DOI: 10.1002/cmdc.201100014

Oxidatively Activated DNA-Modifying Agents for Selective Cytotoxicity

Guorui Li, Tiffany Bell, and Edward J. Merino*[a]

DNA-modifying agents are stalwarts of chemotherapeutic cancer treatments, but require significant design improvements to improve selectivity, minimize side effects, and for their widespread use to continue. Herein we present a novel design strategy in which DNA-modifying agents contain an oxidizable leaving group and a nitrogen mustard. The agents form strong electrophiles specifically when oxidized. Activation, measured by hydrolysis, illustrates that oxidants increase reactivity 1700-fold. Reaction in the presence of 2′-deoxyguanosine leads to the formation of lesions. Cytotoxicity measured in HeLa cells showed that low IC_{50} values require an oxidizable hydroquinone and a nitrogen mustard fragment. Cytotoxicity measurements in 15 cancer cell lines demonstrates that oxidatively activated DNA-modifying agents are highly selective, as the analogue tested has IC_{50} values less than 10 μM for only three of the 15 cell lines; in contrast, cisplatin is highly toxic to 13 of the 15 cell lines. The selective cytotoxicity of oxidatively activated DNA-damaging agents could be useful against kidney cancer cells, as the 786-O cell line model assay resulted in an IC_{50} value of 5 μM.

Introduction

DNA-modifying agents are stalwarts of chemotherapeutic cancer treatments, but require vital design improvements to lower side effects and to continue their widespread use.[1] These agents react with DNA bases, usually at guanine, to halt DNA replication at the reaction site.[2] This reaction, although not selective, ultimately leads to cell death by one of several pathways.[3–5] The two common reactive scaffolds used in alkylating agents, *N*,*N*-bis(2-chloroethyl)amino groups and platinum(II) centers, have led to a variety of chemotherapeutic agents. However, these suffer from reactivity in non-cancerous cells which gives rise to many side effects.[6] Recent work has focused on the synthesis of molecules that attach reactive scaffolds to motifs that enter cancer cells specifically.[7–9] Our research takes an alternative design strategy wherein the reactive scaffold is stable, but is activated by processes that occur more frequently in cancer cells. Thus reactivity, and not uptake, is controlled to induce cytotoxicity more specifically in cancer cells and to lower off-target reactions. One process that has been shown to occur in some cancers is the elevation of reactive oxygen species (ROS). We hypothesize that the design of highly selective DNA-modifying agents can be achieved by oxidative activation.

Oxidative stress, as measured by levels of reactive oxygen, oxidized biomolecules, and enzyme activity, is a hallmark of cancer cells and excised tumors.[10–12] For instance, hypoxic tumors have been shown to experience increases in NADPH oxidases that give off elevated ROS as a byproduct of their catalysis.[13] The mitochondrion is another source of reactive oxygen in cells.[14,15] Mitochondria generate hydrogen peroxide via complex I in oxidative phosphorylation.[16,17] Recently, oxidative stress has been found to play a key role in the activation of several agents either in or close to clinical trials. For instance, mechanisms of both deoxynyboquinone and elesclomol involve oxidative-stress-dependent toxicity.[18,19] Several new chemotherapy strategies are being examined to take advantage of increased reactive oxygen levels within cancerous cells relative to healthy cells.[20–22] Thus, DNA-modifying agents that are activated by ROS can be more specific than the current agents if an appropriate activation strategy can be designed (Figure 1).

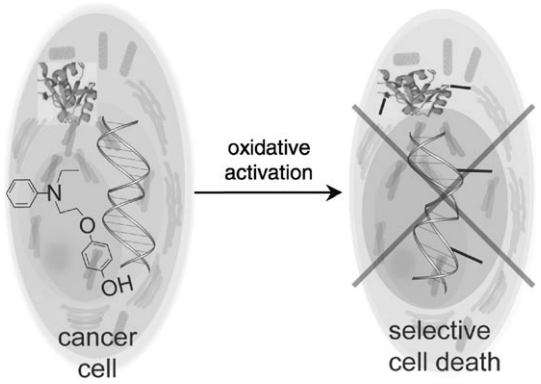
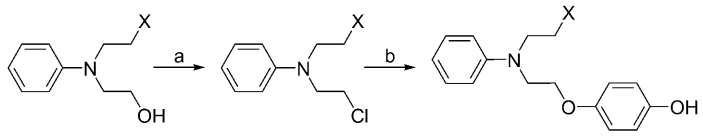


Figure 1. Oxidative-stress-induced cytotoxicity: Many cancers have been shown to possess elevated levels of reactive oxygen. We hypothesize that oxidation-prone leaving groups coupled to DNA-modifying agents can lead to specific control of cytotoxicity by reaction with cellular components.

[a] *G. Li, T. Bell, Prof. E. J. Merino*
Department of Chemistry
University of Cincinnati, Cincinnati, OH 45221-0172 (USA)
Fax: (+1) 513-556-9239
E-mail: merinoed@uc.edu

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201100014.

