ChemPubSoc Europe

DOI: 10.1002/chem.201400090

CHEMISTRY A European Journal Full Paper

Anticancer Drugs

Aromatic Nitrogen Mustard-Based Prodrugs: Activity, Selectivity, and the Mechanism of DNA Cross-Linking

Wenbing Chen, Yanyan Han, and Xiaohua Peng*[a]

Abstract: Three novel H_2O_2-activated aromatic nitrogen mustard prodrugs (**6**–**8**) are reported. These compounds contain a DNA alkylating agent connected to a H_2O_2-responsive trigger by different electron-withdrawing linkers so that they are inactive towards DNA but can be triggered by H_2O_2 to release active species. The activity and selectivity of these compounds towards DNA were investigated by measuring DNA interstrand cross-link (ICL) formation in the presence or absence of H_2O_2. An electron-withdrawing linker unit, such as a quaternary ammonia salt (**6**), a carboxyamide (**7**), and a carbonate group (**8**), is sufficient to deactivate the aromatic nitrogen mustard resulting in less than 1.5% cross-linking formation. However, H_2O_2 can restore the activity of the effectors by converting a withdrawing group to a donating group, therefore increasing the cross-linking efficiency (>20%). The stability and reaction sites of the ICL products were determined, which revealed that alkylation induced by **7** and **8** not only occurred at the purine sites but also at the pyrimidine site. For the first time, we isolated and characterized the monomer adducts formed between the canonical nucleosides and the aromatic nitrogen mustard (**15**) which supported that nitrogen mustards reacted with dG, dA, and dC. The activation mechanism was studied by NMR spectroscopic analysis. An in vitro cytotoxicity assay demonstrated that compound **7** with a carboxyamide linker dramatically inhibited the growth of various cancer cells with a GI_{50} of less than 1 μM, whereas compound **6** with a charged linker did not show any obvious toxicity in all cell lines tested. These data indicated that a neutral carboxyamide linker is preferable for developing nitrogen mustard prodrugs. Our results showed that **7** is a potent anticancer prodrug that can serve as a model compound for further development. We believe these novel aromatic nitrogen mustards will inspire further and effective applications.

Introduction

Chemical agents capable of inducing interstrand cross-links (ICL) have attracted growing interest in chemistry, medicine, and chemical biology. They are used for DNA damage and repair studies and for nucleic acid detection.[1] Some DNA cross-linking agents are used for cancer treatment. Over the past few decades, several research groups have developed novel chemical methods for inducing ICL formation, such as photoirradiation,[2] reduction,[3] oxidation,[4] or fluoride induction.[5] Among these methods, oxidation-induction of ICL formation under physiological conditions is probably least developed.[4] Recently, Greenberg and Zhou's group described a new way for producing ICLs in duplex DNA under mild oxidative conditions by using phenyl selenide precursors.[4a–c] Our group has shown that aryl boronic acids and esters can be used as trigger units for developing ROS-activated DNA cross-linking agents by hydrogen peroxide mediated oxidation.[6] H_2O_2-induced ICL formation could be performed in cells as H_2O_2 is generated endogenously. For example, boronate-based probes have been used for selective detection and imaging of hydrogen peroxide in cells.[7] Thus, boronate-based DNA cross-linking agents may lead to a wide variety of new applications in biology and the life sciences.

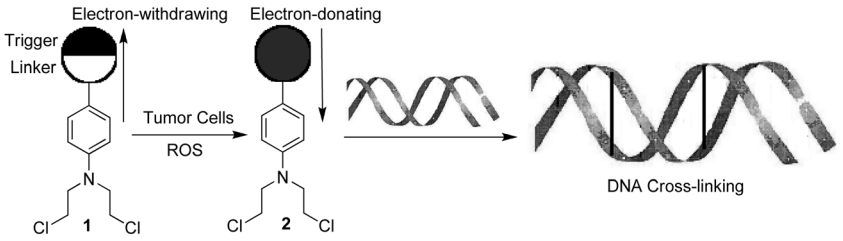
It is well-known that cancer cells exhibit elevated intrinsic oxidative stress.[8] Higher levels of H_2O_2 were found in cancer cells than that in normal ones. Thus, the H_2O_2-inducible cross-linking behaviors of boronate-based agents will provide a novel strategy for tumor-specific damage, which will allow the development of selective anticancer prodrugs. H_2O_2-activated DNA cross-linking agents should comprise two separate functional domains: a ROS-accepting moiety (trigger) and a bifunctional DNA alkylating agent (effector), joined by a "linker system" in such a way that the reaction of the trigger causes a large increase in the cytotoxic potency of the effector. The identification of such compounds requires a systematic design of the efficient triggers, the suitable linkers, and the effectors. In addition, the overall characteristics of the entire prodrug require consideration as well. Recently, our group has reported several arylboronate derivatives that can be activated by hydrogen peroxide to release either mechlorethamine or quinone methides cross-linking DNA.[6] However, these molecules did not show potent anticancer activity, which might be caused by the presence of a quarternary ammonia salt linker. It is well-known that the charged molecules cannot diffuse across cell

[a] Dr. W. Chen, Dr. Y. Han, Prof. X. Peng
Department of Chemistry and Biochemistry
University of Wisconsin-Milwaukee
3210 N. Cramer St, Milwaukee, WI 53211 (USA)
E-mail: pengx@uwm.edu

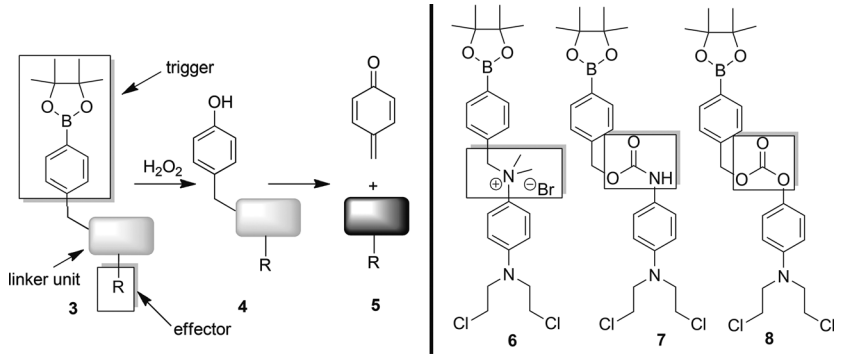
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201400090.

membranes, which is not suitable for drug development. In addition, the structures of the cross-linked DNA adducts were not determined. Thus, there is considerable scope for developing more potent ROS-activated DNA cross-linking agents and providing detailed mechanisms for DNA damages induced by these compounds.

In this work, we investigated the effect of boronates on the activity of aromatic nitrogen mustards that are the major class of traditional alkylating agents that cause cell death by inducing DNA cross-links.[1a,9] Some of them are the frequently used antitumor agents in the clinic, such as bendamustine, melphalan, and chlorambucil. However, like all other anticancer agents, their clinical efficacy has been limited by the toxicity to normal tissues. One of the efforts to improve the selectivity of such agents towards cancer cells is to seek a unique linker-trigger system that can be cleaved under a tumor-specific microenvironment (Scheme 1). For example, a series of dinitrobenzamide nitrogen mustard prodrugs with a nitryl-amidogen linker were exploited to selectively target hypoxic tumors.[3] The selectivity was achieved by conversion of an electron-withdrawing group in the benzene ring to a donating group specifically in cancer cells, which activated the nitrogen mustard. In spite of the advances achieved in this field, the development of prodrugs targeting specific tumor cells is still in high demand. Herein, we report novel aromatic nitrogen mustard prodrugs with a H_2O_2-cleavable trigger bonded to three different linkers. These compounds showed selective DNA cross-linking ability under ROS-containing conditions and enhanced anticancer activity. In addition, the reaction sites of these nitrogen mustards were determined by isolating the alkylating products as well as by varying the DNA sequences.



