

Published in final edited form as:

J Am Chem Soc. 2011 December 7; 133(48): 19278–19281. doi:10.1021/ja2073824.

Hydrogen Peroxide Inducible DNA Cross-Linking Agents: Targeted Anticancer Prodrugs

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Abstract

The major concern for anticancer chemotherapeutic agents is the host toxicity. The development of anti-cancer prodrugs targeting the unique biochemical alterations in cancer cells is an attractive approach to achieve therapeutic activity and selectivity. We designed and synthesized a new type of nitrogen mustard prodrug that can be activated by high level of reactive oxygen species (ROS) found in cancer cells to release the active chemotherapy agent. The activation mechanism was determined by NMR analysis. The activity and selectivity of these prodrugs towards ROS was determined by measuring DNA interstrand crosslinks and/or DNA alkylations. These compounds showed 60–90% inhibition toward various cancer cells, while normal lymphocytes were not affected. To the best of our knowledge, this is the first example of H₂O₂-activated anticancer prodrugs.

Keywords

Interstrand crosslink; H₂O₂-activation; anticancer prodrug; ROS-targeting; arylboronate and arylboronic acid

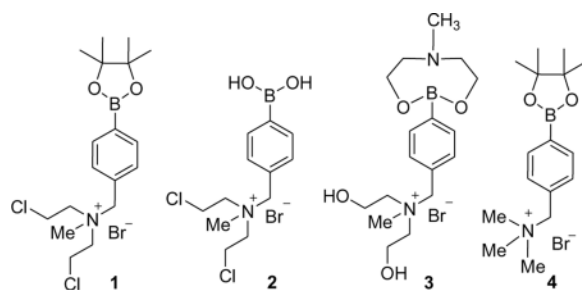
DNA interstrand cross-links (ICLs) are deleterious to cells, because they are unyielding obstruction to replication and transcription.¹ This property is exploited in cancer chemotherapy. ICL-inducing agents such as nitrogen mustards and cisplatin are among the most frequently used antitumor agents in the clinic. However, these agents exhibit severe host toxicity due to their poor selectivity toward cancer cells. One approach to reduce the toxicity of crosslinking agents for normal cells is to trigger the prodrug in tumor cells. Over the past few decades, several research groups have developed novel DNA cross-linking or alkylating agents that can induce ICL formation either by oxidation, reduction, or photolysis.^{2–4} However, there is considerable scope for developing selective agents that can induce DNA crosslinks specifically under tumor-specific conditions. It is believed that the estrogen receptor (ER) is overexpressed in many human breast and ovarian tumors. Essigmann and coworkers have developed tumor-specific toxins by conjugating an ER ligand with a DNA damaging nitrogen mustard or a bifunctional platinum (II) complex to achieve the selective killing of tumor cells while minimizing toxicity to normal tissues.⁵

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Supporting Information. Experimental procedures for all reaction and analyses, preparation and characterization of **1–7**, bioactive evaluation, autoradiograms of Fe-EDTA, and piperidine treatment of cross-linked products and reacted single-stranded DNA.

Cancer cells are also known to exhibit elevated intrinsic oxidative stress.^{6–9} The increased amounts of reactive oxygen species (ROS) can be a therapeutic advantage, because it is an exclusive feature of cancer cells.⁹ Therefore, it is of great interest to develop cross-linking agents that can be activated by ROS found in tumor cells and induce deleterious DNA damages (e.g. ICL formation or alkylation).

