Organic LETTERS

Letter

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Design, Synthesis, and Evaluation of Diazeniumdiolate-Based DNA Cross-Linking Agents Activatable by Glutathione S-Transferase

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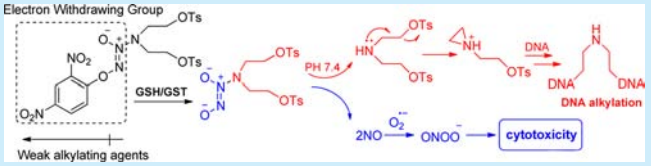
Supporting Information

ABSTRACT: A novel class of O^2-(2,4-dinitrophenyl)-1-[*N*,*N*-bis(2-substituted ethyl)amino]diazen-1-ium-1,2-diolates **4**–**6** were designed, synthesized, and biologically evaluated. The most active compound **6** caused significant DNA damage by releasing *N*,*N*-bis(2-TsO ethyl)amine and two molecules of nitric oxide (NO) after activation by GST/GSH in cancer cells, being more cytotoxic against three cancer cell lines than a well-known diazeniumdiolate-based anticancer agent JS-K, suggesting that the strategy has potential to extend to other O^2-derived diazeniumdiolates to improve anticancer activity.



Nitric oxide (NO) is a short-lived, pleiotropic gas molecule that mediates numerous physiological processes including vasodilation, neurotransmission, and immune response.[1] NO has been shown to have dichotomous (pro- and antitumorigenic) effects on cancer cells, depending on concentration of NO, duration of exposure, chemical redox environment, and cell cycle status of cancer cells.[2] Generally, high concentrations (micromolar to millimolar) of NO can damage biomacromolecules such as proteins, DNA, and lipids and cause cell death by triggering apoptosis.[3] The molecular mechanisms of NO rely on S-nitrosation of thiol-containing proteins via the reaction of the formed nitrosonium ion (NO^+) with the nucleophilic center of the sulfur atom[4] and/or on nitration of tyrosine residue in proteins and guanylic acid moiety in DNA mediated by peroxynitrite, which derives from the reaction of NO with superoxide.[5] Thus, NO has also been considered as a potential anticancer agent, and many NO-based therapies including NO-donating small molecules or nanoparticles have been intensively investigated.[6−8] However, due to its involvement in a myriad of biological processes,[9] release of NO from NO donor compounds should be localized at the tumor site to avoid side effects. Diazeniumdiolates, an important NO donor, spontaneously release two molecules of NO under physiological conditions (pH 7.4, 37 °C) with a range of half-lives from a few seconds to several hours.[10,11] Some O^2-derived compounds such as O^2-2,4-dinitrobenzene[12] and O^2-glycosyl diazeniumdiolates[13] could be enzymatically cleaved in tumor cells to generate diazeniumdiolate anion, which releases NO spontaneously in situ, exhibiting selective and potent antiproliferative activity.[14] JS-K, the representative one, could be attacked by GSH in the presence of glutathione S-transferase (GST) overexpressed in many tumor cells[15] to liberate 4-cabethoxy-PIPERAZI/NO, which immediately releases NO and 4-carbethoxypiperazine.[12,16−18] JS-K exhibited potent antiproliferative activity against various cancer cell lines in vitro and suppressed the growth of human leukemia and prostate cancer xenografts in mice.[19] Further molecular modifications aimed to improve selectivity for GSTπ isozyme and to achieve a better balance between activity and stability have been extensively studied[20−24] with no striking advances yet.

DNA interstrand cross-links (ICLs) are known as the primary mechanism for the cytotoxic activity of many clinically used antitumor drugs[25] such as nitrogen mustard chlorambucil,[26,27] mechlorethamine,[28] ifosfamide,[29] and improsulfan.[30] However, the severe host toxicity exhibited by these drugs continues to be a major problem in cancer chemotherapy. Interestingly, several derivatives of these drugs activated specifically in tumor cells have the potential to reduce toxicity on normal cells. For example, the nitrogen mustards are cytotoxic agents, whereas boron-containing aromatic nitrogen mustard **1** showed lower cellular toxicity due to the electron-withdrawing effect of the boron atom, making the electron cloud around the nitrogen mustard delocalize to the boron and thus inhibit the formation of the electrophilic aziridinium ring. Importantly, the boron ester in **1** could be oxidized to a hydroxyl group with electron-donating effects in the presence of reactive oxygen species (ROS) in cancer cells, regenerating cross-linking activity (Figure 1A).[31]