Our design strategy focuses on selective activation that occurs at the leaving group (Figure 1). Importantly, control of reactivity at the leaving group maximizes flexibility because the remainder of the agent is left intact for further structural optimization. Traditional nitrogen mustards are not selective because they are strong electrophiles, leading to off-target reactivity; the chlorine leaving group has a pK_a value of −4. We instead synthesized agents that are structurally similar to nitrogen mustards. These agents do not have a chlorine leaving group, but instead use a hydroquinone at the leaving group position. The hydroquinone is a poor leaving group, with a pK_a value of 10.3. Owing to the hydroquinone, reactivity via a traditional mechanism is limited. Oxidation of the hydroquinone-coupled DNA-modifying agent forms a strong electrophile and activates reactivity (Figure 1). Therefore, the cytotoxic reactivity of the agent is intimately coupled with the elevated cellular oxidative stress present in many cancers. Several model compounds were synthesized for the examination of our design strategy. Aniline-based nitrogen mustards have been used in a variety of prodrug strategies and clinically relevant alkylating agents.[23] Thus, compounds with this scaffold were synthesized (Scheme 1).



Scheme 1. General synthesis reagents and conditions: a) $SOCl_2$, 40 °C, 1 h; b) K_2CO_3, KI, 80 °C, hydroquinone (5 equiv), 6 h. X = H, OH.

Results and Discussion

Oxidative activation

The oxidative activation of the DNA-modifying agents was investigated. Water, one of the simplest nucleophiles that can serve as a substrate, was examined under various oxidative conditions (Figure 2). A model modified aniline nitrogen mustard analogue, An-Hq, was synthesized in two steps. An-Hq is similar to a nitrogen mustard, except one of the alkylating arms is nonreactive, whereas the second arm possesses a single hydroquinone replacing the chlorine leaving group (Figure 2A).

The hydrolysis of An-Hq as a function of time was quantified by separation of the reaction products by reversed-phase HPLC (Figure 2B). In phosphate buffer hydrolysis was slow, with a half-life of 693 ± 42 h assuming first-order kinetics. To obtain quantifiable data, time points were taken daily over the course of one week. Thus, An-Hq is a poor DNA-modifying agent if not activated by oxidation. Addition of the one-electron oxidant $Na_2Ir_2Cl_6$ led to quantitative hydrolysis of An-Hq with a half-life of 0.4 ± 0.2 h (Figure 2B). The oxidation potential of Ir^{IV} is 0.7 V versus Ag/AgCl, making it an appropriate choice for the oxidation of hydroquinone derivatives.[24] Cells produce ROS from both hydroxyl radical and singlet oxygen. Copper can be used to produce both oxygen species.[25,26] Oxidation of An-Hq by $CuCl_2$–H_2O_2 led to rapid hydrolysis with a half-life of 12.1 ± 0.7 h. The extent of hydrolysis depends on the concentration of hydrogen peroxide, and was slow due to the lack of ascorbate (data not shown). Oxidases such as horseradish peroxidase reduce molecular oxygen to either hydrogen peroxide or water and at the same time oxidize a substrate with an appropriate potential. Thus, we determined the extent of reaction of An-Hq in the presence of horseradish peroxidase as an oxidase. The reaction was too rapid to measure by HPLC, as after 1 min approximately half (53 ± 13%) of the An-Hq was hydrolyzed. The hydrolysis half-life of An-Hq was increased >1700-fold upon addition of oxidative equivalents.

Comparison with standards characterized by RP HPLC was used to verify the hydrolysis reaction products of An-Hq (Figure 2C). In the presence of Ir^{IV}, An-OH (t_R = 19 min) and benzoquinone (t_R = 10 min) were observed. An-OH is the product of hydrolysis, as water has displaced the hydroquinone leaving group. Copper–hydrogen peroxide and peroxidase treatment produced the same products. These data show that once molecules with hydroquinone-based leaving groups enter a cell, oxidases and several forms of ROS will activate reactivity.

Reaction with 2′-deoxyguanosine

Reactivity toward DNA was investigated. By investigating DNA reactivity the likely cytotoxic reaction product will be elucidated (Figure 3B). DNA-modifying agents induce cytotoxicity in cells by modifica-

