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Comparison of the Nucleic Acid Covalent Binding Capacity of Two Nitro-substituted Benzazolo[3,2-*a*]quinolinium Salts Upon Enzymatic Reduction

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Abstract

The DNA binding capacity of two nitro-substituted benzazolo[3,2-*a*]quinolinium chlorides (NBQs), NBQ-38 and NBQ-95, was evaluated upon their enzymatic reduction with hypoxanthine (HX)/xanthine oxidase (XO) under anaerobic conditions. In the presence of 2'-deoxyguanosine (2'-dG) or calf thymus DNA, covalent-addition products were monitored. Reactions of each NBQ with 2'-dG or DNA differed in the NBQ to HX molar ratio. Control reactions, one without HX/XO and another under aerobic conditions, were also analyzed. Adducts were isolated and characterized by high performance liquid chromatography (HPLC) and electrospray ionization-mass spectrometry (ESI-MS). Authentic samples of the reduced forms of these NBQs, identified as ABQ-38 and ABQ-95, were synthesized as standards to monitor bioreduction processes. HPLC analysis showed that the yield of formation of an unknown product (possibly, 2'-dG-NHBQ-38 adduct) from the reaction of NBQ-38 with 2'-dG and DNA was proportional to the HX to NBQ-38 molar ratio. ESI-MS analysis of the DNA hydrolysates showed evidence of an adduct formed upon bioreduction of NBQ-38 by the ions detection at *m/z* 528.3 and 454.8, consistent with chemical structures of a 2'-dG-NHBQ-38 adduct and a fragment ion. DNA adducts were not observed with NBQ-95, although the corresponding bioreduction product ABQ-95 was detected by ESI-MS. This study provides mechanistic information of these bioreductively-activated pro-drugs with potential therapeutic applications.

Keywords

DNA adducts; Bioreductive drugs; benzazolo[3,2-*a*]quinolinium; Hypoxia; Mass spectrometry

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Introduction

Hypoxic environments in solid tumors continue to challenge conventional treatment. Many bioreductive drugs cited in the literature are presumed to be activated when the drug or a drug component (effector) is reduced (Laderoute et al., 1988 and Denny et al., 1996). Furthermore, an enhanced production of hypoxanthine (HX) together with the proteolytic conversion of xanthine deoxydehydrogenase to xanthine oxidase (XO) during ischemia have been implicated in the generation or initiation of a hypoxic and chemically reducing environment (Wardman, 2001).

Recent interest in nitro compounds has focused on therapeutic applications; in particular, their selectivity in the treatment of hypoxic solid tumors (Price et al., 2000 and Borch et al., 2001). Hypoxic solid tumors can show strong resistance to traditional therapeutic approaches such as radiotherapy. Drugs activated in hypoxia by reductase enzymes have the potential, however, to overcome this resistance to radiotherapy. Bioreductive-activated drugs such as nitroarenes, quinones, or those drugs with aromatic *N*-oxide moieties have been known to possess redox properties and to present selective toxicity toward cells with limited oxygen content, such as those within a hypoxic, solid tumor (Wardman, 2001).

The reason for the selectivity of the nitroarene compounds is that in hypoxic tissues, reduction of the nitro group by intracellular metabolic reductases can result in strong electrophilic intermediates and DNA-binding species, i.e. hydroxylamine derivatives. In normoxic tissues, the reduced derivatives could be reoxidized very efficiently by oxygen, thus, inhibiting the formation of DNA-alkylating species (Adams, 1992). Intermediates derived from the bio-oxidation of aromatic amines are known to form covalent DNA adducts in which binding occurs preferentially at the C8 position of the guanine bases. Drugs that interact or bind to DNA could affect cellular processes such as transcription or DNA replication, initiating cell death mechanisms such as apoptosis (Mello et al., 1995).

NBQ-38 (7-ethyl-3-nitrobenzimidazo[3,2-*a*]quinolinium chloride) and NBQ-95 (10-methyl-2-chloro-3-nitrobenzothiazolo[3,2-*a*]quinolinium chloride) (Figure 1 and Table 1) are nitro-containing heterocyclic compounds possessing a positive charge that could facilitate their interaction with cellular organelles. They belong to a new family of unnatural alkaloids known as benzazolo[3,2-*a*]quinolinium salts (BQ). Of these, 3-nitrobenzothiazolo[3,2-*a*]quinolinium chloride (NBQ-2) has shown potential as an anticancer agent (Cox et al., 1982). It has been shown that these planar heterocyclic cationic nitroarenes can be reduced electrochemically or bioreduced in the presence of HX/XO (Cox et al., 1999, Alegria et al., 1993, Alegria et al., 2004). However, nothing is known regarding the possibility of DNA alkylation mediated through nitro reduction by this family of compounds. Therefore, it is of interest to determine whether the electrophilic species, presumably originating from the hydroxylamine, formed in the bioreduction of these NBQs can bind covalently to DNA. A proposed C8-2'dG-NHBQ adduct is presented in Figure 2. This proposed binding site is based solely on evidence from other nitro containing compounds described in the literature (Takamura-Enya, 2003).

