

SYNTHESIS OF ACRIDINE ANALOGUES AS INTERCALATING CROSSLINKERS AND EVALUATION OF THEIR POTENTIAL ANTICANCER PROPERTIES

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Abstract – We synthesized 4,5-bis(halomethyl)acridines, which contain an acridine skeleton for DNA intercalation and two halomethyl groups for DNA crosslinking. 4,5-Bis(bromomethyl)acridine and 4,5-bis(chloromethyl)acridine intercalated in DNA and mediated interstrand DNA crosslinking. Both compounds were cytotoxic to CCRF-HSB-2 cells, a human T cell leukemia cell line. Molecular modeling of 4,5-bis(bromomethyl)acridine intercalated in 5'-GC-3' base pairs of DNA indicated that the bromomethyl group, which mediates alkylation, is in close proximity to N7 of guanine.

INTRODUCTION

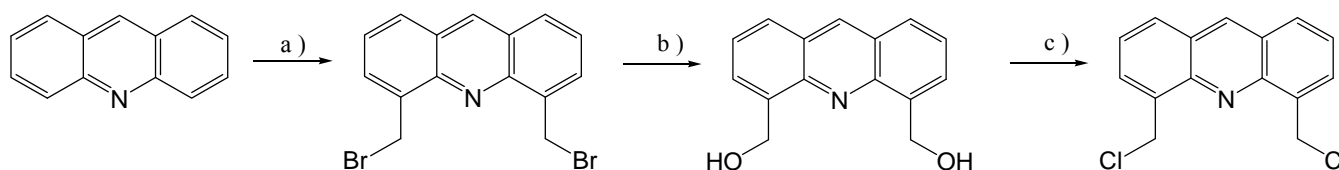
Cisplatin is a typical and widely used anticancer agent that crosslinks DNA. Although it is highly effective, its side-effects, including nausea and renal toxicity, limit its use. We suspect that some of the side-effects are caused by platinum and that a crosslinking agent lacking a heavy metal may remain effective for the treatment of cancer.

DNA intercalating compounds inhibit DNA topoisomerase's ability to mediate local unwinding and untwisting of the DNA double-helix. Two DNA intercalating compounds, acridine and amsacrine, both of which have an acridine skeleton, are well known. Various acridine derivatives have been synthesized and examined for antitumor properties.¹ Hybrids of acridine and nitrogen mustards,^{2,3} nitrosoureas,⁴ and platinum compounds^{5,6} have potent antitumor activity. In particular, hybrids of anthraquinone and nitrogen mustards⁷⁻⁹ are potent antitumor agents in cell lines that are resistant to cisplatin or doxorubicin. Hybrids of DNA crosslinking and intercalating agents are expected to have potent antitumor activity. In this case, DNA intercalation may enhance the affinity of the crosslinking moiety for DNA. In the current study, we synthesized 4,5-bis(halomethyl)acridines as bifunctional intercalating/crosslinking compounds.

These have an acridine skeleton for DNA intercalation and two halomethyl side chains for DNA crosslinking. They also lack a heavy metal. We characterized their DNA crosslinking and intercalating activities using plasmid DNA and examined their ability to inhibit the proliferation of the human leukemia cell line CCRF-HSB-2, which expresses wild-type p53. Finally, we examined the mechanism of intercalation and crosslinking of 4,5-bis(bromomethyl)acridine (BBMA) by molecular modeling.

RESULTS AND DISCUSSION

We designed compounds with an acridine skeleton for DNA intercalation and two halomethyl side-chains for DNA crosslinking. BBMA and 4,5-bis(hydroxymethyl)acridine (BHMA) were synthesized according to the method of Carole et al.^{10,11} Briefly, BBMA was synthesized by reaction of acridine with bromomethylmethylether (BMME) in H₂SO₄, and BHMA was synthesized by hydrolysis of BBMA. 4,5-Bis(chloromethyl)acridine (BCMA) was synthesized by a substitution reaction of BHMA. All compounds were purified by silica gel column chromatography.