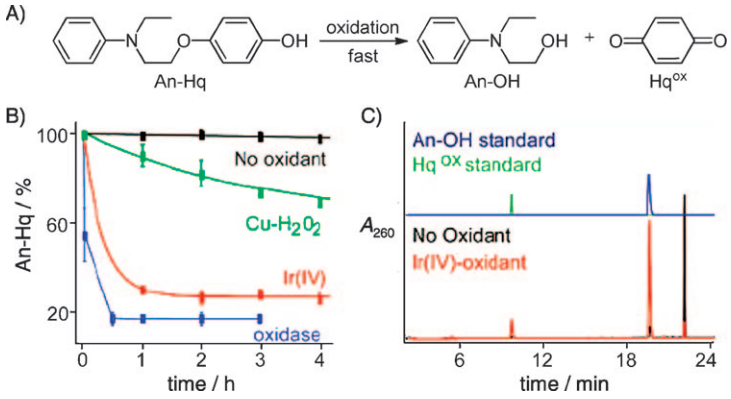


Figure 2. Hydrolysis of an oxidatively activated agent: A) Model compound An-Hq was synthesized. An-Hq possesses a hydroquinone leaving group that reacts slowly with nucleophiles (reaction). In the presence of oxidative stress, An-Hq is activated, leading to rapid reactivity and formation of benzoquinone (Hq^{ox}) and An-OH. B) The hydrolysis of An-Hq was monitored by HPLC at λ 260 nm at the indicated times. In the absence of oxidant (black, top), An-Hq is highly stable in buffered solutions. Addition of $CuCl_2$–H_2O_2 (green) or Ir^{IV} (orange) resulted in rapid hydrolysis. Cellular antioxidant proteins such as peroxidases (blue), can also rapidly oxidize An-Hq. C) The reaction products were identified by comparison with standards. An-Hq elutes at t_R 22 min (black). Addition of an oxidant led to the formation of new products at t_R 19 and 10 min (orange). Comparison with standards of An-OH (top, blue) and Hq^{ox} (top, light green) revealed that hydrolysis occurs via elimination of benzoquinone.

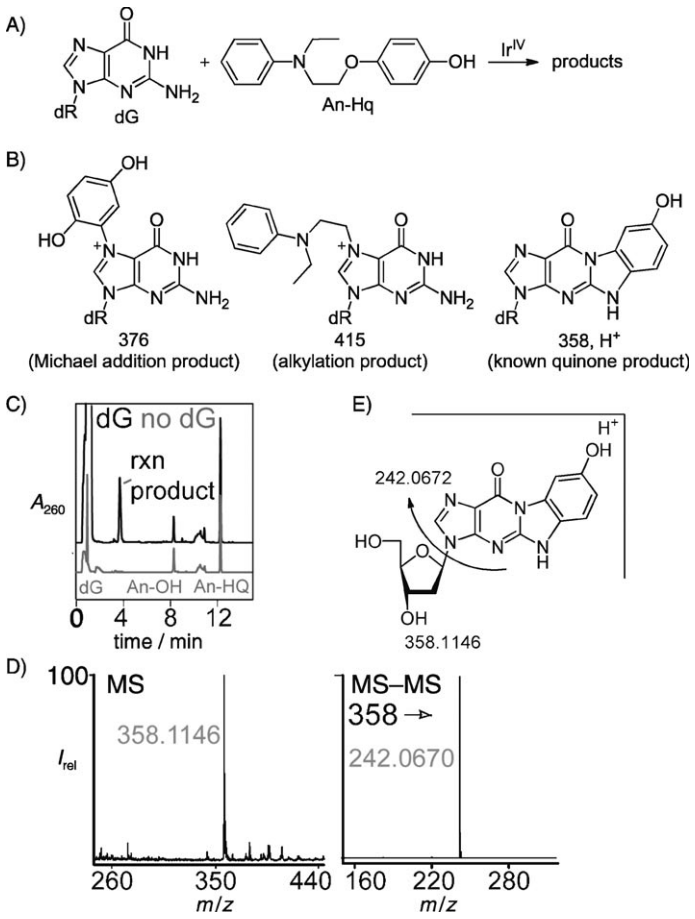


Figure 3. Oxidation-induced modification of 2′-deoxyguanosine: A) The reaction of An-Hq with 2′-deoxyguanosine was analyzed. B) Three possible products with 2′-deoxyguanosine are (from left to right): the Michael addition product, the alkylation product, and a product that has been reported in the literature. C) The reaction was monitored by HPLC at λ 260 nm. In the absence of 2′-deoxyguanosine, hydrolysis proceeds (grey). The presence of the nucleoside leads to formation of a specific product (black). D) The product was partially dried and analyzed by MS. Observed *m/z* ratios are in grey. Injection of the sample showed the product has the same mass and elemental composition as one of the possible products. Collision-induced dissociation led to a single fragment observed. E) Theoretical mass in grey and bold; errors $<$750 ppb. Superimposing the proposed structure shows correlation with the structure. The single fragmentation observed is cleavage at the glycosidic bond.

tion of either guanine or adenine bases (Figure 3A). We therefore probed the reactivity of An-Hq with 2′-deoxyguanosine. An-Hq is a bifunctional molecule that can modify DNA via several reaction pathways; we envisioned three likely routes. Quinone chemistry is well studied, and the predominant reaction product arises via Michael addition. This reaction product occurs after the formation of benzoquinone (Hq^{ox}, Figure 3) via nucleophilic addition of N7 of the guanine base. Moreover, traditional N7-alkylation of the guanine base (Figure 3B) could also be possible. These adducts have masses of M_r 376 and 415 Da, respectively. Finally, a survey of the literature found that some isomers of hydroquinones form guanine lesions in which N1 and N2 have added to produce a substituted phenol (Figure 3B).[27] 2′-Deoxyguanosine was incubated with an iridium oxidant and An-Hq for 7 days. Reaction mixture (5 mg) was then purified by RP HPLC (Figure 3C). In comparison with a control lacking 2′-deoxyguanosine, a reaction product was observed at ~4 min. After several purifications, the product was concentrated threefold (complete drying leads to product degradation, as many modified nucleosides possess labile glycosidic bonds). Interestingly, the product possessed an absorbance at λ 330 nm, indicating the addition of an aromatic group to the guanine base (Supporting Information).