In this study, we developed an analytical LC-ESI-MS method that allowed us to characterize the NHBQ-DNA covalent adducts formed under conditions that mimic the hypoxic tumor environment. Authentic samples of reduced NBQ-38 and NBQ-95, (ABQ-38, 3-amino-7-ethylbenzimidazolo[3,2-*a*]quinolinium chloride and ABQ-95, 3-amino-2-chloro-10-methylbenzothiazolo[3,2-*a*]quinolinium chloride; respectively) were prepared by the photochemically induced cyclization of (*E*)-7-ethyl-2-(5-amino-2-chlorostyryl)benzimidazole and (*E*)-5-methyl-2-(5-amino-2,4-dichlorostyryl)benzothiazole, respectively, and used to monitor the reduction of the NBQs in the hypoxia-mimicking environment. Each reaction was analyzed by ESI-MS to evaluate the formation of 2'dG-NBQ adducts. The presence of the

reduced, ABQ, molecular ion (formed during the dG-NBQ reaction) was taken as an indication of the reductase activity and the potential capability of NBQ's reactive intermediates to bind covalently to nucleic acid in the presence of HX/XO.

Materials and Methods

Chemicals

NBQ-38 (7-ethyl-3-nitrobenzimidazo[3,2-*a*]quinolinium chloride) was prepared as described (Cox et al., 1982, Alegría et al., 1993). NBQ-95 (2-chloro-10-methyl-3-nitrobenzothiazolo [3,2-*a*]quinolinium chloride) was synthesized by a general procedure consisting of a modification of that previously described for the synthesis of 3-nitrobenzothiazolo[3,2-*a*]quinolinium chloride, NBQ-2 (Cox et al., 1982). NBQ-95 was prepared and characterized as follows: Following the general procedure (Cox et al., 1982), (*E*)-5-methyl-2-(2,4-dichloro-5-nitrostyryl)benzothiazole (0.543 g, 1.49 mmol), prepared from the condensation of 2,4-dichloro-5-nitrobenzaldehyde and 2,5-dimethylbenzothiazole as described (16), was photocyclized to give 0.22 g (41%) of NBQ-95 as a yellow solid: mp 236-240°C (dec.); ¹H NMR (300.15 MHz, DMSO-*d*₆): δ ppm 9.54 (s, 1H), 9.37 (s, 1H), 9.06 (s, 1H), 9.00 (s, 2H), 8.59 and 7.86 (AB, 2H, J = 8.4 Hz), 2.74 (s, 3H); UV-*vis* (95% EtOH) λ_{max}/nm (ε): 389 (11196), 380 (11363), 270 (14858), 225 sh (21441) and 202 (28390); Anal. Calcd for C₁₆H₁₀Cl₂N₂O₂S·H₂O: C, 50.14; H, 3.16; N, 7.31%. Found: C, 49.85; H, 3.35; N, 7.00%.

The amino precursors of ABQ-38 and ABQ-95, (*E*)-7-ethyl-2-(5-amino-2-chlorostyryl)benzimidazole & (*E*)-5-methyl-2-(5-amino-2,4-dichlorostyryl)benzothiazole, respectively, were prepared by the reduction of the corresponding nitro derivatives with hydrazine in the presence of nickel boride in dry methanol as described (Lloyd and Nichols, 1986). (*E*)-7-ethyl-2-(5-amino-2-chlorostyryl)benzimidazole was photocyclized as described (Cox et al. 1982) to give 3-amino-7-ethylbenzimidazo[3,2-*a*]quinolinium chloride (ABQ-38) as a yellow solid: ¹H NMR (500.13 MHz, DMSO-*d*₆): δ ppm 9.05 (d, 1H, J = 8.50 Hz), 8.88 (d, 1H, J = 9.0 Hz), 8.56 (d, 1H, J = 9.5 Hz), 8.27 (d, 1H, J = 8.5 Hz), 8.23 (d, 1H, J = 9.50 Hz), 7.90 (t, 1H, J = 7.5 Hz), 7.81 (t, 1H, J = 7.5 Hz), 7.43 (dd, 1H, J = 8.5, 2.5), 7.27 (d, 1H, J = 2.5 Hz), 4.83 (q, 2H, J = 7.0 Hz), 1.48 (t, 3H, J = 7.0 Hz); UV-*vis* (95% EtOH) λ_{max}/nm (ε): 409 (1410), 351 (8104), 336 (9815) and 271 (20866); HRMS calcd for C₁₇H₁₆N₃ (compound without the chloride counter ion) 262.1, found by ESI at m/z 261.9.

