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Role of the Nitro Functionality in the DNA Binding of 3-Nitro-10-methylbenzothiazolo[3,2-*a*]quinolinium Chloride

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Abstract

Interest in DNA binding drugs has increased in recent years due to their importance in the treatment of genome-related diseases, like cancer. A new family of water-soluble DNA binding compounds, the benzothiazolo[3,2-*a*]quinolinium chlorides (BQCl)s, is studied here as potential candidates for chemical treatment of solid tumor cells that may encounter low-oxygen environments, a condition known as hypoxia. These compounds are good DNA intercalators; however, no studies have been made of these compounds under hypoxic conditions. This work demonstrates the importance of the nitro-functionality in the DNA binding of 3-nitro-10-methylbenzothiazolo[3,2-*a*]quinolinium chloride (NBQ-91), which possesses nitro-functionality, and 10-methylbenzothiazolo[3,2-*a*]quinolinium chloride (BQ-106), which does not. Both NBQ-91 and BQ-106 have similar noncovalent binding affinity toward DNA. Dialysis experiments show that NBQ-91 binds DNA under N₂-saturated conditions with increasing concentrations of reducing agent, presumably due to reduction of the nitro-functionality. Conversely, because of the lack of nitro-functionality, the presence of a reducing agent had no effect on BQ-106 binding to DNA under both aerobic and N₂-saturated conditions. Clonogenic assays were performed to determine the quinolinium chloride cytotoxicities under both aerobic (95% air and 5% CO₂) and hypoxic (80% N₂ and 20% CO₂) conditions. The calculated ratios of cellular toxicity under aerobic to hypoxic conditions caused by the same concentration of test agent (CTR values) show greater levels of cell death under hypoxia than under aerobic conditions for mitomycin C (MC) (CTR = 0.7 at 1 μM) and NBQ-91 (CTR = 0.4 at 200 μM) than for BQ-106 (CTR = 1.0 at 200 μM), which agreed with the previously reported data for MC and confirmed the importance of nitro-functionality for reactivity under hypoxic conditions. There was no correlation between noncovalent binding affinity constants and their cytotoxicity under hypoxic conditions. Adduct formation between the NBQ-91 and 2'-dG was also assessed by reacting 2'-dG or DNA with NBQ-91 and BQ-106 under N₂-saturated conditions in the presence of hypoxanthine and xanthine oxidase (HX/XO). DNA covalent adduct formation was analyzed by two techniques: LC-ESI-MS and Sephadex size exclusion column. LC-ESI-MS results clearly indicate the formation of a prominent molecular ion at masses of 266.0 and 530.58 Da, corresponding to the [M + H]²⁺ and [M]⁺ molecular ions of the monitored 2'-dG-NBQ-91 adduct. Results from the Sephadex size exclusion chromatography support these findings because the NBQ-91 elution percentage increases in the presence of HX/XO due to the reduction of the nitro-functionality, which results in covalent binding to DNA. This study reports evidence of the DNA binding capacity of this bio-reductive drug. The preferential N₂-saturated over aerobic conditions for DNA binding makes NBQ-91 a potential bio-reductive compound for hypoxic cell killing.

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Introduction

Biological reductive activation of drugs is a pharmacotherapeutic modality aimed at killing cells with limited oxygen content. This includes anaerobic bacteria occurring in infections (1,2) and hypoxic tumor cells (3). Nitroarene compounds are bioreductive drugs with extensive clinical applications. Examples of these are heterocyclic imidazoles, furans, and mono- and polycyclic aromatics (4). The basis for the selective toxicity of these drugs depends on the reduction of the nitro-group, which can accept up to six electrons to produce the corresponding amine (5) via the nitroso (two electrons) and hydroxylamine (four 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 (6,7). Although the possibility exists that redox cycling by nitroarenes generates cytotoxic effects, the greatest cytotoxicity is usually observed under hypoxic or anaerobic conditions (8). 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. Therefore, searching for compounds with high hypoxic cytotoxicity ratio, that is, agents with higher toxicity under hypoxic than aerobic conditions, is of great clinical importance (8).

In general, DNA is proposed as an important biological target (4). Reduced nitroarenes cause DNA strand breakage, cross-linking, and helix destabilization under hypoxic conditions (9, 10). Reduced nitroarenes also bind covalently to DNA (11,12). In this work, we compare the ability of the novel heterocyclic compounds, 3-nitro-10-methylbenzothiazolo[3,2-*a*]quinolinium chloride and 10-methylbenzothiazolo[3,2-*a*]quinolinium chloride (NBQ-91¹ and BQ-106, respectively, Figure 1), to interact covalently with calf-thymus DNA (CT-DNA) in the presence of hypoxanthine/xanthine oxidase (HX/XO).