In this context, we designed and synthesized a novel class of O^2-(2,4-dinitrophenyl)1-[*N*,*N*-bis(2-substituted ethyl)amino]diazen-1-ium-1,2-diolates **4**–**6** (Figure 1B). We hypothesized that the diazeniumdiolate moiety, acting as an electron-withdrawing group, could make the lone pair of the nitrogen in the secondary amine delocalize to the diazeniumdiolate moiety and thus mask the DNA cross-linking activity of the *N*,*N*-bis(2-leaving group substituted ethyl)amino functionality. Once GSH/GST, highly expressed in cancer cells,[32] attacks the carbon atom

Received: July 27, 2016
Published: October 4, 2016

DOI: 10.1021/acs.orglett.6b02222
Org. Lett. 2016, 18, 5196−5199

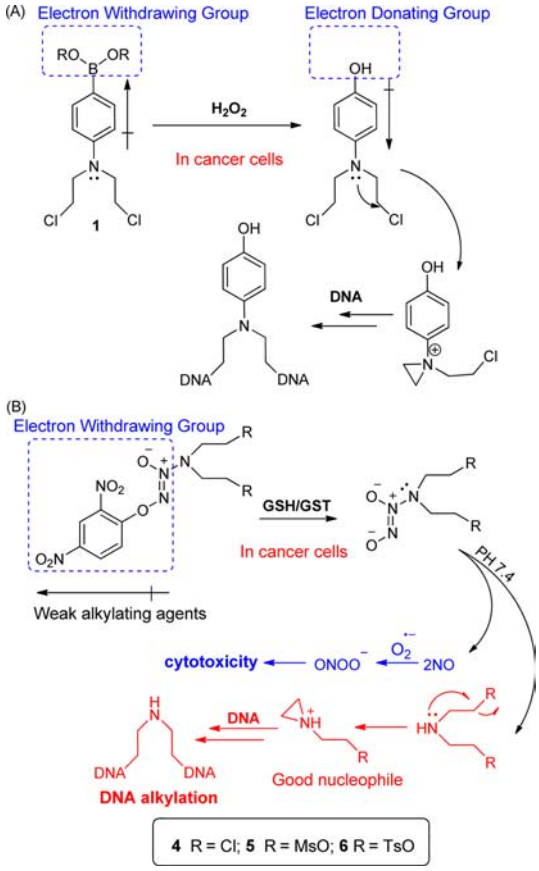


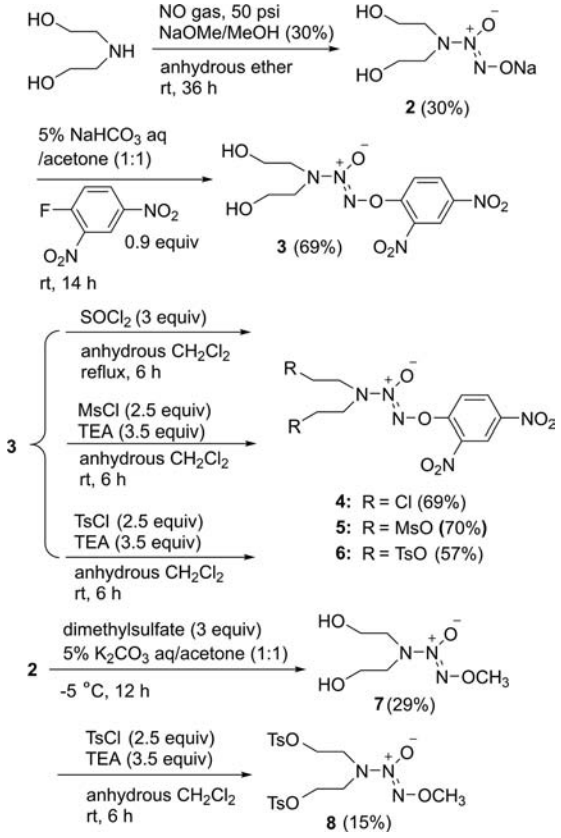
Figure 1. (A) ROS-inducible DNA cross-linking agent **1**; (B) rational design for O^2-(2,4-dinitrophenyl) 1-[*N*,*N*-bis(2-substituted ethyl)-amino]diazen-1-ium-1,2-diolates **4**–**6**.

connecting to O^2 atom on the benzene ring, the diazeniumdiolate moiety would be liberated, which could spontaneously release *N*,*N*-bis(2-substituted ethyl)amine and two molecules of NO. The former may regenerate the DNA cross-linking effect while the latter may display cytotoxicity as reported previously,[13,18−20] leading to synergistic anticancer effects.