Scheme 1. Synthesis of BBMA, BHMA, and BCMA. Reagents and conditions: a) BMME, H₂SO₄, 50 °C, 12 h, under N₂, 66.2% yield; b) CaCO₃, dioxane, H₂O, reflux for 1 h, 75.9% yield; c) ZnCl₂, HCl, 80 °C for 24 h, 34.8% yield.

The ability of bis(halomethyl)acridines to form DNA interstrand crosslinks was determined using plasmid DNA.^{12,13} BBMA and BCMA dose-dependently promoted the formation of crosslinked double-stranded DNA. Partial crosslinking was observed at 1 μM BBMA and complete crosslinking at 10 μM (Figure 1, lanes 3-5). BCMA at a concentration of 10 μM resulted in only partial interstrand crosslinking (Figure 2, lanes 3-7). In contrast, BHMA and acridine did not mediate DNA crosslinking in this assay (Figure 1, lanes 6-11). The activities of BBMA and BCMA were lower than that of the positive control, cisplatin (Figure 1, lanes 12-14). Disappearance of the band generated by 10 μM cisplatin was due to DNA strand breakage. These results suggest that nucleophilic attack of the DNA base by the halomethyl group is essential for DNA crosslinking.

We next examined the stability of BBMA or BCMA in aqueous solution by HPLC. The half-lives of BBMA and BCMA were 2 and 4 h, respectively (Figure 3). This suggests that the interstrand crosslinking activity of BCMA is lower than that of BBMA because BCMA is more resistant to nucleophilic reagents.



Figure 1. DNA interstrand crosslinking activity of BBMA, BHMA, and acridine toward plasmid DNA. Lane 1, nondenatured DNA; lane 2, denatured DNA; lanes 3-5, plasmid treated with 10, 1, and 0.1 μM BBMA, respectively; lanes 6-8, plasmid treated with 10, 1, and 0.1 μM BHMA, respectively; lanes 9-11, plasmid treated with 10, 1, and 0.1 μM acridine, respectively; lanes 12-14, plasmid treated with 10, 1, and 0.1 μM cisplatin, respectively. Reactions were performed for 6 h. DS and SS indicate double-stranded and single-stranded DNA, respectively.

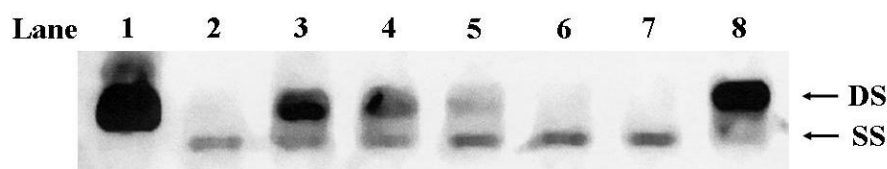


Figure 2. DNA interstrand crosslinking activity of BCMA toward plasmid DNA. Lane 1, nondenatured DNA; lane 2, denatured DNA; lanes 3-7, plasmid treated with 1 mM, 100 μM , 10 μM , 1 μM , and 0.1 μM BCMA; lane 8, plasmid treated with 0.1 μM cisplatin. Reactions were performed for 6 h. DS and SS indicate double-stranded and single-stranded DNA, respectively.

