Reactive Oxygen Species (ROS) Inducible DNA Cross-Linking Agents and Their Effect on Cancer Cells and Normal Lymphocytes

Wenbing Chen,† Kumudha Balakrishnan,‡ Yunyan Kuang,† Yanyan Han,† Min Fu,‡ Varsha Gandhi,‡ and Xiaohua Peng*†

†Department of Chemistry and Biochemistry, University of Wisconsin—Milwaukee, 3210 N. Cramer Street, Milwaukee, Wisconsin 53211, United States
‡Department of Experimental Therapeutics, MD Anderson Cancer Center, Houston, Texas 77030, United States

Supporting Information

ABSTRACT: Reducing host toxicity is one of the main challenges of cancer chemotherapy. Many tumor cells contain high levels of ROS that make them distinctively different from normal cells. We report a series of ROS-activated aromatic nitrogen mustards that selectively kill chronic lymphocytic leukemia (CLL) over normal lymphocytes. These agents showed powerful DNA cross-linking abilities when coupled with H2O2, one of the most common ROS in cancer cells, whereas little DNA cross-linking was detected without H2O2. Consistent with chemistry observation, in vitro cytotoxicity assay demonstrated that these agents induced 40−80% apoptosis in primary leukemic lymphocytes isolated from CLL patients but less than 25% cell death to normal lymphocytes from healthy donors. The IC50 for the most potent compound (2) was ∼5 μM in CLL cells, while the IC50 was not achieved in normal lymphocytes. Collectively, these data provide utility and selectivity of these agents that will inspire further and effective applications.

INTRODUCTION

Making use of the unique property of cancer cells is one of the most important avenues to design targeted anticancer drugs. Many types of cancer cells are under oxidative stress because of their disturbed intracellular redox balance, which makes them distinct from their “healthy” counterparts.1−5 The increased amounts of reactive oxygen species (ROS) can be a therapeutic advantage because it is an intrinsic feature of cancer cells.6−9 Recently, several anticancer agents based on the ROS-mediated mechanisms have been developed to target these specific tumor cells and have shown selective killing of cancer cells.10−14 For example, Huang and co-workers reported that β-phenethyl isothiocyanate10 and 2-methoxyoestradiol11 selectively killed human leukemia cells but not normal lymphocytes by causing further ROS stress in cancer cells. Piperlongumine was also found to selectively kill cancer cells by increasing ROS levels but had little effect on primary normal cells.13,14 Most of the existing ROS-targeting drugs focus on enhancing ROS production to inflict lethal damage. To the best of our knowledge, the drug design for targeting tumor cells containing high levels of ROS via inducing DNA interstrand cross-links (ICLs) is rarely reported.

DNA ICLs are recognized as the primary mechanism for the cytotoxic activity of many clinically useful antitumor drugs, such as chlorambucil, cyclophosphamide, bendamustine, and cisplatin. However, the severe host toxicity exhibited by these anticancer drugs continues to be a major problem in cancer chemotherapy. Prodrugs that are activated specifically in tumor cells have the potential to reduce the toxicity of the cross-linking agents for normal cells. Gates and co-workers demonstrated that several anticancer drugs displayed selective toxicity by releasing DNA damaging species selectively in tumor cells.15−17 Over the past few decades, several research groups have developed novel DNA cross-linking or alkylating agents that can induce ICL formation by oxidation, reduction, or photolysis.18−25 Recently, our group has shown that H2O2-induced DNA cross-linking behaviors provided a novel strategy for tumor-specific damage.26,27 H2O2 is one of the most...
common ROS, which is believed to be produced in large amounts in several human tumor cells.\textsuperscript{1−5} The transformed cells showed more than 10-fold increase in H$_2$O$_2$ levels.\textsuperscript{28a} Different from O$_2$•− or hydroxyl radicals that are extremely unstable, H$_2$O$_2$ has the chemical stability required to establish significant steady-state concentrations in vivo and is uncharged. These properties allow H$_2$O$_2$ to freely diffuse across plasma membranes and to travel to the cells. In addition, other ROS such as O$_2$ can also be reduced to H$_2$O$_2$ in the oxygen metabolism via O$_2$•− generation involved in hypoxia-inducible factor 1 (HIF-1) regulation.\textsuperscript{28b,c} Thus, developing H$_2$O$_2$-activated prodrugs to selectively kill ROS-containing cancer cells can be a potent strategy in cancer chemotherapies.

Such agents should consist of two separate functional domains: an efficient H$_2$O$_2$-responsive moiety “trigger” and a potent cell-damaging functional group “effector”, joined by a linker system in such a way that the reaction of the trigger with H$_2$O$_2$ causes a large increase in the cytotoxic potency of the effector (Scheme 1). The selective reaction of boronic acid or ester derivatives with H$_2$O$_2$ has been applied for fluorescent detection of H$_2$O$_2$, gene expression, point-of-care assay, and prodrug development.\textsuperscript{26,27,29−37} Recently, we have developed two types of H$_2$O$_2$-activated DNA cross-linking agents using boronic acid or ester as “trigger”. One class can release a nitrogen mustard effector upon treatment with H$_2$O$_2$, while the other can produce quinone methides cross-linking DNA. However, both did not show potent anticancer activity. We speculate that these charged molecules may not be suitable for drug development because it is well-known that charged molecules cannot diffuse across cell membrane. Here, we report a novel strategy for creating neutral H$_2$O$_2$-activated prodrugs that showed dramatically increased potency and selective cytotoxicity toward various cancer cells. For the first time we demonstrated that the direct attachment of a boron group to an aromatic ring is sufficient to mask the toxicity of the nitrogen mustard. The potential therapeutic utility has been demonstrated by determining their toxicity and selectivity toward primary leukemic lymphocytes from CLL patients and comparing that with normal lymphocytes from healthy donors.