NBQ-91 belongs to a new family of unnatural alkaloids known as benzazolo[3,2-*a*]quinolinium salts. These compounds are potentially useful anticancer agents that were synthesized to establish a structure-activity relationship (13). Of these, 3-nitrobenzothiazolo[3,2-*a*]quinolinium chloride (NBQ-2) showed a strong potential as an anticancer agent (13). The structural features present in this series of compounds include a planar heteroaromatic system incorporating quaternized nitrogen and a fused benzothiazole nucleus. NBQ-2 displays several interesting biological properties. It shows good antitumor activity against P-388 leukemia and Ehrlich ascites tumors in mice. Moreover, it forms complexes of the intercalative type with DNA (14–16). Also, NBQ-2 effectively blocks DNA, RNA, and protein synthesis in both KB and Ehrlich ascites cells (17). However, there are no data on the possibility of DNA alkylation, mediated by nitro-reduction, by this family of compounds. It is the purpose of this work to determine the importance of the nitro-functionality in the reductive activation of these compounds and consequence of DNA alkylation.

The use of XO in this work is relevant to the bioreductive activation of these quinolinium salts. XO is capable of reducing a wide range of substrates (18). It is found in endothelial cells (19), likely to be active in tumor cells (20), and is also found in nuclei (21). In addition, covalent binding of nitroarenes to DNA via HX/XO reduction is also well-documented (22,23).

¹Abbreviations: NBQ-91, 3-nitro-10-methylbenzothiazolo[3,2-*a*]quinolinium chloride; BQ-106, 10-methylbenzothiazolo[3,2-*a*]quinolinium chloride; NBQ-2, 3-nitrobenzothiazolo[3,2-*a*]quinolinium chloride; CTR, ratios of cellular toxicity under aerobic to hypoxic conditions caused by the same concentration of test agent; HX, hypoxanthine; XO, xanthine oxidase; MC, mitomycin C; *m*-AMSA, 4'-(9-acridinyamino)-methanesulfon-*m*-aniside; DTT, dithiotreitol; DPV, differential pulse voltammetry; PEI-cellulose, polyethyleneimine cellulose; HPLC, high-performance liquid chromatography; ESI, electron spray ionization; MS, mass spectrometry.

Materials and Methods

Chemicals

HX, XO (EC 1.1.3.22), CT-DNA, allopurinol, mitomycin C (MC), 2'-deoxyguanosine (2'-dG), and 4'-(9-acridinyamino)-methanesulfon-*m*-aniside (*m*-AMSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Nanopure water was used for the preparation of all stock solutions, which were stored at 4 °C, with the exception of *m*-AMSA, which was stored at -20 °C until used. Samples were prepared in a buffer solution containing 30 mM phosphate, 140 mM NaCl, and 5 mM KCL (pH 7.4). CT-DNA stock solutions were dialyzed against 30 mM phosphate buffer (pH 7.4) overnight at 4 °C and stored at this temperature. The DNA concentration was determined at 260 nm using $6600 \text{ M}^{-1} \text{ cm}^{-1}$ as the extinction coefficient. Care was always taken to minimize the exposure of quinolinium-containing solutions to light. Mass spectrometry (MS) grade water and methanol were purchased from Sigma.

The compounds BQ-106 and NBQ-91 were synthesized by a general procedure consisting of a modification of that previously described for the synthesis of NBQ-2 (13). Characterization of these compounds is described as follows.

BQ-106

Following the general procedure, the photocyclization of (*E*)-5-methyl-2-(2-chlorostyryl) benzothiazole (0.65 g, 2.27 mmol) gave 0.35 g (54%) of BQ-106 as an off-white solid; mp >200 °C (dec). ^1H NMR (300.15 MHz, DMSO- d_6): δ ppm 9.43 (d, 1H, $J = 8.7$ Hz), 9.09 (s, 1H), 8.94 and 8.81 (AB, 2H, $J = 9.0$ Hz), 8.53 (d, 1H, $J = 8.1$ Hz), 8.51 (dd, 1H, $J = 8.6, 1.5$ Hz), 8.24 (td, 1H, $J = 8.1, 1.7$ Hz), 8.06 (t, 1H, $J = 7.7$ Hz), 7.81 (d, 1H, $J = 8.7$ Hz), 2.72 (s, 3H, -CH₃). ^{13}C NMR (75 MHz, DMSO- d_6): δ ppm 159.7, 140.1, 139.8, 139.2, 136.7, 133.7, 130.9, 130.4, 129.0, 127.1, 126.6, 124.8, 119.9, 119.4, 119.3, 21.5. UV-vis (H₂O) λ_{max} /nm (ϵ): 379 (11622), 365 (11842), 348 sh (8755), 253 (18598), 231 sh (17118), and 223 (17816). Anal. calcd for C₁₆H₁₂ClNS: C, 67.36; H, 4.24; N, 4.91%. Found: C, 67.20; H, 4.58; N, 5.09%.

