Published in final edited form as:

Eur J Med Chem. 2017 June 16; 133: 197–207. doi:10.1016/j.ejmech.2017.03.041.

Hydrogen Peroxide Activated Quinone Methide Precursors with Enhanced DNA Cross-Linking Capability and Cytotoxicity towards Cancer Cells

Yibin Wang^{a,+}, Heli Fan^{a,+}, Kumudha Balakrishnan^b, Zechao Lin^a, Sheng Cao^a, Wenbing Chen^a, Yukai Fan^a, Quibria A. Guthrie^a, Huabing Sun^a, Kelly A. Teske^a, Varsha Gandhi^b, Leggy A. Arnold^a, and Xiaohua Peng^{a,*}

^aDepartment of Chemistry and Biochemistry and the Milwaukee Institute for Drug Discovery, University of Wisconsin-Milwaukee, 3210 N. Cramer Street, Milwaukee, Wisconsin, 53211, United States

^bDepartment of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX 77030, USA. pengx@uwm.edu

Abstract

Quinone methide (QM) formation induced by endogenously generated H₂O₂ is attractive for biological and biomedical applications. To overcome current limitations due to low biological activity of H₂O₂-activated QM precursors, we are introducing herein several new arylboronates with electron donating substituents at different positions of benzene ring and/or different neutral leaving groups. The reaction rate of the arylboronate esters with H₂O₂ and subsequent bisquinone methides formation and DNA cross-linking was accelerated with the application of Br as a leaving group instead of acetoxy groups. Additionally, a donating group placed *meta* to the nascent exo-methylene group of the quinone methide greatly improves H₂O₂-induced DNA interstrand cross-link formation as well as enhances the cellular activity. Multiple donating groups decrease the stability and DNA cross-linking capability, which lead to low cellular activity. A cell-based screen demonstrated that compounds **2a** and **5a** with a OMe or OH group dramatically inhibited the growth of various tissue-derived cancer cells while normal cells were less affected. Induction of H2AX phosphorylation by these compounds in CLL lymphocytes provide evidence for a correlation between cell death and DNA damage. The compounds presented herein showed potent anticancer activities and selectivity, which represent a novel scaffold for anticancer drug development.

*Corresponding Author. pengx@uwm.edu. Phone: 414-229-5221.

⁺These authors contributed equally to this work

ASSOCIATED CONTENT

Supporting Information

Experimental procedures for reactions and analysis, characterization of **1–5**, DNA experiments, and NMR analysis. This material is available free of charge via the internet at <http://pubs.acs.org>.

The authors declare no competing financial interest

Keywords

DNA interstrand cross-linking agents; quinone methide; Hydrogen peroxide activation; Arylboronates; Anticancer activity

INTRODUCTION

Exploiting cellular processes to tissue-specifically generate pharmacological-active agents has found widespread applications in medicinal chemistry and biological science.[1–3] Generation of DNA alkylating agents that take advantage of the unique processes that occur in tumors can be exploited for development of new anticancer drugs and enzyme inhibitors. [1, 4–8] Tumor cells produce high levels of reactive oxygen species (ROS), which makes them distinctly different from normal cells.[9–13] Our group recently introduced a novel prodrug strategy involving H₂O₂-induced DNA cross-linking with a nitrogen mustard cytotoxin for selective destruction of tumor cells.[4, 14–16] This strategy was applied to quinone methide-based prodrugs that can be activated by H₂O₂ to release active quinone methide (QM) capable of cross-linking DNA.[1, 17–19]

Quinone methides are naturally occurring and highly reactive electrophiles with distinct biological activity.[1, 20, 21] Quinone methides occur during several biological processes such as lignification in trees, enzyme inhibition, reactions with phosphodiesterases, DNA alkylation, and DNA cross-linking. They are the ultimate cytotoxins and can be found among many antitumor drugs, DNA alkylators, insecticides, and antibiotics. Some biologically inactive agents can be converted to reactive QMs via cellular processes such as enzymatic oxidation, reduction, or high level of ROS.[1, 21, 22] A variety of chemical methods have also been developed to generate QMs in situ from various precursors,[1, 20] such as photoradiation,[23–28] thermal extrusion reactions,[1, 29] acid- or base-catalyzed reactions,[1, 30] NaIO₄ induction,[31, 32] and fluoride-[33–35] or H₂O₂-mediated reactions. [17–19] Among these methods, photogeneration and H₂O₂ induction are particular important for biological applications. Photo induction is a non-invasive method with high spatio-temporal resolution and control and offers the options of orthogonality. The research groups of Freccero and Zhou reported photo-inducible QM formation from various precursors, including biphenyl or binol quaternary ammonium salts and 2-alkynylphenols. [24, 26, 36–38] Many of these QM precursors showed potent photocytotoxicity in various cell lines.[26, 36, 37] H₂O₂-induced QM generation can occur under physiological conditions and is attractive for *in vivo* applications as H₂O₂ is endogenously generated.[1] Our group have developed several arylboronate analogues that can be activated by H₂O₂ to form QMs and directly alkylate DNA.[18, 19] Further investigations showed that different leaving groups and aromatic substituents strongly affect QM formation and DNA alkylation. [17, 19]

However, most existing arylboronate QM precursors did not show activity towards cancer cells.[17–19] Recently, we observed that positively charged arylboronates showed lower cellular activity than the corresponding neutral molecules, which is due to insufficient cell membrane permeability.[14, 16] In this work, we observed that compound **1a** with Br as a

leaving group was a potential H₂O₂-activated anticancer prodrug (Scheme 1). Thus, we modified **1a** as a lead compound to develop more effective prodrugs by ring substitution and leaving group variation that has been reported to modulate their reaction rates with H₂O₂ and subsequent QM formation.[17, 19, 23, 39] In addition, it has been reported that electron rich aryl organic groups are less likely to be mutagenic, which we expect to be suitable trigger units for developing non-toxic prodrugs[40]. Thus, we modified the boron-containing ring by introducing an electron-donating group in the *ortho* or *para* position or both (**2a–4a**), and employing different neutral leaving groups (**1a,b**, **2a,b** and **5a,b**) (Scheme 1). We further investigated the reactivity and selectivity of these compounds toward H₂O₂ and DNA and evaluated their cytotoxicity in different tissue-derived cancer cells. The effects of different substituents and benzylic leaving group on their reactivity and cytotoxicity have been compared.

RESULTS AND DISCUSSION

Based on our previous work,[41] compounds **2a–5a** with Br as a leaving group were synthesized via borylation of the corresponding 1-bromobenzene **6**, **10**, **13**, or **16** using *n*-butyllithium and isopropoxyboronic acid pinacol ester followed by bromination with *N*-bromosuccinimide (NBS) and azobisisobutyronitrile (AIBN) (Note: To be consistent, the numbering shown for **1a** and **1b** is used for all compounds described in this paper). Compound **6** is commercially available, while **10**, **13**, and **16** were synthesized starting from methoxy substituted benzenes **8**, **12**, and **15**. As previously described,[42] compound **8** was converted to 2,5-dibromo-4,6-dimethoxy-1,3-dimethylbenzene (**9**), which was then debrominated using *n*-butyllithium yielding **10** (Scheme 2B). Similarly, compound **13** was synthesized from trimethoxybenzene **12** (Scheme 2C).[42] Protection of hydroxyl group of **15** using TBDMSCl yielded **16** (Scheme 2D).

We changed the synthetic route for **1b** and **2b**, because any attempts to introduce the acetate functionality at last step were not successful (Scheme 3). The boronate ester was introduced via palladium-catalyzed borylation from 2-bromobenzene analogue **21** or **24**. Compound **21** was prepared from 2-bromoisophthalaldehyde (**19**) via reduction followed by acylation (Scheme 3A), while **24** was synthesized from 2-bromo-5-methoxy-1,3-dimethylbenzene (**22**) via bromination followed by nucleophilic substitution using NaOAc (Scheme 3B). Compound **5b** was obtained from **5a** by replacing Br with OAc using NaOAc in DMF (Scheme 3C).

