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Effects of structural modification on the DNA binding properties and photo-induced cleavage reactivity of propargylic sulfones conjugated with an anthraquinone structure

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Abstract—Propargylic sulfones are known as pH-dependent DNA cleaving agents. We have designed a novel propargylic sulfone conjugated with an anthraquinone structure and evaluated its DNA binding and cleavage characteristics. The propargylic sulfone **3** showed high intercalating ability attributable to anthraquinone chromophore, leading to the efficient alkylation of DNA. The anthraquinone chromophore in **3** also acted as a photosensitizer, and photoirradiation of **3** with DNA induced one-electron oxidation, resulting in the further DNA cleavage. Evaluation of the effect of **3** against EMT6/KU cells revealed that **3** exhibited potent cytotoxicity, even without photoirradiation.

1. Introduction

Propargylic sulfone derivatives have been developed as artificial DNA cleaving agents, which damage DNA bases in a pH-dependent manner.[1] These propargylic sulfones show DNA alkylating ability through isomerization into allenic sulfones under basic conditions and nucleophilic addition of nucleobase, especially guanine bases.[2] Recently, we have synthesized the propargylic sulfones with various planar molecules, such as naphthalene and anthraquinone (AQ), and have evaluated their DNA binding properties and DNA cleavage activity (**1** and **2** in Chart 1).[3] Among these compounds, the propargylic sulfone **2** possessing an AQ chromophore as an intercalative photosensitizer showed sufficient intercalating ability to duplex DNA in addition to photochemical one-electron oxidation functionality.[3d,4] However, this high affinity toward DNA was not reflected in an enhanced DNA alkylating ability. Both the DNA binding constant and molecular modeling suggested that an unfavorable spatial arrangement between activated allenic sulfone and nucleobases, which were derived from the intercalation far from the reaction site of **2**, was an impediment to this compound exhibiting a high DNA alkylating ability.[3d,5]

These results stimulated us to improve the DNA cleavage using propargylic sulfones to obtain DNA cleaving agents with high performance. We designed a new propargylic sulfone (**3** in Chart 1), which maintained a high intercalating ability and had a photo-sensitizing function due to the AQ chromophore located in the neighborhood of the sulfonyl group and the reactive allenic site. This propargylic sulfone **3** showed a considerably high DNA binding and alkylating ability relative to the propargylic sulfones **1** and **2**. This high DNA cleaving ability suggests that **3** can effectively approach duplex DNA, and that the spatial arrangement of the activated allene is suitable for the alkylation of the nucleophilic DNA bases. Upon photoirradiation, **3** induced further DNA cleavage by the AQ-derived

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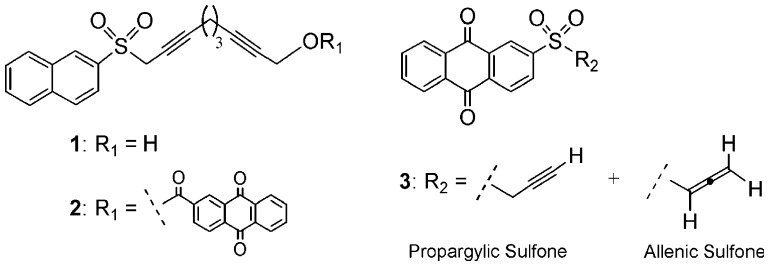


Chart 1. Structures of propargylic sulfone derivatives.

photo-induced one-electron oxidation. Evaluation of the cytotoxicity of **3** against an EMT6/KU cell line showed a sufficient IC_{50} value, even without photoirradiation.