The isolate was subjected to HRMS to identify the product (Figure 3D). Notably, in all cases, errors in ion masses are $<$750 ppb. Therefore, differences in mass such as that between O and NH_2, a difference of 942 ppm, are easily distinguishable and permit assignment of the elemental composition even on dissociated fragments in MS–MS. The spectrum showed a product with a mass of 358.1146 Da and an elemental composition of $C_{16}H_{16}N_5O_5^+$ (theoretical mass: 358.1146 Da, error: 126 ppb). A mass of 358 Da demonstrates the addition of a benzoquinone group with loss of water. The published structure for such a lesion is shown in Figure 3B. Collision-induced dissociation induced fragmentation of the lesion. MS–MS led to a loss of $C_5H_8O_3$ (error: 666 ppb), which is a common fragmentation product of nucleosides at the glycosidic bond, representing the loss of 2′-deoxyribose (Figure 3E). MS–MS confirms that the adduct contains 2′-deoxyguanosine and that modification did not occur on the ribose ring. Thus, the adduct was neither the Michael addition or alkylation product, but rather a guanine lesion modified at two heteroatom positions. Notably, other isomers are possible with connectivity at ring positions, but these isomers are only distinguishable by selective isotopic labeling. The structure listed corresponds to the isomer identified in the literature.[27]

Structural investigation into cytotoxicity

The cytotoxicity of An-Hq and its derivatives were evaluated in HeLa cells (Figure 4). Cell viability was monitored by MTT dye, which forms a purple formazan product in viable cells that are undergoing me-

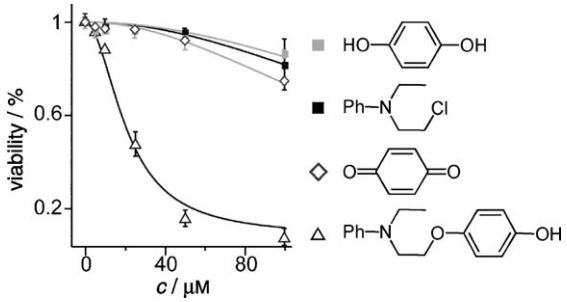


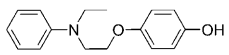
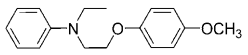
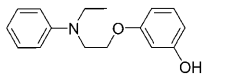
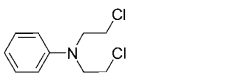
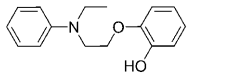
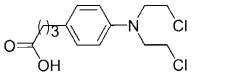
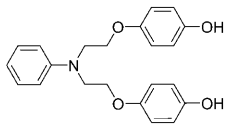
Figure 4. High cytotoxicity requires hydroquinone and a nitrogen mustard: Structures of each compound tested are on the right with the corresponding point. Hydroquinone (■) and the corresponding oxidized form, benzoquinone (◇), were only slightly toxic, with IC_{50} values of 161 and 227 μM, respectively. An analogue equipped with a chlorine leaving group (■) had an estimated an IC_{50} value of 211 μM. The IC_{50} of An-Hq (△) was 23 μM.)

tabolism. Concentrations of An-Hq and its analogues ranged from 0 to 100 μM, and IC_{50} values were calculated with a four-parameter regression analysis of at least three replicates. Errors reported are between two separate experiments; curve-fitting errors were $<25\%$ ($R>0.98$). An-Hq was toxic to HeLa cells with an IC_{50} value of 23 ± 4 μM (Figure 4, △). In contrast, the unmodified nitrogen mustard with a chlorine leaving group (Figure 4, ■) has much lower cytotoxicity, with an IC_{50} value of 211 ± 17 μM. Cytotoxicity was not derived from the hydroquinone portion of An-Hq or from the benzoquinone oxidation product, as IC_{50} values of these compounds were 161 ± 14 (■) and 227 ± 21 μM (◇), respectively (Figure 4). The data taken together illustrate that the hydroquinone and the DNA-modification portion of the molecule are necessary for low IC_{50} values.

The cytotoxicity of several derivatives was monitored in HeLa cells (Table 1). To examine the relationship between oxidation potential and cytotoxicity, several quinone derivatives were synthesized. Resorcinol, with a 1,3-diol, is less readily oxidized than hydroquinone, which has hydroxy groups at the 1- and 4-positions.[28] Resorcinol was attached to An-Cl to create An-Rs. An-Rs has an IC_{50} value of 44 ± 10 μM. Additionally, an analogue was synthesized in which a catechol group is attached to An-Cl. Catechol was recently used in the selective modification of DNA in cancer cells.[29] Catechol, with a 1,2-diol, also shows a decreased IC_{50} value of 71 μM. We synthesized a derivative that is not prone to oxidation: An-Hq-CH_3. An-Hq was methylated with methyl iodide. Hydroquinones oxidize from the phenoxide anion. Methylation does not allow formation of the required anion, and thus prohibits oxidation of hydroquinone. Addition of Ir^{IV} does not produce the oxidation-dependent color change to brown (data not shown). An-Hq-CH_3 showed no detectable toxicity toward HeLa cells (Table 1). These synthesized analogues illustrate that cytotoxicity correlates with oxidation potential.