The primary mechanism for the cytotoxicity of QM prodrugs is formation of DNA interstrand cross-links (ICLs).[1, 20, 21] ICLs are deleterious to cells, because they inhibit transcription and replication.[2, 43] Therefore, the cytotoxicity and DNA sequence selectivity of the proposed prodrugs can be predicted by their ability to induce DNA cross-linking or DNA alkylation in the presence or absence of H₂O₂. In this work, we characterized compounds **1–5** with respect to H₂O₂ sensitivity, the ability to induce DNA ICLs formation, and DNA alkylation.

The reactivity of **1–5** towards DNA was investigated by reacting them with a 49-mer DNA duplex **25** in pH 8 phosphate buffer at 37 °C for 24 h. Initially, 2 mM compounds and 2 mM

H₂O₂ were used for comparison of the DNA cross-linking abilities of **1–5**. ICL formation and yields were analyzed via denaturing polyacrylamide gel electrophoresis (PAGE) with phosphor image analysis (Image Quant 5.2). Among all compounds tested, **1b**, **2a**, **2b**, **5a**, and **5b** did not induce DNA cross-linking in the absence of H₂O₂, indicating that they are not DNA alkylators (Figure 1, lanes 4 and 7–10). In the presence of H₂O₂, efficient ICL formation was observed (20–50%; Figure 1, lanes 12–18), while the presence of H₂O₂ alone did not lead to DNA cross-linking (Figure 1, lane 2). These data clearly demonstrate that these inactive prodrugs can be efficiently activated by H₂O₂ and act as DNA cross-linking agents. Also, compared with their parent compound **1a** (22 ± 5%), **2a** (36 ± 4%) and **5a** (50 ± 5%) greatly improved the ICL yield.

In order to obtain more detailed information about the substituent effects on the DNA cross-linking, we took an in-depth look at the stability and reactivity of **2a–4a**. Comparison of the ICL yields showed that **2a** (36%) is superior to **3a** (20%) and **4a** (0%) as H₂O₂-activated DNA cross-linking agents. In addition, **3a** was not selective and induced ICL formation even without H₂O₂ (17%), while the presence of H₂O₂ only slightly increased ICL yield of **3a** (20%) (Figure 1, lane 5 and SI, Figure S4, lane 2). This might be due to the presence of two methoxy groups greatly enhancing the electrophilicity of this molecule and decreasing its stability. We observed that **3a** was not stable and easily decomposed even at 0 °C. We also observed decomposition of **3a** during ¹³C NMR measurement though it was stable during the short ¹H NMR measurement. Although 3,4,5-trimethoxy analogue **4a** is more stable than **3a**, DNA ICL products were not formed with **4a** at pH 7 or higher pH (Figure 1, lane 14 and SI, Figure S7, lanes 7–9). We did, however, observe that the ICL formation induced by **4a** strongly depended on the pH. Acidic conditions resulted in higher ICL yields than neutral and basic conditions (SI, Figure S7). Rokita and co-workers reported that the methoxy group at the positions-3, -4, or -5 destabilized the nucleoside-QM adducts and favored regeneration of QM that was quenched by water.[23] We propose that the ICL products formed from **4a** may have decreased stability under neutral and basic conditions in comparison to acidic conditions because the presence of three methoxy groups greatly facilitate regeneration of QMs that are finally quenched by water. In order to test our hypothesis, we determined the stability of the ICL products induced by **4a** under acidic and basic conditions. The DNA ICLs were generated at pH 6 with duplex **25** and **4a**, then incubated at pH 6 or pH 8 at 37 °C for 24 h. We did observe that the ICL yields decreased from 16.6 ± 1.0% to 3.0 ± 0.5% at pH 8 while remained unchanged at pH 6 (Figure S7, lanes 19 and 20). These results provide evidence for that a lower ICL yield under basic condition than acidic condition is caused by the decreased stability of the ICL products formed with **4a** as a result of the high reversibility of the QM-nucleoside adducts.

Among the compounds tested, 4-methoxy analogue **2a** is the best H₂O₂-activated DNA cross-linking agent that is chemically stable and does not react with DNA without H₂O₂ but efficiently induced DNA ICL formation in the presence of H₂O₂. It is highly likely that the *meta* position in respect to the QM methylene (position-4) is an ideal site for further modification. In order to see the generality of this phenomenon, we designed and synthesized **5a** with a hydroxyl group at the position-4 and investigated its reactivity toward DNA. As expected, **5a** is relatively stable and inert towards DNA, but can react with H₂O₂ to

form QMs that directly cross-link DNA ($50 \pm 5\%$). This provided another evidence that the electron-donating group on the position-4 favors the ICL formation. Further study showed that the cross-linking yield of **2a** and **5a** were affected by their concentrations, the compound/ H_2O_2 ratio, and the pH of the buffer solution. The best compound: H_2O_2 ratio was 1:1 (SI, Figure S1 and S8) and the cross-linking yield increased with increasing compound concentrations (Figure SI, Figure S2 and S9). The cross-linking was more efficient under basic conditions than acidic conditions (SI, Figure S3 and S10).

In order to fully investigate the effect of the aromatic substituents on DNA ICL formation, we studied the kinetics of DNA cross-linking and determined the reaction rate of these compounds with H_2O_2 (Table 1). The ICL growth induced by **2a** and **5a** followed first-order kinetics, which was similar to the parent compound **1a** (SI Figure S13 A and B). The rate constants for **2a** and **5a** with an electron donating group were 2–3 times the rate constants of **1a** (Table 1). This result indicated that the electron-donating substituent facilitated DNA cross-linking as well as enhanced the cross-linking yield.

Previous studies showed that the mechanism of DNA cross-linking by the arylboronates involved generation of the phenol intermediates followed by spontaneous release of QMs capable of cross-linking DNA.[17–19] We proposed a similar mechanism for **2a** and **5a** (Scheme 4A). QM-trapping experiment with a large excess of ethyl vinyl ether (EVE) as nucleophile confirmed the formation of highly active electrophile QMs which directly cross-link DNA (Scheme 4B,C) (Note: The trapping product **27** was not stable, in particular after purification and concentration. Decomposition and color change were observed during NMR measurement, but we did detect the fragment **28** in mass spectrometry (Scheme 4C and SI, Figure S63). In order to investigate the effect of electron-donating groups on both formation of the phenolic intermediates and QM generation, we determined the reaction rate of these compounds with H_2O_2 by NMR analysis (SI, Figure S14 and S18). A mixture of phosphate buffer (pH 8 in D_2O) and DMSO in a 2:3 ratio was used to ensure good solubility of these compounds and to mimic DNA cross-linking conditions. As hydrolysis of boronate esters to the corresponding boronic acids made analysis more complex, all compounds were first hydrolyzed to the corresponding boronic acids in DMSO/pH8 buffer (10:1) prior to the addition of H_2O_2 as previously described.[17] The rate for the phenolic intermediate formation (oxidation) was estimated from the disappearance of the ^1H -NMR peaks at about 5.0 ppm (peak d) corresponding to $-\text{CH}_2-$ of the precursors. The reaction rates of these compounds with H_2O_2 are in the order of **5a** **2a**>**1a** (Table 1). The rates of QM formation were estimated by the generation of the final products (peak f, its hydrolyzed compounds), which showed a similar trend: **5a** **2a**>**1a** (Table 1). Both methoxy group and hydroxyl groups at the position-4 greatly increased the rate of QM formation. However, QM formation is generally slower than the generation of the corresponding phenol intermediate (A), which indicated that QM formation is the rate-determining step for DNA cross-linking. The results obtained from NMR analysis further supported that the electron-donating group favors the QM formation therefore leading to more efficient DNA cross-linking.