The synthetic route of the target compounds **4**–**6** was depicted in Scheme 1. Sodium 1-[*N*,*N*-bis(2-hydroxyethyl)-amino]diazen-1-ium-1,2-diolate **2**, prepared by the reaction of NO gas (50 psi) with 2,2′-dihydroxydiethylamine in the presence of NaOMe, was treated with 2,4-dinitrofluorobenzene to offer O^2-substituted intermediate **3**. Chlorination of **3** in the presence of sulfoxide chloride with a catalytic amount of dimethylformamide (DMF) in anhydrous dichloromethane produced dichloride **4**. Treatment of **3** with methylsulfonyl chloride in the presence of trimethylamine (TEA) furnished compound **5**, while the reaction of **3** with p-toluenesulfonyl chloride gave compound **6**. Compound **8** was designed and synthesized as a negative control since O^2-methyl is unable to be cleaved in the presence of GSH/GST. Methylation of **2** in the presence of dimethylsulfate in 5% K_2CO_3 aqueous solution and acetone produced compound **7**. Finally, the reaction of **7** with *p*-toluenesulfonyl chloride provided compound **8**. The diethylamine-based mustard compound **11** without the "NONO" moiety was synthesized to evaluate the specific contribution of mustard moiety to the cytotoxicity using a previously reported method (Scheme S1).[33]

We first evaluated the antiproliferative activity of compounds **4**–**6**, **8**, and **11** against human colon carcinoma HCT116 cells, human ovarian OVCAR5 cells, human leukemia HL-60 cells,

Scheme 1. Synthetic Route of Compounds 4–6, 8



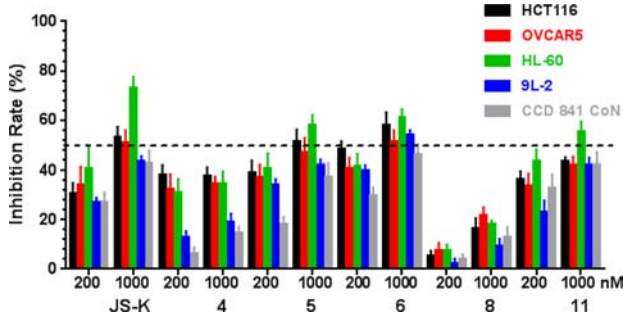


Figure 2. Inhibitory rates of compounds **4**–**6**, **8**, **11**, and JS-K against HCT116, OVCAR5, HL-60, 9L-2, and CCD 841 CoN cell lines. Cells were incubated with the indicated compounds at 200 and 1000 nM for 72 h, and cell proliferation was assessed by the MTT assay.

murine gliosarcoma 9L-2 cells, and human normal colonic epithelial CCD 841 CoN cells by MTT assay using JS-K as a control. As shown in Figure 2, compounds **5** and **6** exhibited comparable or more potent antiproliferative activity against four cell lines relative to JS-K. Compound **4** with a chlorine atom showed weaker antiproliferative activity than mesylate compound **5** and tosylate **6**. O^2-Methyl analogue **8** showed much lower inhibitory activity even at a concentration of 1000 nM, whereas compound **11**, as the diethylamine moiety of **6**, possessed less antiproliferative activity than **6**, suggesting that the antiproliferative activity of compounds **4**–**6** may be attributable to both the mustard and diazeniumdiolate moiety. All target compounds **4**–**6** showed less cytotoxicity on human normal colonic epithelial CCD 841 CoN cells than human colon carcinoma HCT116 cells, indicating somewhat selective activity against cancer cells.