NBQ-91

Following the general procedure, the photocyclization of (*E*)-5-methyl-2-(2-chloro-5-nitrostyryl)benzothiazole (0.20 g, 0.61 mmol) gave 0.146 g (73%) of NBQ-91 as a yellow solid; mp 210–228 °C (dec.). ^1H NMR (500.13 MHz, DMSO- d_6): δ ppm 9.62 (d, 1H, $J = 9.6$ Hz), 9.48 (d, 1H, $J = 2.7$ Hz), 9.12 and 8.99 (AB, 2H, $J = 9.1$ Hz), 9.07 (s, 1H), 8.80 (dd, 1H, $J = 9.6, 2.7$ Hz), 8.59 and 7.86 (d, 1H, $J = 8.3$ Hz), 2.73 (s, 3H). ^1H NMR (300.15 MHz, DMSO- d_6): δ ppm 9.60 (d, 1H, $J = 9.6$ Hz), 9.47 (d, 1H, $J = 2.3$ Hz), 9.10 and 8.96 (AB, 2H, $J = 9.0$ Hz), 9.06 (s, 1H), 8.80 (dd, 1H, $J = 9.6, 2.6$ Hz), 8.56 and 7.86 (AB, 2H, $J = 8.4$ Hz), 2.73 (s, 3H). ^{13}C NMR (125.77 MHz, DMSO- d_6): δ ppm 162.1, 145.8, 140.6, 139.7, 139.2, 139.1, 130.8, 127.3, 126.8, 126.5, 126.3, 125.1, 121.5 (2 C), 119.8, 21.5. UV-vis (95% EtOH) λ_{max} /nm (ϵ) 385 (11465), 376 (11719), 276 (19491) and 201 (24672). Anal. calcd for C₁₆H₁₁ClN₂SO₂·1.5H₂O: C, 53.82; H, 3.95; N, 7.84%. Found: C, 53.79; H, 3.91; N, 7.58%.

NMR Characterization of NBQ-91 and BQ-106

The ^1H and ^{13}C NMR spectra were recorded on a General Electric QE-300 (using a 5 mm C/H dual probe) operating at an observation frequency of 300.15 and 75.48 MHz for ^1H and ^{13}C , respectively, and equipped with Nicolet 1280 data system and a 293-C pulse programmer or on a Bruker DRX500 (using a 5 mm broadband probe) operating at an observable frequency of 500.13 and 125.77 MHz for ^1H and ^{13}C , respectively. Proton data were referenced to either tetramethylsilane (TMS) at δ 0.0 ppm, chloroform (CDCl₃) at δ 7.26 ppm, or methyl sulfoxide (DMSO- d_6) at δ 2.49 ppm. The ^{13}C nuclei were referenced to the

center peak of the 1:1:1 multiplet of deuteriochloroform at δ 77.0 ppm or the multiplet of hexadeuteriomethyl sulfoxide at δ 39.5 ppm.

Cell Culture

The cell line used in this study was the human epidermoid carcinoma cells A-431 (ATCC CRL-1555). In a previous work, it was found that a family of benzazoloquinolinium salts was cytotoxic against a number of cancer cell models including A431, HL-60, U937, K562, HeLa, and HT-29 cells (15). Among the cell lines tested, the human epidermoid A431 cells were among the most sensitive to this family of drugs and, therefore, selected for the present work. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal clone serum (Invitrogen, Inc.) supplemented with 1% antimycotic antibiotic solution and 1% kanamycin (Sigma). Cultures were maintained at 37 °C in a humidified atmosphere of 95% air/ 5% CO₂.