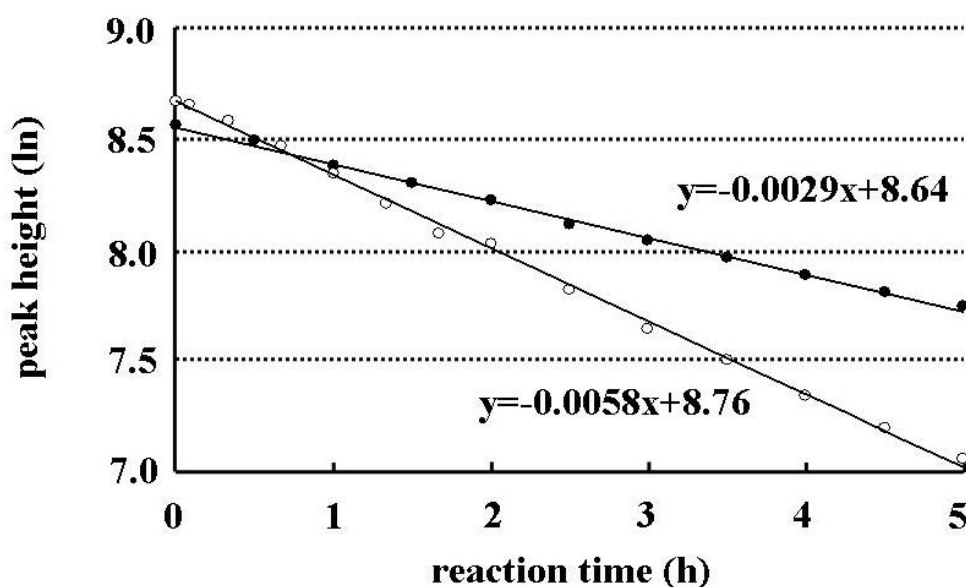


Figure 3. Stability of BBMA (\circ) and BCMA (\bullet) in aqueous solution. Equations indicate the nonlinear least squares fit for each line.

We performed a DNA unwinding assay¹⁴ to assess the ability of BBMA and BCMA to bind DNA. First, plasmid DNA (negatively supercoiled) was converted to the open circular form by treatment with

topoisomerase I. Open circular DNA was then converted to the positively supercoiled form by the binding of an intercalator. The DNA bands formed as a result of intercalation were visualized by electrophoresis (Figure 4). Acridine and BHMA intercalated into DNA at or above 100 μ M (lanes 4), whereas BBMA intercalated into the DNA only at 1 mM (lane 3), and BCMA did not intercalate into DNA at this concentration.

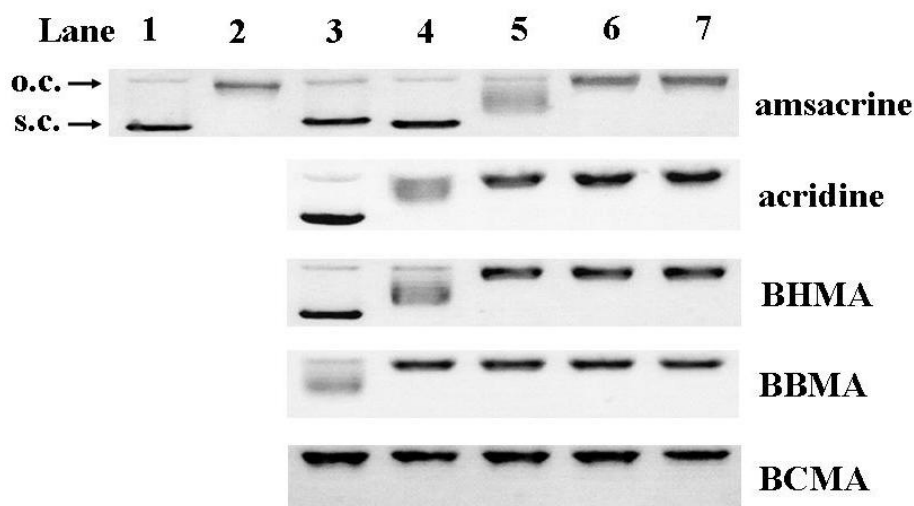


Figure 4. Unwinding of plasmid DNA by BBMA, BCMA, BHMA, acridine, and amsacrine. Lane 1, supercoiled plasmid DNA (s.c.); lane 2, open circular plasmid DNA (o.c.); lanes 3-7; plasmid DNA treated with 1 mM, 100 μ M, 10 μ M, 1 μ M, and 0.1 μ M of each compound. Reactions were performed for 30 min.

