Novel Oxidatively Activated Agents Modify DNA and Are Enhanced by Ercc1 Silencing

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ABSTRACT: Agents that chemically modify DNA form a backbone of many cancer treatments. A key problem for DNA-modifying agents is lack of specificity. To address this issue, we designed novel molecular scaffolds, termed An-Hq and An-Hq₂, which are activated by a hallmark of some cancers: elevated concentrations of reactive oxygen species. Elevated reactive oxygen species are linked to oncogenesis and are found to increase in several aggressive cancers. The agents are quinones that, upon oxidation, form highly electrophilic species. In vitro studies identified the mode of addition to DNA. The aniline portion of An-Hq serves to enhance nucleophilic addition to the ethyl phenyl ether instead of forming common Michael additions. Structural characterization showed that the agents add to 2′-deoxyguanosine at the N2,N3-positions. The product formed is a bulky hydroxy-N2,3-benzetheno-2′-deoxyguanosine adduct. In addition, the oxidatively activated agents added to 2′-deoxyadenosine and 2′-deoxycytidine but not thymidine or 2′-deoxyinosine. These findings are confirmed by primer extension analysis of a 392 base pair DNA. The full-length primer extension product was reduced by 69.0 ± 0.6% upon oxidative activation of An-Hq₂ as compared to controls. Little sequence dependence was observed with 76% of guanine, adenine, and cytosine residues showing an increase in extension stops between 2- and 4-fold above controls. Benzetheno-nucleobase addition to double-stranded DNA was confirmed by LC/MS of a self-complementary oligonucleotide. Experiments were carried out to confirm in vivo DNA damage. Because of the lesion identified in vitro, we reasoned that nucleotide excision repair should be involved in reversing the effects of these oxidatively activated agents and enhance toxicity in Drosophila melanogaster. Using an RNAi-based approach, Ercc1 was silenced, and survival was monitored after injection of an agent. As expected, bulky cross-linking DNA-modifying agents, cisplatin and chlorambucil, showed statistically significant enhanced toxicity in Drosophila with silenced Ercc1. In addition, 5-fluorouracil, which does not produce bulky lesions, showed no selective toxicity. An-Hq and An-Hq₂ showed statistically significant toxicity in Drosophila with silenced Ercc1. Examination of cytotoxicity shows renal carcinoma cell lines as a target of these agents with a median IC₅₀ of 1.8 μM. Taken together, these data show that the designed oxidatively activated agents form distinct, bulky DNA modifications that prove difficult for cancer cells possessing an elevated reactive oxygen species phenotype to overcome. The modification produced is relatively unique among anticancer agents.

INTRODUCTION

The harsh side effects of current chemotherapy treatments are one of the motivating factors for increasing selectivity in novel anticancer agents.7 Agents that chemically modify DNA form the backbone of many cancer treatments. A typical DNA-modifying agent is cisplatin, which is used to treat 50% of cancer patients.7 These agents represent a large portion of current and phased-out anticancer agents. The design of DNA-modifying agents has slowed drastically due to the perception that these agents will have high levels of side effects due to the modification of DNA and subsequent cytotoxicity in nontarget cells. This lack of specificity is termed off-target reactivity, which limits tolerated doses and, therefore, decreases efficacy.7 Instead, these agents have been attached to transport active scaffolds that enhance uptake into target cancer cells.4,5 Side effects, however, are still problematic, and improving selectivity is a major goal in the development of novel anticancer agents. We are developing novel agents to address these problems.

A potential path forward in cancer drug development is to take advantage of a hallmark of many cancer cells, elevated reactive oxygen species (ROS).6,7 Persistent elevation of ROS has been found in numerous cancers, including renal cell carcinoma, melanoma, and leukemia.8−10 ROS occurs in four major endogenous forms within the cell: superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radical.11−13 Mitochondrial dysfunction, common to cancer, is known to increase ROS.14 Increased ROS damages DNA, leading to mutation.15
Consequently, it is no surprise that levels of ROS-induced DNA damage correlate with cancer prognosis.\textsuperscript{16} In turn, these mutations cause enhanced activation of oncogenes.\textsuperscript{17} A critical role for ROS in oncogene-transformed cancer cells has been shown when investigating c-Myc-induced damage.\textsuperscript{18} Furthermore, once the Ras oncogene is locked into an active state, the concentration of cellular antioxidant enzymes is lowered, causing elevated ROS.\textsuperscript{19,20} As a result, several chemical research teams are actively searching for means to utilize elevated ROS to generate selective anticancer agents.\textsuperscript{21–23} Our lab has designed oxidatively activated DNA-modifying agents, which selectivity target the elevated ROS phenotype in cancer cells.\textsuperscript{24}

Our new oxidatively activated agents possess attractive anticancer properties.\textsuperscript{24} These agents, such as 4-((2-(ethyl-(phenyl)amino)ethoxy)phenol (An-Hq) and 4,4′-(2,2′-(phenylanediyldi)bis(ethane-2,1-diyl)bis(oxy))diphenol (An-Hq\textsubscript{2}), utilize a novel mechanism of activation and are highly selective (Figure 1A). The agents are unreactive and stable in water, but they are strongly activated by all the ROS forms, excluding hydrogen peroxide. The addition of oxidative equivalents leads to a rate enhancement of greater than 1700-fold as compared to when no oxidant is present. When reactivity was examined, we found that rather than a nitrogen mustard, our agents were actually oxidatively activated quinones. More specifically, we have demonstrated that these agents are capable of adding to a guanine. The product was a hydroxy-benzetheno-2′-deoxyguanosine (dG) adduct, where the guanine possessed an added phenol.