(*E*)-5-methyl-2-(5-amino-2,4-dichlorostyryl)benzothiazole was photocyclized as described (Cox et al., 1982) to give 3-amino-2-chloro-10-methylbenzothiazolo[3,2-*a*]quinolinium chloride (ABQ-95) as a yellow solid: ¹H NMR (500.13 MHz, DMSO-*d*₆): δ ppm 9.17 (s, 1H), 8.92 (s, 1H), 8.63 (AB quartet, 2H, J = 9.5 Hz), 8.48 (d, 1H, J = 8.5 Hz), 7.76 (d, 1H, J = 8.5 Hz), 7.53 (s, 1H), 6.50 (m, 2H), 2.70 (s, 3H); UV-*vis* (95% EtOH) λ_{max}/nm (ε): 429 (4417), 380 (10387), 282 (22568) and 214 (2650); HRMS calcd for C₁₆H₁₂ClN₂S 299.0 (without the chloride counter ion), found by ESI at m/z 299.0 M⁺.

Hypoxanthine (HX), xanthine oxidase (XO), allopurinol, 2'-deoxyguanosine (2'-dG), 2'-deoxythymidine (2'-dT), 2'-deoxyadenosine (2'-dA), 2'-deoxycytosine (2'-dC), calf thymus DNA, nuclease P1, alkaline phosphatase and phosphodiesterase I were obtained from Sigma-Aldrich (St. Louis, MO). Sodium acetate, zinc chloride and ammonium acetate, water and methanol HPLC grade were obtained from Fisher Scientific (New Jersey). Water (MS grade) was obtained from Waters, Corp. (Caguas, PR). All solvents and water were degassed and filtered prior to use.

Instrumentation

The chromatographic data was acquired with an HPLC system equipment consisting of a binary pump 1525 coupled to an autosampler (717 Plus) and a Dual λ absorbance detector (2487) from Waters, Corporation (Milford, MA). The chromatographic separation was performed using a Discovery C8 4.6 mm $\times$ 250 mm obtained from Supelco, Sigma-Aldrich, St. Louis MO. Collected peaks from the HPLC were then analyzed by ESI-MS/MS on a Micromass Quattro Micro API triple quadrupole mass spectrometer with an ESI source (Micromass, Inc., Manchester, UK).

Reactions of NBQs and 2'-dG

Stock solutions of NBQ-38 and NBQ-95 were prepared in water and dilutions kept in amber vials at 4°C. In order to mimic a hypoxic environment reaction, four 1.5-mL amber vials were connected to a vacuum manifold to provide a continuous flow of argon gas through the reaction vials during the reaction period (1 h). Two controls were also included, one containing all reactants as reaction 1 but under aerobic conditions and one without 2'-dG. Each sample had two syringes connected: one used to connect the vial to the manifold, and the second to release the positive argon pressure and keep a continuous hypoxic environment. After the first 15 min of continued argon flow, the reagents (NBQs, 2'-dG, HX and XO) were added into the vial connected to the manifold. In each of the following reactions, the reagents were diluted with deionized water to a final volume of 1 mL: **Reaction-1** contained 0.2mM of NBQ, 0.6mM of 2'-dG, 20.0mM ammonium acetate buffer (pH 7.4), 0.6mM HX, and 50mU XO/mL; **Reaction-2** 0.2mM NBQ, 0.6mM dG, 20.0 mM ammonium acetate buffer (pH 7.4), 1.2 mM HX, and 100 mU XO /mL; **Reaction-3**, 0.2mM NBQ, 0.6mM dG, 20.0 mM ammonium acetate buffer (pH 7.4), 0.3 mM HX, and 50mU XO /mL; and **Reaction-4** (without HX/XO), 0.2 mM NBQ, 0.6 mM 2'-dG, 20.0 mM ammonium acetate buffer (pH 7.4), and no HX/XO. **Reaction-5** and **Reaction-6** contained all reagents at the same concentrations as in **Reaction-1**, but were performed under aerobic conditions and without 2'-dG as control. In summary, the molar ratios of HX to NBQ used per reactions 1-4 were 3, 6, 1.5 and 0, respectively. After 1 h of reaction, 0.1mM of allopurinol was added via syringe to each vial in order to stop enzymatic activity, and after 15 min the samples were placed at -20°C until ready for HPLC analysis. The above reactions (1-5) were repeated in triplicates.

Reactions of NBQs with DNA and enzymatic digestion

The reaction of NBQs with 100 μ g of Calf thymus DNA was performed similarly as described previously with 2'-dG. Calf thymus DNA stock solutions (1mg/mL) were prepared in filtered deionized water. Once DNA-NBQs reactions were complete, samples were placed in 1.5-mL Eppendorf microtubes for DNA digestion. A control sample with untreated calf thymus DNA was included in the digestion process for assessment of the digestion efficiency. Enzymatic digestions were performed according to the methods presented by Zayas et al., 2006. Samples were then placed on ice and stored at -20°C until HPLC analysis.