Scheme 1. Selective DNA cross-linking agents with a ROS-responsive 'trigger-linker' system. The activity of the nitrogen mustards is masked by introducing an electron-withdrawing 'linker' in the prodrug, while reaction of the trigger unit with ROS converts an electron-withdrawing 'linker' to a donating group resulting in a potent ROS-activated DNA cross-linking agent **2** ('trigger-linker' circle changes from black–white to a homogeneous black color).



Scheme 2. Strategy of prodrug design and structures of compounds **6**–**8**.

H_2O_2, a common ROS in cancer cells, is well known to react with arylboronic ester **3** under physiological conditions to generate phenol derivative **4** that subsequently releases compound **5**.[7d,e,10,11] As the activity of nitrogen mustard depends on the availability of lone-pair electrons on the nitrogen atom,[6b] we expect that the cross-linking ability of the aromatic nitrogen mustard can be tuned by varying the aromatic substituents. On the basis of this, we designed structure **3** with the aromatic nitrogen mustard (effector) connected to the arylboronate (trigger) by an electron-withdrawing group (linker) (Scheme 2). These compounds are expected to be inactive as the activity of nitrogen mustards is shielded by the withdrawing linker units. However, the reaction of the aryboronates with H_2O_2 can activate the aromatic nitrogen mustards by converting a *para* electron-withdrawing to a donating group. Thus, such compounds are expected to be potent selective alkylating agents towards H_2O_2.

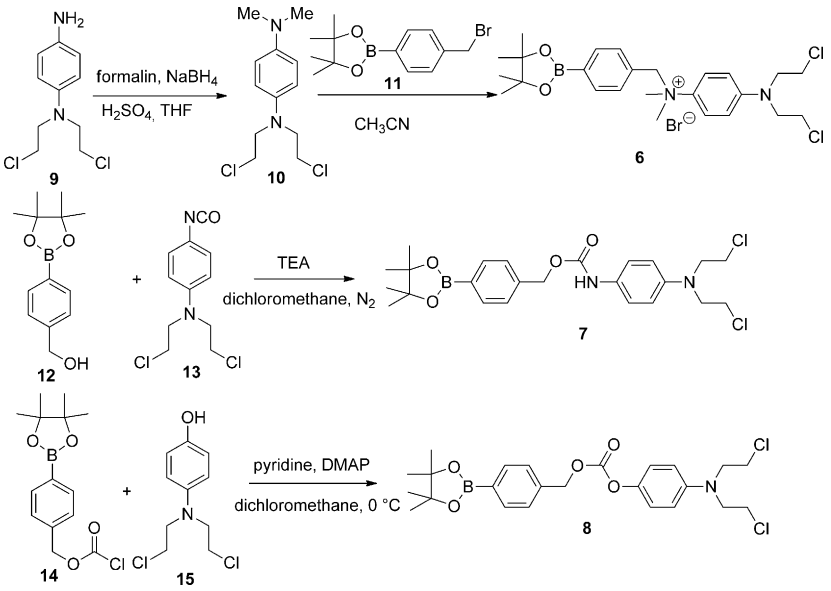
Results and Discussion

Synthesis

To test our hypothesis, we chose to prepare compounds **6**–**8** containing three different electron-withdrawing linker units including a quaternary ammonia salt (**6**), a carboxyamide (**7**), and a carbonate group (**8**). The synthesis of these prodrugs was accomplished in a straightforward fashion from the corresponding aromatic nitrogen mustards **10**, **13**, or **15**, respectively (Scheme 3). Quaternary ammonia salt **6** was obtained from benzyl bromide **11** and compound **10** that was synthesized from the aniline derivative **9**[12] by the Eschweiler–Clarke methylation. Treatment of benzyl alcohol **12** with isocyanatobenzene **13**[13] produced **7** with a quantitative yield. The reaction of benzyl chloroformate **14**[7f] with phenol derivative **15**[12] resulted in compound **8**.

DNA cross-linking

To identify the effective linkers capable of masking the toxicity of the nitrogen mustard and triggering its activity upon H_2O_2



Scheme 3. Synthesis of compounds **6**–**8**.

treatment, we first investigated the activity and selectivity of these compounds by determining their ability to form DNA ICLs in the presence or absence of H_2O_2. Initially, a 49-mer DNA duplex **16** was employed in the DNA cross-linking experiments that were carried out under physiological conditions (Figure 1). ICL formation and cross-linking yields were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) with phosphorimager analysis (Image Quant 5.2) by taking advantage of the differing mobility of ICL products and single-stranded DNA. The selectivity of the ICL formation induced by compounds **6**–**8** towards H_2O_2 is illustrated in Figure 1. In the absence of H_2O_2, very few DNA ICLs (1.0–1.5%) were observed for compounds **6**–**8** (1 mM) (Figure 1, lanes 2, 4, 6). These data indicated that the three electron-withdrawing linkers are sufficient to deactivate the aromatic nitrogen mustards. To our delight, addition of H_2O_2 greatly increased the ICL yields to 20% (Figure 1, lanes 3, 5 and 8). Even at a lower concentration (300 μM) of **6**, the ICL yield was increased about five times (10.8%) in comparison with that without H_2O_2. In a control experiment in which only H_2O_2 was added, ICL formation was not observed (Figure 1, lane 1). Obviously, H_2O_2 is capable of triggering the activity of these prodrugs. As we proposed, the conversion of an electron-withdrawing boron group to a donating hydroxyl group by H_2O_2 produces the phenol derivatives greatly increasing the electron density of mustard nitrogen and therefore facilitating the ICL formation.

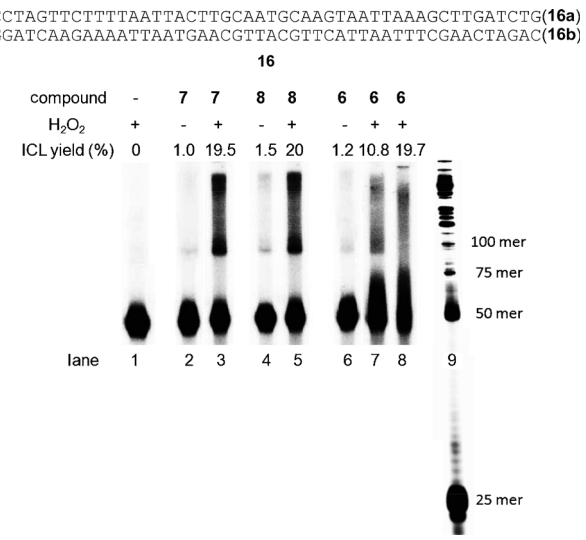


Figure 1. Selective DNA cross-linking ability of **6**–**8** towards H_2O_2. Phosphorimage autoradiogram of denaturing PAGE analysis of the cross-linking reaction of DNA duplex **16** in the presence of **6**–**8** (1.0 mM for lanes 1–6 and 8; 0.3 mM for lane 7) with or without H_2O_2 (all reactions were carried out at room temperature). Lane 9, DNA marker.