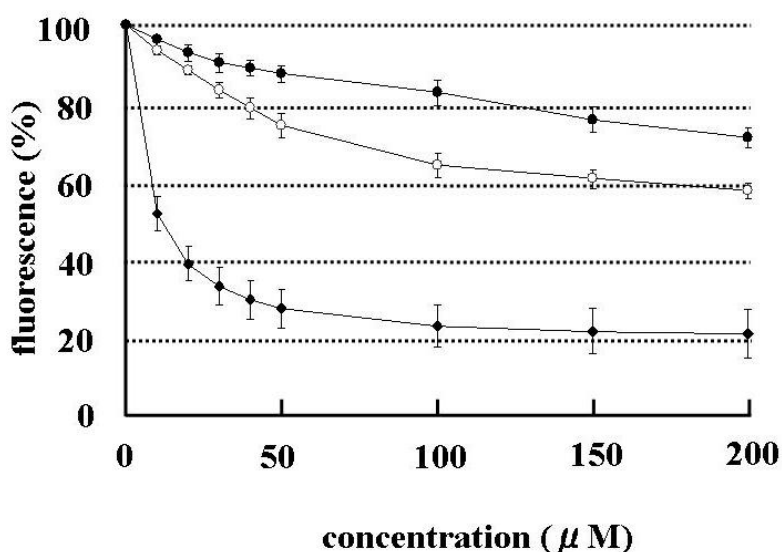


Figure 5. Analysis of BBMA (○), BCMA (●), and amsacrine (◆) by competitive ethidium displacement assay.

We examined the intercalating activity of BCMA by a competitive ethidium displacement assay.¹⁵ The fluorescence of ethidium is enhanced when it is bound to DNA, and the binding of an intercalator reduces the fluorescence. We examined the intercalation of BBMA and BCMA, which were inactive in the unwinding assay. Both BBMA and BCMA dose-dependently reduced the fluorescence (Figure 5), indicating that they intercalate into DNA. However, they were less active than acridine or BHMA in the unwinding assay. We assumed that steric hindrance of halogen atoms in the alkylating functional group reduces their DNA intercalating activity.

Next, we examined the cell antiproliferative activity of each compound in CCRF-HSB-2 cells, a human T cell leukemia cell line.¹⁶ Tokino et al. reported that CCRF-HSB-2 cells, which contain wild-type p53, are very useful for screening for the induction of p53 and for the development of new antitumor agents.¹⁷ The proliferation of these cells was dose-dependently inhibited by BBMA and BCMA (Figure 6). Because BBMA and BCMA bind and intercalate into DNA, we expected that they would be more potent than cisplatin at inducing DNA crosslinking and inhibiting cell proliferation. We found that the IC₅₀ was 1.1 μM for BBMA and 0.5 μM for BCMA, which is lower than for cisplatin (1.9 μM) (Table 1).

Given the results from the DNA interstrand crosslinking assay, we suspect that the antiproliferative activity of the bis(halomethyl)acridines is due not only to DNA interstrand crosslinking but also other mechanisms such as formation of DNA intrastrand crosslinks or inhibition of DNA topoisomerase. Although BCMA was the weakest at mediating DNA interstrand crosslinking, it was the most potent at inhibiting the proliferation of CCRF-HSB-2 cells. We suspect that BCMA is the most effective at crosslinking DNA because it is the most stable in cell culture medium. We are continuing to investigate the mechanism by which bis(halomethyl)acridines inhibit cell proliferation. These studies will employ additional cancer cell lines that are resistant to cisplatin.

Table 1. Inhibition of CCRF-HSB-2 cell proliferation by bis(halomethyl)acridines.