The acetate group (OAc) was used as a good leaving group in a number of inducible DNA cross-linking agents developed by research groups of Freccero and Rokita.[23, 44] We expected that the arylboronic esters with OAc as a benzylic leaving group could be efficient

H₂O₂-inducible DNA ICL agents. Compounds **1b**, **2b**, and **5b** were successfully synthesized and investigated towards H₂O₂ and DNA reactivity. As expected, these compounds can be activated by H₂O₂ to form DNA ICLs. The cross-linking reactions for **1b**, **2b**, and **5b** were complete within 90 mins, which proceeded 2–3 times faster than **1a**, **2a**, and **5a** (Table 1). However, their cross-linking yields (**1b**: 20%; **2b**: 26%; and **5b**: 32%) are lower than the corresponding bromides **1a**, **2a**, and **5a** (22%, 36%, and 50%) (Table 1). Further study showed that ICL formation induced by **1b**, **2b**, and **5b** did not follow the first order kinetics (SI, Figure S13 C–E) and the cross-linking yield did not correlate well with the reaction rates. One possibility might be the hydrolysis of the acetate under the conditions used for DNA cross-linking. In order to test our hypothesis, the NMR analysis was performed with **1b**, **2b**, and **5b** in a mixture of phosphate buffer (pH8 in D₂O) and DMSO in a 2:3 ratio. The results showed that the acetate group was hydrolyzed prior to addition of H₂O₂ (SI, Figure S16–18 B) resulting in low ICL formation for **1b**, **2b**, and **5b**. We also observed that the oxidative deboronation of **1b**, **2b** and **5b** with H₂O₂ was much slower than that of **1a**, **2a** and **5a**. The reaction of **2a** and **5a** with H₂O₂ was completed within 30 min for **2a** and 20 min for **5a** (SI, Figure S14 and 15 E, F), while the reaction of **2b** or **5b** with H₂O₂ were not finished within one hour (SI, Figure S17 and 18 E). However, due to the complexity of the reactions, the rate constants could not be determined. A possible reason for the slower reaction of **2b** and **5b** towards H₂O₂ than **2a** and **5a** might be the formation of the hypercoordinated complexes **A** and/or **B** caused by the interaction between the oxygen of OAc and boron. There are precedents for the formation of such complexes between boron and oxygen or nitrogen.[45] In addition, NMR analysis showed that the resonances corresponding to the pinacol ester for OAc compound **2b** (δ = 1.37 ppm for (C4',5'-H) shifted to upper field than those for bromo compound **2a** (δ = 1.46 ppm for **2a**) (SI Figure S24 and S26). Slightly upper field shift was also observed for ¹³C NMR for the pinacol ester and 3 ppm up field shift observed for the aromatic C1 (SI Figure S27 and S29). These data suggested that coordination of oxygen and boron increased the electron density of boron therefore decreasing the deshielding effect. All of the above observations suggested that the acetate group was not a suitable leaving group for designing novel arylboronic esters as efficient H₂O₂-inducible cross-linking agents.

Having established that these compounds could be effectively activated by H₂O₂ to induce ICL formation, their toxicity towards cancer cells was evaluated. Initially, the ability of **1a** and **2a** to reduce cancer cell growth was determined with 60 human cancer cells lines by National Cancer Institute DTP program. Single dose screening at 10 μ M showed that **2a** induced significant growth inhibition of most cancer cell lines and was more toxic than **1a** (SI, Figure S20 and 21). The growth percentage of most cell lines treated with 10 μ M of **2a** was less than 50%. Thus, dose dependent analysis of **2a** was further evaluated in all 60 human cancer cell lines exhibiting GI₅₀ values of around 2 μ M in most cancer cells (SI, Table S1).

Encouraged by the NCI results, we compared the effects of these compounds in a selected number of cancer cells, such as ovarian cancer SKOV3 cell, breast cancer MDA-MB-468 cell, and seven renal cancer cell lines. The initial test with SKOV3 cells showed that **1b**, **2b**, and **4a** were not cytotoxic, however **1a**, **2a**, and **5a** led to significant cancer cell death with

an IC₅₀ of 6.3 μ M for **1a**, 5.2 μ M for **2a**, and 3.8 μ M for **5a** (SI, Figure S23). Therefore, we focused on the active compounds **1a**, **2a**, and **5a** and compared their cytotoxicity with two clinically used alkylating agents, chlorambucil and melphalan in breast cancer cells MDA-MB-468 and seven different renal cancer cell lines, UO-31, A498, SN12C, 786-0, TK-10, CAKI-1 and ACHN. In general, **1a**, **2a**, and **5a** were more effective than chlorambucil and melphalan in these cell lines. Additionally, **2a** was more potent than **1a** and **5a** in most cells except for CAKI-1 cells that were more sensitive toward **5a** than **1a** and **2a** (Table 2). Among the cells, MDA-MB-468 was the most sensitive cell line in respect to H₂O₂-activated QM prodrugs (Figure 2 and Table 2). Among different renal cancer cell lines, compounds **1a**, **2a**, and **5a** are more cytotoxic to UO-31, A498, SN12C and 786-0 (IC₅₀ of 7.7 μ M–27.8 μ M) than TK-10, CAKI-1 and ACHN (Table 2, SI, Figure S22).

In addition to cell lines, compounds **1a**, **2a**, and **5a** were also tested and compared with chlorambucil and melphalan in lymphocytes obtained from chronic lymphocytic leukemia (CLL) patients (n=3). Dose dependent apoptosis was observed for CLL cells. One representative patient data is provided in Figure 3A. Compounds **1a**, **2a**, and **5a** demonstrated potent cytotoxicity (IC₅₀ – 48.3, 28.4 and 20.8 μ M, respectively) in comparison to chlorambucil and melphalan (IC₅₀ – 98 and 76.9 μ M). We tested the toxicity of compounds **1a**, **2a**, and **5a** in normal lymphocytes obtained from healthy donors (n=2). These compounds exhibited no significant toxicity to normal lymphocytes.

DNA damage response is associated with apoptosis in leukemia cells

With the encouraging data on H₂O₂-induced ICL formation and growth inhibition, we also tested the compounds for the induction of DNA damage response in patient cells. CLL lymphocytes were incubated with 10 μ M of **1a**, **2a**, and **5a** (n=3; 24 hours) and the H2AX phosphorylation at Ser139 was measured (Figure 4A). [46] There was induction of DNA damage response exhibiting heterogeneity among samples (p values 0.45, 0.15, and 0.04 respectively). We next correlated the apoptosis with DNA damage response in the same samples. Our results demonstrated connectivity between DNA damage response and cytotoxicity in CLL cells (Figure 4B–C). Measure of viability of cells and H2AX phosphorylation in **2a**- and **5a**-treated cells obtained from the same patient (n=4; % control) showed a linear correlation (r^2 = 0.8 and 0.97 respectively) between these two end-points.

CONCLUSIONS

In summary, our investigation of H₂O₂-induced reactivity of a series of novel arylboronate esters with DNA revealed that the position and number of electron donating aromatic substituents significantly affected their DNA cross-linking ability and cellular activity. An electron donating group placed *meta* to the QM methylene greatly facilitates QM formation, which is the rate-determining step for DNA cross-linking. Additionally, leaving groups influenced the reaction rate of these boronates with H₂O₂ and subsequent QM formation and DNA cross-linking. Br was observed as a better leaving group than acetate for these arylboronate derivatives. Hydrolysis of the acetates and possible hypercoordination with boronates might be the reasons for slow reaction rates with H₂O₂ as well as QM formation. Sufficient DNA-crosslinking of compounds (**2a** and **5a**) with a donating group at the *meta*

position directly translated to enhanced cellular activity with significant growth inhibition of leukemia, colon cancer, melanoma, and renal cancer cells. Replicationally quiescent CLL lymphocytes also showed induction of H2AX phosphorylation which is correlated with cell death with these compounds. This is a novel example of H₂O₂-induced QM prodrugs (**2a** and **5a**) with potent anticancer activity. These novel molecular scaffolds will be used for further drug design as well molecular probes to elucidate cellular responses of DNA-alkylation.