Characterization of NBQ adducts with 2'-dG and DNA by ESI-MS

Samples were analyzed by HPLC-UV to determine the presence of adducts or new products formed between NBQs and 2'-dG. Areas and height calibration curves with NBQ-38, NBQ-95 and 2'-dG were generated with the final optimized method, resulting in correlation coefficients of 0.995, 0.999, 0.998 (data not shown). The HPLC effluent was monitored at 254 and 370 nm. From each reaction, contents from 40 μ L injections were eluted using a 30 min non-linear gradient from 8% methanol/92% 0.1 M sodium acetate to 100% B in 25 min. By co-chromatographic analysis against the precursors at 370 nm, new (unknown) peaks were monitored and collected for ESI-MS analysis. NBQs absorbed both at 254 nm and 370 nm;

however, nucleic acid, and HX/XO show minimal absorptions at 370 nm, therefore any potential adduct containing NBQ, displayed strong absorption at 370 nm.

Collected unknown peaks were speed vacuum concentrated and re-suspended in 100 μ L of 4:1 water/MeOH. Samples were injected into the MS by direct infusion through capillary tubing at a flow rate of 2 μ L/min. Dilutions of the NBQ and 2'-dG were used for optimization of the tuning conditions. Optimized full scan ESI-MS conditions used were as follow: Positive ionization electrospray mode, pump flow of 2TL/min, capillary voltage (1.56 kV), cone voltage (42 V), extractor (48 V), source temperature (80°C), desolvation temperature 100 (°C) and nitrogen gas flow (100 L/hr). For the parent-daughter analysis argon was used as the collision induced dissociation (CID) gas at collision cell energy of 17 eV.

Results

Analysis of 2'-dG-NHBQ and DNA-NHBQ adduct by HPLC

The set of chromatograms in Figure 3 show the results of the bioreduction of NBQ-38 in the presence of 2'-dG, monitored at 254 nm and 370 nm. Reactions 1-4 differed mainly in the molar concentration ratio of HX to NBQ: 3, 6, 1.5 and 0 per reaction as presented in the experimental section. The results of the reaction monitored at 254 and 370 nm shown in Figure 3 are consistent with the formation of 2'-dG-NHBQ-38 adducts. The unknown peak was collected for further mass characterization and identification of the potential isolated, new 2'-dG-NHBQ-38 adduct(s). The possibility of the new peak in reactions 1 and 2 to be a reduced form of the NBQ-38 was also considered; however, this idea was discarded given the fact that, in the absence of 2'-dG (Reaction-6) but in the presence of HX/XO, this new peak was not formed, indicating the relation of this new peak to the presence of 2'-dG.

In **Reaction-2**, with the higher molar ratio of HX/NBQ, a greater amount of unknown product (adduct) resulted, while **Reaction-3** (as observed in Figure 3), with a lower ratio of HX/NBQ, showed no detectable formation of a potential adduct. **Reaction-4** without HX/XO did not show new peaks indicating a 2'-dG-NHBQ-38 adduct. To support our initial hypothesis, neither **Reaction-5**, containing the same molar ratio of HX to NBQ than **Reaction-1** but performed under aerobic conditions, nor **Reaction-6**, without 2'-dG, presented new or unknown products. In support of our initial hypothesis, neither Reaction-5 (containing the same molar ration of HX to NBQ as reaction-1 but performed under aerobic conditions) nor Reaction-6 (without 2'-dG) presented new or unknown products. Figure 4 shows a comparison between the areas corresponding to the new product formed vs. unreacted NBQ-38 in **Reaction-1** and **Reaction-2**. The chromatograms shown in Figure 5 compare the reactions of NBQ38 with DNA. **Reaction-2** and **Reaction-3** produced new products; however, in contrast with NBQ-38-2'-dG reactions, **Reaction-1** showed no significant adduct formation.

Chromatographic analysis of 2'-dG reactions with NBQ-95, in contrast with NBQ-38, showed no evidence of new products or adducts formation as shown in Figure 6. In order to evaluate any bioreduction of the NBQ-95 and, given the presence of a small shoulder next to the NBQ-95 peak, the peak at retention time 25.00 min. was isolated for ESI-MS analysis. Although 2'-dG reactions with NBQ-95 did not present observable levels of new products or adducts, DNA reactions with NBQ-95 were performed and analyzed in the same way as described for NBQ-38 in order to evaluate the formation of other nucleoside adducts. Evidence of new products was not, however, observed with NBQ-95 and DNA reacting under conditions that mimic an anaerobic environment.

ESI-MS Analysis

The HPLC-isolated new peaks were then prepared for ESI-MS analysis. Samples were speed-vacuum dried and re-suspended in 4:1 H₂O/MeOH, sonicated and direct-injected into an ESI-triple quadrupole MS system. Full scan analysis of NBQ-38 reaction samples was used to monitor the formation of 2'-dG-adducts (calculated mass 527.2), the reduced parent compound (ABQ-38, calculated mass 262.1) or any unreacted NBQ-38 (calculated mass 292.1). The spectrum shown in Figure 7 indicates the presence of a prominent signal at m/z 454.8, representing a 2'-dG-NHBQ-38 adduct fragmentation. A collision-induced dissociation experiment (CID of 528→454) revealed the origin of the m/z 454.8 signal, indicating the $[M+1]^+$ molecular ion m/z 528.2 as its precursor. Although the exact structure of this 454.8 molecular ion is not totally understood, it could represent the loss of part of the ribose group, which is common under ESI conditions (Brown, 2003;B. Van den Driessche et al., 2003;Rindgen, D., et al., 1995).