The possible formation of a benzethenoguanine left several important questions to be addressed. Specifically, we questioned if these new agents were actually DNA-modifying agents. We set out to structurally characterize the lesion produced on nucleosides and on double-stranded DNA (dsDNA) as well as examine the reaction mechanism in vitro (Figure 1A). To determine in vivo DNA adduct formation, studies using a powerful genetic model system,\textsuperscript{25} Drosophila melanogaster, were accomplished (Figure 1B). D. melanogaster has been shown to be an effective, whole-animal model in cancer research and in drug discovery.\textsuperscript{26–29} Many key features of DNA repair and cell death are conserved between Drosophila and mammals.\textsuperscript{30} Approximately 75% of human disease-related genes have a functional orthologue in flies.\textsuperscript{31,32} Moreover, studies on Drosophila have been instrumental in elucidating Ras signaling, with major components conserved in mammals.\textsuperscript{33}

Here, we first investigated the in vitro DNA lesions produced by our novel oxidatively activated agents by nuclear magnetic resonance (NMR). We found that these agents required a nucleoside base amine to initiate reactivity. A primer extension assay was used to explore the lesion produced on a 392-nucleotide PCR product. We discovered that the three nucleosides possessing amines are indeed modified in dsDNA, producing bulky lesions. These agents produce the same adduct on a small dsDNA. Such lesions led us to predict that nucleotide excision repair was an important mechanism in repairing such adducts.\textsuperscript{34} Hence, we used a targeted RNAi knockdown approach based on the GAL4/UAS system (Figure 1B) to evaluate if bulky DNA modification was occurring in vivo.\textsuperscript{35} We silenced the Ercc1 gene, whose protein forms a complex with XPF as a key step in nucleotide excision and double strand break repair pathway.\textsuperscript{36} We found that Drosophila lacking Ercc1 expression showed elevated toxicity toward known DNA-modifying agents, cisplatin and chlorambucil. The elevated Ercc1-dependent sensitivity did not occur when Drosophila were treated with 5-fluorouracil, which does not produce a bulky lesion. These results confirmed D. melanogaster as an effective model organism for ascertaining the genetic mechanisms of DNA-modifying agents. We then examined our novel agents, An-Hq and An-Hq\textsubscript{2}. We demonstrate that flies in which Ercc1 expression was knocked down also show enhanced sensitivity toward the novel agents, particularly An-Hq, supporting a role for these oxidatively activated agents in modifying DNA in vivo.

\section*{Materials and Methods}

\textbf{Synthesis of An-Hq and An-Hq\textsubscript{2}}. The syntheses of An-Hq and An-Hq\textsubscript{2} were accomplished.\textsuperscript{24} NMR (\textsuperscript{1}H and \textsuperscript{13}C) spectra were recorded on Bruker AMX 400 MHz spectrometer. Chemical resonances are reported in ppm (\textsuperscript{1}H) and units using \textsuperscript{13}C and residual \textsuperscript{1}H 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 GF254 (Merck). Compounds were greater than 99% pure according to HPLC.

\textbf{Reaction of An-Hq with Nucleoside Analogues}. For the nucleoside reactivity studies, An-Hq (22 \textmu L dissolved in DMSO, 1 mmol) was added to phosphate buffer (2 mL, 25 mM NaH\textsubscript{2}PO\textsubscript{4} in 5%
The absorbance was monitored at 260 nM. The collected sample was 4 mL/min. Solvent A was 95% water and 5% acetonitrile, and solvent B, 10% over 10 column volumes, 10% to 100% over 20 min, 100% B for 4 min, 0% B over 2 min, and held for 4 min. For mass spectrometry (MS), peaks were collected, dried to remove solvent (if stable; if not, they were directly injected into MS), and then analyzed by LC/MS. All yields were between 0.5% and 3%, depending on the nucleoside adduct.

The MS was performed as follows: isolated products were resuspended in 100 μL of 0.25% acetic acid and 15% acetonitrile. Infusion into the instrument occurred at a rate of 5 μL/min. The mass spectrometry 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 was set to 500000 with a maximum injection time of 1250 ms for FT-ICR full scans. Collision-induced dissociation, MS/MS, was executed in the linear ion trap with an AGC setting of 1000 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.

dG-An-Hq Adduct NMR Analysis. All 1H NMR experiments were carried out on a Bruker DEX-500, 500 MHz instrument. Chemical resonances are reported in δ (ppm) units, using residual 1H signals from deuterated solvents as references. The reaction listed was scaled to 100-fold (total volume, 200 mL). The reaction was incubated in the dark at 25 °C for 4 days. The solvent was evaporated, and the adduct of interest was purified in two stages. First, a Biotage SP1 Flash system, outfitted with a 5.5 g RediSep Rf Gold C18 column, was used to purify the reaction mixture, using a 97% water and 3% methanol buffer A and methanol for buffer B. The gradient was 0% methanol to 100% over 10 column volumes, 10%–100% over 3 column volumes, and held there for 5 column volumes. The sample (~75% pure by HPLC) was collected after 13 column volumes and was lyophilized to remove solvent. The second stage employed HPLC purification, using a Grace Alltima HP C8 semipreparative column (3 μm, 7 mm × 53 mm) at 2.5 mL/min. Solvent A was 95% water and 5% acetonitrile, and solvent B was 95% acetonitrile and 5% water. The gradient was 0% B for 5 min, 30% B over 8 min, 100% B over 3 min, and then held there for 4 min. The absorbance was monitored at 260 nm. The collected sample eluted around 8 min. After HPLC purification, the product was dried and dissolved into 500 μL of DMSO-d6, and evaluated by NMR for 15 h to determine the 1H NMR.