Determination of Cell Survival under Aerobic and Hypoxic Conditions

A-431 cells were seeded in 60 mm $\times$ 15 mm cell plates at a concentration of 8×10^4 cells/mL, in a humidified atmosphere of 95% air and 5% CO₂, the day prior to the assay. On the day of the experiment, the media of each plate were refreshed with new media. To achieve the hypoxic conditions, a Molecular Incubator Chamber (Billups-Rothenberg Inc., Del Mar, CA) was used. This chamber has a reliable airtight seal and fast gas exchange properties, which allowed the rapid creation of a nonfluctuating hypoxic environment. We flushed the chamber with 80% N₂ and 20% CO₂ for 10 min. The chamber was placed inside the incubator at 37 °C for 2 hours. Following the induction of hypoxia, cells were exposed to different concentrations of the test agents, which were injected through the septum in the top lid to conserve the desired hypoxic environment for 1 h at 37 °C. For the aerobic conditions, cells plates were left inside the incubator at 37 °C for the duration of the assay. Treated cells were washed, harvested by trypsinization, and assayed for survival using a clonogenic assay (24). After 7 days in culture, with a change of medium at the third day, the medium was removed, and adherent colonies were fixed and stained with ethanol containing 0.1% crystal violet. Macroscopic colonies consisting of more than 40 cells were counted, and plating efficiencies, defined as the number of microscopic colonies counted divided by the number of cells plated, was determined.

Determination of Half-Wave Reduction Potentials ($E_{1/2}$)

The half-wave reduction potentials at physiological pH were determined for 0.1 mM, nitrogen-purged, quinolinium solutions containing a 1:1 DMSO:aqueous buffer (50 mM phosphate buffer, pH 7.4) mixture, using differential pulse voltammetry (DPV). The buffer salt also served as the supporting electrolyte. A Princeton Applied Research (PAR) Polarographic Analyzer model 264A and a glassy carbon electrode were used in these determinations. An Ag/AgCl electrode was used as the reference electrode ($E' = +0.22$ V vs NHE), and a platinum wire was used as the counter electrode. Differential pulse voltammograms were obtained in the potential range of -2.00 to 0.00 V, using 25 mV of pulse amplitude and a 200 mV/s scan rate. The reduction potential values were obtained from the DPV potential maxima. These are almost similar to the half-wave redox potentials, $E_{1/2}$, in normal polarographic measurements (25).

Determination of the Presence of Reduced Nitroarene Compound Bound to CT-DNA

Samples of 5 mL of N₂-saturated solutions containing 2000 μ M CT-DNA, 100 μ M nitroarene, and different concentrations of HX (ranging from 0 to 200 μ M) and XO (ranging from 0 to 20 mU/mL), where the ratio of HX concentration to XO activity was maintained constant at 10 μ M/mU mL⁻¹, were incubated at 25 °C for 2 h under a N₂-saturated atmosphere. As noted, the activity of XO was increased in proportion to the HX concentration to avoid HX inhibition of XO (26). A glovebag under a N₂-saturated atmosphere was used for the preparation of the

corresponding stock solutions, and these were mixed only after being saturated with nitrogen. After the incubation period, 100 μM allopurinol was added to the sample solution to inhibit XO activity (27). The nitroarene absorbance was then measured, under N_2 -saturated atmosphere, at a maximum absorption wavelength corresponding to a region above the DNA absorption, that is, in the 340–390 nm region. The sample was then submitted to dialysis, using a molecular porous membrane that permits the diffusion of any molecule smaller than 14 kDa, against 1 L of N_2 -saturated 30 mM phosphate buffer under N_2 -saturated conditions for 24 h, with a change of buffer every 8 h. After this period, the absorbance of the dialyzed sample was measured again at the same wavelength region indicated above to detect bound quinolinium species.

Analysis of DNA Covalent Adducts. Sample Preparation

For this purpose, a 200 μM concentration of either NBQ-91 or BQ-106 was incubated under N_2 -saturated conditions in 20 mM ammonium acetate (pH 7.4) with 600 μM 2'-dG or 1 mg CT-DNA/mL in the presence of 0, 300, 600, or 1.2 mM HX in combination with 50, 100, and 200 units XO/mL, respectively. XO in samples was inhibited by the addition of 100 μM allopurinol after 3 h of incubation. DNA-BQCl reactions were then placed in 1.5 mL Eppendorf microtubes for DNA enzymatic digestion. A control (untreated) CT-DNA was included in the digestion process for assessment of the digestion efficiency. Enzymatic digestions were performed according to methods described by Zayas et al. (28). Samples were placed on ice and stored at -20°C . DNA and 2'-dG reaction samples were then analyzed by LC-electron spray ionization (ESI)-MS analysis.