In order to confirm the enzymatic reduction of the nitro group in NBQ-38 and NBQ-95 by HX/XO to the corresponding amine under these conditions, authentic samples of ABQ-38 and ABQ-95 were synthesized. The mass spectrum presented in Figure 8 (C) is that of ABQ-38 and displays signals at m/z 261.9 (parent ion), and m/z 233.8 (parent ion -28 amu). Figure 8A and B display the mass spectra corresponding to reactions of NBQ-38 with 2'-dG and DNA, respectively. As observed the ions at m/z 261.8 in (A), 261.9 in (B), respectively, correspond (within experimental error) to the parent ion of authentic ABQ-38 shown in Figure 8 C. These data confirm the ability of the bioreduction system to convert NBQ-38 to ABQ-38. NBQs and adducted species can be highly sensitive to the conditions applied during the ESI analysis. This helps to explain the dissimilarities of the relative intensities in the spectra, even for the same given sample.

The spectrum presented in Figure 9 represents the full scan ESI analysis of the NBQ-95 with DNA Reaction 2 (A). Spectrum (B) corresponds to that of authentic NBQ-95 (molecular ion m/z 329.0) and (C) represents authentic ABQ-95 (molecular ion m/z 299.0). In spectrum (A) the molecular ion at m/z 329.0 is indicative of un-reacted NBQ-95 and the signal at m/z 299.1 represents bio-reduced NBQ-95. In contrast to reactions of NBQ-38 with DNA, significant molecular ions (or fragment ions) representing the monitored adduct were not observed. The lack of formation of NBQ-95-DNA adducts could be explained by the inability of reactive species to be formed during the bioreduction of NBQ-95, or perhaps a low level of adduct formed which could not be detected by the analytical method.

Discussion

The basis for the selective toxicity of the nitro-substituted benzazolo[3,2-*a*]quinolinium chlorides (NBQ) drugs depends on the reduction of the nitro group, which can accept up to 6 electrons to produce the corresponding amine (Denny, 2000) via the nitroso (2 electrons) and hydroxylamine (4 electrons) intermediates. However, in the presence of air, redox cycling occurs, whereby the nitroarene compound is recovered with the concomitant production of reactive oxygen species (Tocher et al., 1989, Krainev et al., 1998, Sarlauskas et al., 1997). Although the possibility exists that redox cycling by nitroarenes generates cytotoxic effects, the greatest cytotoxicity is usually observed under hypoxic or anaerobic conditions (Wardman, 2001). Bioreductive drugs are activated by intracellular metabolic reduction mediated through various cellular reductases. Usually, bioreduction is favored under hypoxic conditions, and this is why many of these compounds display differential cytotoxicity to hypoxic cells.

Reduction of the nitro group in the NBQs, the object of this study, could result in the formation of an amine as the final product. However, as stated above, the reduction under hypoxic conditions takes place in several discrete steps that generate reactive intermediates capable of

forming covalent adducts with DNA. In the hypoxic microenvironment created in this study, the reduction of the NBQs depends on the hypoxanthine (HX): NBQ ratio. Scheme 1 shows the proposed sequence of events in the anaerobic bioreduction of the nitro group and the potential formation of adducts of purine bases 2'-dG or 2'-Ad. Figure 2 shows a detailed step-by-step mechanism to account for the formation of the proposed C8-2'-dG-NHBQ adducts. The initial attack of an activated hydroxylamine, presumably a nitrenium ion, occurs at N7, followed by a rearrangement to C8. This mechanism is identical to that proposed in the literature (Kennedy, 1997), except that an activated form of the hydroxylamine replaces a nitrenium ion.

To initiate the bioreduction, HX/XO was introduced at three different HX-to-NBQ molar ratios. The ability of XO to reductively activate multiple substrates is documented (Bunting et al., 1980), including the covalent binding of nitroarenes via HX/XO (Fu et al., 1994, Ritter et al., 2002). In the present study, the reaction of NBQ-38 with the highest concentration of HX resulted in the highest level of adduct formation.

The identity of the new product was determined by ESI mass spectrometry. Interestingly, this analytical method could also be used to evaluate evidence for the NBQs reduction process. With NBQ-38, clear evidence of the reduced moiety was found from the molecular ion at m/z 261.9 and fragment ion at m/z 233.8. Evidence of the formation of an 2'-dG-NHBQ-38 adduct was confirmed by the detection of the molecular ion m/z 454.8, resulting from the [M+1] molecular ion m/z 528.3 (Figure 7). Higher levels of adduct formation would have allowed us to better define the structure of this [M+1] 528.3 molecular species; however, the loss of part of the ribose group, which is common under these ESI conditions (Brown, 2003) is a possible explanation.