EXPERIMENTAL SECTION

General Methods

Unless otherwise specified, all chemicals and reagents were commercially purchased and were used as received without further purification. Oligonucleotides were synthesized via standard automated DNA synthesis techniques in a 1.0 μ M scale using commercial 1000Å CPG-succinyl-nucleoside supports. Deprotection of the nucleobases and phosphate moieties as well as cleavage of the linker were carried out under mild deprotection conditions using a mixture of 40% aq. MeNH₂ and 28% aq. NH₃(1:1) at room temperature for 2 h. Oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis. Radiolabeling was carried out according to the standard protocols. Quantification of radiolabeled oligonucleotides was carried out using a Phosphorimage equipped with ImageQuant Version 5.2 software. For all Phosphorimage autoradiogram, the top dark spot that migrated slowest is considered as DNA cross-link product and the bottom dark spot considered as the single-stranded ODN, which were determined using molecular ladder.[15, 19] All cross-linking yields were subtracted from the control experiment with DNA and drug without addition of H₂O₂. ¹H NMR and ¹³C NMR spectra were taken on 300 MHz or 500 MHz spectrophotometer. High resolution mass spectrometry was performed at the University of California-Riverside and University of Wisconsin-Milwaukee Mass Spectrometry Lab on an atmospheric-pressure chemical ionization (APCI) TOF mass spectrometer or electron spray injection mass spectrometer (ESI). The purity was determined by RP-HPLC on a 4.6×250 mm RP-C18 column with 254 nm detection, which confirmed that all compounds had 95% purity.

2-(2,6-Bis(bromomethyl)-4-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2a)—A solution of **7** (1.31 g, 5 mmol), NBS (1.87 g 10.5 mmol), and AIBN (82.1 mg 0.5 mmol) in anhydrous CCl₄ (30 mL) was stirred to reflux with a light for 2 h. The mixture was allowed to cool to room temperature. The solvent was evaporated and 50 mL CH₂Cl₂ added. The organic layer was washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure then purified through column chromatography (30% DCM/Hexane) to give **2a** (0.63 g 30%) as white solid: mp 103–104 °C; ¹H NMR (300 MHz, CDCl₃): δ 6.85 (s, 2H), 4.84 (s, 4H), 3.84 (s, 3H), 1.46 (s, 12H). ¹³C NMR (125 MHz, CDCl₃): δ 160.7, 146.4, 115.7, 84.0, 55.3, 34.1, 25.1. IT-TOF-MS (APCI): *m/z* calcd. for C₁₅H₂₁O₃BBr₂ [M+H]⁺ 419.0026, found 419.0022.

2-(3,5-Dimethoxy-2,6-dimethylphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (11)—To a stirred solution of **10** (1.88 g, 7.7 mmol) in anhydrous THF (40 mL) was added

dropwise a 2.5M solution of *n*-BuLi (3.7 mL, 9.25 mmol) at -78°C via cannula over a 2 min period under argon. The cloudy solution was stirred at -78°C for 30 min. Isopropoxyboronic acid pinacol ester (1.89 mL, 9.25 mmol) was added at -78°C under argon via syringe. The mixture was allowed to stir at -78°C for 30 min, then warmed slowly to room temperature and stirred for 6 h. The mixture was quenched with aqueous 1M HCl solution and extracted with 3×30 mL EtOAc. The organic layer was washed with water and brine, dried over sodium sulfate, concentrated under reduced pressure, and purified through column chromatography (5% EtOAc/Hexane) to give **11** (1.92 g 85%) as white solid: mp $140-141^{\circ}\text{C}$; ^1H NMR (300 MHz, CDCl_3): δ 6.46 (s, 1H), 3.81 (s, 6H), 2.21 (s, 6H), 1.42 (s, 12H). ^{13}C NMR (75 MHz, CDCl_3): δ 156.1, 121.2, 97.2, 83.9, 56.0, 25.1, 14.7. HRMS (ESI): m/z calcd. for $\text{C}_{16}\text{H}_{25}\text{O}_4\text{B}$ $[\text{M}]^+$ 291.1877, found 291.1872.

2-(2,6-Bis(bromomethyl)-3,5-dimethoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (3a)—A solution of **11** (584 mg, 2 mmol), NBS (854 mg, 4.8 mmol), and AIBN (32.8 mg, 0.2 mmol) in anhydrous CCl_4 (10 mL) was stirred to reflux under a light for 2h. The mixture was allowed to cool to room temperature and the solvent evaporated. Then, 20 mL CH_2Cl_2 was added. The organic layer was washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure to give **3a** (120 mg 13%). ^1H NMR (300 MHz, CDCl_3): δ 6.47 (s, 1H), 4.9 (s, 4H), 3.92 (s, 6H), 1.49 (s, 12H). **3a** is not stable enough for ^{13}C NMR and MS.

4,4,5,5-Tetramethyl-2-(3,4,5-trimethoxy-2,6-dimethylphenyl)-1,3,2-dioxaborolane (14)—To a stirred solution of **13** (3.88 g, 14.2 mmol) in anhydrous THF (40 mL) was added dropwise a 2.2 M solution of *n*-BuLi (7.7 mL, 17 mmol) at -78°C via cannula over a 2 min period under argon. The cloudy solution was stirred at -78°C for 30 min. Then, isopropoxyboronic acid pinacol ester (3.47 mL, 17 mmol) was added at -78°C under argon via syringe. The mixture was allowed to stir at -78°C for 30 min, warm slowly to room temperature, and stirred for 6 h. The mixture was quenched with aqueous 1M HCl solution, extracted with 3×30 mL EtOAc, the organic layer was washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure then purified through column chromatography (5% EtOAc/Hexane) to give **14** (3.32 g 73%) as colorless oil. ^1H NMR (300 MHz, CDCl_3): δ 3.91 (s, 3H), 3.79 (s, 6H), 2.28 (s, 6H), 1.41 (s, 12H). ^{13}C NMR (75 MHz, CDCl_3): δ 149.7, 147.2, 130.3, 83.9, 60.7, 60.5, 25.0, 15.1. HRMS (ESI): m/z calcd. for $\text{C}_{17}\text{H}_{27}\text{O}_5\text{B}$ $[\text{M}]^+$ 321.1982, found 321.1983.

2-(2,6-Bis(bromomethyl)-3,4,5-trimethoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (4a)—A solution of **14** (1.28 g, 4 mmol), NBS (1.71 g 9.6 mmol) and AIBN (65.6 mg 0.4 mmol) in anhydrous CCl_4 (30 mL) was stirred to reflux under a light for 2h. The mixture was allowed to cool to room temperature. After evaporation of the solvent followed by addition of 20 mL CH_2Cl_2 , the organic layer was washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure then purified through column chromatography (2.5% EtOAc/Hexane) to give **4a** (0.53 g 28%) as white solid: mp $96-98^{\circ}\text{C}$; ^1H NMR (300 MHz, CDCl_3): δ 4.90 (s, 4H), 3.99 (s, 6H), 3.91 (s, 3H), 1.48 (s, 12H). ^{13}C NMR (75 MHz, CDCl_3): δ 152.7, 147.4, 132.8, 84.5, 61.2, 60.6, 27.6, 25.1. HRMS (ESI): m/z calcd. For $\text{C}_{17}\text{H}_{25}\text{O}_5\text{BBr}_2$ $[\text{M}]^+$ 477.0193, found 477.0179.