2. Results and discussion

Synthesis of the propargylic sulfone **3** is outlined in Scheme 1. Bis(2-anthraquinyl) disulfide **4**, which was prepared according to the previous method,[6] was converted to 2-mercaptoanthraquinone by reduction with $NaBH_4$. The resulting thiol was continuously treated with propargylic bromide in the presence of NaH to give the propargylic sulfide **5**. Finally, oxidation of **5** by *m*-CPBA furnished the synthesis of the desired propargylic sulfone **3** as a mixture of isomers consisting of the propargylic and allenic sulfones. ^1H NMR spectra of **3** in $CDCl_3$ indicated that 30% of the propargylic sulfone was isomerized to the allenic form.[7]

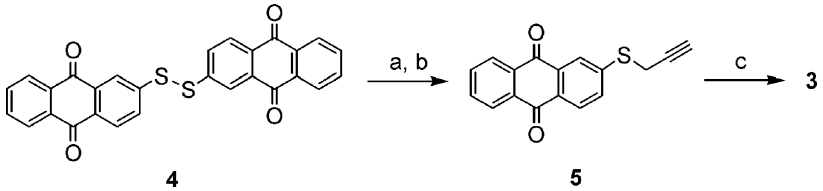
We determined the inhibitory binding constant (K') of **3** against intercalated ethidium bromide (EB) using Salmon Sperm DNA, according to the reported fluorometry (Table 1).[8] An aliquot of EB solution was used to titrate the Salmon Sperm DNA in 5% DMSO-containing H_3BO_3/NaOH buffer solution (pH 8.5) containing various concentrations of **3** by recording the change in fluorescence intensity at maximum emission wavelength (586 nm in this study) upon excitation at 525 nm. As shown in Table 1, **3** showed about a 2.3-fold higher intercalating ability than the control compound **2**, although **3** possesses only a single DNA binding group. The binding constant of **3** is the highest among those of other reported propargylic sulfones ($\sim 7.0 \times 10^4\ M^{-1}$).[3c,d] Thus, it is clear that incorporation of the AQ chromophore in the vicinity of the sulfonyl unit is effective for enhancing the intercalating ability.

We then examined DNA cleavage by the propargylic sulfone **3** using agarose gel electrophoresis of the restriction fragment of circular ΦX174 RFI DNA.[9] As shown in Figure 1, we observed strong DNA cleavage up to 40% on incubation for only 30 min at 37 °C in the presence of **3**. According to our previous report,[3c,d] the propargylic sulfones **1** and **2** required a 2-fold concentration of **3** and incubation for 72 h at 37 °C to achieve a similar 40–60% cleavage of DNA. Thus, the propargylic sulfone **3** has an exceedingly high DNA cleavage activity via the nucleobase alkylation mechanism, consistent with its high DNA binding property.[10] This high affinity of **3** toward DNA was also reflected in its photosensitized cleavage. Photoirradiation of **3** for 10 min at 365 nm during incubation for 30 min at 37 °C induced the one-electron oxidation of DNA, resulting in a considerable enhancement of DNA cleavage of up to 80%, while the control propargylic sulfone **2** required 4 h irradiation to gain a similar level of DNA cleavage.[3d] Thus, we concluded that an appropriate spatial arrangement between the activated allenic sulfone and nucleobase, which is derived from an efficient intercalation, leads to a substantial enhancement of DNA cleavage via alkylation of the nucleobase and photo-induced one-electron oxidation.

Table 1. Binding constants (K') of propargylic sulfones

Compound	$K' \times 10^4\ M^{-1}$
1	3.2
2	3.7
3	8.6
Naphthalene	5.2
9,10-Anthraquinone	11.3

Next, we investigated the base selectivity in the dark and in photo-induced DNA cleavage reactions by the propargylic sulfone **3** using polyacrylamide gel electrophoresis of ^{32}P-5′-end-labeled 24-mer DNA (5′-ATCAAGG TAGAAGGAATGGCTAAA-3′/5′-TTTAGCCATTC CTTCTACCTTGAT-3′).[11] We carried out photoirradiation



Scheme 1. Reagent and conditions: (a) $NaBH_4$, EtOH, THF, rt; (b) NaH, propargyl bromide, THF, rt, 42% (two steps); (c) *m*-CPBA, CH_2Cl_2, 0 °C, 52%.