We examined the mechanism of cytotoxicity further. An-Hq-CH_3 shows that cytotoxicity is induced through oxidation, and not some other enzymatic process such as hydrolysis, because both An-Hq-CH_3 and An-Hq would have the same tendency toward enzymatic degradation. Additionally, we synthesized An-Hq_2. An-Hq_2 produces the same guanine base lesion as An-Hq (Supporting Information), and has an IC_{50} value of 11 ± 4 μM. Importantly, An-Hq_2 is twice as cytotoxic as An-Hq, as upon activation two toxic groups are delivered. The therapeutically useful agent cisplatin had an IC_{50} value similar to that of An-Hq_2, while chlorambucil had a lower cytotoxicity of 76 ± 10 μM. Thus, although An-Hq_2 leads to modification of a single DNA base and does not induce cross-links, it can still lead to equivalent cytotoxicity.

Table 1. Comparison of the cytotoxicity of several synthesized derivatives to that of known DNA-modifying agents in HeLa cells.

Name	Structure	IC_{50} [μM][a]	Name	Structure	IC_{50} [μM][a]
An-Hq		23	An-Hq-CH_3		>300
An-Rs		44	An-Cl_2		10
An-Cat		71	chlorambucil		77
An-Hq_2		11	cisplatin		9

[a] Values determined by MTT assay, with errors from triplicate experiments $<25\%$ of the listed value.

Cytotoxicity in 15 cell lines

These data prompted us to further investigate the cytotoxicity of oxidatively activated DNA-modifying agents toward several cell lines (Table 2). Cisplatin, a traditional alkylating agent, was used for comparison against An-Hq_2. Fifteen cancer cell lines were compared. The cell lines tested are a large set of lines from 11 different cancer types giving high diversity to the cytotoxicity measurements. Importantly, five of the 15 cell lines analyzed had IC_{50} values >200 μM. The oxidative activation of An-Hq_2 showed a much different cytotoxicity profile than the profile obtained from cisplatin (Table 2). In contrast, IC_{50} values >200 μM were observed for only one of the 15 cell lines. Many cells seem to be resistant to the cytotoxic mechanism of An-Hq_2. Furthermore, An-Hq_2 showed important differences in IC_{50} values of the remaining 10 cell lines. The IC_{50} values ranged from 5 to 93 μM with an average of 37 μM and a deviation of 33 μM. Only

Table 2. Comparison of the cytotoxicity of several synthesized derivatives to that of known DNA-modifying agents in HeLa cell.

Cell line	IC_{50} [μM][a]		Cancer
	An-Hq_2	Cisplatin	
786-O	5	2	Kidney
Hek-293	4	10	Kidney
MDA-MB-231	36	>200	Breast
PC-3	>200	8	Prostate
DLD-1	27	5	Colorectal
LOVO	>200	26	Colorectal
PANC-1	>200	6	Pancreatic
BX-PC-3	93	6	Pancreatic
SK-MEL-5	20	4	Melanoma
KB	99	9	Oral
SK-OV-3	33	6	Ovarian
OVCAR3	>200	5	Ovarian
HeLa	10	9	Cervix
Calu-6	>200	2	Lung
NCI-N87	42	4	Gastric

[a] Values determined by MTS assay, with errors from triplicate experiments $<25\%$ of the listed value.

three cell lines were observed with high cytotoxicity (IC_{50} values $<10\,\mu M$). This level of discrimination of An-Hq_2 is in stark contrast to cisplatin cytotoxicity. Cisplatin gave average IC_{50} values of $7\,\mu M$ with a deviation of $6\,\mu M$. IC_{50} values $<10\,\mu M$ were observed in most cells. Cisplatin was highly cytotoxic to 12 of the remaining 14 cell lines. An-Hq_2 had high cytotoxicity against 786-O kidney cancer cells, which are used as a model for renal cell carcinoma. Therefore, oxidation-activated DNA-modifying agents showed highly selective cytotoxicity.

Conclusions

In summary, our data demonstrate that oxidatively active leaving groups can be used to control the cytotoxicity of DNA-modifying agents. Oxidation-activated DNA-modifying agents are unreactive until activated by oxidative stress. ROS and oxidases can serve to activate these types of agents. The reactivity of a given agent is tuned by the oxidation potential of the leaving group; the substitution pattern of the nitrogen is intact and may be further modified. These agents likely modify DNA by the addition of a phenol to guanine at two ring positions such as N1 and N2. Thus, these large structural modifications likely induce the observed cytotoxicity. Importantly, despite not forming cross-links, oxidatively activated DNA-modifying agents have cytotoxicity similar to that of clinically relevant agents. Therefore, oxidatively activated DNA-modifying agents represent a design improvement over traditional alkylating agents and may help address the problem of severe side effects caused by off-target reactivity in noncancerous cells. This conclusion is supported by data that show one third of the cell lines tested exhibit limited cytotoxicity, whereas many other cell lines have low ($>50\,\mu M$) cytotoxicity.