LC-ESI-MS Analysis

Samples were analyzed by high-performance liquid chromatography (HPLC)-ESI-MS to determine the presence of adducts formed between BQCl and 2'-dG. HPLC separation analysis was performed in a Waters Alliance 2695 HPLC dual Pump equipped with an autosampler coupled to a Waters 996 PDA. HPLC analysis was monitored at a wavelength range of 254–370 nm. The mobile phase consisted of MS grade H_2O (A) and MeOH (B), and the stationary phase consisted of a Supelcosil LC-18-S 15 cm $\times$ 3 mm, 5 μM (Supelco). From each reaction, 20 μL injections were eluted using a 30 min nonlinear gradient from 98% H_2O /2% MeOH to 100% B in 25 min at a flow rate of 350 $\mu\text{L}/\text{min}$. Samples were then analyzed by ESI-MS through the LC interphase into a Micromass Quattro Micro ESI mass spectrometer using full scan positive ionization analysis and characterization of the monitored adducts.

Dilutions of the BQCl and 2'-dG were used for optimization of the tuning conditions. Optimized full scan ESI-MS conditions used were as follows: positive ionization mode and mass range set from 100 to 600 Da; drying gas, N_2 ; flow rate, 200 L/h; capillary voltage, 3.08 kV; cone voltage, 70 V; extractor, 5 V; source temperature, 100°C ; and desolvation temperature, 200°C .

Sephadex Size Exclusion Chromatography

Different solutions (2 mL) were prepared as follows: (i) a 100 μM concentration of the respective benzothiazolo[3,2-*a*]quinolinium chloride; (ii) a 100 μM concentration of the respective benzothiazolo[3,2-*a*]quinolinium chloride and 2000 μM CT-DNA; (iii) a 100 μM concentration of the respective benzothiazolo[3,2-*a*]quinolinium chloride, 300 μM HX, and 300 mU/mL XO; and (iv) a 100 μM concentration of the respective benzothiazolo[3,2-*a*]quinolinium chloride, 300 μM HX, 300 mU/mL XO, and 2000 μM DNA. These were prepared in septum-sealed reaction bottles containing two syringes: one used to connect the bottle to the nitrogen tank and the second to release the positive nitrogen pressure and keep a continuous hypoxic environment. These solutions were then purged with nitrogen (N_2) for 7 min to achieve a N_2 -saturated environment. A reducing agent was added at a concentration of 300 μM HX

and 30 mU/mL XO. The solutions were purged with N₂ for 5 additional min, covered with aluminum paper, and then incubated at 25 °C for 12 h. After the reaction period, 100 μM allopurinol was added to each sample solution to inhibit XO activity, before taking the absorption spectra for each solution. Samples were then submitted to Sephadex size exclusion chromatography to detect BQCl covalent binding to DNA.

The Sephadex resin was prepared by dissolving 10 g of the resin and 1 g of NaCl in 120 mL of water, stirring for 3 h, and storing at 4 °C for 24 h. The columns were prepared the next day, saturated in the same solution, and stored at 4 °C until used.

Sample solutions were applied to the Sephadex column, and eluents were collected before extraction with chloroform followed by ethyl ether and overnight ethanol precipitation. The following day, each solution was centrifuged, and the precipitated DNA was resuspended in PBS followed by absorption spectra recording to detect the covalently bound BQCl to the eluted CT-DNA.

Determination of BQCl-CT-DNA Binding Constants

DNA binding constants were determined by a competition method with the well-known DNA intercalator ethidium bromide (EB), as described by Armitage et al. (29), which is based on the original work by Sari et al. (30). Samples consisted of 2 μM DNA in 2.5 mL and were titrated with different EB concentration solutions ranging from 2.5×10^{-7} to 3.73×10^{-6} M. Samples were allowed to equilibrate by stirring for 5 min. Fluorescence spectra were obtained using a Photon Technology International Spectrofluorimeter (Princeton, NJ). An excitation wavelength of 510 nm was used, and emission was detected at 600 nm. The concentration of the free EB, C_F , was obtained from eq 1:

$$C_F = C_T \left[\frac{(I/I_0) - P}{1 - P} \right] \quad (1)$$

where I and I_0 are the fluorescence intensities in the presence and absence of DNA, respectively (30–32), and P is the ratio of the observed fluorescence quantum yield of the probe in bound form to that in the free form. The value of P was obtained from a plot of I/I_0 vs $C_T/[DNA]$ such that the limiting fluorescence yield is given by the y-intercept and C_T is the total concentration of agent used. The amount of bound agent C_B is equal to $C_T - C_F$.