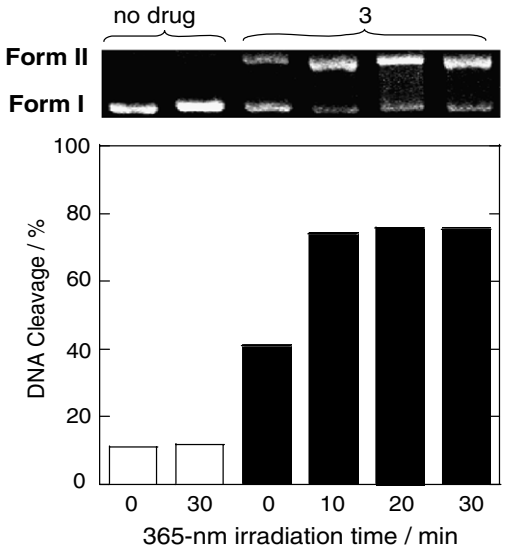


Figure 1. Gel picture and scanning densitometry results of DNA cleavage by **3**. ΦX174 RFI DNA (50.0 μM) in the presence of **3** was photoirradiated for 10 min during incubation at 37 °C for 30 min in 20% acetonitrile-containing TAE buffer solution (pH 8.5) and then analyzed by 1% agarose gel electrophoresis and ethidium bromide stain.

and incubation of sample solutions containing DNA and **3**, and treated the resulting mixture with hot piperidine to visualize the alkaline labile damage. The representative gel is shown in Figure 2a. Upon incubation at 37 °C in the dark, **3** caused DNA cleavage at each guanine base (lane 3 in Fig. 2a), while **3** did not cause any DNA cleavage with neither photoirradiation nor incubation. This result indicates that **3** cleaves DNA by alkylation at guanine bases,[2] consistent with our previous reports.[3d] Moreover, on incubation with photoirradiation, an enhancement of DNA cleavage at 5′-G of GG sequences, which is known to be a sink of positive charge migration through DNA, was observed (lane 4 in Figs. 2a and b).[4] In view of this, it is most likely that **3** also cleaves DNA strands by photo-induced one-electron oxidation in addition to the alkylation mechanism. In a control experiment, we carried out DNA cleavage using **3** on photoirradiation at 0 °C to suppress DNA cleavage by alkylation mechanism. We observed typical DNA cleavage by one-electron oxidation mechanism (see Figure S1), indicating that the alkylation and the photo-induced one-electron oxidation proceed independently, resulting in DNA cleavage under the present conditions.

We then assessed the cytotoxicity of **3** toward EMT6/KU tumor cells (murine mammary carcinoma). An MTT (3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyltetrazolium bromide) assay was employed to assess the cytotoxicity test in vitro.[12] The IC_{50} value was defined as the concentration required to reduce the viability of the cells by 50% and was evaluated using the MTT assay on exposure to propargylic sulfones. Figure 3 shows the survival curve of the EMT6/KU cells incubated in the presence of **3** or **1** at various concentrations in air containing 5% CO_2 for 72 h at 37 °C in the dark conditions. Consistent with DNA cleavage via alkylation, the