Subsequently, we investigated the effect of drug concentration, ratio of drug to H_2O_2, and pH value. In the presence of H_2O_2, DNA cross-linking induced by compounds **6**–**8** was observed at a concentration as low as 10 μM (about 2.4% ICL yield) and 1 mM of drugs resulted in 20–26% of cross-linked DNA (see Figure S1 and Table S1 in the Supporting Information). It is known that a single unrepaired ICL is sufficient to kill an eukaryotic cell and approximately 40 unrepaired ICLs can kill a mammalian cell.[14] So we believe that these compounds could be potent selective alkylating agents. A higher ratio of H_2O_2 to drug (from 1:1 to 2:1) resulted in more efficient ICL formation (see Table S2 and Figure S2 in the Supporting Information). The H_2O_2-inducible activity of **7** and **8** is pH-dependent. The most efficient ICL formation was observed under physiological pH (7.0–7.5). Acidic conditions (pH 5 and 6) and basic conditions (pH 8–9) resulted in lower ICL yields (see Table S3 and Figure S3 in the Supporting Information). It is likely that acidic conditions suppressed oxidative deboronation[15] and the activity of the nitrogen mustards,[16] while basic conditions increased the coordination of boronate ester and boronic acid with hydroxide anion, resulting in a negative charge in the boron group, which slightly blocked the reaction of boron with H_2O_2.[10]

The time course study of ICL formation induced by compounds **7** and **8** showed that the DNA cross-linking formation was completed within 24 h (Figure 2). The ICL growth followed first-order kinetics with a rate constant (k_{ICL}) of $2.25 \pm 0.10 \times 10^{-5}$ s^{-1} for **7** and $2.69 \pm 0.25 \times 10^{-5}$ s^{-1} for **8**, respectively.

It is well-known that ICL induced by nitrogen mustards mainly occurred at N-7 of dG,[17] thus the stability and reaction sites of the ICL products were examined to provide further insight into the reactivity of compounds **7** and **8**. We isolated

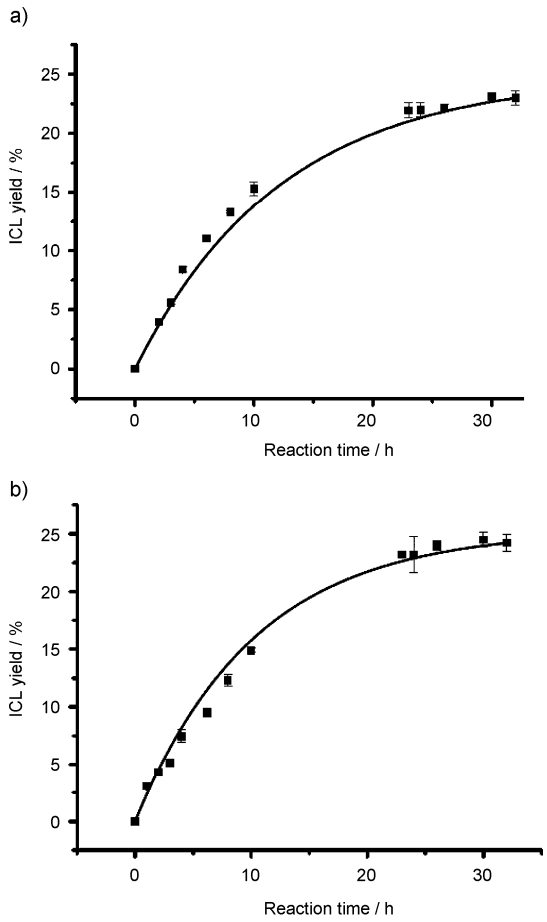


Figure 2. Rate of ICL growth from **16** upon treatment with **7**/H_2O_2 (a) or **8**/H_2O_2 (b).

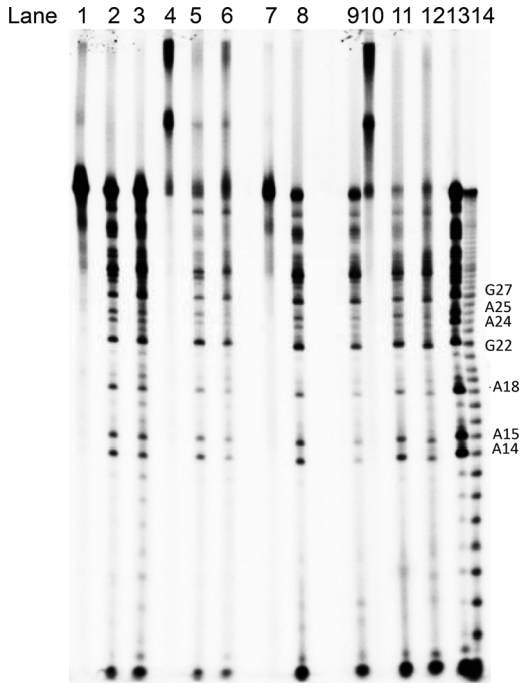


Figure 3. Phosphorimage autoradiogram of 20% denaturing PAGE analysis of the isolated ICL product and single-stranded DNA upon heating in piperidine or phosphate buffer (The ICL product and single-stranded DNA were isolated from reaction of duplex **16** with **7** or **8** in the presence of H_2O_2, **16a** was radiolabeled at the 5′-terminus). Lanes 1-3: single-stranded DNA induced by **7**/H_2O_2; lanes 4-6: ICL induced by **7**/H_2O_2; lanes 7–9: single-stranded DNA induced by **8**/H_2O_2; lanes 10-12: ICL induced by **8**/H_2O_2; lanes 1, 4, 7, and 10: control (no treatment); lanes 2, 5, 8, and 11: treated by heating at 90 °C in buffer (pH 7.0); lanes 3, 6, 9, and 12: treated by heating at 90 °C in piperidine; lane 13: G+A sequencing of **16a**; lane 14: Fe·EDTA (EDTA=ethylenediaminetetraacetate) treatment of **16a**.

the ICL products and monoalkylated single-stranded DNA, which were heated in a phosphate buffer (pH 7) or in 1.0 M piperidine (90 °C). Piperidine is known to induce cleavage with N-7 alkylated purines according to the Maxam–Gilbert sequencing procedure.[4c,18] The DNA ICLs were completely destroyed after heating for 30 min, which led to obvious cleavage bands at dGs and dAs, such as G97, G96, G90, A89, G71, G58, A57, G52, G44, G40, A39-37, A31, G27, G22, A18, A15, A14, and G6 (Figure 3 and Figures S4–S6). Our results indicated that the alkylation induced by **7** and **8** mainly occurred at the purine sites, which is consistent with previous observation. Similar cleavage patterns were observed with single-stranded DNA which showed that in addition to ICL formation, compounds **7** and **8** also induced an intrastrand cross-link that is deleterious to cells as well.

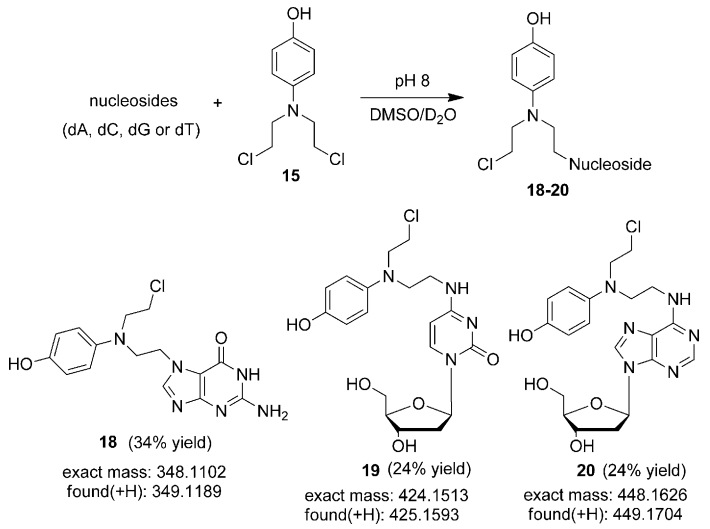
To investigate whether the ICL could occur with pyrimidines, we tested the ICL reaction with duplex **17** containing dCs/dTs in one strand and dGs/dAs in the complimentary strand. Obvious ICL formation was observed when duplex **17** was treated with **7**/H_2O_2 (7.5%) or **8**/H_2O_2 (11%). Although the ICL yields with **17** were not as high as that with **16**, these results clearly showed that ICL induced by **7** or **8** not only occurred at purine sites but also at pyrimidine sites (see Figure S7 in the Supporting Information). One of the possible reasons for the lower ICL yield obtained from duplex **17** (21-mer) than from **16** (49-mer) is shorter DNA sequences. To the best of our knowledge, this is the first report that nitrogen mustard derivatives could also cross-link pyrimidines.[17]