Scatchard analysis was done according to the McGhee and von Hippel formalism (33), eq 2,

$$r/C_f = K(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1} \quad (2)$$

where r is the ratio $C_B/[DNA]$, K is the intrinsic association constant, and n is the binding size in base pairs. A plot of r/C_f vs r was best fitted using nonlinear regression, and the best values of K and n were obtained. In the absence of quinolinium, the binding constant is the actual binding constant of EB, K_{EB} . The apparent binding constant is then used to calculate the equilibrium binding constant of the quinolinium, K_Q , using eq 3,

$$K = \frac{K_{EB}}{1 + K_Q C_{f,Q}} \quad (3)$$

where $C_{f,Q}$ is approximately equal to the total concentration of quinolinium (29,30).

Determination of Uric Acid Formation

Uric acid production was quantified by C-18 reverse phase HPLC using two Alltech analytical C-18 μ Bondapak C₁₈ (3.9 mm $\times$ 300 mm) columns connected in series and eluting with 50 mM phosphate buffer:methanol (99:1 v/v) at a flow rate of 1.0 mL/min. A Waters analytical HPLC system equipped with a 600 solvent delivery pump, UV-vis tunable detector was used. The absorbance of the eluent mixture was monitored at 300 nm.

Absorption Spectra

These were measured using a Beckman DU 650, diode array UV-vis spectrophotometer (Fullerton, CA).

Results and Discussion

Absorption Spectra Changes and Uric Acid Formation upon Reduction

In the absence of reducing agent, a broad absorption peak with a maximum at 375 nm was observed for NBQ-91 (only the wavelength region above 300 nm was of interest since below this region the species HX, X, and uric acid will also have a contribution) (Figure 2). Upon addition of HX/XO to NBQ-91, a broad shoulder builds up with a maximum at 361 nm and another at 400 nm (Figure 2). This observation, together with the evidence for uric acid formation (Figure 3), indicates that NBQ-91 was reduced by HX/XO as previously reported for other nitroquinolinium salts (28,34). A broad absorption spectrum with two maxima at 365 and 379 nm and a shoulder at 350 nm was observed for BQ-106 in the absence of reducing agent. The addition of HX/XO to BQ-106 produced essentially no changes in the absorption spectrum of this quinolinium, implying that no detectable reaction was occurring (Figure 2). In addition, only a small amount of uric acid formation was also detected in the presence of BQ-106, indicating that a very small fraction of this compound was being reduced apparently due to the lack of nitro-substitution (Figure 3). These observations are consistent with the smaller redox potential of BQ-106 as compared to that of NBQ-91 (Table 1). The fact that uric acid formation occurs at a smaller rate in the presence of DNA reveals that after binding to DNA there is an insufficient amount of compound available for reduction (see below).

Binding to DNA in the Presence of HX/XO. Absorption Spectra Analysis of Dialyzed Samples after HX/XO Reduction

After HX/XO reduction of NBQ-91 and BQ-106 in N₂-saturated solutions containing CT-DNA, followed by inhibition and dialysis, absorption peaks were observed above 375 nm for both BQCl samples, indicating the presence of DNA-bound species (Figure 4). No absorbance of HX, X, or uric acid occurs at wavelengths above 350 nm. The observed peaks were much more intensive for NBQ-91 than for BQ-106 after dialysis, indicating that more NBQ-91 is bound to DNA than BQ-106 after reduction. In fact, the absorption spectrum of the bound NBQ-91 resembles more that of the reduced derivative, that is, a broad maximum followed by a broad shoulder at a longer wavelength (Figure 2), although shifted to longer wavelengths. Also, an increase in the measured absorbance of the bound NBQ-91, but not for BQ-106, was observed with increasing HX concentrations (Figure 5). The wavelengths used for experiments corresponding to Figure 5 (400 nm for NBQ-91 and 380 nm for BQ-106) were selected so that there were no changes in extinction coefficients of the quinolinium salt with increasingly higher concentrations of HX/XO, in the presence of DNA, before dialysis. The fact that the bound NBQ-91 spectrum resembles that of the reduced NBQ-91 indicates that the observed increase in absorbance with an increase in HX concentration, after dialysis, is due to an increase in the concentration of the reduced DNA-bound species. The observed increase in DNA-bound NBQ-91 with increase in the HX:NBQ-91 mol ratio is expected since more of the hydroxylamine derivative should be formed with an increase in that ratio; thus, more binding

of NBQ-91 is expected. Hydroxylamine derivatives are known to alkylate DNA bases under hypoxia or anoxia (4,8).