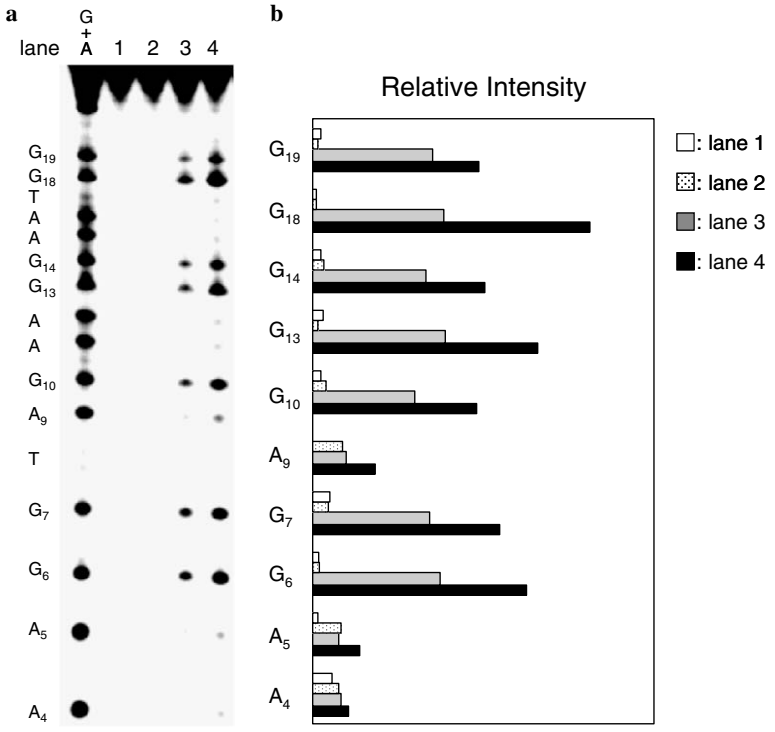


Figure 2. (a) An autoradiogram of a denaturing sequencing gel for the ^{32}P-5′-end-labeled 24-mer DNA after alkylation and photooxidation in the presence of **3**. Duplex DNA was cleaved by **3** (20 μM) in 20% acetonitrile-containing TAE buffer solution (pH 8.5). After piperidine treatment (90 °C, 20 min), the samples were electrophoresed through a denaturing 15% polyacrylamide/7 M urea: lane G+A, Maxam–Gilbert sequencing lane; lane 1, control DNA, with piperidine treatment only; lane 2, neither photoirradiation nor incubation in the presence of **3**; lane 3, incubated for 4 h at 37 °C in the presence of **3**; and lane 4, photoirradiated at 365 nm for 4 h at 37 °C in the presence of **3**. (b) The histogram representing relative intensities of cleavage bands obtained by densitometry assay of lanes 1–4 in (a).

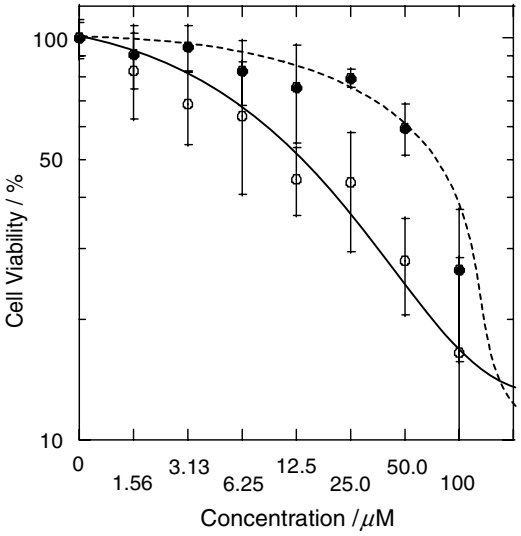


Figure 3. Cytotoxicity of propargylic sulfones **1** and **3** on EMT6/KU tumor cells as a function of drug concentration. EMT6/KU tumor cell was incubated with **1** and **3** at each concentration as observed after incubation in 0.4% DMSO-containing MEM at 37 °C for 72 h under 5% CO_2 atmosphere and then analyzed by MTT assay: (●) **1**, (○) **3**.

cytotoxic effect of **3** (IC_{50} = 12.7 μM) was higher than that of **1** (IC_{50} = 70.1 μM). Thus, these results clearly indicate that the progress of DNA alkylating ability enhanced the cytotoxicity of the propargylic sulfone remarkably. We also attempted to evaluate the cytotoxic effect of **3** upon photoirradiation. However, we could not obtain an IC_{50} value, because the low optical permeability of medium prevented the direct excitation of **3**, which was administered to cells.

3. Conclusions

In summary, we have designed and synthesized a novel propargylic sulfone derivative **3** conjugated with an anthraquinone chromophore. We have confirmed that **3** effectively binds to DNA duplex by intercalation, leading to a quite efficient DNA cleavage through alkylation and photo-induced one-electron oxidation of guanine bases. These results strongly suggest that an appropriate spatial arrangement of the reaction site between **3** and nucleobase in DNA is a key factor for the enhanced DNA cleavage. Furthermore, propargylic sulfone **3** showed potent cytotoxicity against EMT6/KU cells, even without photoirradiation. In view of the DNA binding affinity and cleavage characteristics of **3**, a strategy based on the molecular design protocol established in this study would be applicable to the development of more potent and practical antitumor agents.