5′-dGCCTAGTTCTTTTAATTACTTGCAATGCAAGTAATTAAAGCTTGATCTG(**16a**)
3′-dCGGATCAAGAAAATTAATGAACGTTACGTTCATTAATTTCGAACTAGAC(**16b**)
16

5′-dCCCTTCTTTTCTTTTCTTCCC(**17a**)
3′-dGGGAAGAAAAGAAAAGAAGGG(**17b**)
17

Nucleoside alkylation selectivity

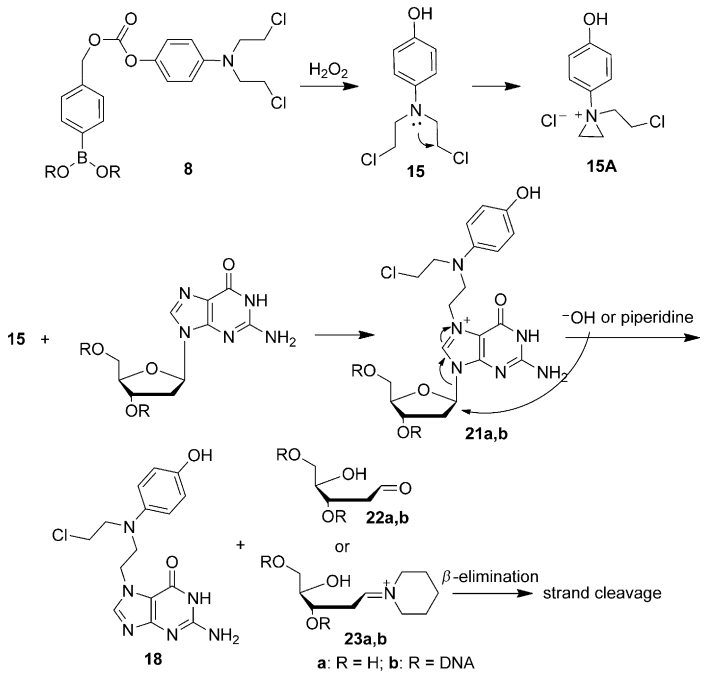
To gather the direct proof for the DNA cross-linking sites and to examine the reactivity of these drugs towards DNA, we performed a monomer reaction by treating the activated drug with four canonical nucleosides (dA, dC, dG, and dT) in a DMSO/PBS (pH 8) solution. Since the reaction of **8** with H_2O_2 releases compound **15** that is the direct DNA cross-linking agent, **15** was employed in the monomer reaction. After a two-day reaction, the new products were observed with dG, dC, and dA but not with dT. After separation and purification,



Scheme 4. Reactions of mononucleosides with compound **15**.

18 (34%), **19** (24%), and **20** (24%) were obtained and characterized by NMR spectroscopy and HRMS (Scheme 4). Collectively, our results suggested that compounds **7** and **8** could alkylate dC, dG, and dA upon hydrogen peroxide treatment. Thus, we conclude that the ICL induced by the aromatic nitrogen mustards occurred with both purines and pyrimidines.

On the basis of the monomer reaction, the mechanism for DNA alkylation was proposed (Schemes 5 and 6). We propose that compound **18** resulted from a deglycosylation of the corresponding N7 adduct of dG (**21a**) (Scheme 5). A similar mechanism was proposed for the DNA ICL reaction induced by compound **8**. Treatment of the inactive **8** with H_2O_2 generated **15** which underwent an intramolecular nucleophilic substitution reaction to form highly electrophilic intermediate **15A**. It is well known that N-7 of dG is the most active alkylation site among all nucleosides. Thus, dG in DNA easily reacts with **15A** generating monoalkylated single-stranded adduct **2b**. Subsequently, other nucleotides (dG, dA, or dC) in the complementary strand react with **21b** producing DNA ICLs (Scheme 5). N-7 alkylated dG adducts are labile under basic conditions or in the presence of strong nucleophiles such as piperidine. For example, a deglycosylation of **21a,b** easily occurred generating **18** and sugar residues **22a,b** or **23a,b** under these conditions (Scheme 5). Compound **23b** further induced strand cleavage after β-elimination. Therefore, cleavage bands were observed at dGs when monoalkylated ODNs or ICLs were heated in 1.0 M piperidine (Figure 3).



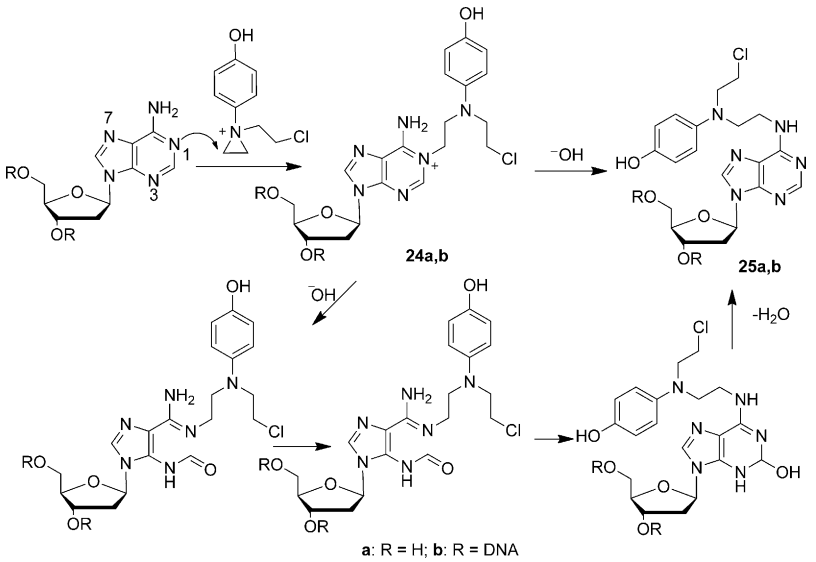
Scheme 5. Mechanism for DNA alkylation at dG and deglycosylation of N7-alkylated dG adduct under basic conditions.