We designed and synthesized a series of H$_2$O$_2$-activated boron-containing aromatic nitrogen mustard prodrugs (1−3) with two linker systems and various leaving groups. Compounds 1a−f and 2 contain a nitrogen mustard group directly bonded to the aromatic ring (Scheme 2A). The electron-withdrawing boronate group decreases the electron density of the benzene ring and makes the lone pair of the nitrogen mustard delocalize to boron (D). Therefore, these prodrugs do not form the electrophilic aziridinium ring E and are not deleterious to cells with low ROS levels (Scheme 2B). However, the oxidation of the carbon–boron bond by H$_2$O$_2$ followed by a transformation to a hydroxyl group can trigger an increased electron release to the nitrogen of the mustard moiety (B);\textsuperscript{28} this facilitates the formation of a highly electrophilic aziridinium ring C capable of cross-linking DNA. Compound 3 contains a withdrawing carbonyl group that can reduce the toxicity of the nitrogen mustard. We assumed that release of the amine effector would occur upon activation of 3 by H$_2$O$_2$.\textsuperscript{30−32}

Scheme 1. Selective DNA Cross-Linking Agent with a ROS-Responsive “Trigger” and an “Effector”

Scheme 2. (A) Structures of the Designed Prodrugs and (B) Mechanism of Targeting ROS-Containing Cancer Cells
RESULTS

Synthesis of 1a–f, 2, and 3. The synthesis of compounds 1–3 is shown in Scheme 3. Compounds 1a–f and 2 were synthesized starting from p-bromoaniline (4). 2-Chloroethanol was first coupled with 4 using calcium carbonate as a base yielding 6. Palladium-catalyzed borylation of 6 provided boronate intermediate 8 which reacted with MsCl resulting in dimesylate mustard 1f at 80% yield. Nucleophilic substitution of 1f with 1.0 equiv of lithium chloride or lithium bromide afforded 1a, 1d or 1b, 1e respectively. Compound 1d was converted to 1c by further treatment with lithium bromide (Scheme 3A). For the synthesis of boronic acid 2, compound 6 was first converted to dichloromustard 9 via mesylation and chlorination (Scheme 3B), and treatment of 9 with butyllithium

Figure 1. Comparison of the H$_2$O$_2$-inducible activity and selectivity of 1a–f and 2. Phosphorimage autoradiogram of denaturing PAGE analysis of the cross-linking reaction of DNA duplex 12 in the presence of 1a–f or 2 (1.0 mM) (all reactions were carried out at room temperature).
and trisopropyl borate was followed by hydrolysis that yielded boronic acid. Compound 3 was obtained via an amidation reaction of bis(2-chloroethyl)amine hydrochloride and chloroformate which was synthesized from 10 and triphosgene (Scheme 3C).

Selective DNA Cross-Linking Ability. Initially we investigated their DNA cross-linking abilities and selectivity by allowing cross-linkers to react with DNA duplex 12 which contains GNC sequences at the terminus. First, we studied the effect of the carbamate linker (3) on the activity of nitrogen mustard. As we expected, 3 did not induce ICL formation in the absence of H2O2, which indicated that a carbamate linker is sufficient to deactivate the nitrogen mustard. To our surprise, DNA ICLs were not formed in the presence of 3 and H2O2. Obviously, a carbamate linker is not suitable for constructing H2O2-inducible DNA cross-linking agents.

Next, we studied the reactivity of 1a−f and 2 toward 12 (Figure 1). In the absence of H2O2, no obvious DNA ICLs were observed for 1a−e and 2, while 1f induced 22% DNA cross-linking. Compound 1f contains two mesylate groups, while the others have one (1d and 1e) or no mesylate group (1a−c and 2). The better leaving property of the mesylate group may cause the higher reactivity of 1f compared to others in a H2O2-free system. These data indicated that boron groups are sufficient to deactivate dihalogen or halogenmesylate mustards but cannot completely mask the reactivity of dimesylate mustard 1f. The addition of H2O2 triggered the activity of 1a−e and 2, leading to efficient ICL formation (37%−49%). Similarly, the cross-linking yield of 1f was increased 3-fold upon H2O2 activation. It is worth mentioning that the ICL was not observed when the DNAs were treated with H2O2 only (Figure 1, lane 2). As we proposed, the conversion of an electron-withdrawing boron group to a donating hydroxyl group by H2O2 increases the electron density of nitrogen mustard and therefore facilitates the ICL formation (Scheme 2B). DNA ICLs induced by 1a−f and 2 were observed at a concentration as low as 50 μM (~3% ICL yield), and the optimum ratio of drug to H2O2 was 1:2 (Tables S1−S7 and Figures S2−S3). The best selectivity and activity were observed under physiological pH and temperature (pH 7.0−8.0 and 37.0−38.0 °C) (Figure S4 and S5). The ICL formation induced by these compounds followed first-order kinetics with a rate constant (kICL) ranging from (2.45 ± 0.25) × 10−5 s−1 to (5.42 ± 0.15) × 10−5 s−1 (Figure S6). Among these agents with different leaving groups, compounds with the methyl mesylate group showed a higher H2O2-inducible DNA cross-linking ability than those having halogen groups with an order of 1f > 1d > 1e > 1a > 1b > 1c. Upon treatment of H2O2, 1f with two mesylate groups resulted in 66% DNA ICLs, while 1d and 1e with one mesylate group and one halogen group produced 47% and 48% ICL, respectively. Lastly only 35%−37% yields were observed with 1a, 1b, and 1c with two halogen groups. Besides that, no obvious different reactivity was found in compounds with bromine or chlorine groups (1d vs 1e, 1a vs 1b vs 1c).