Solvolysis of An-Hq in Methanol Using Na3IrCl6 as the Oxidant. An-Hq (10 mg, 0.039 mmol) dissolved in methanol (2 mL). Na3IrCl6·4H2O (43 mg, 0.078 mmol) and N,N-disopropylethylamine (4.5 mg, 0.039 mmol) were added to the mixture. The mixture was rehydrated in methanol and reacted at room temperature for 0.5 h. The reaction was filtered through silica gel and washed with acetonitrile. The filtrate was concentrated, and the residue was purified by flash chromatography on silica gel to give the product (9 mg, 0.017 mmol, 90% yield) as a yellow oil. 1H NMR (CDCl3, 400 MHz): δ 7.23 (t, J = 8.2 Hz, 2 H), 6.80 (d, J = 10.4 Hz, 2 H), 6.69 (m, 3 H), 6.26 (d, J = 10.0 Hz, 2 H), 3.76 (t, J = 6.2 Hz, 2 H), 3.54 (t, J = 6.2 Hz, 2 H), 3.41 (q, J = 7.1 Hz, 2 H), 3.36 (s, 3 H), 0.87 (t, J = 7.1 Hz, 3 H). HRMS (ESI, positive): m/z calcd for C17H22NO3 [M + H]+: 288.1600; found, 288.1614.

DNA Damage Visualization on a 392-Nucleotide PCR Product. Primer extension experiments were performed using a 392-nucleotide dsDNA, synthesized from pUC19 plasmid vector (New England Biolabs). A 30 cycle PCR amplification (55 °C for 30 s, 75 °C for 45 s, and 95 °C for 30 s) was performed, using a 19-nucleotide forward primer, GGCGCTCTGGATCTACGG, starting at the nucleotide at position 287 of the vector, and a 19-nucleotide reverse primer, ATACGCAACGCCTCTCTC, starting at position 672. The oligonucleotide was purified using a Cycle Pure Kit (Oligene BioTek), yielding a final concentration of 40 μg/μL. DNA was reacted with the agent, An-Hq2, and Na3IrCl6 for 5 h at 37 °C (720 μg of DNA, 0.2 mM sodium phosphate, pH 8, 0.5 mM An-Hq2, and 2 mM iridium). For the preincubated control, DNA was added 24 h after the agent and the oxidant were mixed. The end products were benzoxazine and N-phenyldiethanolamine. Each sample of reacted DNA was then added to the primer extension mix [1x vent buffer, 100 μM dNTP, 200 nM primer, 0.05 U/μL vent(exo-) DNA polymerase]. The primer was fluorescently labeled with IRDye700. Twelve cycles were accomplished (55 °C for 15 s, 72 °C for 1 min, and 95 °C for 30 s). Denaturing load dye was then added to the samples, and a 12% denaturing PAGE gel was performed. The gel was visualized using the Odyssey Infrared Imaging System (LiCor) with 169 μm resolution and the 700D channel. Sequencing was performed by standard methods, using manual sequencing of the 392-nucleotide PCR product and acylo-terminators, except that the fluorescently labeled M13 primer was substituted. Experiments were performed in triplicate, and standard errors were calculated.

LC/MS Product Analysis of Reaction of An-Hq, with Oligonucleotide. High-purity oligonucleotides were purchased from Eurofin MWG operon. Electrophoresis showed the oligonucleotide to be greater than 95% pure. The DNA sequence was 5′-GGCGCAAATGCGCGC-3′. The DNA was annealed in 25 mM sodium phosphate. Prior to reaction, the DNA was desalted and placed in 5 mM ammonium acetate buffer, pH 8.0. The 100 μL reaction contained 2 mM DNA, 10 mM An-Hq2, and 10 mM a (diacetoxyiodo)benzene oxidant. The reaction was left at room temperature in the dark for 3 days. Analysis by mass spectrometry utilized a Thermo Scientific LTQ-FT, a hybrid instrument consisting of a linear ion trap and a Fourier transform ion cyclotron resonance mass spectrometer. The injection volume was 10 μL. Liquid chromatography was accomplished using a Waters Symmetry C18 5 μm, 2.1 mm × 150 mm column, Finnigan Surveyor MS pump, and Finnigan Micro AS autosampler. The flow rate was 200 μL/min, and the gradient ranged from 2% (v/v) acetonitrile in 5 mM ammonium formate to 15% over 35 min. Autogain control was used and set at 500000 with a maximum injection time of 1250 ms for FT-ICR full scans. FT-ICR full scans were acquired in the negative ion mode at 100000 resolving power at m/z 400. Mass accuracy errors were below 500 ppb for full scan.

Drosophila Stocks and Husbandry. Drosophila were maintained on standard cornmeal, agar, and molasses media at 25 °C under a 12:12 h light:dark cycle. To induce targeted gene silencing, the following RNAi line was obtained from the Vienna Drosophila RNAi Center: 5′-UAS-Ercc1RNAi (v12622VDRC). This line was crossed to the daughterless-GAL4 (da-GAL4) driver line to ubiquitously inactivate gene expression. The isogenic host strain, w1118 (60100VDRC), was crossed to da-GAL4 as a control for genetic background effects. The da-GAL4 line was kindly provided by Dr. Mike Grotewiel (Virginia Commonwealth University).

RNAi-Mediated Suppression of Targeted Genes. To confirm knockdown efficiency of the transgenic RNAi approach, RT-PCR assays were conducted. For each sample, 15 adult male Drosophila were homogenized, and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Total RNA was then DNase treated using DNA-free (Ambion, Austin, TX), according to manufacturer’s instructions. Total RNA (0.5 μg) was reverse transcribed using the AccScript High Fidelity First Strand cDNA synthesis kit (Agilent Technologies, Santa Clara, CA), and the resulting cDNA was used in RT-PCR assays. Primers were as follows: Ercc1-F 5′-CGTCGTCTGA-GCTGCGC-3′ and Ercc1-R 5′-CTCAGGAAACGCCTGTTCTG-3′. QuantumRNA β-actin Internal Standards (Ambion) were used to amplify β-actin (control) according to the manufacturer’s instructions, following optimization of the β-actin:competitor ratio. PCR products were separated electrophoretically on a 2% agarose gel and visualized with ethidium bromide. RT-PCR assays on two independent RNA isolations per genotype were performed.