4. Experimental

4.1. General

^1H and ^{13}C NMR spectra were measured with JEOL JNM-AL-300 (300 MHz), JEOL JMN-EX-400 (400 MHz), and JEOL JNM-A-500 (500 MHz) spectrometers at room temperature. All samples were dissolved in $CDCl_3$. Coupling constant (J value) is reported in hertz. The chemical shifts are expressed in part per million downfield from tetramethylsilane, using residual chloroform (δ = 7.24 in ^1H NMR, δ = 77.0 in ^{13}C NMR) as internal standard. Multiplicity is designed as singlet (s), doublet (d), triplet (t), quartet (q) or multiplet (m). FAB Mass spectra were recorded on a JEOL JMS-SX102A spectrometer. Fluorescence spectra were recorded on a Hitachi F-2000 and Shimadzu RF-5300PC. Photoirradiation at 365 nm was carried out using a ULTRA-VIOLET PRODUCTS NTFL-40 transilluminator. An ATTO sequencing gel electrophoresis apparatus and a GIBCO BRL Model S2 sequencing gel electrophoresis apparatus were used for agarose gel electrophoresis and polyacrylamide gel electrophoresis (PAGE), respectively. The gels were analyzed by densitometry with an ATTO Lane Analyzer (version 3). A Microplate Reader (Model 550, BIO-RAD) and a data analysis software, MPM III (BIO-RAD), were used to calculate 50% inhibition concentration (IC_{50}), defined as the concentration required to reduce the viability of cells by 50%.

4.2. Materials

Reagents were purchased from Aldrich, Wako Pure Chemical Industries, and Nacalai Tesque, and used without purification. Reactions were monitored by thin-layer chromatography (TLC) on silica gel plates (Merck silica gel 60 F_{254}). Column chromatography was carried out on Wakogel C-300 (Wako Pure Chemical Industries). Salmon Sperm DNA and supercoiled ΦX174 RFI DNA were used as received from GIBCO BRL and TAKARA SHUZO CO., LTD, respectively. The oligo-deoxynucleotides, T4 polynucleotide kinase, and [γ-^{32}P]ATP (10 mCi/mL) were used as received from Invitrogen, NIPPON GENE (10 U/μL), and Amersham, respectively.

4.3. 3-Anthraquinylthiopropyne (5)

Compound **4** (85.2 mg, 0.17 mmol) was dissolved into dry THF (2.5 mL) with sodium borohydride (12.0 mg, 0.3 mmol), EtOH (2 mL). The mixture was stirred at 0 °C for 1 h. The reaction was quenched by $NaHCO_3$ and extracted with EtOAc. The extract was washed with brine, dried with anhydrous $MgSO_4$, and filtered. Removal of solvents in vacuo gave the crude 2-mercaptoanthraquinone. The resulting crude product was dissolved into dry THF (2 mL) with sodium hydride (20 mg, 0.5 mmol). The mixture was stirred at 0 °C for 1 h and then added 3-bromo-1-propyne (0.05 mL, 0.66 mmol) with stirring for additional 0.5 h. The reaction was quenched by cold water and extracted with EtOAc. The extract was washed with brine, dried with anhydrous $MgSO_4$, and filtered. Removal of solvents in vacuo gave the crude product. Purification by silica gel column chromatography with 1% MeOH/$CHCl_3$ gave **5** (39.4 mg, 42%) as a mixture of isomers consisting of 3-anthraquinylthiopropyne and 1-anthraquinylthiopropadiene as a yellow solid. This mixture was impossible to be purified: mp 127.5–130 °C; 3-anthraquinylthiopropyne: ^1H NMR ($CDCl_3$, 400 MHz)

δ 8.29–8.18 (m, 5H), 7.80–7.77 (m, 2H), 3.79 (d, J = 2.8 Hz, 2H), 2.28 (t, J = 2.4 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 182.6, 182.2, 134.2, 133.9, 133.9, 131.7, 131.2, 127.7, 127.7, 127.1, 127.1, 124.4, 124.1, 78.8, 72.4, 50.8, 20.6; 1-anthraquinylthiopropadiene: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.70–7.68 (m, 7H), 6.04 (t, J = 6.0 Hz, 1H), 5.10 (d, J = 6.4 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 211.4, 182.7, 182.3, 145.8, 144.6, 133.5, 133.5, 133.4, 133.4, 133.1, 130.8, 130.6, 130.5, 128.7, 82.4, 78.2, 68.1; FAB-MS (NBA) m/z 279 [(M+H)$^+$]; HR-MS calcd for $C_{17}H_{11}O_2S$ [(M+H)$^+$]: 279.0474. Found: 279.0478.