In order to understand whether the location of GNC sequences affects the cross-linking efficiency, we synthesized duplex 12’ with GNCs in the middle of the sequence and investigated ICL formation of duplex 12’ induced by 1a/H2O2 and 1b/H2O2 (1 mM). The cross-linking yield of 12’ (27% ± 3.5) was slightly lower than that of duplex 12 (35% ± 4.0), but both are within the experimental error of each other (Figure S7). These data showed that the activity and selectivity of these agents can be achieved with a variety of DNA sequences. It is necessary to point out that smearing bands were observed when the cross-linking yield was high and/or when there were multiple cross-linking sites or alkylating sites, while such phenomena were not observed when the yields were low (Figures S6F and S8).

Subsequently, we investigated the activity of 1a, 1d, and 2 toward other ROS including tert-butyl hydroperoxide (TBHP), OCl−, HO−, BuO−, O2−, and NO (Figure S8). Among these, H2O2 is the most efficient ROS that triggers the activity of these prodrugs, while TBHP, OCl−, and O2− also slightly activate 1a, 1d, and 2. In the presence of H2O2 these compounds induced 30−47% ICL formation, while much lower ICL yields were observed with other ROS (0.9−6.6% for OCl−, 1.0−3.6% for TBHP, and 5−15% for O2−) (Figure 2). This is consistent with previous reports about the selective reaction of boronic acids and their esters toward H2O2.26,30

![Figure 2](Image 324x81 to 564x262)

Figure 2. ICL formation induced by 1a, 1d, and 2 upon treatment with various ROS (1 mM of drugs and ROS were used).

NMR Detection of Activation of 1a and 2 by H2O2. Activation of 1a and 2 by H2O2 and the formation of hydroxyl analogue 13 were confirmed by NMR analysis (Figures 3−5). The reaction of 1a (20 μmol) or 2 (20 μmol) with H2O2 (30 μmol) was carried out in a mixture of 400 mM deuterated potassium phosphate buffer (pH 8.0) (50 μL) and DMSO-d6 (450 μL). In the presence of H2O2, oxidative deboronation of 1a and 2 occurred to give 1b and 2, respectively, with an 80% yield. The structural changes were monitored by 1H NMR and 13C NMR spectra (Figures 3−5). The reaction of 1a with H2O2 resulted in the formation of 1b, which can be further transformed to 13 via the reaction of 1b with H2O2 and the following reaction (Scheme 3D). The formation of 1b was confirmed by 1H and 13C NMR spectra and the observation of a new peak at δ = 7.11 ppm in the 1H NMR spectrum and a new peak at δ = 114.7 ppm in the 13C NMR spectrum. The reaction of 2 with H2O2 resulted in the formation of 13, which can be further transformed to 1b via the reaction of 13 with H2O2 and the following reaction (Scheme 3D). The formation of 13 was confirmed by 1H and 13C NMR spectra and the observation of a new peak at δ = 7.31 ppm in the 1H NMR spectrum and a new peak at δ = 114.7 ppm in the 13C NMR spectrum. The reaction of 1a with H2O2 and the following reaction resulted in the formation of 13, which can be further transformed to 1b via the reaction of 13 with H2O2 and the following reaction (Scheme 3D). The formation of 13 was confirmed by 1H and 13C NMR spectra and the observation of a new peak at δ = 7.31 ppm in the 1H NMR spectrum and a new peak at δ = 114.7 ppm in the 13C NMR spectrum.

![Figure 3](Image 347x399 to 542x549)

Figure 3. Activation of 1a and 2 by H2O2.
1a occurred yielding alkylation agent 13 and boronic acid (1C), which was evidenced by the appearance of $C_1$-H (δ 1.26 for 1a and δ 1.14 for 1C) (Figure 4). Compound 1C was further hydrolyzed to pinacol (1D, δ 1.06). The intermediate 1B was too active to be detected. The conversion of 1a to 13 was so fast that more than 80% of 1a to 13 was completed within 30 min, and >95% of 13 was formed after 2 h, which showed that the aryl boronates developed in this work are efficient $H_2O_2$-responsive trigger units. To ensure the role of $H_2O_2$ in activation, a control experiment was performed by incubating 1a in potassium phosphate buffer in the absence of $H_2O_2$ (1.0 M deuterated potassium phosphate buffer/DMSO-d$_6$ = 2:450:48).