The smaller extent of BQ-106 binding to DNA, after HX/XO reduction and extensive dialysis, as compared to that of NBQ-91, under N₂-saturated conditions, is in agreement with the low amount of uric acid formed in the presence of BQ-106, as compared to that for NBQ-91. A small anaerobic ferricytochrome *c* reduction rate increase by a quinolinium derivative without nitro-substitution, in the presence of xanthine/XO, has been previously reported, which suggests the possibility of BQ-106 reduction by HX/XO (35). Because of the lack of a nitro-group, BQ-106⁺ (represented here as the cation of BQ-106, that is, without the accompanying chloride anion), remains essentially unreduced until a large amount of HX/XO has been added. This shifts equilibrium 6 to the reduced quinolinium species, BQ-106 (reactions 4–6).

The decrease in absorbance of the dialyzed sample should be caused by a weaker adherence of BQ-106 to DNA after BQ-106⁺ losing its positive iminium charge upon reduction, which would be consistent with noncovalent binding of BQ-106 to DNA. The importance in the noncovalent DNA binding process of the positive charge at the iminium nitrogen has been previously demonstrated for EB (36) and NBQ-2 (14).

Control experiments in which NBQ-91 was reduced by HX/XO under aerobic conditions showed that increasing the HX concentration had no significant effect on binding to DNA (Figure 5). This may be explained by the “futile” reduction of NBQ-91 by HX/XO, whereby the one-electron-reduced NBQ-91 species transfers an electron to oxygen; thus, further reduction to the nitroso or hydroxylamine derivatives is inhibited or intercepted by oxygen (37). Redox cycling by nitrobenzazolo[3,2-*a*]quinolinium derivatives, with the consequent increase in the rate of superoxide production in the presence of xanthine/XO, has been previously reported (35). A decrease in absorbance after high HX concentrations (data not shown) suggests NBQ-91 degradation due to the production of reactive oxygen species (Scheme 1, represented here as the cation of NBQ-91, that is, without the accompanying chloride anion). Similar results were obtained upon aerobic HX/XO reduction of BQ-106.

LC-ESI-MS Detection of the 2'-dG Adduct of NBQ-91

The formation of a 2'-dG-NBQ-91 covalent adduct was monitored by LC-ESI-MS analysis. Results of the chromatographic analysis for the reactions of NBQ-91 with 2'-dG as presented in Figure 6 show the presence of XO (2.10 min), HX (2.48 min), NBQ-91 (7.47 min), and 2'-dG (12.20 min). Small unknown peaks were also observed. The formation of the 2'-dG-NBQ-91 was then monitored by ESI-MS analysis. The parent NBQ-91 was first analyzed by direct injection in the positive ionization mode to confirm its ionization pattern and potential fragmentation. As indicated in Scheme 1, NBQ-91⁺ is a cation with a molecular ion of 295.33 Da (Figure 7). The ESI analysis of the reaction of NBQ-91⁺ with 2'-dG was performed to monitor the formation of a 2'-dG-NBQ-91 adduct with molecular mass of 530.58 Da. The results as shown in Figure 8, however, indicated a strong molecular ion at *m/z* 266.0 corresponding to the [M + H]²⁺ molecular ion of a 2'-dG-NBQ-91⁺. The reported reaction in

Figure 6 and Figure 8 was the reaction where the HX:NBQ-91 molar ratio was the highest (6:1). Similar chromatograms were observed for reaction with lower HX:NBQ-91 molar ratios; however, the intensity of the monitored ion was less prominent or not detected at lower levels of HX:NBQ-91 ratio. The adduct-detected region in Figure 6 refers to the area where the ion at m/z 266.0, corresponding to the $[M + H]^{2+}$ molecular ion reported in Figure 7, was detected upon LC-ESI-MS analysis. A region rather than a specific RT is mentioned, given the slight variations observed within reactions with different HX:NBQ-91 molar ratios for the detection of the monitored molecular ion. For the monitoring of a DNA-NBQ-91 adduct formation, enzymatic digestions of DNA were performed prior to the LC analysis. As shown in Figure 9, the chromatographic analysis presents the separation of the digestion enzymes, NBQ-91 and nucleosides. As with the 2'-dG reactions, LC-ESI-MS analysis was applied to detect the formation of the potential 2'-dG-NBQ-91 adduct at similar mass. As observed in Figure 10, a prominent ion at m/z 266.0 was observed (as with the 2'-dG reaction). However, an additional significant molecular ion was observed at m/z 530.7, corresponding to the $[M]^+$ molecular ion of 2'-dG-NBQ-91 adduct (expected MW of 530.58). In this reported reaction of NBQ-91 with CT-DNA, the HX:NBQ-91 molar ratio was also 6:1 as in the 2'-dG-NBQ-91 reaction presented above. Similar reactions and analysis were performed with BQ-106 (analogue without the nitro-group) to confirm the effect of the nitro-group on the adduct formation. Results from the LC-ESI-MS analysis confirmed that the absence of the nitro-group in BQ-106 inhibits the formation of the 2'-dG adduct, at least under the studied conditions, since no significant molecular ion (or potential fragments) corresponding to the adduct of interest was detected regardless of the HX:BQ-106 ratio.