	BBMA	BCMA	BHMA	acridine	cisplatin
IC₅₀ (μM)	1.1	0.5	>100	>100	1.9

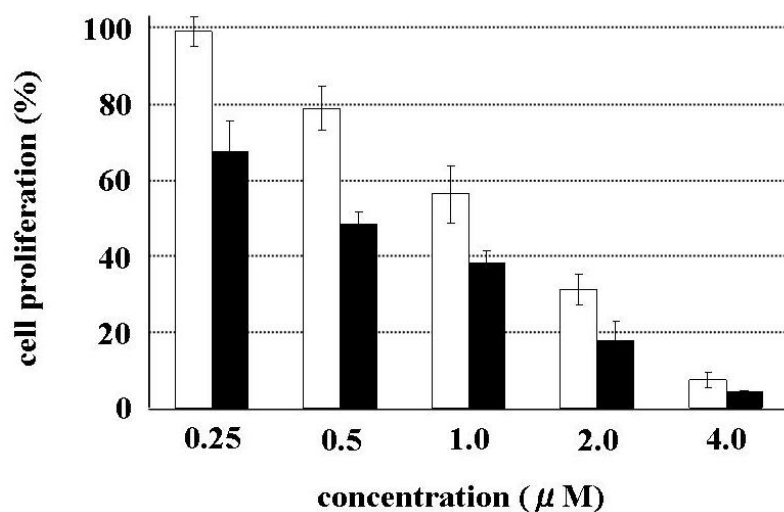


Figure 6. Antiproliferative activity of bis(halomethyl)acridines in CCRF-HSB-2 cells. Open bars, treated with BBMA; solid bars, treated with BCMA. Reactions were performed for 24 h.

Our results suggest that some of the antiproliferative activity of bis(halomethyl)acridines is due to DNA intercalation, which, in turn, allows DNA interstrand crosslinking. Therefore, we examined how BBMA intercalates into DNA using molecular modeling software. For DNA alkylating agents, the major product forms at N7-G in the major groove.¹⁸ Accordingly, we expected that BBMA would form DNA interstrand crosslinks between the N7-G of complementary DNA strands. We therefore included the 3'-GC-5' base pairs as a part of the intercalation site. Modeling showed that the two bromomethyl groups, respectively, are positioned at 3.941 Å and 4.569 Å from N7-G and that, due to intercalation, BBMA is in a position that allows it to easily form DNA interstrand crosslinks (Figure 7). Thus, introduction of an acridine skeleton increased the affinity for DNA, enhancing DNA interstrand crosslinking activity.

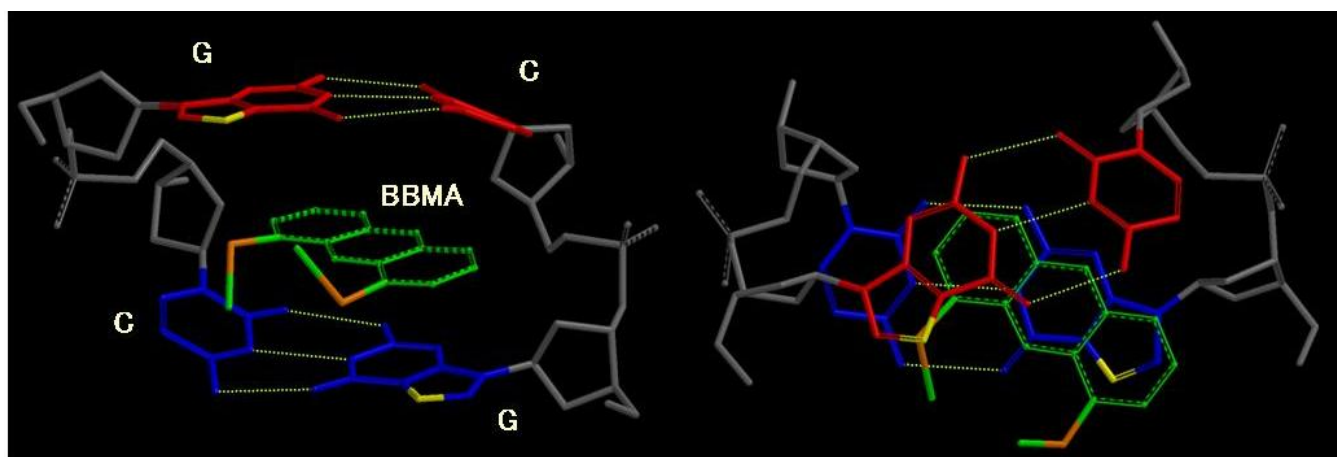


Figure 7. Intercalation of BBMA in 3'-GC-5' base pairs. Complexes are shown in a projection plane parallel (left) and orthogonal (right) to the helix axis. Green, BBMA; orange, bromomethyl group in BBMA; yellow, N7 of guanine.