Figure 4. $^1$H NMR analysis of the activation of 1a (40 mM) by $H_2O_2$ (60 mM): (A) $^1$H NMR analysis of 1a in 400 mM phosphate buffer (pH 8) (50 μL)/DMSO-d$_6$ (450 μL); (B) 30 min after the addition of $H_2O_2$; (C) 24 h after 1a was incubated in 4.0 mM deuterated potassium phosphate buffer in the absence of $H_2O_2$ (1.0 M deuterated potassium phosphate buffer/DMSO-d$_6$ = 2:450:48).

Figure 5. $^1$H NMR analysis of the activation of 2 (40 mM) by $H_2O_2$ (60 mM): (A) 5 min after the addition of $H_2O_2$; (B) 2 h after the addition of $H_2O_2$ [the reaction was carried out in 1.0 M deuterated phosphate buffer (pH 8) (100 μL)/DMSO-d$_6$ (450 μL)]; (C) an authentic sample of compound 13 in D$_2$O (100 μL)/DMSO-d$_6$ (450 μL).
within 2 h (Figure 5). Overall, the prodrugs developed in this work are sensitive to H$_2$O$_2$ under physiological conditions.

In order to confirm that $13$ was the direct alkylating agent generated from $1a$ or $2$ with H$_2$O$_2$, we isolated $13$ from the reaction of $2$ with H$_2$O$_2$ in potassium phosphate buffer (Figure S1). The reaction was so efficient that 85% (isolated yield) of $13$ was obtained after 2 h. Compound $13$ was characterized by $^1$H NMR, $^{13}$C NMR, and high resolution mass spectrometry (Supporting Information). Its reactivity with DNA was also converted to $13$ within 2 h (Figure 5).

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Figure 6. Comparison of specific cross-linking sites caused by $1a$/H$_2$O$_2$, $13$, and mechlorethamine. Phosphorimage autoradiogram of 20% denaturing PAGE analysis of the ICL products upon heating in piperidine phosphate buffer. The ICL products were produced by incubation of duplex 12 with $1a$/H$_2$O$_2$, $13$, or mechlorethamine. $12a$ was radiolabeled at 5′-terminus. Lanes 1–3, $1a$/H$_2$O$_2$; lanes 4–6, compound $13$; lanes 7–9, mechlorethamine; lanes 1, 4, 7, control (no treatment); lanes 2, 5, 8, treated by heating at 90 °C in buffer (pH 7.0); lanes 3, 6, 9, treated by heating at 90 °C in piperidine; lane 10, G + A sequencing; lane 11, Fe-EDTA treatment of $12$.

Figure 7. Comparison of the anticancer activities of prodrugs with the ones releasing mechlorethamine. Each cell line was grown in two plates and treated with drug (10 μM) for 48 h at 37 °C, 5% CO$_2$, 95% air, and 100% relative humidity. The growth percents were determined by NCI-60 DTP human tumor cell line screen: 40 (A) $1a$ vs $14$; (B) $2$ vs $15$. 
Table 1. Cytotoxicities of 1a, 1c, 1d, and 2