The most common ROS include hydrogen peroxide (H_2O_2), superoxide anions ($\text{O}_2^{\cdot-}$), and hydroxyl radical. Among these, H_2O_2 has the chemical stability required to establish significant steady-state concentrations *in vivo* and is uncharged. Compared with their normal counterparts, cancer cells have increased level of H_2O_2 (up to $0.5 \text{ nmol}/10^4 \text{ cells/h}$).^{6,10} These factors make H_2O_2 an ideal candidate as a therapeutic target for the development of ROS-activated prodrugs. Such agents should consist of two separate functional domains: a H_2O_2 -accepting moiety ('trigger') and an 'effector', joined by a linker system in such a way that the reaction of the trigger with H_2O_2 causes a large increase in the cytotoxic potency of the effector. The aryl boronic acids and their esters are well known to be cleaved by H_2O_2 .¹¹ This reactivity provides a chemospecific, biologically compatible reaction method for detecting endogenous H_2O_2 production. Chang's group has used boronic esters for the development of H_2O_2 -activated fluorescent probes for imaging H_2O_2 in cells.¹² Boronic acids and esters do not appear to have intrinsic toxicity issues, and the end product, boric acid, is considered non-toxic to humans.¹³ All of this information encouraged us to use arylboronates or boronic acids as the trigger units for the development of H_2O_2 -activated anticancer prodrugs. We chose nitrogen mustard as the effector to create a more broadly-applicable strategy. Therefore, we designed and synthesized prodrugs of nitrogen mustards (**1** and **2**), investigated their inducible reactivities, and compared these activities with their analogs **3** and **4**.



Compounds **1–3** were synthesized starting from 4-(bromomethyl)phenylboronic acid pinacol ester (**5**) (Scheme 1). Treatment of **5** with *N*-methyl-diethanolamine yielded **3**, which was converted to **2** by using thionyl chloride. Compound **1** was prepared by the reaction of **5** with *N,N*-bis(2-chloroethyl)methyl ammine (**7**, HN2). In a similar way, **4** was synthesized (Supporting Information (SI), Scheme S2).

It was known that the ICLs are the source of the cytotoxicity of nitrogen mustards. Thus, the activity of **1** and **2** was investigated by determining their ability to form DNA interstrand crosslinks using a 49 mer DNA duplex **8**. The DNA cross-linking experiments were carried out in phosphate buffer (pH = 7.5). ICL formation and crosslinking yield were analyzed via denaturing polyacrylamide gel electrophoresis (PAGE) with phosphorimager analysis (Image Quant 5.2) by taking advantage of the differing mobilities of ICL products and single stranded DNA. In the absence of H_2O_2 , no ICL was observed with **1** and **2** (Figure 1, lane 2 and 10), which indicates the toxicity of nitrogen mustard mechlorethamine (**7**) is masked in the prodrugs. When **8** was treated with **1** or **2** in the presence of H_2O_2 , efficient crosslink formation was observed (11–43%) (Figure 1, lanes 5–9 and 13–17). DNA crosslinking by **1** and **2** was observed at a concentration of H_2O_2 as low as $50 \mu\text{M}$ (lanes 3 and 11). This

clearly shows that **1** and **2** are non-toxic to DNA, but can be activated by H₂O₂ to release the DNA damaging agent **7**. The best ratio of drug to H₂O₂ is 2:1 (SI, Figure S1). ICL growth followed first-order kinetics. The observed rate constant for ICL formation induced by **1** ($k_{\text{ICL}} = (4.6 \pm 0.4) \times 10^{-5} \text{ s}^{-1}$, $t_{1/2} = 4.2 \text{ h}$) was within experimental error of that induced by **2** ($k_{\text{ICL}} = (4.7 \pm 0.3) \times 10^{-5} \text{ s}^{-1}$, $t_{1/2} = 4.1 \text{ h}$) (SI, Figure S2).

As H₂O₂ is not the only ROS in biological system, we studied the inducible activity of **1** and **2** towards other ROS, such as *tert*-butylhydroperoxide (TBHP), hypochlorite (OCl⁻), hydroxyl radical, *tert*-butoxy radical, superoxide (O₂⁻), and nitric oxide. The activation of **1** and **2** to release nitrogen mustard is highly selective for H₂O₂ over other ROS, which is demonstrated by the selective ICL formation (Figure 2 and SI, Figure S3). In the presence of H₂O₂, compounds **1** and **2** induced efficient ICL formation (35%), while less than 5% ICLs were observed with other ROS. The selective reaction of phenylboronate or boronic acid derivatives (**1** and **2**) with H₂O₂ is consistent with the observation of Chang's group.^{12e}