(3,5-Bis(bromomethyl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy) (tert-butyl) dimethylsilane (18)—A solution of **17** (4.1 g, 11.8 mmol), NBS (5.11 g, 28.4 mmol) and AIBN (194 mg, 0.118 mmol) in anhydrous CCl₄ (100 mL) was stirred to reflux under a light for 2h. The mixture was allowed to cool to room temperature. After evaporation of the solvent followed by addition of 100 mL CH₂Cl₂, the organic layer was washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure then purified through column chromatography (20% DCM/Hexane) to give **18** (2.67 g 44%) as white solid: mp 81–82 °C; ¹H NMR (300 MHz, CDCl₃): δ 6.79 (s, 2H), 4.80 (s, 4H), 1.47 (s, 12H), 1.00 (s, 9H), 0.23 (s, 6H). ¹³C NMR (125 MHz, CDCl₃): δ 157.1, 146.3, 121.9, 84.0, 34.0, 25.7, 25.1, 18.2. IT-TOF-MS (APCI): *m/z* calcd. for C₂₀H₃₃O₃BSiBr₂ [M+H]⁺ 519.0736, found 519.0736.

3,5-Bis(bromomethyl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenol (5a)—A solution of **18** (2.6 g, 5.0 mmol) and TBATB (0.24 g, 0.5 mmol) in MeOH (50 mL) was stirred for one day, then another portion of TBATB (0.24 g, 0.5 mmol) was added to the solution. The mixture was stirred for one more day, evaporated, and purified through column chromatography (20% DCM/Hexane) to give **5a** (0.72 g 36%) as white solid: mp 195–195 °C; ¹H NMR (300 MHz, CDCl₃): δ 6.78 (s, 2H), 4.79 (s, 4H), 1.46 (s, 12H). ¹³C NMR (125 MHz, CDCl₃): δ 156.8, 146.7, 117.1, 84.1, 33.7, 25.1. HRMS (ESI): *m/z* calcd. for C₁₄H₁₉O₃BBr₂ [M]⁺ 402.9825, found 402.9814.

(2-Bromo-1,3-phenylene)bis(methylene) diacetate (21)—To a solution of **20** (1.08 g, 5 mmol) in CH₂Cl₂ (25 mL) was added Et₃N (2.02 g, 20 mmol), pyridine (1.58 g, 20 mmol), and acetyl chloride (1.56 g, 20 mmol) at 0 °C. The mixture was allowed to warm up to room temperature and stirred overnight. The reaction mixture was washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure, then purified through column chromatography (2.5% EtOAc/Hexane) to give **21** (1.13 g, 75%) as white solid: mp 51–52 °C; ¹H NMR (300 MHz, CDCl₃): δ 7.43–7.36 (m, 3H), 5.25 (s, 4H), 2.17 (s, 6H). ¹³C NMR (75 MHz, CDCl₃): δ 170.6, 136.1, 129.4, 127.4, 124.3, 66.0, 20.9. IT-TOF-MS (APCI): *m/z* calcd. for C₁₂H₁₃O₄Br [M+H]⁺ 301.0070, found 301.0060.

(2-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-1,3-phenylene)bis(methylene) diacetate (1b)—A mixture of **21** (301 mg, 1 mmol), bis(pinacolato)diboron (508 mg, 2 mmol), KOAc (589 mg, 6 mmol), and PdCl₂(dppf) (49 mg, 0.06 mmol) in 1,4-dioxane (20 mL) was refluxed under argon overnight and cooled to room temperature. Then, water was added and the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure then purified through column chromatography (50% DCM/Hexane) to provide **1b** (121.9 mg, 35%) as white solid: mp 43–44 °C; ¹H NMR (300 MHz, CDCl₃): δ 7.36–7.39 (m, 3H), 5.28 (s, 4H), 2.08 (s, 6H), 1.39 (s, 12H). ¹³C NMR (75 MHz, CDCl₃): δ 170.6, 141.0, 130.0, 129.2, 84.1, 66.6, 24.9, 21.1. IT-TOF-MS (ESI): *m/z* calcd. for C₁₈H₂₅O₆B [M+NH₄]⁺ 366.2086, found 366.2084.

(2-Bromo-5-methoxy-1,3-phenylene)bis(methylene) diacetate (24)—A mixture of **23** (0.71 g, 1.9 mmol) and NaOAc (0.79 g, 9.6 mmol) were suspended in DMF (20 mL) and

heated for 8 h at 80 °C. The mixture was allowed to cool to room temperature, diluted with EtOAc, and washed with water and brine. The organic layer was dried over sodium sulfate and concentrated under reduced pressure then purified through column chromatography (10% DCM/Hexane) to give **24** (0.51 g, 81%) as white solid: mp 81–82 °C; ¹H NMR (300 MHz, CDCl₃): δ 6.96 (s, 2H), 5.21 (s, 4H), 3.84 (s, 3H), 2.18 (s, 6H). ¹³C NMR (75 MHz, CDCl₃): δ 170.5, 158.9, 137.0, 114.9, 114.2, 65.9, 55.6, 20.9. IT-TOF-MS (ESI): *m/z* calcd. for C₁₃H₁₅O₅Br [M+H₂O]⁺ 348.0203, found 348.0200.

(5-Methoxy-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1,3-

phenylene)bis(methylene) diacetate (2b)—A mixture of **24** (331 mg, 1 mmol), bis(pinacolato)diboron (508 mg, 2 mmol), KOAc (589 mg, 6 mmol), and PdCl₂(dppf) (49 mg, 0.06 mmol) in 1,4-dioxane (20 mL) was refluxed under argon overnight and cooled to room temperature. Water was added and the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure then purified through column chromatography (50% DCM/Hexane) to provide **2b** (147.5 mg, 39%) as white solid: mp 37–38 °C; ¹H NMR (300 MHz, CDCl₃): δ 6.91 (s, 2H), 5.29 (s, 4H), 3.85 (s, 4H), 2.10 (s, 6H), 1.37 (s, 12H). ¹³C NMR (75 MHz, CDCl₃): δ 170.7, 160.9, 143.6, 114.5, 83.8, 66.6, 24.8, 21.1. IT-TOF-MS (ESI): *m/z* calcd. for C₁₉H₂₇O₇B [M+NH₄]⁺ 396.2192, found 396.2186.

(5-Hydroxy-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1,3-

phenylene)bis(methylene) diacetate (5b)—A mixture of **5a** (100 mg, 0.248 mmol) and NaOAc (101.5 mg, 1.238 mmol) were suspended in DMF (10 mL) and heated for 8 h at 80 °C. The mixture was allowed to cool to room temperature, diluted with EtOAc, washed with water and brine, dried over Na₂SO₄, and concentrated under reduced pressure then purified through column chromatography (5% EtOAc/DCM) to give **5b** (27 mg, 31%) as white solid: mp 178–179 °C; ¹H NMR (300 MHz, CDCl₃): δ 6.86 (s, 2H), 5.28 (s, 4H), 2.10 (s, 6H), 1.37 (s, 12H). ¹³C NMR (75 MHz, CDCl₃): δ 171.9, 157.1, 144.0, 115.7, 83.8, 66.3, 24.8, 21.1. IT-TOF-MS (ESI): *m/z* calcd. for C₁₈H₂₅O₇B [M+NH₄]⁺ 382.2035, found 382.2035.

QM Trapping Assay for 2a—A solution of **2a** (50 mg) in a mixture of CH₃CN (3 mL) and 1 M potassium phosphate buffer (52 μL, pH 8) was incubated at 37 °C for 30 min in the presence of excess ethyl vinyl ether (EVE). Then, H₂O₂ (2 equivalent of **2a**) was added to the reaction mixture that was stirred at 37 °C for 24 h, then evaporated. Water (2 mL) was added to the residue and extracted with ethyl acetate (3 × 5 mL). The organic phase was combined, dried over anhydrous Na₂SO₄, and evaporated. The crude product was purified through column chromatography with 0–50% EtOAc in hexane to provide the QM-EVE adduct **26**.

(2-Ethoxy-6-methoxychroman-8-yl)methanol (26)—Colorless oil, 15% yield (4.3 mg). ¹H NMR (300 MHz, CDCl₃): δ 6.76 (s, 1H), 6.62 (s, 1H), 5.34 (s, 1H), 4.59–4.49 (m, 2H), 4.05–3.95 (m, 1H), 3.77 (s, 1H), 3.73–3.68 (m, 1H), 3.04–2.93 (m, 1H), 2.67–2.59 (m, 1H), 2.08–1.97 (m, 2H), 1.22 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (500 MHz, CDCl₃): δ 152.9,

126.0, 124.0, 115.2, 114.0, 96.8, 63.8, 55.7, 29.0, 26.4, 20.9, 15.1. IT-TOF-MS (ESI): m/z calcd. for $C_{13}H_{18}O_4$ $[M+H]^+$ 239.1278, found 239.1285.