DOI: 10.1021/acs.orglett.6b02222
Org. Lett. 2016, 18, 5196−5199

Table 1. IC_{50} Values of Compounds 5, 6, 11, and JS-K against HCT116, OVCAR5, HL-60, 9L-2, and CCD 841 CoN Cells[a]

	IC_{50} (nM)			
compd	5	6	11	JS-K
HCT116	1060 ± 92.4	386.6 ± 41.7	1201 ± 108.4	698.1 ± 83.4
OVCAR5	1316 ± 125	971.7 ± 104.2	1867 ± 224.2	1021 ± 114.7
HL-60	485.5 ± 62.3	350.5 ± 29.7	613.7 ± 88.5	280.1 ± 32.5
9L-2	2003 ± 235	735.5 ± 89.3	1631 ± 191.3	1995 ± 214.6
CCD 841 CoN	2371 ± 285.3	1447 ± 132.2	1603 ± 211.7	1161 ± 126.8

[a]Cells were incubated with indicated compounds at different concentrations for 72 h, and cell proliferation was assessed by the MTT assay. Data were expressed as the mean ± SD of each group of cells from five individual experiments.

The IC_{50} values of compounds **5** and **6**, mustard compound **11**, as well as positive control JS-K against four cancer cells lines and one normal cell line were then measured using the MTT assay, and the results are summarized in Table 1. Although JS-K (280.1 ± 32.5 nM) exhibited somewhat more potent antiproliferative activity than compounds **5** (485.5 ± 62.3 nM), **6** (350.5 ± 29.7 nM), and **11** (613.7 ± 88.3 nM) against HL-60 cells, compound **6** exhibited much more potent antiproliferative activity against HCT116, OVCAR5, and 9L-2 cell lines than compound **5**, **11**, and JS-K. Compound **6** (735.5 ± 89.3 nM) significantly inhibited the growth of 9L-2 cells relative to **5** (2003 ± 235 nM), **11** (1631 ± 191.3 nM), and JS-K (1995 ± 214.6 nM). Importantly, **6** showed better selective activity against human colon carcinoma HCT116 cells relative to human normal colonic epithelial CCD 841 CoN cells than other peer compounds. These results suggest that the substituted diethylamines might possess potential cross-linking activity, which could be beneficial for antiproliferative activity of target compounds.

The NO release behaviors of **6** along with JS-K in HCT116 were further examined using both NO-sensitive fluorophore, 4-amino-5-(methylamino)-2′,7′-difluorofluorescein diacetate (DAF-FM DA),[34] and Griess reagent.[35] It was observed that **6** showed more significant fluorescence (Figure 3A) and produced

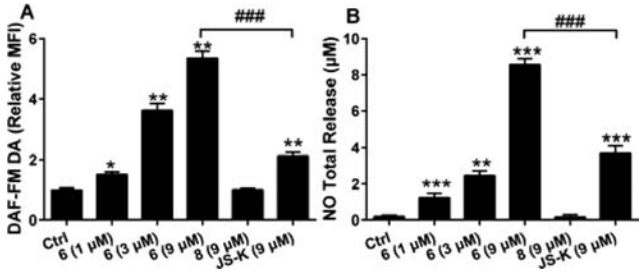


Figure 3. (A) HCT116 cells was treated with the indicated concentrations of compound **6**, **8**, and JS-K for 8 h, stained with DAF-FM DA, and analyzed by fluorescence-activated cell sorting (FACS). (B) Analyzed by Griess assay. Data shown here were representative of three different experiments. Data are presented as means ± SD ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group in HCT116 cells. $^{\#\#\#}P < 0.001$.

greater amounts of nitrite (Figure 3B) in a dose-dependent manner in HCT116 cells than JS-K, whereas **8** could not release NO in HCT116 cells. Furthermore, the studies of decomposition kinetics of **6** clarified the GST/GSH specific activation (Figure S1), which has been well studied for JS-K.[17]

Next, DNA ICL activity of compound **6** and JS-K in the 9L-2 cell line was examined using a well-established alkaline comet assay.[36] As visually shown in Figure 4, **6** exhibited significant DNA ICL activity in a dose-dependent manner, and the obvious