| tumor type       | cell line       | 1a  | 1c  | 1d  | 2   |
|------------------|-----------------|-----|-----|-----|-----|
| leukemia         |                 |     |     |     |     |
|                 | CCRF-CEM        | 3.34| 5.03| 4.01| 3.27|
|                 | HL-60(TB)       | 4.66| 5.11| 3.88| 2.88|
|                 | K-562           | 17.2| 22.6| 19.0| 15.8|
|                 | MOLT-4          | 3.48| 3.69| 3.74| 2.90|
|                 | RPMI-8226       | 10.90|19.40|14.7 |8.59 |
|                 | SR              | 0.63| 0.66| 0.63| 0.48|
| non-small-cell   |                 |     |     |     |     |
| lung             | A549/ATCC       | 2.69| 4.88| 4.98| 0.89|
|                 | EKVX            | 18.6 |22.5 |24.5 |15.4 |
|                 | HOP-62          | 10.9 |10.9 |8.85 |8.48 |
|                 | HOP-92          | 9.24| 12.80|11.5 |10.50|
|                 | NCI-H226        | 12.9| 11.9 |10.4 |10.3 |
|                 | NCI-H23         | 4.57| 5.38 |4.70 |3.36 |
|                 | NCI-H322M       | 14.7| 32.5 |28.2 |15.1 |
|                 | NCI-H460        | 0.33| 0.42 |0.49 |0.23 |
|                 | A549/ATCC       | 2.69| 4.88| 4.98| 0.89|
|                 | EKVX            | 18.6 |22.5 |24.5 |15.4 |
|                 | HOP-62          | 10.9 |10.9 |8.85 |8.48 |
|                 | HOP-92          | 9.24| 12.80|11.5 |10.50|
|                 | NCI-H226        | 12.9| 11.9 |10.4 |10.3 |
|                 | NCI-H23         | 4.57| 5.38 |4.70 |3.36 |
|                 | NCI-H322M       | 14.7| 32.5 |28.2 |15.1 |
|                 | NCI-H460        | 0.33| 0.42 |0.49 |0.23 |
| colon cancer     |                 |     |     |     |     |
|                 | COLO 205        | 11.60|11.40|11.00|7.26 |
|                 | HCC-2998        | 14.9| 24.0 |14.6 |12.1 |
|                 | HCT-116         | 11.60|13.90|11.10|9.37 |
|                 | HCT-15          | 13.20|17.10|13.50|9.46 |
|                 | HT29            | 13.8| 18.8 |14.6 |11.9 |
|                 | KM12            | 14.9| 31.3 |25.8 |15.1 |
|                 | SW-620          | 11.30|13.90|11.90|8.39 |
| CNS              |                 |     |     |     |     |
|                 | SF-268          | 4.61| 4.90 |5.39 |4.72 |
|                 | SF-295          | 2.11| 2.64 |2.99 |1.36 |
|                 | SF-539          | 5.70| 6.37 |4.83 |3.39 |
|                 | SNB-19          | 8.06| 10.60|10.20|8.00 |
|                 | SNB-75          | 7.98| 10.70|5.85 |3.21 |
| melanoma         |                 |     |     |     |     |
|                 | LOX IMVI        | 5.17| 6.60 |4.72 |3.13 |
|                 | MALME-3M        | 17.5| 18.4 |14.0 |19.7 |
|                 | M14             | 5.10| 7.86 |5.68 |4.77 |
|                 | MDA-MB-435      | 14.2| 16.5 |15.3 |13.0 |
|                 | SK-MEL-2        | 22.1| 20.8 |21.5 |19.0 |
|                 | SK-MEL-28       | 21.0| 20.6 |15.6 |13.5 |
|                 | SK-MEL-5        | 14.5| 13.7 |12.8 |10.9 |
|                 | UACC-257        | 11.6| 13.4 |13.0 |11.6 |
|                 | UACC-62         | 6.12| 9.38 |5.83 |4.71 |
| ovarian          |                 |     |     |     |     |
|                 | IGROV1          | 15.6| 19.1 |13.6 |18.1 |
|                 | OVCAR-3         | 14.6| 15.0 |13.4 |12.0 |
|                 | OVCAR-4         | 17.4| 14.4 |13.9 |12.1 |
|                 | OVCAR-5         | 21.6| 25.9 |22.9 |16.0 |
|                 | OVCAR-8         | 7.83| 11.90|8.20 |4.20 |
|                 | NCI/ADR-RES     | 5.33| 6.69 |6.52 |2.69 |
|                 | SK-OV-3         | 8.03| 9.25 |8.96 |3.81 |
| renal            |                 |     |     |     |     |
|                 | 786-0           | 5.35| 8.95 |6.55 |4.04 |
|                 | A498            | 2.81| 5.87 |8.59 |3.43 |
|                 | ACHN            | 3.64| 3.40 |3.03 |1.75 |
|                 | CAKI-1          | 2.78| 3.52 |3.10 |1.44 |
|                 | RXF 393         | 10.3| 10.4 |5.71 |2.55 |
|                 | SN12C           | 4.11| 4.45 |4.40 |2.08 |
|                 | TK-10           | 16.7| 22.6 |18.0 |15.5 |
|                 | UO-31           | 5.78| 6.51 |6.18 |6.05 |
| prostate         |                 |     |     |     |     |
|                 | PC-3            | 14.30|17.70|15.10|15.10|
|                 | DU-145          | 4.25| 6.69 |4.52 |4.89 |
| breast           |                 |     |     |     |     |
|                 | MCF7            | 4.12| 5.03 |4.44 |1.89 |
|                 | MDA-MB-231/ATCC | 16.5| 22.8 |22.1 |16.6 |
|                 | HS 578T         | 26.2| 27.4 |24.1 |31.4 |
|                 | BT-549          | 10.5| 12.1 |12.0 |6.63 |
|                 | T-47D           | 10.70|12.00|8.49 |6.29 |
studied. The cross-linking efficiency of 1a/H2O2 (50%) or 2/H2O2 (55%) was close to that of 13 (52%) (Figure S9), which also supported our conclusion.

**Determination of Specific Cross-Linking Sites and the Stability of Cross-Linked Products.** The stability and reaction sites of the ICL products were examined to provide further insight into the reactivity of compounds 1a–e and 2.

The reaction sites of DNA alkylation can be determined by investigating their heating stability under basic and/or neutral conditions. It was reported that the ICL induced by nitrogen mustards usually occurred at N-7 of dGs.38 Piperidine is known to induce cleavage with N-7 alkylated purines upon heating.39

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**Table 1. continued**

| tumor type | cell line | 1a | 1c | 1d | 2 |
|------------|-----------|----|----|----|---|
| MDA-MB-468 |           | 1.60 | 1.49 | 1.07 | 0.51 |