QM Trapping Assay for 5a—To a solution of **5a** (100 mg) in CH_3CN , 83.6 mg potassium phosphate and excess EVE were added. Then, H_2O_2 (2 equivalent of **5a**) was added to the reaction mixture that was stirred at 37 °C for 24 h, then evaporated. Water (2 mL) was added to the residue and extracted with ethyl acetate (3×5 mL). The organic phase was combined, dried over anhydrous Na_2SO_4 , and evaporated. The crude product was purified through column chromatography with 10% EtOAc in hexane to provide the QM-EVE adduct **27**.

8-(Bromomethyl)-2-ethoxychroman-6-ol (27)—Colorless oil, 20% yield (13.7 mg). 1H NMR (300 MHz, DMSO- d_6): δ 6.63 (s, 1H), 6.46 (s, 1H), 5.31 (s, 1H), 4.59-4.50 (q, 2H, $J = 9.3$ Hz), 3.92-3.82 (m, 1H), 3.69-3.58 (m, 1H), 2.79-2.68 (m, 1H), 2.55-2.49 (m, 1H), 1.96-1.80 (m, 2H), 1.13-1.08 (t, $J = 6.9$ Hz, 3H). ^{13}C NMR (500 MHz, DMSO- d_6): δ 150.7, 143.1, 125.9, 123.9, 116.8, 115.8, 96.7, 63.4, 30.5, 26.4, 20.6, 15.5. Decomposition was observed during NMR measurement. We also observed that the solution changed from colorless to orange after NMR analysis. The instability also made the mass analysis of **27** $C_{12}H_{15}O_3Br$ more difficult. The molecular ion $[M]^+$ (286.0205) peak was not observed. However, the fragmentation of **28** $[M-Br]^+$ 207.1021 was detected, found 207.0979.

Interstrand cross-link formation with duplex DNA 25—The ^{32}P -labelled oligonucleotide (0.5 μM) was annealed with 1.5 equiv of the complementary strand by heating to 65 °C for 3 min in a solution of 10 mM potassium phosphate buffer (pH 7) and 100 mM NaCl, followed by cooling slowly to room temperature overnight. The ^{32}P -labeled oligonucleotide duplex (2 μL , 0.5 μM) was mixed with 1 M NaCl (2 μL), 100 mM potassium phosphate (2 μL , pH 6–8), H_2O_2 (2 μL), and compounds **1–5** (concentration range: 100 μM to 2 mM in 6 μL CH_3CN). Then, the appropriate amount of autoclaved distilled water was added to give a final volume of 20 μL . The reaction was incubated at room temperature for 24 h and quenched by an equal volume of 90% formamide loading buffer, then subjected to 20% denaturing polyacrylamide gel electrophoresis.

Cell inhibition study of 1a, 2a and 5a towards tumor cells—The *in vitro* cancer cell screening with 60 human cancer cell lines was performed at the National Cancer Institute (NCI Developmental Therapeutics Program). The procedure details can be found in NCI website: <http://dtp.nci.nih.gov/branches/btb/ivclsp.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 were inoculated into 96 well microtiter plates in 100 μL at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines.

Cytotoxicity study of 1a, 2a, and 5a towards tumor cells—The human tumor cell lines were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 384-well microtiter plates in 20 μL at plating densities ranging from 5,000 to 10,000 cells/well. After cell inoculation, the microtiter plates were

incubated at 37°C, 5 % CO₂, 95% air, and 100% relative humidity for 2–3 h prior to addition of drugs.

Drugs were solubilized in DMSO at 20 mM concentration and serially diluted ten times each time 50% dilution in DMSO in a 384-well plate. Then 200 nL of the serially diluted drug solution was added to the cell plate (1:100 dilution) using FreedomEVOware two times 100 nL transfer. Following drug addition, the plates were incubated for an additional 48 h at 37°C, 5% CO₂, 95% air, and 100% relative humidity. After 48 hours, 20 µL CellTiter-Glo Luminescent solution was added to the cell plate. The plate was then incubated at room temperature for 10 mins before the luminescent was measured with Infinite M1000.

Patients and healthy donors

CLL or normal lymphocytes were separated from peripheral blood by Ficoll-hypaque gradient method and suspended in 10% autologous plasma in RPMI media. All patient and healthy donor participants had signed written informed consent forms in accordance with the Declaration of Helsinki, and the laboratory protocols were approved by the Institutional Review Board at the UT MD Anderson Cancer Center.

Measurement of cell viability

CLL cell viability (measure of apoptosis) was measured by annexin V/propidium iodide (PI) binding method.

Measurement of H2AX Phosphorylation

The lymphocytes harvested after incubation with compounds were washed with PBS once and fixed in 6–8 mL ice-cold ethanol (70%) and stored at –20°C until analysis for H2AX phosphorylation. The cells were then permeabilised and stained with FITC tagged H2AX^{Ser139} antibody (Biolegend, San Diego CA) and measured in the flow cytometer according to manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health [1R15CA152914-01 and R03 DA031090], Great Milwaukee Foundation (Shaw Scientist Award), the University of Wisconsin Milwaukee Research Growth Initiative, the University of Wisconsin Milwaukee Research Foundation (Catalyst grant), the Lynde and Harry Bradley Foundation, the Richard and Ethel Herzfeld Foundation, and Wisconsin Applied Research Grant (ARG) Award. This work was supported in part by the Alliance grant from CLL Global Research Foundation.

Abbreviations

AIBN	azobisisobutyronitrile
APCI	atmospheric pressure chemical ionization
CLL	chronic lymphocytic leukemia

DCM	dichloromethane
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
EVE	ethyl vinyl ether
FITC	fluorescein isothiocyanate
HRMS	high resolution mass spectrometry
ICLs	interstrand cross-links
IT-TOF-MS	Ion-Trap-Time-of-Flight-Mass-Spectrometer
NBS	<i>N</i> -bromosuccinimide
NL	normal lymphocytes
PAGE	denaturing polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PI	propidium iodide
QM	quinone methide
ROS	reactive oxygen species
TBATB	tetrabutylammonium tribromide
TBDMSCl	tert-Butyldimethylsilyl chloride

References

1. Cao S, Peng X. Exploiting endogenous cellular process to generate quinone methides in vivo. *Curr. Org. Chem.* 2014; 18:70–85.
2. Noll DM, Mason TM, Miller PS. Formation and repair of interstrand cross-links in DNA. *Chem. Rev.* 2006; 106:277–301. [PubMed: 16464006]
3. Hagen H, Marzenell P, Jentzsch E, Wenz F, Veldwijk MR, Mokhir A. Aminoferrocene-based prodrugs activated by reactive oxygen species. *J. Med. Chem.* 2012; 55:924–934. [PubMed: 22185340]
4. Peng X, Gandhi V. ROS-activated anticancer prodrugs: a new strategy for tumor-specific damage. *Ther. Deliv.* 2012; 3:823–833. [PubMed: 22900465]
5. Johnson KM, Parsons ZD, Barnes CL, Gates KS. Toward hypoxia-selective DNA-alkylating agents built by grafting nitrogen mustards onto the bioreductively activated, hypoxia-selective DNA-oxidizing agent 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine). *J. Org. Chem.* 2014; 79:7520–7531. [PubMed: 25029663]
6. Tercel M, Lee AE, Hogg A, Anderson RF, Lee HH, Siim BG, Denny WA, Wilson WR. Hypoxia-selective antitumor agents. 16. Nitroarylmethyl quaternary salts as bioreductive prodrugs of the alkylating agent mechlorethamine. *J. Med. Chem.* 2001; 44:3511–3522. [PubMed: 11585455]