In order to provide further insight into the reactivity of **1** and **2**, we examined the stability and reactivity of purified ICL products and single stranded DNA isolated from the reaction mixture (**8a'** and **8b'**). The stability of DNA alkylation products depends upon the reaction site. The cross-links formed from **1** and **2** were almost completely destroyed upon heating at 90 °C (pH 7.2) for 30 min (SI, Figure S4). When the ICL was treated with 1 M piperidine (90 °C, 30 min), strong DNA cleavage bands were observed with all dGs and weaker bands with dAs (SI, Figures S5 and S6). These results are consistent with the reaction of nitrogen mustard mainly occurring at N7- of dG.¹⁴ The alkaline hydrolysis of N7-alkylated purines produces formamidopyrimidines that are labile to heating in piperidine.^{2d, 15} It was reported that nitrogen mustard forms ICLs in 5'-dGC or 5'-dGNC sequences.¹⁴ Therefore, the possible cross-linking sites are G1-G97, G22-G76, G27-G71, G40-G58, or G1-G96, G49-G52 (SI, Figure S5, lanes 4, 8, 14, 20). The alkylation was also observed with all DNA guanine units in single-stranded DNA **8a'** and **8b'** (SI, Figure S5, lanes 3, 7, 13, 19). In a control experiment, **8** was treated with drug alone (**1** or **2**) or H₂O₂ alone, no cleavage band was observed (SI, Figure S5, lanes 1, 2, 11, 12, 17, 18). This indicated that **1**, **2**, or H₂O₂ alone do not induce DNA damage under the conditions used in our experiments. When duplex **8** was treated with **3** or **4** in the presence of H₂O₂ followed by 1 M piperidine (90 °C, 30 min), there was no DNA cleavage band observed (SI, Figure S7). These data showed that the released quinone methide by **3** or **4** did not produce detectable DNA alkylations under our experimental conditions. Rokita *et al* has shown that adducts formed between quinone methides and deoxynucleosides were reversible, and the labile alkylating products tended to decompose with short half-life time and were difficult to be isolated.¹⁶ Therefore, the detected ICL formation or alkylation induced by **1** or **2** in the presence of H₂O₂ was exclusively induced by **7** released from **1** and **2**. In a control experiment, we examined the cross-linking efficiency of nitrogen mustard **7** alone (47% ICLs), which was identical to those induced by **1** and **2** (43%) upon H₂O₂-activation (SI, Figure S8).

The masked toxicity of nitrogen mustard in **1** and **2** was caused by the positive charge developed on the nitrogen (**A**) that strongly decreases the electron density of mustard nitrogen required for alkylation (Scheme 2). The release of tertiary amine **C** is triggered by the oxidation of the carbon-boron bond initiated by nucleophilic attack by H₂O₂ (**A** → **B**), followed by deboronation (**B** → **C**). The lone pair developed on **C** can form highly electrophilic aziridinium ring **D** by intramolecular displacement of the chloride by the amine nitrogen. **D** greatly facilitates the DNA alkylation and crosslinking formation. The activation of **1** and **2** by H₂O₂ (**A** → **B**) and the release of **C** were further confirmed by NMR analysis of the reaction of **1** and **2** with H₂O₂.