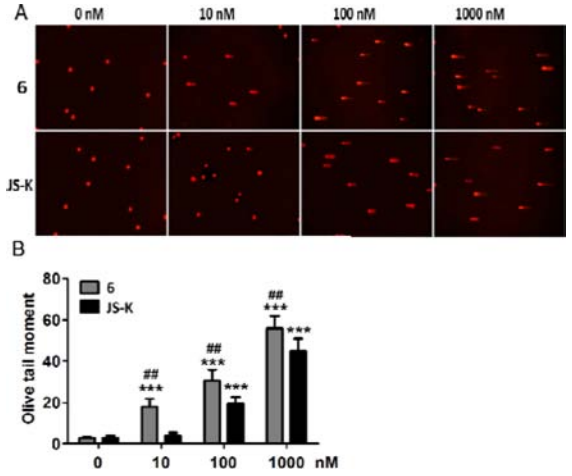


Figure 4. (A) Effect of **6** on 9L-2 cell DNA damage. 9L-2 cells were treated with **6** (0, 10, 100, 1000 nM) or JS-K (0, 10, 100, 1000 nM) for 2 h, cells were collected, and alkaline comet electrophoresis was performed. (B) The quantitative analysis was performed with the comet analysis software CASP, and the olive tail moment was employed to evaluate DNA damage. Assays were repeated three times, and data were expressed as mean ± SD. $^{***}p < 0.001$, vs control, $^{\#\#}p < 0.01$, vs the same concentration of JS-K.

cellular DNA damage is observed as comet tails at the low concentration of 10 nM. In comparison, JS-K showed much lower DNA damage activity than **6**, and no obvious cellular DNA damage was observed at 10 nM. Notably, the diazeniumdiolate moiety, liberated from **6**, could simultaneously release the *N*,*N*-bis(2-TsO-ethyl)amino moiety and two molecules of NO shown in Figure 1B. As a nitrogen mustard analogue, the former may display potent and synergetic activity together with the latter, making **6** more active in inducing DNA damage than JS-K.

As an important class of NO donors, diazeniumdiolate, has attracted significant attention because of its capability to specifically release NO into tumor cells after rational O^2-modification, generating selective anticancer activity.[14] To the best of our knowledge, the modification of secondary amine moiety in diazeniumdiolates to enhance the cytotoxicity has not yet been reported. In this study, we designed and synthesized a novel class of O^2-(2,4-dinitrophenyl) 1-[*N*,*N*-bis(2-Cl, MsO, and TsO ethyl)amino]diazen-1-ium-1,2-diolates **4**–**6**, respectively. All compounds exhibited moderate to potent antiproliferative activity against cancer HCT116, OVCAR5, HL-60, and 9L-2 cell lines. Among them, the most active compound **6** showed more potent activity than its diethylamine compound **11** and JS-K. In sharp contrast, the analogue of **6**, O^2-methyl diazeniumdiolate **8**, which was reluctant to liberate diazeniumdiolate anion in cancer cells, showed much lower inhibitory activity even at the concentration of 1000 nM. Compound **6** was able to release

DOI: 10.1021/acs.orglett.6b02222
Org. Lett. 2016, 18, 5196–5199

NO in HCT116 cells in a dose-dependent manner, whereas **8** failed to release NO in HCT116 cells. Importantly, in comparison with JS-K, **6** showed better selective inhibition of HCT116 cancer cells while sparing noncancer cell CCD 841 CoN cells. Further alkaline comet assay indicated that **6** exhibited more potent DNA ICL activity than JS-K. Collectively, these results support the proposed mechanism of action as shown in Figure 1B; i.e., in the absence of GSH/GST, the diazeniumdiolate moiety in **6** masked its DNA ICLs activity, but in the presence of GSH/GST in cancer cells the *N*,*N*-bis(2-TsO-ethyl)amino moiety could be generated upon diazeniumdiolate moiety release to exert potent DNA ICL activity together with cytotoxicity of NO synergistically to enhance the anticancer activity. The assessment of in vivo anticancer activity for **6** will be conducted in the due course.

Importantly, the modification of the secondary amine moiety in the diazeniumdiolate to the substituted diethyl amines with inducible DNA cross-linking effects could be potentially expanded to other diazeniumdiolate compounds such as O^2-derived diazeniumdiolate activated by basic natural amino acids via a β-elimination cleavage reaction,[37] DT-diaphorase,[38] and nitroreductase[39] to improve the anticancer activity. Our findings may provide better insight into the design of anticancer drug pertaining to diazeniumdiolate-based NO donors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02222.

Experimental procedures and compound characterization for all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (Nos. 81273378, 21372261, and 81673305) and Jiangsu Province Funds for Distinguished Young Scientists (BK20160033). Part of the work was supported by the opening project (SKL201513SIC) of the State Key Laboratory of High Performance Ceramics and Superfine Microstructure, Program for New Century Excellent Talents in University (NCET-13-1033), and the Jiangsu Shuang Chuang team.

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DOI: 10.1021/acs.orglett.6b02222
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