Microinjection of Drosophila. Microinjections were performed on individual adult male Drosophila between 3 and 7 days old.
Individuals were held in position for microinjection using a gentle vacuum. A thin pulled glass micropipet attached to a Picospritzer III (Parker Hannifin, Cleveland, OH) was used to deliver anticancer agents into the fly abdomen. A 0.5 μL volume of agent was injected at ~40 psi of compressed air. Agents were dissolved in 100% DMSO, and solutions were made fresh daily. Blue food dye (0.125 mg/mL) was added to the solutions to confirm the delivery of the agent. Agents tested included An-Hq, An-Hq 2, chlorambucil, cisplatin, and 5-fluorouracil. Microinjections of the vehicle alone (control) were also performed. For each agent, 10 individuals of each genotype were injected at a 10 mM concentration. A concentration of 10 mM was used because preliminary analysis showed this to be effective at eliciting a response. Postinjection, flies were placed on fresh standard Drosophila media at 25 °C and on a 12:12 h light-dark cycle. Individuals were scored for survival after 1 day and followed for a total of 7 days to examine long-term effects of the agents. To analyze the significance of the data, χ2 was calculated.

**RESULTS**

**In Vitro Reactions with Nucleosides and Oligonucleotides.** Our data established that An-Hq and An-Hq 2 are highly selective cytotoxic agents against certain cancers. As is the new convention,30 the numbering for dG is retained throughout this manuscript. The numbering for the An-Hq phenol derivatives is denoted by a double prime, even when discussing the phenol and the remaining moieties of the molecule. Initially, An-Hq was oxidized by Na2IrCl6 oxidant to determine if addition occurred. We found that the hydroquinone was the active portion of the molecule. Initially, An-Hq was reacted with Na2IrCl6 to determine if addition occurred. We found that the mass of the product corresponded to the formation of a benzetheno-dG. Previous literature demonstrates that benzoquinone can add to dG and produce a 3′-hydroxy-1,N2-benzetheno-dG.41 The first step in the reaction mechanism is a Michael addition. Thus, we probed the ability of An-Hq to react and form the same benzetheno-dG adduct and find it to be different.

To determine if these agents modify DNA as benzoquinone, An-Hq was oxidized in the presence of methanol, and the product was determined. Na2IrCl6 was used to induce oxidation in methanol (Figure 2A). Solvolysis by methanol serves as a simple nucleophile and as a means to trap the site of nucleophilic addition. Upon oxidation, An-Hq was 90% oxidized to a new compound after 15 min. 1H NMR was used to analyze the isolated product; the region between 3 and 8 ppm is shown in Figure 2A. There are 18 protons in this region, indicating a product with an additional methoxy group. The singlet peak at 3.4 ppm with an integration of 2.9 corresponds to the added methoxy group. The aromatic region is symmetric, indicating that addition did not occur at C3″ or C2″ of the phenol through Michael addition. We used MS to ensure a product consistent with a single methanol addition. Therefore, An-Hq does not behave like benzoquinone. Instead, oxidative activation leads to a potent electrophile, via addition at C4″. This confirms that the DNA lesion produced may be different than benzoquinone, and these agents are not simply releasing benzoquinone as their mechanism of action.

As there was a difference in reactivity due to oxidative activation, we investigated the lesion produced through NMR analysis (Figure 2B). For consistency, the NMR is labeled starting from A for both products. The DNA lesion formed upon incubation of An-Hq was compared to that formed with benzoquinone. The product of An-Hq, Na2IrCl6, and dG in water-phosphate buffer was used to isolate the dG adduct. The oxidant used was Na2IrCl6 because it possesses the correct potential to oxidize the agents but not dG.42 The reaction of benzoquinone and dG has been established by Jowa et al. as well as by Chenna and Singer.41,43 We followed the procedure, which entails incubation of dG in dimethylformamide (DMF) and potassium carbonate. This nonaqueous system formed the benzetheno adduct in high yield. After reaction, we purified the products to greater than 95% purity levels (see Figure S1 in the Supporting Information). Analysis by positive ion MS showed products with an m/z value of 358.1146 and an elemental composition of C16H16N5O5. Figure 3. MS/MS shows deglycosylation, indicating that the ribose portion of the nucleoside was not modified (see Figure S2 in the Supporting Information).

Reaction of benzoquinone revealed addition at N1,N2 as had been previously observed (Figure 2B, bottom). Ten resonances between the 4–10 ppm are shown. The B shift was a singlet at 9.4 ppm and was ascribed to the terminal phenol hydrogen at C1″. Two strong singlets, C and D, were observed at 8.0 ppm. These singlets were the C8-hydrogen and the C2″ hydrogens, with the C2″ being more shielded. Importantly, the C8-hydrogen indicated that the five-membered ring of guanine was not modified. Accordingly, N7 was not the addition site. Resonances E and F were the remaining C5″ and C6′′ singlet phenol hydrogens. Taken together, the three phenol resonances