Initially, the reaction of **1** and **2** with H₂O₂ was performed in 10 mM deuterated potassium phosphate buffer (pH 7.5). However, the reaction was too fast to observe all intermediates. Compound **1** was oxidized by 97% within 5 min and spontaneously released **7** (SI, Figure S9B). After 2 h, **1** was completely consumed and converted to **7** (SI, Figure S9C). Similarly, the activation of **2** with H₂O₂ followed by the release of **7** was complete within 2 h (SI, Figure S9D–F). When the reaction of **1** with H₂O₂ was carried out in a mixture of DMSO/D₂O, we were able to observe all intermediates (Scheme 3 and Figure 3A–D). The integral change of C₂-H, C₃-H and C₄'-H indicated the kinetic transformation of compound **1** into nitrogen mustard **7**. Compound **1** was transformed to **1B** by 5% after 2 h (Figure 3A), as evidenced by the presence of δ 7.35 (doublet), δ 6.83 (doublet), and δ 4.53 (singlet). Subsequently, 1, 6-benzyl elimination of **1B** took place leading to quinone methide that spontaneously reacted with H₂O. This was evidenced by the appearance of C₂-H (δ 7.09, doublet), C₃-H (δ 6.70) and C₄'-H (δ 4.33) of compound **9** (2% within 5 h) (Figure 3B). Compounds **1B** and **9** were obtained in 32% and 20% within 24 h (Figure 3C). Nitrogen mustard **7** was released in about 25%, as shown by the shift of δ 4.13 (multiplet) to δ 3.96 (triplet) and δ 3.06 (singlet) to δ 2.84 (singlet) (Figure 3C). These data are consistent with those of the authentic sample (Figure 3D). Compound **1** was activated by H₂O₂ to release nitrogen mustard **7** in approximate 95% yield within 4 days in DMSO/D₂O (SI, Figure S10B). However, NMR analysis 4 days after the addition of H₂O₂ to **2** did not show any change with compound **2** in DMSO/D₂O. It is highly likely that the weak acidity of boronic acid group in **2** inhibits the oxidative ability of H₂O₂. It is precedented that the reaction between arylboronic acid and hydrogen peroxide was pH-dependent.^{11, 17}

Having established that the prodrugs **1** and **2** could be effectively passivated and activated by H₂O₂, the ability of these compounds to inhibit cancer cell growth was evaluated. Both compounds inhibited various types of cancer cells at 10 μ M. They showed about 90% inhibition toward SR cells (Leukemia cell), 85% inhibition toward NCI-H460 (Non-small Cell Lung Cancer cells), and 66% inhibition toward CAKI-1, and 57% toward SN12C (Renal Cancer cells) (Figure 4A).¹⁸ However, compound **3** is less toxic to these cells. The toxicity of **1** and **2** is highly likely caused by the release of nitrogen mustard after tumor-specific activation. In order to determine the selectivity, we evaluated the toxicity of **1** and **2** towards non-cancer cells. Normal lymphocytes obtained from three healthy donors were incubated without or with 10 μ M of compounds **1** and **2**; untreated samples were used as time-matched controls. In all the 3 samples studied, compared to time-matched controls, there was no increase in apoptosis observed between 24 – 72 hrs (Figure 4B and SI, Figure S11).

In conclusion, two prodrugs of nitrogen mustard coupled with an arylboronate or boronic acid demonstrated an effective way to mask the cytotoxicity of cancer chemotherapeutic agents and selectively release them in the presence of H₂O₂. The activity and selectivity were measured by crosslinking or alkylation of DNA as well as by evaluating their ability to inhibit cancer cell growth and toxicity towards normal cells. To the best of our knowledge, we are the first to report anticancer prodrugs (**1** and **2**) that can be activated by ROS to release DNA crosslinking agents. Such compounds are non-toxic but are highly likely to undergo tumor-specific activation to generate toxic species in cancer cell. They offer novel ways to improve the therapeutic effectiveness and selectivity of current anticancer agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