7. Mauger AB, Burke PJ, Somani HH, Friedlos F, Knox RJ. Self-immolative prodrugs: candidates for antibody-directed enzyme prodrug therapy in conjunction with a nitroreductase enzyme. *J. Med. Chem.* 1994; 37:3452–3458. [PubMed: 7932574]
8. Duan JX, Jiao H, Kaizerman J, Stanton T, Evans JW, Lan L, Lorent G, Banica M, Jung D, Wang J, Ma H, Li X, Yang Z, Hoffman RM, Ammons WS, Hart CP, Matteucci MP. Potent and highly selective hypoxia-activated achiral phosphoramidate mustards as anticancer drugs. *J. Med. Chem.* 2008; 51:2412–2420. [PubMed: 18257544]
9. Hileman EO, Liu J, Albitar M, Keating MJ, Huang P. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemother. Pharmacol.* 2004; 53:209–219. [PubMed: 14610616]
10. Lim SD, Sun C, Lambeth JD, Marshall F, Amin M, Chung L, Petros JA, Arnold RS. Increased Nox1 and hydrogen peroxide in prostate cancer. *Prostate.* 2005; 62:200–207. [PubMed: 15389790]
11. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* 1991; 51:794–798. [PubMed: 1846317]
12. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev. Drug Discov.* 2009; 8:579–591. [PubMed: 19478820]
13. Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H, Chiao PJ, Achanta G, Arlinghaus RB, Liu J, Huang P. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell.* 2006; 10:241–252. [PubMed: 16959615]
14. Chen W, Balakrishnan K, Kuang Y, Han Y, Fu M, Gandhi V, Peng X. Reactive oxygen species (ROS) inducible DNA cross-linking agents and their effect on cancer cells and normal lymphocytes. *J. Med. Chem.* 2014; 57:4498–4510. [PubMed: 24801734]
15. Chen W, Han Y, Peng X. Aromatic nitrogen mustard-based prodrugs: activity, selectivity, and the mechanism of DNA cross-linking. *Eur. J. Chem.* 2014; 20:7410–7418.
16. Kuang Y, Balakrishnan K, Gandhi V, Peng X. Hydrogen peroxide inducible DNA cross-linking agents: targeted anticancer prodrugs. *J. Am. Chem. Soc.* 2011; 133:19278–19281. [PubMed: 22035519]
17. Cao S, Christiansen R, Peng X. Substituent effects on oxidation-induced formation of quinone methides from arylboronic ester precursors. *Eur. J. Chem.* 2013; 19:9050–9058.
18. Cao S, Wang Y, Peng X. ROS-inducible DNA cross-linking agent as a new anticancer prodrug building block. *Eur. J. Chem.* 2012; 18:3850–3854.
19. Cao S, Wang Y, Peng X. The leaving group strongly affects H₂O₂-induced DNA cross-linking by arylboronates. *J. Org. Chem.* 2014; 79:501–508. [PubMed: 24378073]
20. Freccero M. Quinone Methides as Alkylating and Cross-Linking Agents Mini-Reviews in Organic Chemistry. 2004; 1:403–415.
21. Wang P, Song Y, Zhang L, He H, Zhou X. Quinone methide derivatives: important intermediates to DNA alkylating and DNA cross-linking actions. *Curr. Med. Chem.* 2005; 12:2893–2913. [PubMed: 16305478]
22. Thompson D, Moldeus P. Formation and reactivity of a quinone methide in biological systems. *Adv. Exp. Med. Biol.* 1991; 283:589–596. [PubMed: 2069027]
23. Weinert EE, Dondi R, Colloredo-Melz S, Frankenfield KN, Mitchell CH, Freccero M, Rokita SE. Substituents on quinone methides strongly modulate formation and stability of their nucleophilic adducts. *J. Am. Chem. Soc.* 2006; 128:11940–11947. [PubMed: 16953635]
24. Wang P, Liu R, Wu X, Ma H, Cao X, Zhou P, Zhang J, Weng X, Zhang XL, Qi J, Zhou X, Weng L. A potent, water-soluble and photoinducible DNA cross-linking agent. *J. Am. Chem. Soc.* 2003; 125:1116–1117. [PubMed: 12553783]
25. Di Antonio M, Doria F, Mella M, Merli D, Profumo A, Freccero M. Novel naphthalene diimides as activatable precursors of bisalkylating agents, by reduction and base catalysis. *J. Org. Chem.* 2007; 72:8354–8360. [PubMed: 17892300]
26. Verga D, Nadai M, Doria F, Percivalle C, Di Antonio M, Palumbo M, Richter SN, Freccero M. Photogeneration and reactivity of naphthoquinone methides as purine selective DNA alkylating agents. *J. Am. Chem. Soc.* 2010; 132:14625–14637. [PubMed: 20863115]

27. Percivalle C, La Rosa A, Verga D, Doria F, Mella M, Palumbo M, Di Antonio M, Freccero M. Quinone methide generation via photoinduced electron transfer. *J. Org. Chem.* 2011; 76:3096–3106. [PubMed: 21425810]
28. Richter SN, Maggi S, Mels SC, Palumbo M, Freccero M. Binol quinone methides as bisalkylating and DNA cross-linking agents. *J. Am. Chem. Soc.* 2004; 126:13973–13979. [PubMed: 15506758]
29. Britt PF, Buchanan AC 3rd, Cooney MJ, Martineau DR. Flash vacuum pyrolysis of methoxy-substituted lignin model compounds. *J. Org. Chem.* 2000; 65:1376–1389. [PubMed: 10814099]
30. Ashot Merijan PDG. Quinone Methides. Base-Catalyzed Condensation Reactions of Hydroxybenzyl Alcohols and Ethers. *J. Org. Chem.* 1965; 30:3965–3967.
31. Hong IS, Greenberg MM. DNA interstrand cross-link formation initiated by reaction between singlet oxygen and a modified nucleotide. *J. Am. Chem. Soc.* 2005; 127:10510–10511. [PubMed: 16045337]
32. Weng X, Ren L, Weng L, Huang J, Zhu S, Zhou X, Weng L. Synthesis and biological studies of inducible DNA cross-linking agents. *Angew. Chem. Int. Ed. Engl.* 2007; 46:8020–8023. [PubMed: 17853519]
33. Rokita SE, Yang J, Pande P, Greenberg WA. Quinone Methide Alkylation of Deoxycytidine. *J. Org. Chem.* 1997; 62:3010–3012. [PubMed: 11671669]
34. Veldhuyzen WF, Lam YF, Rokita SE. 2'-Deoxyguanosine reacts with a model quinone methide at multiple sites. *Chem. Res. Toxicol.* 2001; 14:1345–1351. [PubMed: 11559052]
35. Veldhuyzen WF, Pande P, Rokita SE. A transient product of DNA alkylation can be stabilized by binding localization. *J. Am. Chem. Soc.* 2003; 125:14005–14013. [PubMed: 14611237]
36. Di Antonio M, Doria F, Richter SN, Bertipaglia C, Mella M, Sissi C, Palumbo M, Freccero M. Quinone methides tethered to naphthalene diimides as selective G-quadruplex alkylating agents. *J. Am. Chem. Soc.* 2009; 131:13132–13141. [PubMed: 19694465]
37. Doria F, Richter SN, Nadai M, Colloredo-Mels S, Mella M, Palumbo M, Freccero M. BINOL-amino acid conjugates as triggerable carriers of DNA-targeted potent photocytotoxic agents. *J. Med. Chem.* 2007; 50:6570–6579. [PubMed: 18047263]
38. Doria F, Lena A, Bargiggia R, Freccero M. Conjugation, Substituent, and Solvent Effects on the Photogeneration of Quinone Methides. *J. Org. Chem.* 2016; 81:3665–3673. [PubMed: 27035895]
39. Weinert EE, Frankenfield KN, Rokita SE. Time-dependent evolution of adducts formed between deoxynucleosides and a model quinone methide. *Chem. Res. Toxicol.* 2005; 18:1364–1370. [PubMed: 16167827]
40. Hansen MM, Jolly RA, Linder RJ. Boronic Acids and Derivatives-Probing the Structure-Activity Relationships for Mutagenicity. *Org. Process Res. Dev.* 2015; 19:1507–1516.
41. Wang Y, Liu S, Lin Z, Fan Y, Wang Y, Peng X. Photochemical Generation of Benzyl Cations That Selectively Cross-Link Guanine and Cytosine in DNA. *Org. Lett.* 2016; 18:2544–2547. [PubMed: 27191599]
42. Bugarin A, Connell BT. *Organometallics.* 2008; 27:4357–4369.
43. Gniazdowski M, Cera C. The Effects of DNA Covalent Adducts on in Vitro Transcription. *Chem. Rev.* 1996; 96:619–634. [PubMed: 11848767]
44. Veldhuyzen WF, Shallop AJ, Jones RA, Rokita SE. Thermodynamic versus kinetic products of DNA alkylation as modeled by reaction of deoxyadenosine. *J. Am. Chem. Soc.* 2001; 123:11126–11132. [PubMed: 11697955]
45. Hall, DG. *Boronic Acids: Preparation and Applications in Organic Synthesis and Medicine.* WILEY-VCH Verlag GmbH & Co.; KGaA, Weinheim: 2005. Structure, Properties, and Preparation of Boronic Acid Derivatives. Overview of Their Reactions and Applications.
46. Podhorecka M, Skladanowski A, Bozko P. H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy. *J Nucleic Acids.* 2010; 2010