When tested in a diverse panel of 15 cell lines, oxidatively activated DNA-modifying agents induce high cytotoxicity in 786-O renal cell carcinoma, which requires more therapeutic options.[30] Renal cell carcinoma is marked by substantial changes to redox homeostasis such that oxidative stress has an important role in the growth and development of this type of cancer.[31,32] Reactive oxygen-activated DNA-modifying agents directly contradict the paradigm that they cannot be designed without high levels of nonspecific reactivity, as the agents synthesized in this work do show selective cytotoxicity.

Experimental Section

Most commercially supplied reagents were used without further purification. Chemicals and enzymes were obtained from Sigma–Aldrich or Thermo-Fisher unless otherwise noted. All synthesized molecules were >95% pure for intermediates and 98% pure for cytotoxicity measurements as analyzed by HPLC. ^1H and ^{13}C NMR spectra are available in the Supporting Information. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX 400 MHz spectrometer. Chemical shifts (δ) are reported in ppm, with ^{13}C and residual ^1H signals from deuterated solvents as references. High-resolution mass spectra (ESI) were recorded on a Micromass Q-TOF 2 (Waters). Analytical thin-layer chromatography (TLC) was performed on silica gel 60 GF_{254} (Merck). Column chromatography was conducted on silica gel (230–400 mesh). All synthesized compounds were additionally analyzed by IR spectroscopy and HRMS.

Hydrolysis studies

Hydrolysis reactions were performed in 1.5 mL total volume with 0.6 mM analogue, 5% CH_3CN, 25 mM phosphate buffer (pH 7.4) in a 2 mL glass HPLC tube. Time points were obtained by repetitive injection of 5 μL into a Beckman Coulter System Gold instrument with an autosampler equipped with a diode array detector. Solvent A was 98% H_2O and 2% CH_3CN; solvent B was 95% CH_3CN and 5% H_2O. The column used was an Agilent Zorbax SB-C_{18} (5 μm, 4.6×150 mm). The gradient was 0% B for 1 min, 10% B over 8 min, 100% B over 8 min. Absorbance was monitored at λ 260 nm. $CuCl_2$, Na_2IrCl_6, and peroxide concentrations were 0.1 mM, 1.2 mM, and 50 mM, respectively. Horseradish peroxidase was used at a concentration of $0.02\,U\,\mu L^{-1}$. Each experiment was performed in at least duplicate; half-lives are reported. Curve fitting for hydrolysis in H_2O involved a first-order fit, while other half-lives are empirical. Time points were taken as indicated. Purification of the DNA reaction column was carried out by using an Alltech C_{18} Rocket (7 ID, 53 mm, 3 μm) on a 5 mg reaction. Solvent A was 95% H_2O and 5% CH_3CN; solvent B was 95% CH_3CN and 5% H_2O. The gradient was 0% B for 1 min and then 100% B over 20 min. The collected fraction was vacuum dried to one third the volume. MS identification was performed directly on an HPLC fraction that contained 0.25% acetic acid and 15% CH_3CN. Analyses involved infusion directly into the instrument at $5\,\mu L\,min^{-1}$. MS analysis was performed on a Thermo-Fisher Scientific LTQ-FT, a hybrid instrument consisting of a linear ion trap and a Fourier transform ion cyclotron resonance mass spectrometer. The entire eluent was introduced into the LTQ-FT using the standard electrospray ionization source for the instrument, with a spray voltage of 5 kV and a capillary temperature of 275 °C. Autogain control (AGC) was used and set at 500000 with a maximum injection time of 1250 ms for FT-ICR full scans. Collision-induced dissociation, MS–MS, was executed in the linear trap with an AGC setting of 10000 and a maximum injection time of 500 ms. FT-ICR full scans were acquired in the positive ion mode at 100000 resolving power at m/z 400.

Cell culture and cytotoxicity measurements

Human cervical carcinoma (HeLa) cells were obtained from the ATCC for cell culture. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, $100\,U\,mL^{-1}$ penicillin, and $100\,\mu g\,mL^{-1}$ streptomycin (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. MTT assays were performed by seeding cells at a density of 2×10^5 cells per well in a 24-well plate and incubation at 37 °C for 5 h. Cells were then treated for 65 h with indicated concentrations of freshly dissolved compounds. The medium containing compounds was discarded, and fresh medium containing 20 μL MTT ($5\,mg\,mL^{-1}$) was added to each well and incubated for an additional 4 h. The medium was removed. After adding 200 μL DMSO to each well, the optical densities at λ 570 nm were determined. Cytotoxicity data are expressed as IC_{50} values obtained from the fit to a four-parameter sigmoid. All R values were >0.98, and standard errors of the three replicates were <20%. Experiments on 15 cell lines were performed by CrownBio Inc. (Santa Clara, CA, USA) according to a similar MTS assay (Promega), according to the manufacturer's protocol. Briefly, each cell line (obtained from ATCC) was plated on a 96-well plate and grown. An-Hq_2 was incubated with each cell line for 3 days, and cell viability was monitored at λ 490 nm.