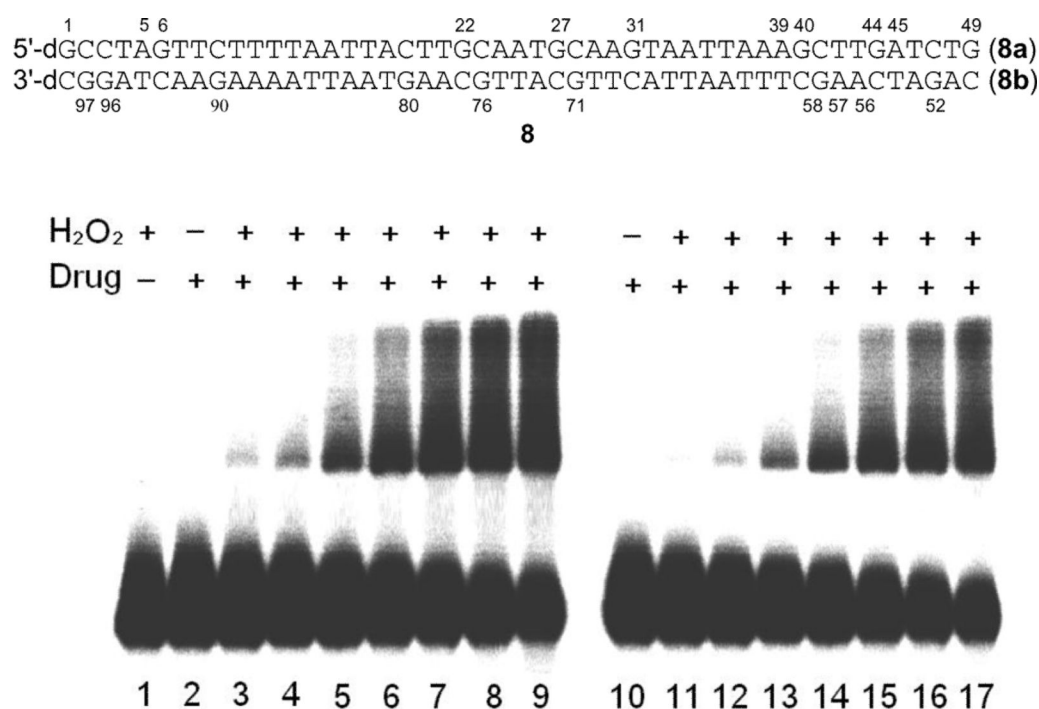
Acknowledgments

We are grateful for financial support of this research from the National Cancer Institute (1R15CA152914-01) and UWM start-up funds (XP). This research was supported in part by the National Cancer Institute (-CA136411) Lymphoma SPORE (VG).

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18. Data were obtained from Developmental Therapeutics Program at the National Cancer Institute (NCI-60 DTP Human Tumor Cell Line Screen).

**Figure 1.**

Concentration dependence of compounds **1** and **2** for DNA cross-link formation upon H₂O₂-activation. Lane 1 without drug; lanes 2–9 with drug **1**: lane 2 without H₂O₂ (cross-linking yield 0%); lane 3: 50 μ M H₂O₂ + 100 μ M **1** (2.2%); lane 4: 100 μ M H₂O₂ + 200 μ M **1** (5%); lane 5: 250 μ M H₂O₂ + 500 μ M **1** (11%); lane 6: 500 μ M H₂O₂ + 1.0 mM **1** (18%); lane 7: 1.0 mM H₂O₂ + 2.0 mM **1** (28%); lane 8: 1.5 mM H₂O₂ + 3.0 mM **1** (36%); lane 9: 2.0 mM H₂O₂ + 4.0 mM **1** (42%); lanes 10–17 with drug **2**: lane 10 without H₂O₂ (0%); lane 11: 50 μ M H₂O₂ + 100 μ M **2** (2.0%); lane 12: 100 μ M H₂O₂ + 200 μ M **2** (4%); lane 13: 250 μ M H₂O₂ + 500 μ M **2** (11%); lane 14: 500 μ M H₂O₂ + 1.0 mM **2** (17%); lane 15: 1.0 mM H₂O₂ + 2.0 mM **2** (27%); lane 16: 1.5 mM H₂O₂ + 3.0 mM **2** (35%); lane 17: 2.0 mM H₂O₂ + 4.0 mM **2** (43%).