An NH_2-N^6 alkylated dA adduct **20** might result from the rearrangement of the N-1 adduct **24a** by a proposed mechanism shown in Scheme 6.[4c,19a,d,e] It was reported that the basicity of the ring nitrogen of N9-substituted adenine decreased in the order N1 > N7 > N3.[20] Thus, alkylation with **15A** preferentially occurred at N1 of dA providing adduct **24b**, which further reacted with other nucleobases in the complementary strand to form DNA ICLs. Meanwhile, the N1-alkylated adduct **24a,b** can rearrange to the N6-alkyladenine **25a,b**, which is facilitated under basic conditions (Scheme 6).[19] However, we could not exclude that the DNA alkylation might also occur at N7 or N3 of dA in DNA.[19c]

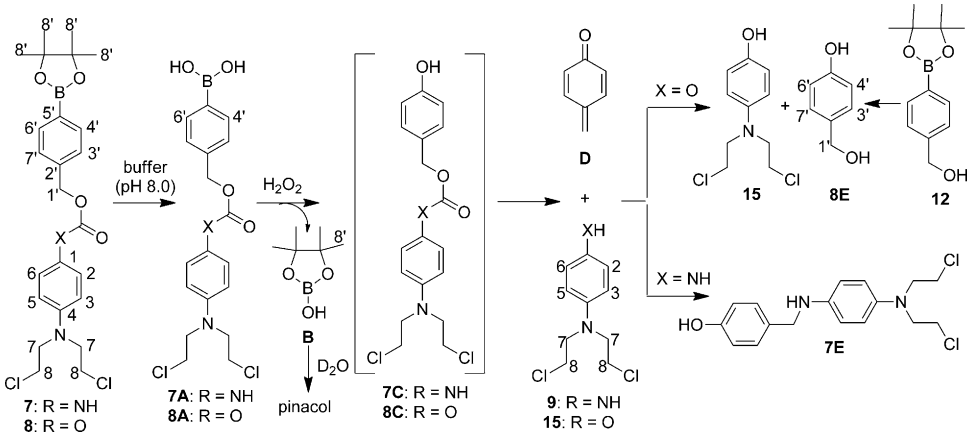
There are very few reports about the reaction of nitrogen mustards with pyrimidine nucleosides.[17] We found that the aromatic nitrogen mustard **15** can react with dC but not with dT. The structure of the monomer adduct **19** suggested that the alkylation of dC occurred at N4 (Scheme 4).

Determination of the activation mechanism by NMR spectroscopic analysis

NMR spectroscopic analysis of the monomer reactions was used to determine the activation mechanism of compounds **7** and **8** by H_2O_2 (Scheme 7). The reaction of **7** or **8** with H_2O_2 was carried out in a mixture of 1 mM deuterated potassium phosphate buffer (pH 8.0) (50 μL) and [D_6]DMSO (450 μL). Without addition of H_2O_2, hydrolysis of boronate ester **8** easily occurred in the phosphate buffer yielding boronic acid **8A** and pinacol. This was evident by the appearance of $C_{4',6'}$–H (**8A**, $\delta = 7.78$–7.77 ppm) and $C_{8'}$–H (pinacol, $\delta = 1.05$ ppm) (see Figure S8 in the Supporting Information). After the addition of H_2O_2, oxidative deboronation occurred leading to the formation of **8C** and 4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-ol (**B**) ($\delta = 1.13$ ppm, $C_{8'}$–H), which was further hydrolyzed to pinacol. The formation of the direct alkylating agent **15** was indicated by the appearance of $\delta =$



Scheme 6. DNA alkylation induced by **8** occurred at dA.



Scheme 7. Activation of **7** and **8** by H_2O_2.

7.11–6.68 ppm for $C_{2,3,5,6}$–H and $\delta = 3.61$–3.56 for $C_{7,8}$–H. The quinone methide intermediate **D** rapidly hydrolyzed to **8E** which was evidenced by appearance of $C_{1'}$–H ($\delta = 4.33$ ppm) and $C_{3',4',6',7'}$–H ($\delta = 7.11$–6.68 ppm). The formation of **8E** was proved by an authentic sample obtained from the reaction of **12** with H_2O_2, the peaks of which matched exactly with that of **8E** and pinacol (see Figure S9 in the Supporting Information). The conversion of **8** to **8E** was so fast that about 73% of **8E** was generated within 30 min, and more than 93% of **8** was consumed within 2 h. These data showed that the arylboronates developed in this work are efficient H_2O_2-responsive trigger units. Different from **8**, formation of **8E** was not observed with **7** upon treatment with H_2O_2. Instead, the final product **7E** was produced by the reaction of **D** and **9** possibly due to the presence of a more nucleophilic amino group or from an intramolecular reaction of **7C** (see Figure S10 in the Supporting Information). Overall, the prodrugs developed in this work are sensitive to H_2O_2 under physiological conditions.

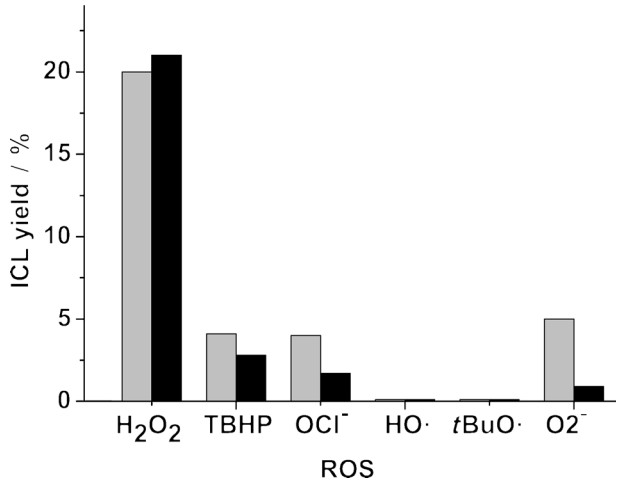


Figure 4. ICL formation induced by **7**–**8** (1 mM) upon treatment with various ROS. ■: **7**; ■: **8**.

The linker units developed are suitable for the future design of ROS-activated DNA cross-linking agents.

DNA cross-links towards different ROS

Finally, we investigated the activity of compounds **7** and **8** towards other ROS including *tert*-butylhydroperoxide (TBHP), hypochlorite (OCl^-), hydroxyl radical ($HO^•$), *tert*-butoxy radical ($tBuO^•$), and superoxide (O_2^-). Among these, hydrogen peroxide is the most efficient ROS that can trigger the activity of these prodrugs, while TBHP, OCl^-, and superoxide also slightly activate them (Figure 4). In the presence of H_2O_2, these compounds induced about 20% ICL formation, while less than 5% ICL yields were observed with OCl^-, TBHP, and superoxide). No ICL formation was induced by hydroxyl radical and *tert*-butoxy radical. The selectivity of arylboronate esters towards hydrogen peroxide is consistent with previous reports.[6]

Cytotoxicity assay

As two representatives, the cytotoxicity of compounds **6** and **7** were further evaluated in biolog-

ical systems. Initially, their ability for inhibiting cancer cell growth was determined at a single dose of 10 μM in the cell lines of leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer. Compound **6** did not show obvious toxicity in all cell lines tested, while compound **7** induced significant growth inhibition of most cancer cell lines (see Figure S11–S12 in the Supporting Information). The growth percentage of most cell lines treated with **7** was less than 50%. These data indicated that a neutral carboxyamide linker is preferable for developing ROS-activated prodrugs. The toxicity of **7** was further evaluated against the 60 cell line panel at five concentration levels to determine the GI_{50}. The GI_{50} of **7** in most cancer cell lines is less than 1 μM (Table 1). In particular, higher toxicities by **7** were observed towards leukemia, lung cancer, breast cancer, and renal cancer. It was reported that these cell lines have high intracellular concentrations of ROS that can more efficiently activate the ROS-activated prodrugs.[8] Our results showed that **7** is a potent anticancer prodrug that can be used as a lead compound for further development.

Table 1. Cytotoxicities induced by **7**.