Synthesis

***N*-(2-Chloroethyl)-*N*-ethylaniline (An-Cl)**. $SOCl_2$ (8.8 mL, 121 mmol) was added dropwise over 20 min to a solution of 2-(*N*-ethyl-*N*-phenylamino)ethanol (10 g, 60 mmol) and CH_2Cl_2 (150 mL). The mixture was held at reflux during addition, and the solution turned from colorless to yellow and then to brown. After the addition was complete, the mixture was heated at reflux for one hour. After cooling to room temperature, the mixture was quenched carefully with cold, saturated aqueous K_2CO_3 (150 mL). The mixture was then extracted with CH_2Cl_2, and the combined organic layer was washed with saturated aqueous K_2CO_3, washed with brine, and dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography with hexane/CH_2Cl_2 (4:1) to provide An-Cl (5.54 g, 30 mmol, 50%) as a yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.28 (m, 2H), 6.74 (m, 3H), 3.65 (m, 4H), 3.46 (q, J = 7.2 Hz, 2H), 1.22 ppm (t, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 147.02, 129.56, 116.61, 111.90, 52.45, 45.44, 40.56, 12.57 ppm; IR (KBr): $\tilde{\nu}$ = 2971, 2891, 1599, 1505, 1353, 1269, 748, 693 cm^{-1}; HRMS (ESI+) *m/z* calcd for $C_{10}H_{15}ClN$ $[M+H]^+$: 184.0893, found: 184.0836.

4-{2-[Ethyl(phenyl)amino]ethoxy}phenol (An-Hq). A mixture of An-Cl (500 mg, 2.7 mmol), K_2CO_3 (750 mg, 5.4 mmol), and KI (90 mg, 0.54 mmol) in DMF (50 mL) was bubbled with argon for 10 min. Hydroquinone (600 mg, 5.4 mmol) was added to the mixture under argon. The reaction mixture was heated and stirred at 80 °C for 6 h. After cooling to room temperature, the solvent was evaporated, and the residue was quenched with 100 mL H_2O. The mixture was adjusted to pH ~ 7 with dilute HCl. The mixture was then extracted with EtOAc, the combined organic layer was washed with H_2O and then brine, and was dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography with CH_2Cl_2 to provide An-Hq (400 mg, 1.55 mmol, 57%) as a brown oil. ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 8.92 (s, 1H), 7.14 (m, 2H), 6.75–6.64 (m, 6H), 6.57 (m, 1H), 3.98 (t, J = 5.8 Hz, 2H), 3.62 (t, J = 5.8 Hz, 2H), 3.41 (q, J = 7 Hz, 2H), 1.09 ppm (t, J = 7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 152.85, 149.74, 147.71, 129.49, 116.24, 115.73, 112.13, 66.20, 49.80, 45.69, 12.31 ppm; IR (KBr): $\tilde{\nu}$ = 3400, 2970, 1598, 1508, 1230, 1035, 827, 749, 694 cm^{-1}; HRMS (ESI+) *m/z* calcd for $C_{16}H_{20}NO_2$ $[M+H]^+$: 258.1494, found: 258.1436; HRMS (ESI+) *m/z* calcd for $C_{16}H_{19}NO_2Na$ $[M+Na]^+$: 280.1313, found: 280.1250.

3-{2-[Ethyl(phenyl)amino]ethoxy}phenol (An-Rs). A mixture of An-Cl (500 mg, 2.7 mmol), K_2CO_3 (750 mg, 5.4 mmol), KI (90 mg, 0.54 mmol) in DMF (50 mL) was bubbled with argon for 10 min. Resorcinol (600 mg, 5.4 mmol) was added to the mixture under argon. The reaction mixture was heated and stirred at 80 °C for 6 h. After cooling to room temperature, the solvent was evaporated, and the residue was quenched with 100 mL H_2O. The mixture was adjusted to pH ~ 7 with dilute HCl. The mixture was then extracted with EtOAc, and the combined organic layer was washed with H_2O and then brine, and dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography with CH_2Cl_2 to provide An-Rs (380 mg, 1.48 mmol, 55%) as a light-yellow oil. ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 9.38 (s, 1H), 7.15 (t, J = 8 Hz, 2H), 7.04 (t, J = 8 Hz, 1H), 6.70 (d, J = 8 Hz, 2H), 6.57 (t, J = 7.2 Hz, 1H), 6.32 (m, 3H), 4.02 (t, J = 5.8 Hz, 2H), 3.64 (t, J = 5.8 Hz, 2H), 3.44 (q, J = 7 Hz, 2H), 1.10 ppm (t, J = 7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 155.14, 151.95, 142.80, 125.44, 124.66, 111.56, 107.52, 103.46, 102.10, 97.43, 60.60, 44.77, 40.83, 7.34 ppm; IR (KBr): $\tilde{\nu}$ = 3390, 2971, 1598, 1505, 1149, 748, 687 cm^{-1}; HRMS (ESI+) *m/z* calcd for $C_{16}H_{20}NO_2$ $[M+H]^+$: 258.1494, found: 258.1534.