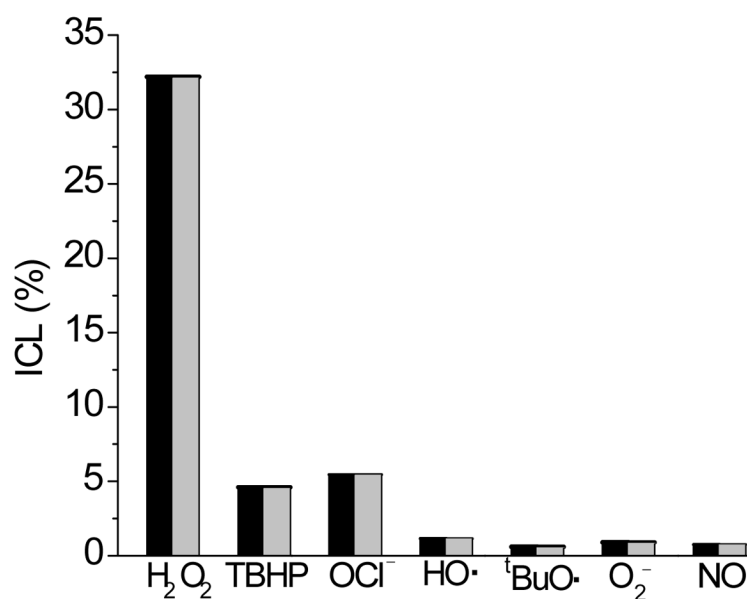
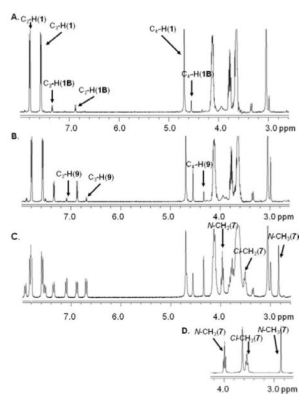


Figure 2.
ICL formation induced by **1** and **2** (2 mM) upon treatment with various reactive oxygen species (ROS) at 1 mM (black bar – compound **1**; grey bar –compound **2**).

**Figure 3.**

^1H NMR analysis of the activation of **1** by H_2O_2 in a mixture of $\text{D}_2\text{O}/\text{DMSO}$. (A) 2 h after addition of H_2O_2 (1.5 equiv.); (B) 5 h after addition of H_2O_2 ; (C) 24 h after addition of H_2O_2 ; (D) ^1H NMR of **7** in a mixture of $\text{D}_2\text{O}/\text{DMSO}$.

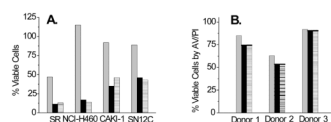
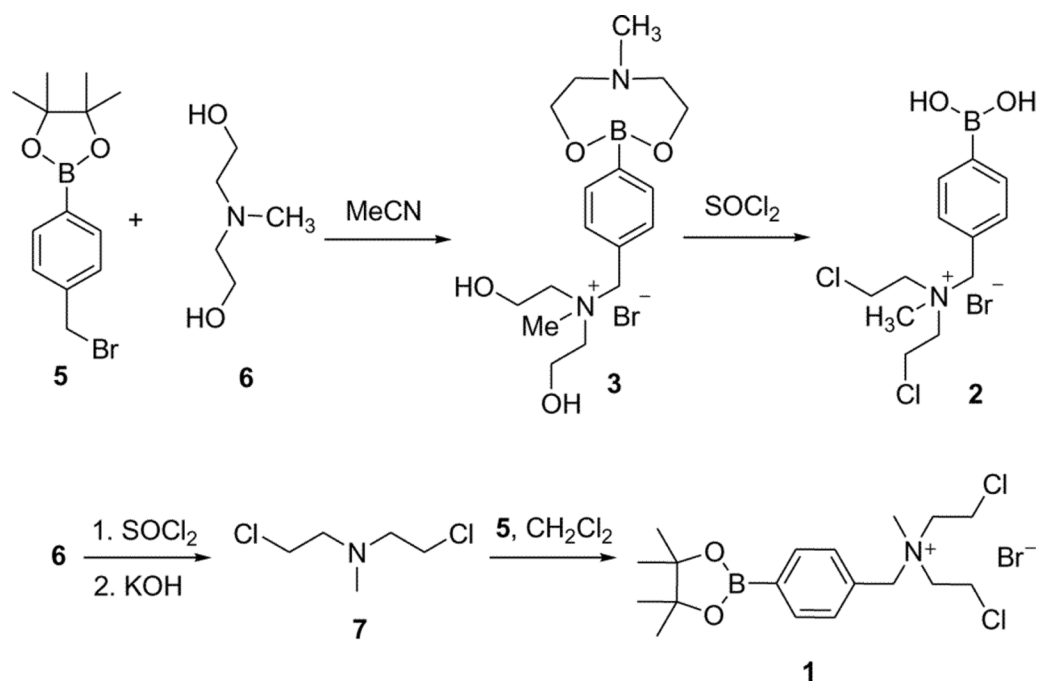
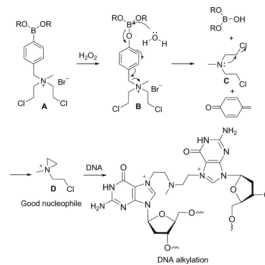