EXPERIMENTAL

¹H NMR spectra were obtained on a Jeol JNM-A500 spectrometer using tetramethylsilane as an internal reference. Mass spectra were determined on a Shimadzu GCMS-QP5050A spectrometer. Acridine and BMME (purest grade available) were purchased from Wako Pure Chemical, Inc. (Osaka, Japan). Silica gel 60 was purchased from Merck & Co. (Rahway, NJ, USA). The pBR322 plasmid DNA and topoisomerase I were purchased from Takara-Bio, Inc. (Tokyo, Japan). Calf thymus DNA was purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). LiChrosorb RP-18 column was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). RPMI1640 medium was purchased from Nissui Seiyaku Inc. (Tokyo, Japan). Fetal calf serum was purchased from JRH Bioscience (Lenexa, KS).

BBMA

BMME (500 mg, 4 mmol) was added to a solution of acridine (179 mg, 1 mmol) in concentrated (95%) H₂SO₄ (10 mL) at 50 °C. The mixture was maintained at 50 °C under nitrogen for 12 h and cooled on ice for 1 h. The mixture was extracted with CHCl₃, and the organic phase was dried over Na₂SO₄. The solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography with 6:1 hexane/AcOEt to obtain BBMA (241.5 mg, 66.2% yield) as a yellow powder. Mp 152-153 °C (lit.¹⁰: 156 °C). ¹H-NMR (CDCl₃) δ: 8.79 (1H, s), 8.00 (2H, d, *J* = 8.6Hz), 7.94 (2H, d, *J* = 6.7Hz), 7.52 (2H, dd, *J* = 8.6Hz, 7.3Hz), 5.43 (4H, s). MS *m/z*: 365 (M⁺).

BHMA

BBMA (138.0 mg, 0.38 mmol) was dissolved into 20 mL of dioxane, and CaCO₃ (2 g, 20 mmol) in 20 mL of water was added. The mixture was refluxed for 1 h. Next, the mixture was allowed to cool to rt and filtered. The solvent was evaporated, and the residue was purified by silica gel column chromatography with 1:1 hexane/AcOEt to obtain BHMA (68.9 mg, 75.9% yield) as yellow needles. Mp 156-158 °C. ¹H-NMR (CD₃OD) δ: 8.95 (1H, s), 8.02 (2H, d, *J* = 7.9Hz), 7.86 (2H, d, *J* = 6.7Hz), 7.57 (2H, dd, *J* = 8.5Hz, 6.7Hz), 5.40 (4H, s). MS *m/z*: 239 (M⁺).

BCMA

BHMA (23.9 mg, 0.1 mmol) was dissolved in concentrated (35% to 37%) HCl (5 mL), and zinc chloride (60 mg, 0.44 mmol) in HCl (5 mL) was added. The mixture was stirred at 80 °C for 24 h. Next, the mixture was allowed to cool to rt and extracted with CH₂Cl₂. The organic phase was dried over Na₂SO₄. The solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography with 2:1 hexane/AcOEt to obtain BCMA (9.6 mg, 34.8% yield) as a yellow powder. Mp 163-165 °C. ¹H-NMR (CDCl₃) δ: 8.80 (1H, s), 8.01 (2H, d, *J* = 7.9Hz), 7.96 (2H, d, *J* = 7.3Hz), 7.56 (2H,

dd, $J = 6.7\text{Hz}$, 8.5Hz), 5.52 (4H, s). HRMS: 275.0243 (calcd for $\text{C}_{15}\text{H}_{11}\text{NCl}_2$: 275.0269(M^+)). *Anal.* Calcd for $\text{C}_{15}\text{H}_{11}\text{NCl}_2 \cdot \text{CH}_3\text{COOC}_2\text{H}_5$: C, 62.13; H, 5.21 ; N, 3.81. Found: C, 62.06; H, 5.44; N, 3.44.