2-{2-[Ethyl(phenyl)amino]ethoxy}phenol (An-Cat). A mixture of An-Cl (500 mg, 2.7 mmol), K_2CO_3 (750 mg, 5.4 mmol), KI (90 mg, 0.54 mmol) in DMF (50 mL) was bubbled with argon for 10 min. Catechol (600 mg, 5.4 mmol) was added to the mixture under argon. The reaction mixture was heated and stirred at 80 °C for 6 h. After cooling to room temperature, the solvent was evaporated, and the residue was quenched with 100 mL H_2O. The mixture was adjusted to pH ~ 7 with dilute HCl. The mixture was then extracted with EtOAc, and the combined organic layer was washed with H_2O and then brine, and dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography with CH_2Cl_2 to provide An-Cat (400 mg, 1.55 mmol, 57%) as a white solid. ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 8.86 (s, 1H), 7.15 (t, J = 8 Hz, 2H), 6.90 (d, J = 8 Hz, 1H), 6.78 (m, 5H), 6.57 (t, J = 7.2 Hz, 1H), 4.07 (t, J = 6.0 Hz, 2H), 3.68 (t, J = 6.0 Hz, 2H), 3.45 (q, J = 6.8 Hz, 2H), 1.10 ppm (t, J = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 148.00, 146.35, 145.81, 129.47, 122.24, 120.06, 117.24, 115.04, 112.86, 67.44, 49.79, 45.65, 12.19 ppm; IR (KBr): $\tilde{\nu}$ = 3462, 2926, 2869, 1594, 1500, 1258, 740, 695 cm^{-1}; HRMS (ESI+) *m/z* calcd for $C_{16}H_{20}NO_2$ $[M+H]^+$: 258.1494, found: 258.1465; calcd for $C_{16}H_{19}NO_2Na$ $[M+Na]^+$: 280.1313, found: 280.1345.

***N*-Ethyl-*N*-[2-(4-methoxyphenoxy)ethyl]aniline (An-Hq-CH$_3$)**. MeI (1 mL, 16 mmol) was added to a mixture of An-Hq (78 mg, 0.3 mmol) and K_2CO_3 (95 mg, 0.69 mmol) in acetone (50 mL). The reaction mixture was heated at reflux for 48 h. After cooling to room temperature, the solvent was evaporated, and the residue was quenched with 50 mL H_2O. The mixture was then extracted with EtOAc, and the combined organic layer was washed with H_2O then brine, and dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography with hexane/CH_2Cl_2 (2:1) to provide An-Hq-CH$_3$ (49 mg, 0.18 mmol, 60%) as a yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.23 (m, 2H), 6.83 (m, 4H), 6.73 (d, J = 8.4 Hz, 2H), 6.68 (t, J = 7 Hz, 1H), 4.07 (t, J = 6.4 Hz, 2H), 3.77 (s, 3H), 3.70 (t, J = 6.4 Hz, 2H), 3.47 (q, J = 7 Hz, 2H), 1.20 ppm (t, J = 7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 153.93, 152.91, 147.62, 129.38, 115.95, 115.40, 114.67, 111.80, 66.01, 55.74, 49.76, 45.59, 12.30 ppm; HRMS (ESI+) *m/z* calcd for $C_{17}H_{22}NO_2$ $[M+H]^+$: 272.1645, found: 272.1646.

4,4′-[2,2′-(Phenylazanediyl)bis(ethane-2,1-diyl)bis(oxy)]diphenol (An-Hq$_2$). A mixture of An-Cl_2 (500 mg, 2.29 mmol), K_2CO_3 (634 mg, 4.59 mmol), and KI (76 mg, 0.46 mmol) in DMF (50 mL) was bubbled with argon for 10 min. Hydroquinone (1.32 g, 12 mmol) was added to the mixture under argon. The reaction mixture was heated and stirred at 80 °C for 6 h. After cooling to room temperature, the solvent was evaporated, and the residue was quenched with 100 mL H_2O. The mixture was adjusted to pH ~ 7 with dilute HCl. The mixture was then extracted with EtOAc, and the combined organic layer was washed with H_2O then brine, and dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (15:1) to provide An-Hq$_2$ (150 mg, 0.41 mmol, 18%) as a yellow oil. ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 8.91 (s, 2H), 7.16 (t, J = 7.8 Hz, 2H), 6.78–6.72 (m, 6H), 6.66–6.60 (m, 5H), 4.02 (t, J = 5.8 Hz, 2H), 3.75 ppm (t, J = 5.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 152.87, 149.60, 147.31, 129.50, 116.55, 116.08, 115.56, 111.79, 65.91, 50.99 ppm; IR (KBr): $\tilde{\nu}$ = 3366, 2926, 1598, 1508, 1229, 826, 750 cm^{-1}; HRMS (ESI+) *m/z* calcd for $C_{22}H_{24}NO_4$ $[M+H]^+$: 366.1700, found: 366.1701.

Acknowledgements

We thank the University of Cincinnati for startup funding. We also thank the University of Cincinnati Faculty Research Grants Program for funding E.J.M. and the University of Cincinnati Post-doctoral Fellow Research Program for funding G.L.

Keywords: chemotherapy · DNA modification · nitrogen mustard · oxidative stress · reactive oxygen species

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Received: January 14, 2011
Revised: January 24, 2011
Published online on March 4, 2011