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**Figure 2.** An-Hq and its derivatives react with guanine differently than benzoquinone. (A) Quinones can react via two paths: C4″ addition (black) and C2″-Michael addition (gray). The product for each path is shown. To determine the reaction path, the product from oxidation in methanol was isolated, and the NMR and MS are shown. Oxidative activation of An-Hq in methanol proceeds via C4″ addition based on the gray NMR assignments and integrals. (B) Top: NMR of the reaction between An-Hq, dG, and an iridium oxidant in phosphate buffer. On the left is the reaction with the assigned NMR resonances superimposed on the product. On the right is the H1-NMR between 3 and 10 ppm. The assignments are in gray. An N1-proton signal is observed. Bottom: The reaction between dG, base, and benzoquinone according to literature. On the left is the reaction with assignments. On the right is the H1-NMR. Importantly, the N1 peak at around 10 ppm is missing from the benzoquinone reaction product NMR. The different reaction path of An-Hq leads to a different guanine lesion.
Figure 3. An-Hq reacts with nucleoside base amines. (A) Analogues that react. Reactions with An-Hq (left), HPLC analysis (center), and mass spectrometry (right). dG, 2'-deoxyadenosine, 2’-deoxycytidine, and 7-deaza-dG all form products with similar spectra (inset). For HPLC analyses, the gray chromatograms are control reactions. The gray traces include no An-Hq, no oxidant, and a minus An-Hq and oxidant in the reaction mixture. The reaction trace (black) contains An-Hq, oxidant, and the nucleoside analogue. Purification and mass spectrometry of the products indicate each has an added phenol. 2’-Deoxynosinosine had two phenols added. (B) Analogues that do not react. 2'-Deoxynosinosine, which lacks an N2-amine, does not react. Thymidine does not form identifiable adducts. Importantly, this provides evidence that the N2-amine is necessary for addition on the nucleoside.

Analysis of the An-Hq-dG adduct was accomplished in a similar manner (Figure 2B, top). Again, 10 resonances are observed with some key differences. Several resonances were identical to the 3’-hydroxy-1,N2-benzetheno-dG adduct. Shift D, the C8-hydrogen, confirmed that An-Hq does not modify N7 on the five-membered ring. Likewise, resonances B, G, H, I, and J directly overlap (compare Figure 2B, top and bottom), indicating that modification was not at the ribose. The NMR identified several distinct hydrogens. Two new hydrogen peaks were seen. First, the A shift at 9.9 ppm was a strong singlet. Such a strongly deshielded hydrogen can only come from the amido-hydrogen at N1. In addition, the K-shift at the N2 hydroxyl was observed at 4.3 ppm. Importantly, shift A (bolded in Figure 2B) indicated that the N1 position was no longer part of the phenol-dG adduct. This was interesting because, if taken with the appearance of the C8-hydrogen, the NMR reveals that the phenol added to N2 and N3. To further support our data that An-Hq is producing a different hydroxy-benzetheno-dG adduct, we determined stability in acid. It has been observed that N2,3-benzetheno-dG adducts are prone to degradation by incubation in acid since a weaker glycosidic bond is present.44 Incubation at pH 1 and elevated temperatures led to degradation of the adduct when compared to the 3’-hydroxy-1,N2-benzetheno adducts (see Figure S4 in the Supporting Information). It should be noted that several tautomers can be drawn that assign correctly to the NMR data (see Figure S5 in the Supporting Information). In addition, a proposed mechanism to form the guanine adduct is listed in Figure S5 in the Supporting Information. Importantly, these data showed that An-Hq generates a distinct lesion from benzooquinone. The distinct lesion stemmed from the oxidative activation mechanism of An-Hq.

We performed a series of HPLC analyses to determine which DNA bases are modified by An-Hq (Figure 3). Nucleosides were incubated with An-Hq and Na3IrCl6. After the incubation period, reactions were analyzed by HPLC with UV detection set to 260 nm. For each nucleoside, a reaction chromatogram (black) and control chromatograms (gray) are shown. Each of the controls excludes a single reaction component: An-Hq, dG, and Na3IrCl6. The reaction of dG with An-Hq is shown on the top left of Figure 3. A clearly observable product is seen at 13 min when comparing the controls and reaction traces. The product has a characteristic three band absorbance spectrum (inset). MS analysis showed a predominate ion at m/z of 358, and the elemental composition is C16H16N5O5 with less than 300 ppb error. It should be noted that there is a small product at 18 min, which possessed an m/z value equal to the deglycosylation product (data not shown). To the top right in Figure 3, we examined 7-deaza-dG for reactivity. The NMR revealed that N7 was not involved in the reaction. On the basis of these data, substitution of dG with 7-deaza-dG should not interfere with formation of reaction product. By performing the same reaction with 7-deaza-dG, a new series of products are observed at 15 min. Again, these products have similar UV absorbance. The MS spectra had a mass change of 1 amu or C16H15N5O5+, which is correct for a 7-deaza-dG adduct with an added phenol. These data proved that N7 is not involved in the reaction. To further validate the NMR results, we reacted 2’-deoxynosinosine with An-Hq (Figure 3B, bottom left). 2’-Deoxynosinosine lacks N2 and, according to the NMR results, should not react. The reaction was prepared identically to the

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reaction with dG. We saw no product formation, further confirming the NMR results that N2 is required for product formation.

We examined An-Hq adduct formation with each nucleoside. Analysis of the reaction between 2′-deoxyadenosine and An-Hq revealed a new product at 15 min (Figure 3, middle left). The absorbance was similar with the dG adduct formed. The product was formed in low yield. The yield was \( \sim 0.4\% \). The MS analysis for this adduct gave a mass indicative that two phenols had added (addition of 188 amu). The addition of two phenols has been observed in the literature involving quinone addition to nucleosides.\(^{43}\) The reaction with 2′-deoxycytidine (Figure 3, middle right) gave even less product, which limited characterization to HPLC. Finally, we did not observe any adduct formation upon incubation with thymidine (Figure 3, bottom right). The key difference is that thymidine lacks a nucleoside aryl amine. Altogether, these data demonstrate that the oxidative activation of An-Hq and its derivatives generates an electrophile that can add to exocyclic amino groups of adenine, guanine, and cytosine. The yield of these reactions on double-stranded DNA will differ from nucleosides as the aryl amines of adenine and cytosine are accessible from the major groove, while N2 of guanine is accessible from the minor groove.