Figure 4.

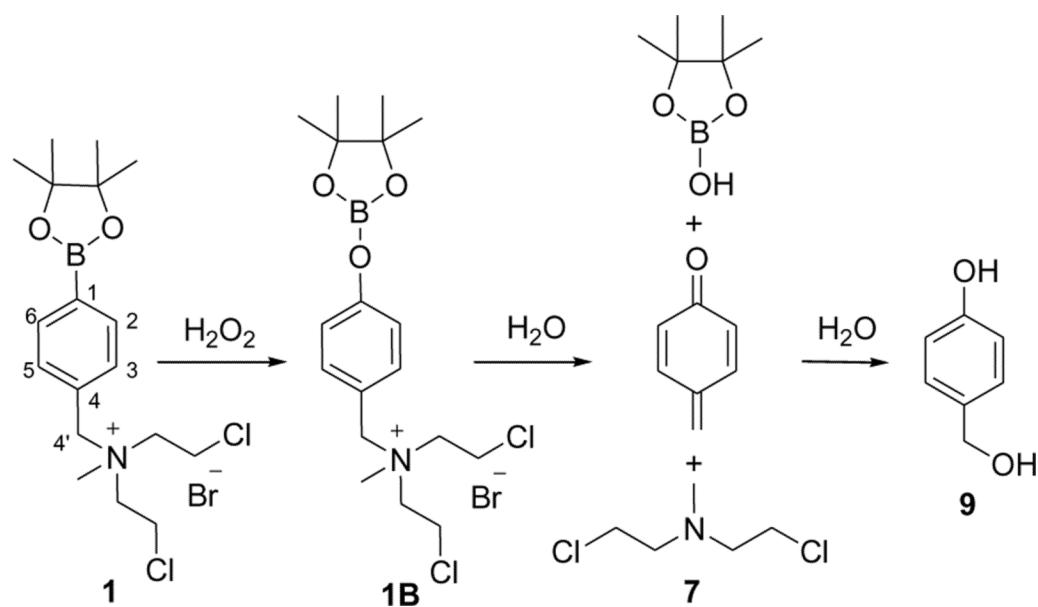
Effect of compounds **1**, **2**, and **3** on cancer cells and normal lymphocytes: (A) Four human cancer cells (SR, NCI- H460, CAKI-1, and SN12C) were incubated with 10 μ M of compounds **1**, **2**, and **3** for 48 h (grey bar - **3**; black bar - **1**; lined bar - **2**); (B) Normal lymphocytes obtained from healthy donors (n=3) were incubated with 10 μ M of **1** and **2** for 48 h. Time matched control samples are set up concurrently (grey bar - control; black bar - compound **1**; lined bar -compound **2**).



Scheme 1.
The synthesis of 1–3.



Scheme 2.
ICL formation induced by **1** and **2** upon H₂O₂-activation.

**Scheme 3.**Release of nitrogen mustard by **1** upon treatment with H_2O_2 .