and T denote dideoxy sequencing of the DNA substrate. DNA cleavage is indicated by bands in the lanes containing high drug concentrations. The dark band just below the *Pvu*II cut site, which is present in all cleavage reactions, is a labeling artifact.

served to be 38% on both days 15 and 18. The lower dose of 11 mg/kg also showed significant effects. Makaluvamine I was much more toxic *in vivo* and only two of the five mice survived one injection of 11 mg/kg. Still, following that one injection a significant anti-tumor effect was seen in the survivors (T/C 34% at day 15, the next lower dose of makaluvamine I, 5 mg/kg, showed no effect). The positive control data are shown for an MTD dose of etoposide of 50 mg/kg.

Quantification of DNA cleavage

The makaluvamines examined in this study are shown in Figure 1. Two assays were used to quantify topoisomerase II cleavage complex formation by the makaluvamines. The first used DNA sequencing technology to measure the ability of the compounds to increase topoisomerase II site-specific cleavage in an end-labeled fragment of plasmid DNA. Dose-related cleavage for makaluvamines 1-3 could be shown in pUC 19 DNA at nucleotide A329 (Figure 3). This was a cleavage site common to the two positive control drugs, etoposide and mitoxantrone. While topoisomerase II cleavable complex formation could be demonstrated for the makaluvamines using this assay, the relative degree of this formation was clearly less than was seen for etoposide or mitoxantrone. These positive control compounds showed seven and 10 prominent cleavage sites in this stretch of DNA. The makaluvamines, on the other hand, showed only one or two relatively weak cleavage sites (Figure 3).

A second assay was developed to quantify the drug-induced topoisomerase II cleavage in a whole plasmid. This assay gives a better relative measure of topoisomerase II cleavable complex formation because cleavage activity is measured for a much longer (approximately 7.25 kbp) stretch of DNA (versus the 200 bp or so visualized by the DNA sequencing procedure). Figure 4 shows an agarose gel containing drug- and topoisomerase II-cleaved DNA. The individual bands of DNA were excised from the gel and counted for radioactivity in order to calculate their fractional contribution to the total DNA in the lane. Table 1 presents the makaluvamine-induced plasmid cleavage as a percentage of the cleavage induced by an equimolar concentration of etoposide (100 μM). In the assay the percent of DNA cleavage varied amongst the makaluvamines from a high of 54% for makaluvamine I

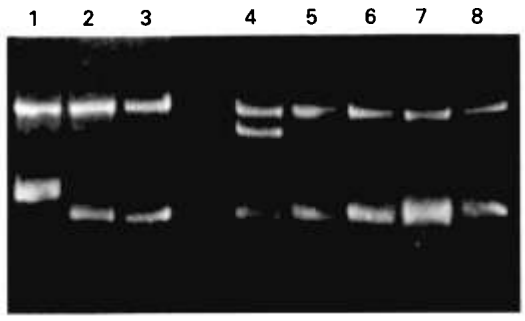

Figure 4. DNA cleavage assayed by agarose gel electrophoresis. Electrophoresis was carried out in the presence of ethidium bromide. Lane 1, substrate DNA only. Lanes 2 and 3, reactions of substrate DNA and topoisomerase II without and with 1% DMSO, respectively. Lanes 4–8 reactions of DNA substrate and topoisomerase II with 91 μM etoposide (lane 4), makaluvamine A (lane 5), makaluvamine C (lane 6), makaluvamine D (lane 7) or makaluvamine E (lane 8) in 1% DMSO. DNA can be seen to migrate from the top to bottom of the gel in four bands. The top band is nicked circular DNA. The next band down, most prominent in the etoposide reaction (lane 4), is linear DNA. The band second to the bottom is negatively supercoiled DNA substrate (present in lane 1). The fastest migrating band (at the bottom of lanes 2–8) is relaxed but intact circular DNA that was supercoiled by the ethidium bromide in the electrophoresis buffer.