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**Figure 8.** Evaluation of cytotoxicity in primary leukemia cells (A) and normal lymphocytes (B) at 24 h (upper panel) and 48 h (lower panel). Leukemic lymphocytes were obtained from peripheral blood of patients with CLL (n = 4). Normal lymphocytes were obtained from peripheral blood of age-matched healthy donors (n = 3). Incubations were carried with or without (con) 10 μM compounds 1a, 1c, or 2 for 24 or 48 h, and the apoptosis induction was measured by annexin/PI binding assay. Each line represents one patient. The p value was obtained from Student’s t tests (two tailed) performed using the GraphPad Prism5 software (GraphPad Software, Inc., San Diego, CA).
Thus, we examined the stability of DNA cross-linking products formed by these compounds in phosphate buffer (pH 7) or in 1.0 M piperidine (90 °C). The DNA ICLs were completely destroyed after heating for 30 min which led to obvious cleavage bands at dGs and dAs (Figure 6 and Figures S10–S13). These results are consistent with the observation that the reaction of nitrogen mustard mainly occurred at N7 of purines. However, in addition to major cleavage bands at the purine sites, we also observed some weak ones at pyrimidine nucleotides (e.g., 7–9, 16, 17, 19, 20, 59, 60, 65, 66, 68, and 72) as shown in Figure 6 and Figures S9–S11. Compared with other nitrogen mustards compounds,22 such as mechlorethamine (Figure 6, lanes 7–9) and 13 (Figure 6, lanes 4–6), the cross-linking products induced by 1a–e and 2 in the presence of H2O2 showed similar cleavage patterns as those induced by 13 (lanes 4–6) but were a little different from mechlorethamine. In a separate experiment, the ICL products and the drug-treated single stranded DNA were isolated from the reaction mixture and heated in neutral phosphate buffer or 1.0 M piperidine. Similar cleavage patterns were observed for ICL products and single stranded DNA (Figures S14 and S15). These data showed that apart from ICL formation, intrastrand cross-linking and/or monoalkylations were also possible.

**Evaluation of the Cytotoxicities in Cell Lines.** Since the activity of nitrogen mustards were effectively masked in 1a–e and 2 but can be selectively triggered by H2O2 to induce efficient ICL formation, their cytotoxicity and selectivity were further evaluated in biological systems.46 All of these agents showed significant growth inhibition of the cell lines tested. The growth percentages of most cell lines were less than 50% at a single dose of 10 μM. For comparison, 3 was also tested. However, no obvious toxicity was observed, which is consistent with the DNA cross-linking study. Furthermore, we compared the anticancer activity of the aromatic nitrogen mustards 1a and 2 with that of 14 and 15 which released the simplest nitrogen mustard mechlorethamine.29 Compounds 1a and 2 showed a much higher growth-inhibitory effect on tumor cells than 14 and 15 (Figure 7). Although the precise mechanism underlying the higher toxicity of 1a and 2 is not clearly understood yet, one of the possible reasons we considered is that 1a–e and 2 are neutral molecules that are expected to diffuse across a cell membrane better than positively charged 14 and 15.

Considering that different halogen groups (Br and Cl) in these compounds did not show much difference on the reactivity toward DNA and cytotoxicity toward cancer cell lines, compounds 1a, 1c, 1d, and 2 were chosen as representative compounds to evaluate their GI50 (Table 1). All four compounds exhibited a high level of toxicity to the cell lines tested, such as leukemia, non-small-cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer. Although the GI50 toward these cell lines range from 0.23 to 31.4 μM, most of these compounds have a GI50 of less than 5 μM. In particular, they are more toxic toward leukemia, non-small-cell lung cancer, CNS cancer, renal cancer, and breast cancer than colon cancer, melanoma, and ovarian cancer. For example, a GI50 of about 1 μM was observed with cell lines SR (leukemia), NCI-H460 (non-small-cell lung cancer), and MDA-MB-468 (breast cancer). It was reported that leukemia, lung cancer, and breast cancer contain cells that proliferate under conditions of oxidative stress and have high intracellular concentrations of ROS.41–44 It is very likely that these compounds can be more efficiently activated in these cells and therefore can lead to higher toxicity. In our initial DNA ICL study, compounds 1d and 2 showed a little higher inducible DNA cross-linking ability than 1a, but no obvious difference was observed with their cytotoxicity. Compound 2 with a boronic acid group had a little higher activity toward most cell lines than the other three with boronic esters (1a, 1c, and 1d), possibly due to its better water solubility (log P1a = 2.8, log P2 = 2.5; log P was determined in 1-octanol and PBS).

**Apoptosis of CLL Cells or Normal Lymphocytes.** Given that these compounds showed significant cytotoxicity in several cell lines, we investigated the selectivity of representative compounds (1a, 1c, and 2) in primary samples obtained from patients with CLL. CLL cells contain high levels of ROS and therefore should be effectively targeted by these agents. As expected, all three compounds (1a, 1c, and 2) induced significant amount of apoptosis in all samples tested. We observed a time (Figure 8A, 24 and 48 h, n = 4) and dose dependent apoptosis (Figure 9, 24 h, n = 3) in these samples.

![Figure 9. Dose-dependent apoptosis of CLL cells or normal lymphocytes with produgs 1a, 1c, 2. Leukemic lymphocytes obtained from peripheral blood of patients with CLL (n = 3). Normal lymphocytes obtained from peripheral blood of age-matched healthy donors (n = 3). Incubations were carried with 1a, 1c, or 2 for 24 h, and the apoptosis induction was measured by annexin/PI binding assay: (A) CLL cells with 1a; (B) CLL cells with 1c; (C) CLL cells with 2; (D) normal lymphocytes with 2 for 24 h.](image-url)

Compared to compounds 1a and 1c, compound 2 demonstrated increased activities in CLL samples. The IC50 for the most potent compound (compound 2) was between 5 and 6 μM.