With NBQ-95, 2'-dG covalent adducts were not observed at any of the applied HX-to-NBQ ratios. We propose that steric hindrance or electronic effects of the Cl atom in NBQ-95 could interfere with nitro group reduction, or the binding of a reduced nitro group to 2'-dG and DNA. The presence of low amounts of ions from reduced NBQ-95 (ABQ-95) in the mass spectrum was taken as an indication that the drug is bio-reduced in the presence of HX/XO, as with NBQ-38. The absence of unknown peaks (adducts) in the HPLC analysis monitored at 370 nm suggests the low reactivity of the reactive species formed during the bioreduction toward 2'-dG.

In conclusion, we have demonstrated that the novel nitro-substituted benzazolo[3,2-*a*]quinolinium chloride NBQ-38 has the potential to bind to DNA in the presence of HX/XO and under anaerobic conditions. This study provides mechanistic information about these bio-reductively activated pro-drugs with potential therapeutic applications.

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References

- Adams GE. Failla Memorial Lecture. Redox, radiation, and reductive bioactivation. *Radiat Res* 1992;132:129–139. [PubMed: 1438693]
- Alegria AE, Cox O, Santiago V, Colon M, Reyes Z, Zayas L, Rivera LA, Dumas JA. Reductive activation of benzazolo[3,2-*a*]quinolinium chlorides. *Free Radic Biol Med* 1993;15:49–56. [PubMed: 8395453]

- Alegria AE, Flores W, Cordones E, Rivera L, Sanchez-Cruz P, Cordero M, Cox O. Reductive activation and thiol reactivity of benzazolo[3,2-*a*]quinolinium chlorides. *Toxicology* 2004;199:87–96. [PubMed: 15147783]
- Van den Driessche B, Lemiere Filip, Van Dongen Walter, Esmans Eddy L. Alkylation of DNA by melphalan: investigation of capillary liquid chromatography-electrospray ionization tandem mass spectrometry in the study of the adducts at the nucleoside level. *Journal of Chromatography B* 2003;785:21–37.
- Báez A, González FA, Vázquez D, Waring MJ. Interaction between 3-nitrobenzothiazolo[3,2-*a*]quinolinium and DNA. *Biochem Pharmacol* 1983;32:2089–2094. [PubMed: 6870936]
- Borch RF, Liu J, Joswig C, Baggs RB, Dexter DL, Mangold GL. Antitumor activity and toxicity of novel nitroheterocyclic phosphoramidates. *J Med Chem* 2001;44:74–77. [PubMed: 11141090]
- Brown K, Harvey CA, Turteltaub WW, Shields SJ. Structural characterization of carcinogen-modified oligodeoxynucleotide adducts using matrix-assisted laser desorption/ionization mass spectrometry. *J Mass Spectrom* 2003;38:68–79. [PubMed: 12526008]
- Bunting JW, Laderoute KR, Norris D. Specificity of xanthine oxidase for nitrogen heteroaromatic cation substrates. *Can J Biochem* 1980;58:49–57. [PubMed: 6893010]
- Casiano CA, Báez A. Effects of the antitumor drugs 3-Nitrobenzothiazolo[3,2-*a*]quinolinium and fagaronine in nucleic acid and protein synthesis. *Biochem Pharmacol* 1986;35:679–685. [PubMed: 3511918]
- Cox O, Dumas J, Rivera L, García C, Alegría A. Electrochemical and electron spin resonance studies of selected benzazolo[3,2-*a*]quinolinium salts. *J Heterocyclic Chem* 1999;36:943–947.
- Cox O, Jackson H, Vargas VA, Báez A, Colón JI, González BC, De León M. Synthesis and biological activity of benzothiazolo and benzoxazolo[3,2-*a*]quinolinium salts. *J Med Chem* 1982;25:1378–1381. [PubMed: 7143376]
- Denny WA. The role of hypoxia-activated prodrugs in cancer therapy. *Lancet Oncol* 2000;1:25–29. [PubMed: 11905684]
- Denny WA, Wilson WR, Hay MP. Recent development in design of bioreductive drugs. *Br J Cancer* 1996;74:S32.
- Fu PP, Herreno-Saenz D, Von Tungeln LS, Lay JO, Wu YS, Lai JS, Evans FE. DNA adducts and carcinogenicity of nitro-polycyclic aromatic hydrocarbons. *Environ Health Perspect* 1994;102(Suppl 6):177–183. [PubMed: 7889844]
- Kennedy SA, Novak M, Kolb BA. Reactions of ester derivatives of carcinogenic *N*-(4-biphenyl) hydroxylamine and the corresponding hydroxamic acid with purine nucleosides. *J Am Chem Soc* 1997;119:7654–7664.
- Kraiev AG, Williams TD, Bigelow DJ. Enzymatic reduction of 3-nitrotyrosine generates superoxide. *Chem Res Toxicol* 1998;11:495–502. [PubMed: 9585480]
- Laderoute K, Wardman P, Rauth A. Molecular mechanisms for the hypoxia-dependent activation of 3-amino-1,2,4-benzotriazine-1,4-dioxide. *Biochem Pharmacol* 1988;37:1487–1493. [PubMed: 3128984]
- Lloyd DH, Nichols DE. Nickel boride/hydrazine reduction of aromatic and aliphatic nitro compounds. Synthesis of 4-(benzyloxy)indole and α -alkyltryptamines. *J Org Chem* 1986;51:4294–4295.
- Mello JA, Lippard SJ, Essigmann JM. DNA adducts of *cis*-diamminedichloroplatinum(II) and its *trans* isomer inhibit RNA polymerase II differentially in vivo. *Biochemistry* 1995;34:14783–14792. [PubMed: 7578087]
- Price, T.; Walton, MI.; Titley, J.; Cunningham, D.; Workman, P. Studies on the mechanism of action of the cyclin dependent kinase inhibitors 9-nitro and 10-bromopauillone in human colon tumour cell lines. *Clin Cancer Res; Proceedings of the 11th NCI-EORTC-AACR Symposium on New Drugs in Cancer Therapy*; 6, November; 2000. Published as a Supplement to
- Rindgen D, Turesky RJ, Vouros P. Determination of in vitro formed DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine using capillary liquid chromatography/electrospray ionization/tandem mass spectrometry. *Chem Res Toxicol* 1995;8:1005–1013. [PubMed: 8605282]
- Ritter CL, Culp S, Freeman J, Marques M, Beland F, Malejka-Giganti D. DNA adducts from nitroreduction of 2,7-Dinitrofluorene, a mammary gland carcinogen, catalyzed by rat liver or mammary gland cytosol. *Chem Res Toxicol* 2002;15(4):536–544. [PubMed: 11952340]