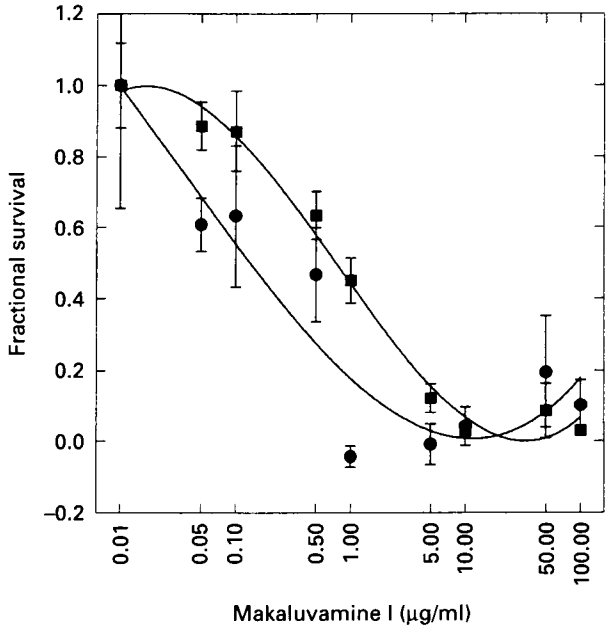

Figure 5. Differential cytotoxicity of makaluvamine I. The IC_{50} of makaluvmine I was approximately 0.4 μM in the DNA double-strand break repair-deficient xrs-6 cells (●), but only approximately 2 μM in the DNA repair-competent AA8 cells (■).

to essentially undetectable levels for makaluvamine V. In general, agreement between the two assays was good. For instance, makaluvamine D gave a poor signal in the sequencing gel and was weak in the plasmid cleavage assay, while makaluvamines E, I, etc., gave readily detectable signals. An exception was makaluvamine C, which gave readily detectable cleavage in the whole plasmid assay but a relatively poor signal in the sequencing gel.

Discussion

There is some disagreement in the literature as to whether makaluvamines target topoisomerase II.[1-5] The results presented here show unequivocally that they have the ability to promote topoisomerase II DNA cleavage *in vitro*. It is therefore possible that this activity contributes to the cytotoxicity of some of these compounds. The observation that DNA double-strand break repair-deficient cells are supersensitive to the makaluvamines (Figure 5) is also consistent with the hypothesized topoisomerase II cleavage complex formation. The fact that, as a class, the makaluvamines are not as topoisomerase II active as the standard compounds etoposide and mitoxantrone might account for some of the discrepancy in the literature.

In a previous *in vivo* xenograft experiment makaluvamines A, C and F were tested against the ovarian tumor line OVCAR3.[1] They were compared to vincristine in that experiment. Vincristine was administered at the maximally tolerated dose under a daily dosing schedule. At a dose of 5 mg/kg on days 3, 5 and 9, makaluvamines A and C appeared the most effective, yielding %T/Cs of 62 and 55% on day 14, respectively. The superior activities of makaluvamines H and I, reported here, is consistent with the idea that topoisomerase II cleavable complex formation contributes to the anti-cancer activity of the makaluvamines since H and I are far superior cleavage complex formers *in vitro*.

Nevertheless, it must be remembered that the makaluvamines are DNA-active compounds and under some conditions can react chemically to cleave DNA (e.g. reductive conditions).[1] It is also possible that other unsuspected mechanisms could contribute to the DNA cleavage detectable in DNA repair-deficient cell lines. Therefore, it is possible that the DNA-directed activity of the makaluvamines could be expressed as cytotoxicity without the effects of topoisomerase II. Future experiments in which cellular topoisomerase II is selectively inhibited during makaluvamine exposure might clarify this question. If such inhibition could protect xrs cells from the characteristic enhanced toxicity of the makaluvamines then topoisomerase II would be more directly implicated in makaluvamine cytotoxicity.

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