4.4. 3-Anthraquinylsulfonyl propyne (3)

5 (9.4 mg, 0.034 mmol) was dissolved into dry CH_2Cl_2 with *m*-CPBA (16.7 mg, 0.068 mol). The mixture was stirred at 0 °C for 2 h. The reaction was quenched by $NaHCO_3$ and extracted with EtOAc. The extract was washed with brine, dried with anhydrous $MgSO_4$, and filtered. Removal of solvents in vacuo gave crude product. Purification by silica gel column chromatography with 10% EtOAc/hexane gave **3** (6.8 mg, 52%) as a mixture of isomers consisting of 3-anthraquinylsulfonyl propyne and 1-anthraquinylsulfonyl propadiene as ocher solid. The mixture was also impossible to be purified: mp 105.5–108.0 °C; 3-anthraquinylsulfonyl propyne: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.39–8.29 (m, 4H), 7.88–7.85 (m, 3H), 4.06 (d, J = 2.7 Hz, 2H), 2.39 (t, J = 2.8 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 181.3, 134.8, 134.9, 134.8, 134.8, 133.8, 133.7, 133.2, 132.2, 130.2, 129.9, 128.4, 128.3, 127.6, 84.8, 48.2, 29.7; 1-anthraquinylsulfonyl propadiene: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.90 (d, J = 2.0 Hz, 2H), 8.53–8.47 (m, 3H), 8.06 (t, J = 2.0 Hz, 1H), 7.41 (t, J = 8.0 Hz, 1H), 6.30 (t, J = 6.4 Hz, 1H), 5.55 (d, J = 6.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 210.4, 181.8, 181.4, 146.5, 142.6, 136.9, 136.4, 134.7, 134.2, 134.0, 132.4, 130.9, 128.8, 128.6, 128.2, 126.9, 77.2; FAB-MS (NBA) m/z 311 [(M+H)$^+$]; HR-MS calcd for $C_{17}H_{11}O_4S$ [(M+H)$^+$]: 311.0378. Found: 311.0374.

4.5. Measurements of DNA binding ability

An aliquot of approximately 300 μM ethidium bromide (EB) solution was used to titrate the Salmon Sperm DNA in 5% DMSO-containing H_3BO_3/NaOH buffer solution (pH 8.5) at various concentrations of an propargylic sulfone with recording the change of fluorescence intensity at maximum emission wavelength with the excitation at 525 nm.

Intrinsic and observed DNA binding constants, K and K_{obsd}, at various concentrations of propargylic sulfones bearing anthraquinone chromophore were evaluated from the slope of the Scatchard plot for the binding of EB to DNA in the absence and presence of propargylic sulfones. The relationship between K and K_{obs} is shown in Eq. 1

$$\frac{1}{K_{obs}} = \frac{1}{K} + \left(\frac{K}{K'}\right) \times C \qquad (1)$$

where K' is the inhibitory DNA binding constant of the propargylic sulfones and C is the concentration of those unbound compounds that are approximately equal to the total concentration before titration of EB. The value of K' was calculated from a plot of $1/K_{obs}$ versus C.