- Sarlauskas J, Dickancaite E, Nemeikaite A, Anusevicius Z, Nivinskas H, Segura-Aguilar J, Cenas N. Nitrobenzimidazoles as substrates for DT-diaphorase and redox cycling compounds: their enzymatic reactions and cytotoxicity. *Arch Biochem Biophys* 1997;346:219–229. [PubMed: 9343369]
- Tocher JH, Knight RC, Edwards DI. Electrochemical characteristics of nitroheterocyclic compounds of biological interest II. Nitrochloroamphenicol. *Free Rad Res Commun* 1989;5:319–326.
- Vivas-Mejia PE, Rodriguez-Caban JL, Diaz-Velazquez M, Hernandez-Perez MG, Cox O, Gonzalez FA. DNA binding-independent anti-proliferative action of benzazolo[3,2-*a*]quinolinium DNA intercalators. *Mol Cell Biochem* 1997;177:69–77. [PubMed: 9450647]
- Takamura-Enya T, Ishikawa S, Mochizuki M, Wakabayashi K. Development for an efficient synthesis method of 2'-deoxyguanosine-C8 adducts with several amino/nitro-arenes. *Nucleic Acids Res* 2003; (Suppl 3):23–4. [PubMed: 12519940]
- Vivas-Mejia PE, Cox O, Gonzalez FA. Inhibition of human topoisomerase II by anti-neoplastic benzazolo [3,2-*a*]quinolinium chlorides. *Mol Cell Biochem* 1998;178:203–212. [PubMed: 9546601]
- Wardman P. Electron transfer and oxidative stress as key factors in the design of drugs selectively active hypoxia. *Curr Med Chem* 2001;8:739–761. [PubMed: 11375747]
- Zayas B, Stillwell SW, Wishnok JS, Trudel LJ, Skipper P, Yu MC, Tannenbaum SR, Wogan GN. Detection and quantification of 4-ABP adducts in DNA from bladder cancer patients. *Carcinogenesis*. 2006in press (Access published online on August 21, 2006)

Abbreviations

BQ	benzazolo[3,2- <i>a</i>]quinolinium chloride
NBQ, or BQ-NO₂	nitrobenzazolo[3,2- <i>a</i>]quinolinium chloride
ABQ or BQ-NH₂	aminobenzazolo[3,2- <i>a</i>]quinolinium chloride
NBQ-38	7-ethyl-3-nitrobenzimidazo[3,2- <i>a</i>]quinolinium chloride
NBQ-95	10-methyl-2-chloro-3-nitrobenzothiazolo[3,2- <i>a</i>]quinolinium chloride
ABQ-38	3-amino-7-ethylbenzimidazolo[3,2- <i>a</i>]quinolinium chloride
ABQ-95	3-amino-2-chloro-10-methylbenzothiazolo[3,2- <i>a</i>]quinolinium chloride
2'-dG	2'-deoxyguanosine
2'-dG-NHBQ-38	Adduct formed in the bio-reduction of NBQ-38 in the presence of 2' dG or DNA
HX	hypoxanthine
XO	xanthine oxidase
ESI-MS	Electrospray Ionization Mass Spectrometry

CID

Collision-induced dissociation

Figure 1.
Structure of NBQs (NBQ-38 and NBQ-95) studied and their corresponding amino derivatives, ABQs, (ABQ-38 and NBQ-95) used to monitor the bio-reduction process.