With the ability to modify three out of the four nucleosides through different parts of the helix, we set out next to investigate our agents’ reactivity with dsDNA, a biologically relevant substrate. Reaction of An-Hq, with \( \text{Na}_2\text{IrCl}_6 \) and a large DNA strand would allow us to identify specific DNA base sequences susceptible to damage by the agents (Figure 4). A 392-nucleotide section of the pUC19 plasmid, positions 287–678, was amplified by PCR. The fluorescently labeled primer used in our studies overlaps at positions 370–389. Vent(exo-) polymerase was used for both extension and manual sequencing. The nucleotides listed in Figure 4 sequence lanes correspond to the template strand. For example, bands in the G lane are equivalent to the G nucleotides in the 392-nucleotide template. Agent and oxidant are required to stop primer extension. When the unmodified DNA was extended, a full-length PCR product of 301 bp was observed. Comparison of the unreacted primer and full-length product allowed calculation of percent yield of the primer extension (Figure 4A). Several controls were utilized to identify unique damage produced by the activated An-Hq. The controls included a minus Na\(_2\)IrCl\(_6\) control (−OX), the end products produced after An-Hq oxidation (EP), a prequenched reaction control (PQ), and a negative control containing only DMSO (NR). Each control yielded 3.1–3.2% full-length product and demonstrated that oxidative activation is required. The end products gave by An-Hq oxidation produced a 3.1 ± 0.4% extension yield, similar to the minus oxidant control. This control established that the end products do not cause extension stops in high yield. When the 392-nucleotide DNA was incubated with already oxidized An-Hq, a similar yield of 3.2 ± 0.1% full-length extension product was observed. The no reagent control yielded a 3.1 ± 0.1% extension yield. As a positive control, we incubated the 392-nucleotide DNA with cisplatin. Cisplatin is a known DNA-modifying agent and, therefore, will induce replication stops at DNA damage sites. As expected, we observed no full-length extension product in the cisplatin positive control. Next, An-Hq was tested to further investigate its DNA-modifying capabilities. Treatment of the 392-nucleotide DNA with An-Hq followed by oxidative activation led to a reduction in the full-length product from % full-length product

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\begin{array}{cccccc}
\text{RXN} & \text{EP} & \text{NR} & \text{PQ} & \text{CIS} \\
-OX & 3.1 & 3.2 & 3.1 & 3.1 & 0.0 \\
\end{array}
\]

Figure 4. An-Hq\(_2\) modifies dsDNA in vitro. (A) Primer extension from a 392-nucleotide dsDNA resulted in a full-length extension product. Controls lanes include lacking oxidant (−OX), incubation with the end products (EP), a prequenched reaction (PQ), and a negative control containing only DMSO (NR). Control lanes did not alter the amount of full-length product produced. Reaction with either cisplatin (CIS) or An-Hq\(_2\) (RXN) in the presence of an oxidant led to a loss of full-length extension. (B) Gel showing sequence-dependent damage. Sequence lanes on left. Unmodified DNA and controls gave similar patterns of extension stops. DNA incubated with cisplatin led to extension stops at polyguanine sequences. In contrast, An-Hq\(_2\) and oxidant caused damage at most sequences. (C) Quantification of An-Hq\(_2\) damage. Extension stops in the reaction (black) and in the prequenched control (light gray) were compared to a thymine sequence lane (T, dark gray). A decrease in extension stops was observed at thymine sequences.
3.2% to 1.0 ± 0.2% or a decrease of 69 ± 0.6%. The decline in product formation shows that An-Hq₂ and its derivatives modify dsDNA and stop the progression of the DNA polymerase in vitro.

The primer extension assay provides further evidence that damage induced by An-Hq₂ and its derivatives is not only guanine specific (Figure 4B). The positive control, cisplatin, reacts at guanine repeats. This can be seen in the C3S lane of the gel, where the largest replication stop was at position 413. The corresponding sequence is 5′-GGGG. The negative controls (−OX, EP, PQ, and NR) had limited replication stops. We attributed these stops to the polymerase’s inability to pass certain structural features on the hard to replicate cloning region of pUC19. In contrast, activated An-Hq₂ in the RXN passed certain structural features on the hard to replicate cloning polymerase in vitro.

An-Hq₂ formed the benzetheno adduct on a dsDNA in vitro (Figure 5). The total ion chromatogram can be seen at the top, black, is the ion chromatogram for 1244 m/z. The middle, gray, shows the total ion current for the reaction. The middle, gray, shows the ion chromatogram for the unmodified DNA. The unmodified DNA is observed at 12.0 min and predominates the total ion current. The bottom, black, is the ion chromatogram for 1244 m/z. This ion mass corresponds to the DNA with a single benzetheno modification in the triply negatively charged state. The modified DNA was found to elute at 13.5 min and is also observed in total ion current chromatogram. (B) The top shows the sequence of the DNA. FT-MS spectra at 12.0 min show unmodified DNA with a mass of 1214.208 for the triply charged and 1821.8176 for the doubly charged unmodified oligonucleotide. (C) FT-MS spectra at 13.5 min show a modified DNA with masses of 1244.2129 for the triply charged and 1866.821 for the doubly charged state. These masses correspond to a DNA with a single benzetheno modification. The top structure shows the DNA sequence with a modified guanine, although the actual guanine on which modification occurred is unknown. Experimental masses are in black, while theoretical masses are in gray.

Reactivity with DNA was further explored in vitro using a 12-nucleotide oligonucleotide. The sequence chosen was a self-complementary sequence of 5′-GGCGAATTGCGC-3′, which has a molecular weight of 3646 g/mol with an elemental composition of C₁₁₂H₁₄₉N₄₆O₇₁P₁₁. Reaction with An-Hq₂ will produce addition of one benzetheno group to the oligonucleotide (Figure 5C). The only other major ion observed in the 12.0 min FT-MS at m/z 1252. We should note that the modification can be at many of the nucleotide positions on the dsDNA, since modification at any location would give the same mass. This result is significant as it proves that An-Hq₂ can add to dsDNA and form a lesion of the same mass shown in the nucleoside studies.