To further assess the selectivity of these compounds toward cancer cells, we evaluated their toxicity toward normal lymphocytes isolated from peripheral blood of age-matched healthy donors (Figure 8B, 24 and 48 h, n = 3). Interestingly, 1a, 1c, and 2 resulted in comparatively less apoptosis, suggesting that the selective action toward cancer cells and the IC50 were not achieved in normal lymphocytes. Dose-dependent apoptosis of normal lymphocytes with produg 2 was achieved at 24 h. Compound 1a at 24 h demonstrated % median for con-9S, treated-93 (p = 0.226) and at 48 h the %
Conclusions.

In summary, a series of ROS-activated aromatic nitrogen mustards with different leaving groups have been successfully synthesized. The boronate ester group sufficiently masks the activity of the aromatic nitrogen mustards which can be restored upon H$_2$O$_2$ treatment. The activation mechanism of these prodrugs by hydrogen peroxide was determined by NMR analysis. Among these agents with different leaving groups, compounds with methyl mesylate group showed more potent inducible DNA cross-linking ability than that with halogen groups, while there was no obvious difference in the reactivity of compounds with bromine or chlorine group. The stability study revealed that DNA cross-linking and/or alkylation induced by these agents mainly occurred with purine nucleotides. Consistent with the chemistry observation, in vitro cytotoxicity assay in respective cell lines demonstrated that these reagents exhibited effective killing of cancer cells with a concentration as low as or less than 1.0 μM. Higher toxicities were observed in cell lines, such as SR (leukemia), NCI-H460 (non-small-cell lung cancer), and MDA-MB-468 (breast cancer). In addition, these compounds showed selective toxicity toward primary leukemic lymphocytes from patients with chronic lymphocytic leukemia (40–80% apoptosis), while they were less toxic to normal lymphocytes from healthy donors (less than 25% cell death). The cellular study with or without an ROS quencher showed that these agents function through ROS-dependent mechanisms. Collectively, these data provide utility and selectivity of these agents which should inspire further and effective application in potential cancer chemotherapies.

## EXPERIMENTAL SECTION

General Information. Unless otherwise specified, chemicals were purchased from Aldrich or Fisher Scientific and were used as received without further purification. T$_4$ polynucleotide kinase was purchased from New England Biolabs. Oligonucleotides were synthesized via standard automated DNA synthesis techniques using an Applied Biosystems model 394 instrument in a 1.0 μmol scale using commercial 1000 A CPG-succinyl-nucleoside supports. Deprotection of the nucleobases and phosphate moieties and cleavage of the linker were carried out under mild deprotection conditions using a mixture of 40% aqueous MeNH$_2$ and 28% aqueous NH$_3$ (1:1) at room temperature for 2 h. [γ-^32P]ATP was purchased from Perkin-Elmer Life Sciences. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics phosphorimager equipped with ImageQuant, version 5.2, software. $^1$H NMR and $^{13}$C NMR spectra were taken on either a Bruker DRX 300 or DRX 500 MHz spectrophotometer. Silicon reagents were used in CDCl$_3$ as internal standard. High resolution mass spectrometry was performed at the University of Kansas Mass Spectrometry Lab or University of California-Riverside Mass Spectrometry Lab. The purity was determined by RP-HPLC on a 4.6 mm × 250 mm RP-C18 column with 277 nm detection, which confirmed that all compounds had ≥95% purity.

Synthesis. 2,2’-(4-Bromophenylazanediyl)diethanol (6). A mixture of 4-bromoaniline (17.1 g, 0.01 mol), 2-chloroethanol (20 mL), CaCO$_3$ (20.0 g), and NaI (1.4 g) in 250 mL of water was heated to reflux overnight, then extracted with dichloromethane and washed with water. After evaporation of the solvent, the residue was purified by column chromatography (hexane/ethyl acetate = 1:2) to afford white solid 6 (18 g, 70%), mp 78–80 °C. $^1$H NMR (CDCl$_3$, 300 MHz): δ 3.62 (t, $J = 4.8$ Hz, 4H), 3.88 (t, $J = 4.8$ Hz, 4H), 6.76 (d, $J = 8.4$ Hz, 2H), 7.38 (d, $J = 8.4$ Hz, 2H). $^{13}$C NMR (CDCl$_3$, 75 MHz): δ...
N-Bis(2-chloroethyl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylalanine (1a). A mixture of If (926 mg, 2 mmol) and LiCl (84 mg, 2 mmol) in acetonitrile (5 mL) was stirred at 60 °C for 18 h. After removal of solvent, the residue was purified by column chromatography (hexane/ethyl acetate = 1:1) followed by recrystallization from EtOAc to afford white solid 1a (233 mg, 34%). 1H NMR (CDCl3, 300 MHz): δ 1.35 (s, 12H), 3.62 (t, J = 6.9 Hz, 2H), 6.70 (d, J = 8.7 Hz, 2H); 7.72 (d, J = 8.7 Hz, 2H). 13C NMR (CDCl3, 75 MHz): δ 24.8, 40.4, 50.3, 53.2, 66.4, 83.1, 111.2, 136.7, 148.5. HRMS-ES (m/z) [M + Na]+ calcd for C10H15BNO2Cl2, 262.0567; found, 262.0573.