5'-dGCCTAGTTCTTTTAATTACTTGCAATGCAAGTAATTAAAGCTTGATCTG (**25a**)
 3'-dCGGATCAAGAAAATTAATGAACGTTACGTTTCATTAATTTGGAAGTAGAC (**25b**)
25

Figure 1.

H₂O₂-induced DNA ICL formation by compounds **1–5**. Lane 1: DNA only (cross-linking yield 0%); lane 2: DNA with 100 μ M H₂O₂ (0%); lane 3–10 without H₂O₂: lane 3: 2 mM **1a** (0%); lane 4: 2 mM **2a** (0%); lane 5: 2 mM **3a** ($17 \pm 3\%$); lane 6: 2 mM **4a** (0%); lane 7: 2 mM **5a** (0%); lane 8: 2 mM **1b** (0%); lane 9: 2 mM **2b** (0%); lane 10: 2 mM **5b** (0%); lane 11–18 with H₂O₂: lane 11: 2 mM **1a** ($22 \pm 5\%$); lane 12: 2 mM **2a** ($36 \pm 4\%$); lane 13: 2 mM **3a** ($20 \pm 2\%$); lane 14: 2 mM **4a** (0%); lane 15: 2 mM **5a** ($50 \pm 5\%$); lane 16: 2 mM **1b** ($20 \pm 3\%$); lane 17: 2 mM **2b** ($26 \pm 3\%$); lane 18: 2 mM **5b** ($32 \pm 4\%$); [H₂O₂] = 2 mM (Reaction mixture was incubated at 37°C for 48 h).

Figure 2.

Comparison of inhibition activity of **1a**, **2a**, and **5a** with that of chlorambucil and melphalan in breast cancer cell line MDA-MB-468. The Fluorescence polarization (FP) assay was conducted in 384-well microplates (Corning, #3570). Compounds transfer into 20 μ l assay solution was accomplished using a stainless steel pin tool (V&P Scientific) delivering 200 nl of compound at different concentrations. Incubation time is 48 h. Inhibition of cell growth was detected by fluorescence polarization using a M1000, Tecan reader at excitation/emission wavelength of 630/685 nm. IC₅₀ value (μ M) was calculated using the following non-linear regression equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$.

Figure 3.

A. CLL lymphocytes were obtained from the peripheral blood of CLL patients (n=3) and incubated with increasing concentrations of compounds **1a**, **2a**, **5a**, chlorambucil and melphalan (1, 5, 10, 20, 50, and 100 μ M). The viability of CLL cells (measure of cytotoxicity) was measured by annexin/PI staining method. IC₅₀ data for one representative patient data is provided. **B.** Normal lymphocytes were obtained from corresponding normal donors (n=2) and incubated with 10 μ M concentration of compounds **1a**, **2a**, and **5a** (24 hours) and the % viable cells was measured by annexin/PI binding assay. One representative donor data is provided. NL – normal lymphocytes.

Figure 4.

A. CLL lymphocytes obtained from patients (n=4) were untreated or treated with **1a**, **2a** and **5a** (10 μ M; 24 hrs) and the DNA damage response was measured by the induction of H2AX phosphorylation. **B–C.** % control values of apoptosis data (measured by annexin/PI binding assay) and the H2AX phosphorylation data obtained for the same samples (n=4), treated with **2a** (**B**) and **5a** (**C**) were correlated. Linear regression analysis and paired student's t-tests (two tailed) were performed by GraphPad Prism 6 software (GraphPad Software, Inc. San Diego, CA).

Scheme 1.
Structures of the designed compounds 1–5

Scheme 2.
Synthesis of 2a (A), 3a (B), 4a (C), and 5a (D)

Scheme 3.
Synthesis of 1b (A), 2b (B), and 5b (C)

Scheme 4. Mechanism of H_2O_2 -induced ICL formation and QM trapping reaction

Scheme 5.
The possible models for boronate hypercoordination

Table 1

Kinetics of ICL formation and monomer reaction

Compds	Disappearance of starting materials ^a		QM formation ^a		Kinetics of ICL formation ^b		
	Time of completion (min)	k_{obs} ($10^{-5} s^{-1}$)	Time of completion (min)	k_{obs} ($10^{-5} s^{-1}$)	Time of completion (min)	k_{obs} ($10^{-5} s^{-1}$)	ICL (%)
1a	60	39.0 ± 1.5	60	9.5 ± 0.2	600	8.8 ± 1.3	22 ± 3
2a	42	56.7 ± 4.3	50	38.3 ± 2.2	240	23.7 ± 1.4	36 ± 4
5a	40	61.7 ± 3.2	40	58.3 ± 4.3	300	20.1 ± 1.5	50 ± 5
1b	240	19.4 ± 1.2	240	n.d. ^c	90 ^d	n.d. ^c	20 ± 3
2b	480	9.1 ± 0.6	480	n.d. ^c	90 ^d	n.d. ^c	26 ± 3
5b	480	8.3 ± 0.5	480	n.d. ^c	90 ^d	n.d. ^c	32 ± 4

^aThe rate constants for monomer reactions was determined by NMR analysis in a mixture of DMSO and a deuterated phosphate buffer pH 8 (3:2) (SI Figures S14–S19).^bThe rate constants for ICL formation was determined in a phosphate buffer pH 8, where the cross-linking reaction was performed.^cn.d.: The rate constants were not determined due to the complexity of the reactions.^dDue to quick hydrolysis of the acetate groups at pH 8 buffer, the observed reaction time for DNA cross-linking reaction was much shorter than that of the monomer reactions.

Table 2

The Cytotoxicity of **1a**, **2a** and **5a** towards renal and breast cancer cell lines

Tumor type	Cell line	IC ₅₀ (μ M)				
		1a	2a	5a	Chlorambucil	Melphalan
renal cancer	UO-31	25.3	10.8	19.5	40.7	42.9
	A498	33.3	18.1	21	280	135
	SN12C	27.0	18.6	24.4	135	71
	786-0	20.3	10.8	27.8	55.5	19.2
	TK-10	21.4	16.2	35.4	n.d.	54.5
	ACHN	26.9	20.0	35.7	133	52.1
breast cancer	CAKI-1	50.3	n.d.	38.3	n.d.	n.d.
	MDA-MB-468	12.0	9.0	11.0	34.4	48.7