**Genetic Knockdown of Nucleotide Excision Repair.** Although An-Hq₂ and its derivatives can modify dsDNA, it was not known if DNA modification occurs within a cellular context. Therefore, we turned to *D. melanogaster*, a powerful whole-animal genetic model. Because of the DNA lesion identified in vitro, we reasoned that nucleotide excision repair should be an important means to repair this adduct if it was occurring in vivo. Thus, our prediction was that the knockdown of *Erc1*, a gene in the nucleotide excision repair pathway, would sensitize Drosophila to oxidatively activated agents. This sensitization would result as limited avenues for repair would remain, even at low basal levels of An-Hq₂ activation. Furthermore, we predicted that we would see an expedient response to DNA-modifying agents. DNA-damaging agents, due to their reactive nature, are rapidly metabolized in cells and plasma. For example, cisplatin is hydrolyzed in blood within minutes to hours to its active forms. The active agents quickly react with DNA, glutathione, thioredoxin, or protein to produce irreversible adducts.
Chlorambucil, which rapidly produces DNA lesions in minutes.\textsuperscript{48}

As a first step in testing our hypotheses, we began by validating Drosophila as a suitable model for the study of agents with known mechanisms of action. The effects of three agents, cisplatin, chlorambucil, and 5-fluorouracil, were examined. Specifically, we conducted a targeted transgenic RNAi knock-down experiment using the GAL4/UAS system in which expression of \textit{Ercc1} was silenced. We confirmed silencing of \textit{Ercc1} expression under the da-GAL4 driver using RT-PCR (Figure S7 in the Supporting Information). Because of the rapid induction of these agents’ mechanisms of action, we monitored the tolerated doses of these agents in Drosophila over the course of 7 days. To do so, we recorded differences in survival relative to the control, following delivery of either the DNA-modifying agent or the vehicle control.\textsuperscript{35} In the majority of injections with healthy animals with the just the vehicle alone, death was noted in approximately 20%. This was attributed to the stress of the injection process and was expected.

First, we examined the effects of cisplatin, which cross-links proximal guanines to create helix-distorting adducts. When cross-linking occurs, nucleotide excision repair is required to correct this adduct. In fact, ERCC1 activity can be used as a prognostic marker of cisplatin-based cancer treatments.\textsuperscript{49,50} As predicted, when Drosophila lacking \textit{Ercc1} expression (da-GAL4/UAS-\textit{Ercc1RNAi}) were injected with cisplatin, a rapid reduction in survival was observed relative to the control line (da-GAL4/+ by day 1 ($X^2 = 5.0, P < 0.025$; Figure 6A). On day 1, 40% of flies expressing \textit{Ercc1RNAi} had died in comparison to none of the control flies. The 40% difference in Drosophila lacking \textit{Ercc1} is significant when compared to injection of vehicle by day 1 ($X^2 = 5.0, P < 0.025$). No significant difference in survival was observed between vehicle and cisplatin-injected Drosophila when \textit{Ercc1} was not silenced. Finally, over the 7 day period, we observed reduced survival in both genotypes with agent delivery consistent with the highly toxic nature of cisplatin. Hence, our \textit{Ercc1} results in \textit{D. melanogaster} accurately replicate the observations of cisplatin and \textit{Ercc1} in cancer.

To further confirm that the Drosophila-based assay is sensitive to DNA-modifying agents producing bulky lesions, we examined the response of Drosophila lacking \textit{Ercc1} expression toward chlorambucil. Chlorambucil is a nitrogen mustard that will produce bulky DNA–DNA cross-links, which require \textit{Ercc1} for repair. In the case of chlorambucil, a significant difference in response to the agent was observed on day 1 between Drosophila lacking \textit{Ercc1} expression and the control line ($X^2 = 3.8, P < 0.05$; Figure 6B). This suggests that flies with wild-type \textit{Ercc1} expression levels can tolerate the formation of cross-links and that Drosophila lacking \textit{Ercc1} have much higher sensitivity, with 50% death on day 1. All of the Drosophila lacking \textit{Ercc1} expression that received chlorambucil died after 4 days as compared to 80% survival in wild-type Drosophila. Additionally, a significant difference in the survival of flies lacking \textit{Ercc1} expression was observed when the flies were injected with either chlorambucil or the vehicle ($X^2 = 6.7, P < 0.001$). However, no significant survival difference was observed between agent and vehicle in wild-type flies. This further supports our model by demonstrating that \textit{Ercc1} is vital to the repair of bulky lesions induced from chlorambucil.

We investigated a second agent to provide additional validation of our model system. The second agent, 5-fluorouracil, is mainly repaired through base excision repair, as it signals the incorrect incorporation of uracil. Consequently, we did not expect downregulation of \textit{Ercc1} to affect its mechanism of action; therefore, it can be considered a negative control. When similar experiments were conducted with 5-fluorouracil, we found no significant discrimination between the flies with \textit{Ercc1} silencing and controls (Figure 6C). On day 1, none of the injected control Drosophila had died. Similarly, in the \textit{Ercc1} knockdown, only 20% had died upon 5-fluorouracil injection, after which no more Drosophila perished. Survival after agent delivery did not significantly differ from injection of
the vehicle alone for either genotype. Thus, little change in mortality is observed upon treatment with 5-fluorouracil and is indicative of DNA modification occurring and being repaired. Therefore, we have validated that Drosophila, as a model organism, can be used to determine if an agent is inducing DNA damage in vivo.