Tumor type	Cell Line	GI_{50} [μM]
leukemia	CCRF-CEM	0.63
	HL-60(TB)	0.62
	MOLT-4	1.73
	SR	0.44
non-small cell lung cancer	A549/ATCC	3.21
	HOP-92	0.39
	NCI-H23	2.05
	NCI-H460	0.53
	NCI-H522	2.64
CNS	SF-268	1.73
	SF-295	1.80
	SF-539	2.20
	SNB-75	0.73
	LOX IMVI	1.8
melanoma	M14	3.86
	UACC-257	1.89
	UACC-62	2.26
renal	786-0	2.72
	ACHN	0.57
	CAKI-1	0.48
	RXF 393	1.43
	UO-31	2.50
prostate	DU-145	2.19
breast	MCF7	0.65
	MDA-MB-468	1.08

Conclusion

We have developed a potent ROS-activated anticancer prodrug that shows high cytotoxicity. The aromatic nitrogen mustard was employed as the effector, the activity of which is masked in the prodrugs by connecting to an arylboronate by an electron-withdrawing linker. Such nontoxic prodrugs can be activated by H_2O_2 that converts the electron-withdrawing boronate group to a donating hydroxyl group. The activation mechanism was studied by NMR spectroscopic analysis. The activity and selectivity have been determined by measuring DNA ICLs with or without H_2O_2 as well as by evaluating their ability to inhibit cancer cell growth. Less than 2% of ICL products were observed with these prodrugs in the absence of H_2O_2 while the ICL yields increased to more than 20% with the addition of H_2O_2. The stability and reaction sites of the ICL products were also determined. We, for the first time, reported that alkylation induced by nitrogen mustards not only occurred at the purine sites but also at pyrimidine sites. This is supported by isolation and determination of the monomer adducts formed between nitrogen mustard analogue **15** and three canonical nucleosides dA, dC, and dG. Compound **6** with a charged linker showed less toxicity than those containing a neutral linker unit. Among the prodrugs tested, compound **7** with a carboxyamide linker displayed the highest cytotoxicity with a GI_{50} of less than 1 μM for most cell lines tested. Our results showed that H_2O_2-activated DNA cross-linking agent **7** is a potent anticancer prodrug that can be used as a lead compound for further development. In addition, a carboxyamide linker is preferable for developing ROS-activated prodrugs. This study reveals a novel way of creating targeted anticancer prodrugs to improve the therapeutic effectiveness and selectivity of current anticancer agents.

Experimental Section

N^1,N^1-Bis(2-chloroethyl)benzene-1,4-diamine (10)

A mixture of **9** (2.5 g, 15 mmol) and sodium borohydride (1.4 g, 37.5 mmol) was added to a solution of formalin (3.7 g, 37.5 mmol) and conc. H_2SO_4 (3 mL) in THF. The resulting mixture was stirred at room temperature for 1.5 h. After being quenched with NaOH solution, extracted with dichloromethane, and washed with water, the mixture was dried over Na_2SO_4 followed by filtration, and the residue was purified by column chromatography (hexane/ethyl acetate = 6:1) to afford **10** (75% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 2.88$ (s, 6H), 3.62 (d, $J = 3.6$, 8H), 6.73–6.81 ppm (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 40.9$, 42.1, 54.5, 115.3, 115.9 ppm; HRMS-ES: *m/z*: calcd for $C_{12}H_{19}N_2Cl_2$: 261.0290 $[M+H]^+$; found: 261.0920.

4-[Bis(2-chloroethyl)amino]-*N*,*N*-dimethyl-*N*-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl]benzenaminium bromide (6)

Compound **10** (520 mg, 2 mmol) and **11** (600 mg, 2 mmol) were mixed in acetonitrile (10 mL) at room temperature and the mixture was stirred overnight. After removal of the solvent, ether (5 mL) and CH_2Cl_2 (5 mL) were added and the product was filtered and washed with more ether (10 mL). Compound **6** (1.0 g, 89% yield); ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 1.28$ (s, 12H), 3.51 (s, 6H), 3.76–3.81 (m, 8H), 4.98 (s, 2H), 6.88 (d, $J = 9.3$, 2H), 7.10 (d, $J = 8.1$, 2H), 7.58–7.62 ppm (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 25.1$, 41.6, 52.0, 53.3, 72.0, 84.5, 112.4, 123.1, 131.8, 132.5, 134.1, 134.8, 147.7 ppm; HRMS-ES: *m/z*: calcd for $C_{25}H_{36}BN_2O_2Cl_2$: 477.2241 $[M-Br]^+$; found: 477.2262.

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl 4-[bis(2-chloroethyl)amino]phenylcarbamate (7)

Compound **12** (468 mg, 2 mmol) and TEA (323 μL, 2.5 mmol) were dissolved in dichloromethane (20 mL) at 0 °C under N_2, and then

a solution of **13** (645 mg, 2.5 mmol) in dichloromethane (5 mL) was added dropwise. After stirring at the same temperature for 3 h, the solvent was evaporated, and the residue was purified by column chromatography (hexane/ethyl acetate = 5:1) to afford **7** (95% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.37 (s, 12H), 3.63 (t, J = 6.3, 4H), 3.72 (t, J = 6.3, 4H), 6.51 (brs, 1H), 6.67 (d, J = 8.7, 2H), 7.26 (brs, 1H), 7.41 (d, J = 7.8, 2H), 7.42 (d, J = 7.8, 2H), 7.84 ppm (d, J = 7.8, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 24.9, 40.5, 53.8, 66.8, 83.9, 112.8, 121.5, 127.3, 135.0, 139.2 ppm; HRMS-ES: m/z: calcd for $C_{24}H_{32}BN_2O_4Cl_2$: 493.1827 $[M+H]^+$; found: 493.1841.

4-[Bis(2-chloroethyl)amino]phenyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonate (8)

A solution of **14** (466 mg, 2 mmol) in dichloromethane (2 mL) was added dropwise to a mixture of **15** (1.2 g, 4 mmol), dry pyridine (320 μL, 4 mmol), and 4-dimethylaminopyridine (DMAP) (24 mg, 0.2 mmol) in dichloromethane (50 mL) at 0 °C. After stirring for 13 h, 1 N HCl (aq.) was added to the resulting mixture and organic phase was separated and the water phase was extracted with dichloromethane. The combined organic phase was dried over Na_2SO_4, evaporated and purified by column chromatography (hexane/ethyl acetate = 5:1) to afford **8** (33% yield). ^{1}H NMR ([D$_6$]DMSO, 300 MHz): δ = 1.31 (s, 12H), 3.72 (s, 8H), 5.27 (s, 2H), 6.76 (d, J = 9.0, 2H), 7.06 (d, J = 9.0, 2H), 7.45 (d, J = 8.1, 2H), 7.72 ppm (d, J = 8.1, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 25.1, 41.5, 52.7, 69.8, 84.2, 122.7, 122.4, 127.8, 135.1, 138.9, 142.3, 144.9, 154.0 ppm; HRMS-ES: m/z: calcd for $C_{24}H_{32}BNO_5Cl_2$: 494.1667 $[M+H]^+$; found: 494.1657.

Reactions of mononucleosides with compound 15

Compound **15** (47 mg, 0.2 mmol) and dA, dC, dG, or dT (0.1 mmol) were dissolved in a mixture of DMSO/H_2O (500/50 μL) respectively, followed by the addition of PBS (pH 8.0, 5 μL, 1 M). The mixtures were shaken for 2 days at room temperature. After evaporation of the solvent, the residues were purified by column chromatography (dichloromethane/CH_3OH = 8:1–4:1) to afford the adducts.

2-Amino-7-{2-[(2-chloroethyl)(4-hydroxyphenyl)amino]ethyl}-1*H*-purin-6(7*H*)one (18)

Yield: 34%; ^{1}H NMR ([D$_6$]DMSO, 300 MHz): δ = 3.43 (d, J = 6.6, 2H), 3.60 (d, J = 6.6, 4H), 4.26 (d, J = 6.6, 2H), 6.15 (s, 2H), 6.63 (d, J = 9.3, 2H), 6.70 (d, J = 9.3, 2H), 7.82 (s, 1H), 8.68 (s, 1H), 10.79 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 41.8, 44.5, 52.4, 53.3, 108.3, 115.4, 116.4, 140.4, 144.2, 149.5, 153.1 ppm; HRMS-ESI/APCI: m/z: calcd for $C_{15}H_{17}ClN_6O_2$: 425.1586 $[M+H]^+$; found: 425.1593.