4.6. Evaluation of DNA cleavage activity

DNA cleavage studies on the propargylic sulfones were performed by the use of supercoiled, covalently closed, circular ΦX174 RFI double-stranded DNA (form I). An aliquot solution of 50.0 μM/bp (micromolar per base pair) solution of ΦX174 RFI DNA and 5 μM propargylic sulfone **3** in 20% acetonitrile-containing TAE buffer (pH 8.5) (total volume 10 μL) was photoirradiated with a transilluminator (λ_{ex} = 365 nm) at 37 °C. Reaction mixtures were photoirradiated for 10 min during incubation at 37 °C for 30 min and then analyzed by gel electrophoresis (1% agarose gel, ethidium bromide stain). DNA cleavage was indicated by the formation of relaxed circular DNA (form II) and linearized DNA (form III). The gels were placed on a UV transilluminator (λ_{ex} = 365 nm) and photographed with Polaroid 667 film. The relative densities of various DNA bands on a picture were quantified by densitometry with an ATTO Lane Analyzer. The percentage of DNA cleavage was calculated by following Eq. 2:

$$\text{DNA cleavage}/\% = \frac{[\text{form II}] + 2[\text{form III}]}{[\text{form I}] + [\text{form II}] + 2[\text{form III}]} \qquad (2)$$

In order to evaluate the percentage of DNA cleavage with good accuracy, the assays in the present study were performed under conditions that concentrations of propargylic sulfones were adjusted so as not to induce form III.

4.7. Identification of DNA cleavage sites

The oligo-deoxynucleotide (ODNs, 400 pmol strand concentration) was 5′-end-labeled by phosphorylation with 4 μL [γ-^{32}P]ATP and 4 μL T4 poly nucleotide kinase using stranded procedures.[11] The 5′-end-labeled ODNs were recovered by ethanol precipitation and further purified by 15% preparative denaturing gel electrophoresis and isolated by the crush and soak method. ^{32}P-5′-end-labeled ODNs were hybridized to the complementary stranded (2.0 μM, strand concentration) in TAE buffer at pH 8.5. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The ^{32}P-5′-end-labeled ODN duplex containing propargylic sulfones **3** in 20% acetonitrile-containing TAE buffer (pH 8.5) (total volume 15 μL) was irradiated with a transilluminator at 37 °C, and then incubated at 37 °C. Reaction mixtures after incubation were precipitated by 800 μL ethanol with the addition of 10 μL of 3 M sodium acetate, 10 μL of 1 mg/1 mL Herring sperm DNA. The DNA thus precipitated was washed with 100 μL of 80% cold ethanol and dried in vacuo. The purified DNA was resolved in 50 μL of water or 50 μL of 10% piperidine (v/v), heated at 90 °C for 20 min, and evaporated under

reduced pressure. The radioactivity of the samples was then assayed using an Aloka 1000 liquid scintillation counter and the dried DNA pellets were resuspended in 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). All reactions, along with Maxam–Gilbert G+A sequencing reactions, were heat-denatured at 90 °C for 3 min and quickly chilled on ice. The samples (0.8–1.0 μL, $5–20 \times 10^3$ cpm) were loaded onto 12% polyacrylamide/7 M urea sequencing gels and electrophoresed at 1900 V for about 2 h, transferred to a cassette, and stored at −80 °C with Fuji X-ray film (RX-U). The gels were analyzed by densitometry with an ATTO Lane Analyzer (version 3). The intensities of the spots resulting from piperidine treatment were determined by volume integration.

4.8. Assessment of cytotoxicity toward EMT6/KU cells

EMT6/KU cells were cultured in Eagle's minimum essential medium (MEM) containing 12.5% fetal bovine serum (FBS). Each propargylic sulfone was dissolved in the 0.4% DMSO-containing MEM to prepare solutions with various concentrations. Compound was dissolved in small amount of DMSO in advance to solve certainly. Cell suspension was planted into a 96-well flat-bottomed microplate, followed by incubation with propargylic sulfone at various concentrations. After 72 h incubation at 37 °C in a 5% CO_2 incubator, 10 μL of MTT (3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyltetrazolium bromide) solution was added to each well, followed by further incubation for 3 h. And then, 100 μL/well of acidified 2-propanol (0.04 N HCl) was added. The optical densities of plates were observed by a Microplate Reader (Model 550, BIO-RAD) at 570 nm. 50% Inhibition concentration (IC_{50}), defined as the concentration required to reduce the viability of cells by 50%, was calculated using a data analysis software, MPM III (BIO-RAD).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.02.036.

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