Finally, we investigated if modification of DNA by our oxidatively activated agents was being repaired in the Drosophila model by a pathway involving Ercc1. Experiments similar to those using other DNA-modifying agents were accomplished, using An-Hq and An-Hq2. Because of the bulky Drosophila model by a pathway involving oxidatively activated agents was being repaired in the death) on day 1 as compared to the control line (\( \chi^2 = 5.5, P < 0.003 \)). These results were not unique to An-Hq; An-Hq2 showed similar trends with flies lacking Ercc1 showing a significant reduction in survival (50% death) on day 1 as compared to the control line (\( \chi^2 = 3.8, P < 0.05 \); Figure 7B) and compared to the delivery of vehicle alone (\( \chi^2 = 6.7, P < 0.01 \)). Wild-type Drosophila did not differ significantly when treated with An-Hq2 versus vehicle on day 1. These data emphasize that our novel oxidatively activated agents induce DNA modification in vivo and strongly support their role as DNA-damaging agents with highly selective cytotoxicity.

**Potency Against Renal Carcinoma Cells.** We then sought to elucidate a target cell type for these novel agents. In our previous work, an MTT assay was used to quantify potency against several types of cancer cell lines. Interestingly, many of the cancer cells tested displayed weak potency against when treated with An-Hq2. 24 Two renal cancer cells displayed a low IC\(_{50}\) value. We, therefore, assessed whether renal cells were sensitive to An-Hq2 treatment. An-Hq2 was evaluated for effects on viability using a sulforhodamine B total protein content assay at the NCI Developmental Therapeutics Program. Seven renal cancer cell lines were examined as follows: 786-O, A498, ACHN, CAKI-1, RXF 393, SN12C, and UO-31. Data at the NCI were then fit to sigmoid, and the IC\(_{50}\) and fitting error were determined (Table 1). Most renal cancer cells tested were sensitive to An-Hq2 with a median IC\(_{50}\) value of 1.8 μM. Importantly, some cell lines display high sensitivity since the IC\(_{50}\) values in ACHN and CAKI-1 cells were 360 ± 90 and 370 ± 40 nM, respectively. One cell line, SN12C, displayed low potency with an IC\(_{50}\) value of 21 ± 1.2 μM. Because six of the seven renal carcinoma cell lines had IC\(_{50}\) values below 5 μM, we infer that this cancer is targeted by oxidatively activated agents.

## CONCLUSION

DNA-modifying agents are highly used in anticancer treatments, even though many are nonselective. We have designed agents in which activation occurs by the elevated ROS that is present within some cancers. Previously, we demonstrated that our designed agents were selective for certain cancer cell types. Expansion of these studies in renal cell carcinoma cell lines shows that these agents can have potency in the midnanomolar range. This is an important finding because several research teams are utilizing ROS-activated agents.7 51 For example, boronic esters limit off-target effects of nitrogen mustards and protease inhibitors.52–55 Our work shows renal cancer cells as a natural target for these approaches. In this manuscript, we explored the mechanism of action of these agents through evaluation of the DNA lesion produced. These novel oxidatively activated agents behave much differently than current DNA-modifying agents. They are not nitrogen mustards, nor are they a simple quinine. Instead, what occurs is that the aniline ring tethered to the quinone induces nucleophilic addition to the 4′-carbon by DNA. DNA addition occurs on any exocyclic amine-bearing residue, which means they are capable of modifying three out of the four DNA bases.

**Table 1. IC\(_{50}\) of An-Hq2 against Renal Cancer Cells**

<table>
<thead>
<tr>
<th>Renal cancer cell</th>
<th>IC(_{50}) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>786-O</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>A498</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>ACHN</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>CAKI-1</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>RXF 393</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>SN12C</td>
<td>21.0 ± 1.2</td>
</tr>
<tr>
<td>UO-31</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

*IC\(_{50}\) values were measured using sulforhodamine B total protein assay.

**Figure 7. Ercc1 silencing in D. melanogaster results in sensitivity toward oxidatively activated agents.** (A) An-Hq or (B) An-Hq2 was injected into D. melanogaster in which Ercc1 expression was silenced (da-GAL4/UAS-Ercc1\(^{RNAi}\)) and compared to flies with wild-type Ercc1 expression (da-GAL4/+). For each genotype, survival with injection of vehicle alone was also assessed.

\[ \chi^2 = \, ]
Our primer extension experiment revealed that novel modification is occurring, likely at the minor groove for guanine and in the major groove for adenine and cytosine. The end product is a bulky hydroxy-N2,3-benzetheno-dG addition that is apt to be a strong replication stop. By making such an array of lesions, these oxidatively activated agents are expected to impart a strong need for DNA repair. Thus, DNA repair, which is altered in many cancers, is an essential component to the cytotoxic mechanism of action of our novel anticancer agents.

Our Drosophila model has shown that loss of Ercc1 function plays a major role in the repair of these bulky DNA lesions. It should be noted that to assess the cellular DNA-modifying ability of our novel agents, we have validated a rapid assay. In fact, the detection of DNA lesions formed from anticancer agents is a challenging process. This difficulty is due to the fact that only a few lesions need to form to impart cytotoxicity. The RNAi-mediated silencing of Ercc1 in Drosophila can serve as a simple means to determine if a therapeutic is a DNA damage agent. This study illustrates that DNA-modifying agents can be selective, even if they form highly disruptive lesions.

### ASSOCIATED CONTENT

5 Supporting Information

Figures of HPLC of benzethenoguanine adducts after purification, MS/MS of An-Hq-benzetheno-dG, NMR of N1,N2-benzetheno-dG + D20, rapid degradation of hydroxy-N2,N3-benzetheno-dG, tautomers and mechanism of N2,N3-benzetheno-dG, LC/MS of An-Hq-oligonucleotide adduct, and RNAi-mediated silencing of Ercc1 expression. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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### ABBREVIATIONS

ROS, reactive oxygen species; dG, 2′-deoxyguanosine; An-Hq, 4-[(2-ethylphenyl)amino]ethoxy)phenol; An-Hq2, 4′,4′-(2,2′-(phenylazanediy1)bis(ethane-2,1-diyl)bis(oxy))diphenol; NMR, nuclear magnetic resonance

### REFERENCES