4-{2-[(2-Chloroethyl)(4-hydroxyphenyl)amino]ethylamino}-1-[(2*R*,4*S*,5*R*)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]pyrimidin-2(1*H*)one (19)

Yield: 24%; ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 2.03–2.06 (m, 1H), 2.17–2.19 (m, 1H), 3.46–3.51 (m, 4H), 3.53–3.59 (m, 2H), 3.64–3.65 (m, 2H), 3.82 (d, J = 3.0, 1H), 4.07–4.10 (m, 2H), 4.21 (s, 1H), 5.13 (s, 1H), 5.33 (d, J = 4.5, 1H), 6.06 (dd, J = 6.0, J = 9.0, 1H), 6.62 (d, J = 9.0, 2H), 6.70 (d, J = 9.0, 2H), 7.89 (s, 1H), 8.73 (s, 1H), 9.64 ppm (brs, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 41.2, 41.9, 47.5, 49.7, 52.9, 61.2, 61.7, 70.2, 86.8, 88.4, 114.7, 115.0, 116.3, 116.4, 140.3, 140.9, 148.7, 149.8, 158.5, 170.9 ppm; HRMS-ESI/APCI: m/z: calcd for $C_{19}H_{26}ClN_4O_5$: 349.1174 $[M+H]^+$; found: 349.1189.

(2*R*,3*S*,5*R*)-5-(6-{2-[(2-Chloroethyl)(4-hydroxyphenyl)amino]ethylamino}-9*H*-purin-9-yl)-2-(hydroxymethyl)tetrahydrofuran-3-ol (20)

Yield: 24%; ^{1}H NMR ([D$_6$]DMSO, 500 MHz): 2.36–2.38 (m, 1H), 2.58–2.61 (m, 1H), 3.47–3.48 (m, 2H), 3.52–3.53 (m, 1H), 3.53–3.55 (m, 2H), 3.59–3.60 (m, 1H), 3.61–3.64 (m, 2H), 3.89–3.90 (m, 1H), 4.40–4.41 (m, 1H), 4.51–4.52 (m, 2H), 5.10 (s, 1H), 5.50 (s, 1H), 6.32 (t, J = 6.0, 1H), 6.57 (d, J = 8.5, 2H), 6.61 (d, J = 8.5, 2H), 8.31 (s, 1H), 8.72 (s, 1H), 8.95 (brs, 1H), 9.85 ppm (brs, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 42.1, 48.8, 49.0, 52.5, 54.2, 61.6, 70.8, 84.5, 88.7, 116.2, 116.3, 117.3, 119.4, 139.8, 142.9, 146.6, 148.3, 150.4, 151.1 ppm; HRMS-ESI/APCI: m/z: calcd for $C_{20}H_{27}ClN_6O_4$: 449.1699 $[M+H]^+$; found: 449.1704.

Acknowledgements

We are grateful for the financial support for this research from the National Cancer Institute (grant: 1R15A152914–01), Chronic Lymphocytic Leukemia Research Consortium (grant: 2 PO1 CA 81534), the Great Milwaukee Foundation (Shaw Scientist Award), and the UWM Research Growth Initiative (grant: RGI101X234).

Keywords: anticancer drugs · aromatic nitrogen mustard · DNA · cross-linking · hydrogen peroxide

[1] a) D. M. Noll, T. M. Mason, P. S. Miller, *Chem. Rev.* **2006**, *106*, 277–301; b) M. Weng, Y. Zheng, V. Jasti, E. Champeil, M. Tomasz, Y. Wang, A. Basu, M. Tang, *Nucleic Acids Res.* **2010**, *38*, 6976–6984; c) X. Peng, M. M. Greenberg, *Nucleic Acids Res.* **2008**, *36*, e31; d) X. Peng, A. Ghosh, B. V. Houten, M. M. Greenberg, *Biochemistry* **2010**, *49*, 11–19; e) J. Wang, H. Cao, C. You, B. Yuan, S. Gupta, C. Nishigori, L. J. Niedernhofer, P. J. Brooks, Y. Wang, *Nucleic Acids Res.* **2012**, *40*, 7368–7374; f) Y. Jiang, H. Hong, H. Cao, Y. Wang, *Biochemistry* **2007**, *46*, 12757–12763; g) Q. Zhang, Y. Wang, *J. Am. Chem. Soc.* **2003**, *125*, 12795–12802; h) H. Sun, X. Peng, *Bioconjugate Chem.* **2013**, *24*, 1226–1234.

[2] a) P. Wang, R. Liu, X. Wu, H. Ma, X. Cao, P. Zhou, J. Zhang, X. Weng, X. Zhang, J. Qi, X. Zhou, L. Weng, *J. Am. Chem. Soc.* **2003**, *125*, 1116–1117; b) S. N. Richter, S. Maggi, S. C. Mels, M. Palumbo, M. Freccero, *J. Am. Chem. Soc.* **2004**, *126*, 13973–13979; c) S. Colloredo-Mels, F. Doria, D. Verga, M. Freccero, *J. Org. Chem.* **2006**, *71*, 3889–3895; d) D. Verga, M. Nadai, F. Doria, C. Percivalle, M. D. Antonio, M. Palumbo, S. N. Richter, M. Freccero, *J. Am. Chem. Soc.* **2010**, *132*, 14625–14637; e) C. Percivalle, A. L. Rosa, D. Verga, F. Doria, M. Mella, M. Palumbo, M. D. Antonio, M. Freccero, *J. Org. Chem.* **2011**, *76*, 3096–3106; f) Y. Kuang, H. Sun, J. C. Blain, X. Peng, *Chem. Eur. J.* **2012**, *18*, 12609–12613; g) F. Doria, S. N. Richter, M. Nadai, S. Colloredo-Mels, M. Mella, M. Palumbo, M. Freccero, *J. Med. Chem.* **2007**, *50*, 6570–6579.

[3] a) G. J. Atwell, S. Yang, F. B. Pruijn, S. M. Pullen, A. Hogg, A. V. Patterson, W. R. Wilson, W. A. Denny, *J. Med. Chem.* **2007**, *50*, 1197–1212; b) R. S. Singleton, C. P. Guise, D. M. Ferry, S. M. Pullen, M. J. Dorie, J. M. Brown, A. V. Patterson, W. R. Wilson, *Cancer Res.* **2009**, *69*, 3884; c) A. V. Patterson, D. M. Ferry, S. J. Edmunds, Y. Gu, R. S. Singleton, K. Patel, S. M. Pullen, K. O. Hicks, S. P. Syddall, G. J. Atwell, S. Yang, W. A. Denny, W. R. Wilson, *Clin Cancer Res.* **2007**, *13*, 3922; d) M. Di Antonio, F. Doria, M. Mella, D. Merli, A. Profumo, M. Freccero, *J. Org. Chem.* **2007**, *72*, 8354–8360.

[4] a) I. S. Hong, M. M. Greenberg, *J. Am. Chem. Soc.* **2005**, *127*, 10510–10511; b) I. S. Hong, H. Ding, M. M. Greenberg, *J. Am. Chem. Soc.* **2006**, *128*, 485–491; c) X. Peng, I. S. Hong, H. Li, M. M. Seidman, M. M. Greenberg, *J. Am. Chem. Soc.* **2008**, *130*, 10299–10306; d) X. Weng, L. Ren, L. Weng, J. Huang, S. Zhu, X. Zhou, L. Weng, *Angew. Chem.* **2007**, *119*, 8166–8169; *Angew. Chem. Int. Ed.* **2007**, *46*, 8020–8023.