1-(2-Bromoethyl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylalanine (1b). A mixture of If (926 mg, 2 mmol) and LiBr (170 mg, 2 mmol) in acetonitrile (5 mL) was stirred at 60 °C for 20 h. After removal of solvent, the residue was purified by column chromatography (hexane/ethyl acetate = 1:1) to afford white solid 1b (277 mg, 31%). 1H NMR (DMSO-d6, 300 MHz): δ 1.24 (s, 12H), 3.10 (s, 3H), 3.71−3.72 (m, 4H), 3.77 (t, J = 5.4 Hz, 2H), 6.75 (d, J = 8.4 Hz, 2H), 7.49 (d, J = 8.4 Hz, 2H). 13C NMR (CDCl3, 75 MHz): δ 24.9, 37.5, 40.4, 50.3, 53.2, 66.4, 83.1, 111.2, 136.7, 148.5. HRMS-ES (m/z) [M + Na]+ calcd for C10H15BNO2Br, 244.0290; found, 244.0297.

Detection of DNA Cross-Linking. ICL formation and crosslinking yields were analyzed via denaturing polyacrylamide gel electrophoresis (PAGE) with phosphorimager analysis. The DNA−DNA cross-linking abilities of these compounds were investigated by reacting with a 3P-labeled 49-mer oligonucleotide 12 (Figure 1) and then subjected to 20% denaturing PAGE analysis. The 32P-labeled oligonucleotide 12a (1.0 μM) was annealed with 1.5 equiv of the complementary strand 12b by heating to 65 °C for 3 min in a buffer of 10 mM potassium phosphate (pH 7) and 100 mM NaCl, followed by slow-cooling to room temperature overnight. The 3P-labeled duplex DNA (2.0 μL, 1 μM) was mixed with 1.0 μM NaOH (100 μL) and 10 mM potassium phosphate (2 μL, pH 8), 10 μM 50 mM H2O2 (2 μL), and 10 μM to 50 mM compounds 1a−f and 2 (resulting in a concentration range of 1 μM to 5 μM), and appropriate amount of autoclaved distilled water was added to give a final volume of 20 μL.
The reaction mixture was incubated at room temperature for 16 h and then quenched by an equal volume of 90% formamide loading buffer. Finally it was subjected to 20% denaturing polyacrylamide gel analysis.

Cell Lines. The in vitro cancer cell screen was performed at the National Cancer Institute (NCI Developmental Therapeutics Program). The procedure details can be found in NCI Web site: http://dtp.nci.nih.gov/branches/btb/wclsp.html (Methodology of the in Vitro Cancer Screen). The human tumor cell lines were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells are inoculated into 96-well microtiter plates in 100 μL at plating densities ranging from 5000 to 40 000 cells/well depending on the doubling time of individual cell lines.

CLL Cells and Normal Lymphocytes. Leukemic lymphocytes were isolated from fresh peripheral blood sample obtained from patients with CLL. Separate laboratory protocols were used to obtain blood samples from patients with CLL and healthy donors. All individuals signed written informed consent forms in accordance with the Declaration of Helsinki and with the laboratory protocols approved by the institutional review board at the University of Texas MD Anderson Cancer Center.

(A) Isolation of CLL and Normal Lymphocytes. Whole blood was collected in heparinized tubes and diluted 1:3 with cold PBS (0.135 M NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8 mM Na2HPO4 [pH 7.4]) and layered onto Picoll-Hypaque (specific gravity, 1.086; Life Technologies, Grand Island, NY). The blood was then centrifuged at 433 g for 20 min, and mononuclear cells were removed from the interface. Cells were washed twice with cold PBS and resuspended in 10 mL of RPMI 1640, supplemented with 10% autologous plasma. A Coulter channelyzer (Coulter Electronics, Hialeah, FL) was used to determine cell number and the mean cell volume. The CLL or normal lymphocytes were suspended in medium at a concentration of 1 × 105 cells/mL, and fresh cells were used for all experiments.

(B) Measurement of Apoptosis. Cell death is evaluated by flow cytometry analysis with the use of annexin V–PI double staining, CLL or normal lymphocytes in suspension are incubated with 10 μM compounds, and the cell death was measured by annexin V binding assay. Time matched control samples with no drug are also maintained.

ROS, reactive oxygen species; CLL, chronic lymphocytic leukemia cell; ICL, interstrand cross-link; MsCl, methanesulfonyl chloride; TBHP, tert-buty1 hydroperoxide; SR, leukemia cell; NCI-H460, non-small-cell lung cancer cells; MDA-MB-468, breast cancer cells; NAC, N-acetyl cysteine.

ABBREVIATIONS

ROS, reactive oxygen species; CLL, chronic lymphocytic leukemia cell; ICL, interstrand cross-link; MsCl, methanesulfonyl chloride; TBHP, tert-butil hydroperoxide; SR, leukemia cell; NCI-H460, non-small-cell lung cancer cells; MDA-MB-468, breast cancer cells; NAC, N-acetyl cysteine.

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