Figure 2. Proposed structure and mechanism for the formation of the C8-2'-dG-NHBQ adduct. The mechanism involves the reaction of an activated hydroxylamine intermediate formed during the bio-reduction of a BQ-NO₂ according to Scheme 1.

Figure 3.

Relative peak intensities from HPLC analyses of NBQ-38 and 2'-dG reactions. From each reaction, contents from 40 μ L injections were eluted using a 30 min non-linear gradient from 8% methanol/92% 0.1 M sodium acetate to 100% B in 25 min. The NBQ-38 retention time is ca. 15.3 min at 254 and 370 nm. An unknown peak is identified clearly in Reactions 1 and 2 with retention time ca. 16.3 min. Both peaks were collected for ESI- MS analysis and potential adduct characterization.

Figure 4.

Comparison of the relative areas from unreacted NBQ-38 and a new, unknown peak (possible C8-2'-dG-NHBQ adduct) observed at 370 nm for NBQ-38 and 2'-dG Reactions 1 and 2.

Figure 5.

Relative peak intensities from the HPLC analyses of reactions of NBQ-38 and calf thymus DNA in comparison with 2'-dG, Reaction 1. From each reaction, contents from 40 μ L injections were eluted using a 30 min non-linear gradient from 8% methanol/92% 0.1 M sodium acetate to 100% B in 25 min. The NBQ-38 retention time is ca. 15.2 min at 254 and 370 nm. An unknown peak is identified clearly in reactions 2 and 3 with retention time ca. 16.3 min. Both were collected for MS analysis.

Figure 6.

Relative peak intensities from the HPLC analyses of reactions of NBQ-95 and 2'-dG reactions. From each reaction, contents from 40 μ L injections were eluted using a 30 min non-linear gradient from 8% methanol/92% 0.1 M sodium acetate to 100% B in 25 min. NBQ-95 had a retention time $\approx$ 25.0 min at 254 and 370 nm. Unknown peaks suggesting NBQ-95-2'-dG adducts formation were not identified.

Figure 7.

Daughter ion analysis of DNA reaction with NBQ-38. A full scan analysis of the DNA reaction with NBQ-38 presented a prominent molecular ion m/z 454.8. To confirm if the molecular ion at m/z 454.8 represented a fragment ion of the monitored C8-2'-dG-NHBQ-38 adduct of m/z 528.3, a daughter ion analysis was performed. For the analysis argon was used as the collision induced dissociation (CID) gas at collision cell energy of 17 eV. The molecular ion m/z 528.3 was then identified as the precursor for the fragment ion m/z 454.8.

Figure 8.

Full Scan ESI-MS analysis of Reaction 2 of NBQ-38 with 2'-dG (A) and Reaction-2 of NBQ-38 with DNA (B). The ESI spectrum of an authentic sample of ABQ-38 displaying a molecular ion at 261.9 m/z is shown in (C). The presence in (A) and (B) of the molecular ions at m/z 261.8 and 261.9 respectively, and fragments similar to those observed in (C) are evidence of the bioreduction of NBQ-38.

A- NBQ-95 with DNA Reaction-2

Figure 9.

Full scan ESI-MS analysis of Reaction 2 of NBQ-95 with DNA (A). A molecular ion at 329.0 is indicative of un-reacted NBQ-95. The molecular ion at m/z 299.1 represents a bio-reduced NBQ-95. Spectrum (B) represents authentic NBQ-95 and (C) represents authentic ABQ-95, corresponding to a reduced NBQ-95.

a) HX/XO in an argon saturated environment

Scheme 1.

Proposed bioreduction of NBQs under hypoxic conditions environment and adduct formation.

Table 1

Exact mass of NBQs (NO₂-BQ), their reduced species ABQ (NH₂-BQ), and the expected adduct with 2'-dG during the bioreduction of the NBQs.

Compound	Exact Mass ^a	Exact Mass ^{a,b} of the C8-2'-dG-NHBQ Adduct
NBQ-38	292.1	527.2
NBQ-95	329.0	564.1
ABQ-38 ^c	262.1	--
ABQ-95 ^c	299.0	--

^a After the loss of the chloride ion.

^b Proposed adduct formed from the reaction of 2'-dG with the reactive species resulting from the bio-reduction of NBQ-38 and NBQ-95 (see Fig. 2)

^c NH₂-BQ, synthesized in an unequivocal fashion for assessment of a bioreduction process and comparison with the expected bio-reduction product.