[5] a) W. F. Veldhuyzen, P. Pande, S. E. Rokita, *J. Am. Chem. Soc.* **2003**, *125*, 14005 – 14013; b) H. Wang, S. E. Rokita, *Angew. Chem.* **2010**, *122*, 6093 – 6096; *Angew. Chem. Int. Ed.* **2010**, *49*, 5957 – 5960; c) J. Wu, R. Huang, T. Wang, X. Zhao, W. Zhang, X. Weng, T. Tian, X. Zhou, *Org. Biomol. Chem.* **2013**, *11*, 2365 – 2369.

[6] a) C. Sheng, Y. Wang, X. Peng, *Chem. Eur. J.* **2012**, *18*, 3850 – 3854; b) Y. Kuang, K. Balakrishnan, V. Gandhi, X. Peng, *J. Am. Chem. Soc.* **2011**, *133*, 19278 – 19281; c) S. Cao, Y. Wang, X. Peng, *J. Org. Chem.* **2014**, *79*, 501 – 508; d) S. Cao, X. Peng, *Curr. Org. Chem.* **2014**, *18*, 70 – 85.

[7] a) E. W. Miller, A. E. Albers, A. Pralle, E. Y. Isacoff, C. J. Chang, *J. Am. Chem. Soc.* **2005**, *127*, 16652 – 16659; b) B. C. Dickinson, C. J. Chang, *J. Am. Chem. Soc.* **2008**, *130*, 9638 – 9639; c) E. W. Miller, O. Tulyathan, E. Y. Isacoff, C. J. Chang, *Nat. Chem. Biol.* **2007**, *3*, 263 – 267; d) L. C. Lo, C. Y. Chu, *Chem. Commun.* **2003**, 2728 – 2729; e) D. Srikun, E. W. Miller, D. W. Domaille, C. J. Chang, *J. Am. Chem. Soc.* **2008**, *130*, 4596 – 4597; f) C. Chung, D. Srikun, C. S. Lim, C. J. Chang, B. R. Cho, *Chem. Commun.* **2011**, *47*, 9618 – 9620.

[8] a) T. P. Szatrowski, C. F. Nathan, *Cancer Res.* **1991**, *51*, 794 – 798; b) S. Toyokuni, K. Okamoto, J. Yodoi, H. Hiai, *FEBS Lett.* **1995**, *358*, 1 – 3; c) P. T. Schumacker, *Cancer Cell* **2006**, *10*, 175 – 176; d) S. D. Lim, C. Sun, J. D. Lambeth, F. Marshall, M. Amin, L. Chung, J. A. Petros, R. S. Arnold, *Prostate* **2005**, *62*, 200 – 207; e) M. Zieba, M. Suwalski, S. Kwiatkowska, G. Piasecka, I. Grzelewska-Rzymowska, R. Stolarek, D. Nowak, *Respir. Med.* **2000**, *94*, 800 – 805; f) N. S. Brown, R. Bicknell, *Breast Cancer Res.* **2001**, *3*, 323 – 327; g) D. Trachootham, H. Zhang, W. Zhang, L. Feng, M. Du, Y. Zhou, Z. Chen, H. Pelicano, W. Plunkett, W. G. Wierda, M. J. Keating, P. Huang, *Blood* **2008**, *112*, 1912 – 1922; h) G. K. Veni, D. B. Rao, M. Kumar, B. Usha, M. Krishma, T. R. Rao, *Rec. Res. Sci. Tech.* **2011**, *3*, 55 – 58.

[9] S. R. Rajski, R. M. Williams, *Chem. Rev.* **1998**, *98*, 2723 – 2795.

[10] D. G. Hall, *Structure, Properties, and Preparation of Boronic Acid Derivatives. Overview of Their Reactions and Applications, in Boronic Acids: Preparation and Applications in Organic Synthesis and Medicine*, Wiley-VCH, Weinheim, **2006**.

[11] a) J. L. Major Jourden, S. M. Cohen, *Angew. Chem.* **2010**, *122*, 6947 – 6949; *Angew. Chem. Int. Ed.* **2010**, *49*, 6795 – 6797; b) G. G. Lewis, M. J. DiTucci, S. T. Phillips, *Angew. Chem.* **2012**, *124*, 12879 – 12882; *Angew. Chem. Int. Ed.* **2012**, *51*, 12707 – 12710.

[12] H. B. Zhang, J. J. Xue, X. L. Zhao, D. G. Liu, Y. Li, *Chin. Chem. Lett.* **2009**, *20*, 680 – 683.

[13] N. Kapuriyaa, K. Kapuriya, H. Dong, X. Zhang, T. C. Chou, Y. T. Chen, T. C. Lee, W. C. Lee, T. H. Tsai, Y. Naliapara, T. L. Su, *Bioorg. Med. Chem.* **2009**, *17*, 1264 – 1275.

[14] a) N. Magana-Schwencke, J. A. Henriques, R. Chanet, E. Moustacchi, *Proc. Natl. Acad. Sci. USA* **1982**, *79*, 1722 – 1726; b) P. D. Lawley, D. H. Phillips, *Mutat. Res.* **1996**, *355*, 13 – 40.

[15] a) H. G. Kuivila, *J. Am. Chem. Soc.* **1954**, *76*, 870 – 874; b) H. G. Kuivila, A. G. Armour, *J. Am. Chem. Soc.* **1957**, *79*, 5659 – 5662.

[16] A. Polavarapu, J. A. Stillabower, S. G. W. Stubblefield, W. M. Taylor, *J. Org. Chem.* **2012**, *77*, 5914 – 5921.

[17] a) K. Hemmimki, S. Kallama, *Reactions of nitrogen mustards with DNA in Carcinogenicity of Alkylating Cytostatic Drugs* (Eds: D. Schmahl and J. M. Kaldor), IARC Publication No. 78, Oxford University Press, Oxford, pp. 55 – 70, **1987**; b) O. P. Pieper, L. C. Erickson, *Carcinogenesis* **1990**, *11*, 1739 – 1746; c) P. Brookes, P. D. Lawley, *Biochem. J.* **1961**, *80*, 496 – 503.

[18] a) A. M. Maxam, W. A. Gilbert, *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 560 – 564; b) K. Haraguchi, M. O. Delaney, C. J. Wiederholt, A. Sambandam, Z. Hantosi, M. M. Greenberg, *J. Am. Chem. Soc.* **2002**, *124*, 3263 – 3269.

[19] a) J. W. Jones, R. Robins, *J. Am. Chem. Soc.* **1963**, *85*, 193 – 201; b) P. D. Lawley, P. Brookes, *Biochem. J.* **1963**, *89*, 127 – 138; c) C. J. Burrows, J. G. Muller, *Chem. Rev.* **1998**, *98*, 1109 – 1151; d) M. Freccero, R. Gandolfi, M. Sarzi-Amade, *J. Org. Chem.* **2003**, *68*, 6411 – 6423; e) C. Percivalle, F. Doria, M. Freccero, *Current Organic Chemistry* **2014**, *18*, 19 – 43.

[20] a) J. E. Šponer, J. Leszczynski, F. Glahé, B. Lippert, J. Šponer, *Inorg. Chem.* **2001**, *40*, 3269 – 3278; b) G. Kampf, L. E. Kapinos, R. Griesser, B. Lippert, H. Sigel, *J. Chem. Soc. Perkin Trans. 1* **2002**, *2*, 1320 – 1327; c) C.-J. Chang, J. D. Gomes, S. R. Byrn, *J. Org. Chem.* **1983**, *48*, 5160 – 5164.

Received: January 8, 2014
Published online on May 7, 2014