DNA interstrand crosslinking assay

DNA crosslinking activity was assayed using a DNA interstrand crosslinking assay. DMSO was freshly distilled from calcium hydride before use (bp₁₄ 76 °C). Linearized pBluescript[®] DNA (460 ng) was treated with the test compound dissolved in DMSO at 37 °C for 6 h. After the reaction, the DNA was precipitated with EtOH (95%) and cooled for 24 h before being centrifuged for 20 min. The supernatant was removed, and the sample was washed with EtOH (75%), and spun for 20 min. The supernatant was removed, and lyophilized to dryness. The DNA was then dissolved in separation buffer (30% DMSO and 1 mM EDTA), heated at 95 °C for 5 min, and immediately placed in an ice bath. The DNA samples were separated by 1% agarose gel electrophoresis, and the DNA bands were visualized by staining with ethidium bromide.

DNA unwinding assay

DMSO was freshly distilled from calcium hydride before use (bp₁₄ 76 °C). Negatively supercoiled pBR322 DNA (0.25 µg/reaction) was first relaxed at 37 °C by incubation with 5 units of calf thymus topoisomerase I at pH 8.0 for 30 min. Next, the DNA was treated with the test compound dissolved in DMSO at pH 8.0 for 30 min at 37 °C. The reaction was terminated by the addition of sodium dodecyl sulfate (0.5% final concentration) followed by dilution and extraction to remove drugs. The DNA samples were separated by 1% agarose gel electrophoresis, and the DNA bands were visualized by staining with ethidium bromide.

Competitive ethidium displacement assay

To a mixture of calf thymus DNA and ethidium bromide (final concentration of 1.26 µM) at pH 7.0, test compound in DMSO was added, and the intensity of fluorescence (excitation at 520 nm, emission at 600 nm) was recorded. The remaining fluorescence was expressed as a percentage to the initial fluorescence. The final fluorescence was obtained by subtraction of the value measured in the absence of ethidium bromide from the value measured in the presence of ethidium bromide.

Stability of BBMA and BCMA

The stability of BBMA and BCMA toward nucleophilic reagents was examined by measuring the decomposition rate in aqueous solution. Test compound (final concentration of 10 µM) was added to an aqueous solution (70:30 mixture of 0.01 M sodium phosphate buffer [pH 7.4] and MeCN), and 2 µL of

the solution was injected into the HPLC system at regular intervals. A sample of test compound was analyzed on a LiChrosorb RP-18 column (5 μm), eluted with an 80:20 mixture of MeCN and water.

Cell proliferation assay

The antiproliferative activity of the compounds was assayed using a cell proliferation assay. The cell line used in this study, CCRF-HSB-2 (a human T cell leukemia cell line), was provided by RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured in RPMI1640 supplemented with 10% fetal calf serum at 37 °C in an atmosphere containing 5% CO₂. Cells were collected when they were nearly confluent and diluted to a concentration of 3×10^5 cell/mL with culture medium. Aliquots (3 mL) of cell suspension were dispensed into each well of a 6-well plate. After incubation for 24 h at 37 °C in an atmosphere containing 5% CO₂, the cells were treated with the test compound dissolved in DMSO. Each well received 3 μL of the dissolved test compound. After incubation for 24 h at 37 °C in an atmosphere containing 5% CO₂, Trypan blue-excluding cells were counted.

Molecular modeling

Molecular mechanics calculations were performed using the MMFF94s force field in Spartan'04 (Wavefunction, Inc., Irvine, CA). To produce compound-DNA complexes, 5'-GC-3' base pairs and the vacant intercalation pocket were manually assigned. BBMA was placed into the major groove of the DNA double helix. The resulting complex was subjected to energy minimization using the MMFF94s force field. During energy minimization, the BBMA structure was allowed to move, while the base pairs were fixed.

CONCLUSION

The tricyclic aromatic compounds BBMA and BCMA mediate DNA interstrand crosslinking, intercalate into DNA, and potentially inhibit the proliferation of CCRF-HSB-2 cells. Of these two compounds, BCMA appeared to be more effective at inhibit the growth of CCRF-HSB-2 cells. Molecular modeling suggested that DNA intercalation due to the acridine skeleton enhanced the formation of interstrand crosslinks. On the basis of these findings, we propose bis(halomethyl)acridines as novel anticancer lead compounds.

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