In relation to the possible structure of the 2'-dG-NBQ-91 adduct, several possibilities exist that agree with a molecular mass of 530.58 Da. Some of those are shown in Figure 11. These will be produced by the reaction of the hydroxylamine derivative of NBQ-91 with 2'-dG. However, the actual structure of this adduct is, at present, unknown.

Sephadex Size Exclusion Chromatography

To support the LC-ESI-MS findings of the DNA covalent adducts formed, Sephadex size exclusion chromatography was performed. Solutions were prepared containing DNA, NBQ-91 or BQ-106, and HX/OX and the respective controls. After the appropriate reaction period, absorption spectra were recorded before and after column elution. The results indicated that only NBQ-91 was not retained in the size exclusion column after reaction with DNA in the presence of HX/XO, due to the reduction of the nitro-functionality, which resulted in covalent binding of NBQ-91 to DNA (see the Supporting Information).

Noncovalent Binding Constants of BQCs to DNA

No significant difference, within experimental error, was detected between the CT-DNA binding constant of NBQ-91 and that of BQ-106, in the absence of reducing agent (Table 1). A previous report observed that noncovalent DNA binding by other quinolinium compounds of this series occurs by intercalation (15). The similarity between the binding constants may be due to the structural similarities on both compounds. Thus, the difference in covalent DNA binding ability between BQ-106 and NBQ-91 is not related to the noncovalent DNA binding properties of these compounds.

Cell Survival under Aerobic and Hypoxic Conditions

The cell line A-431, a human epidermoid carcinoma, was used to determine the cytotoxicities of NBQ-91 and BQ-106 using MC and *m*-AMSA as model agents. A salient feature of the molecular mechanism of action of MC is that it exists as a pro-drug, and both its DNA cross-linking and monoalkylating activities require the reduction of the quinone ring to hydroquinone, which transforms MC into a highly reactive alkylating species (38). Under

hypoxic conditions, MC shows greater cytotoxicity than under aerobic normoxic conditions. *m*-AMSA is a well-known DNA intercalator. It causes double-strand breaks in DNA and inhibits topoisomerase II activity (39). Its activity toward DNA does not depend on the presence of reducing agents; therefore, it shows no preference under aerobic or hypoxic conditions. The cytotoxicity of each compound under aerobic and hypoxic conditions was determined by measuring the surviving fraction of the cells using clonogenic assays. Results show greater levels of cell death under hypoxia than under aerobic conditions for MC and NBQ-91 (Table 2). This observation agrees with the previously reported data for MC and demonstrates the importance of the nitro-functionality in the toxicity under hypoxic conditions of this type of compounds.

In summary, we have established that a novel nitro-substituted heterocyclic compound, NBQ-91, binds covalently to DNA in the presence of HX/XO under N₂-saturated conditions and that the nitro-functionality is essential for this activity. Cell survival assays further confirm the importance of this functionality under hypoxic conditions. Future studies will focus on testing other compounds in this series for DNA covalent binding, upon reduction, under both hypoxic and normoxic conditions, as well as performing aerobic vs hypoxic cytotoxicity tests on tumor cell lines in a search for new water-soluble nitroarenes with low CTR values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NBQ-91 **BQ-106**

Figure 1.
Quinolinium salts used in this study.

Figure 2. Absorption spectra of solutions containing 30 mM phosphate buffer (pH 7.4) and (A) NBQ-91 without HX/XO (continuous line), NBQ-91 and 2 mol of HX per mol of NBQ-91, and 20 mU XO/mL (broken line) and (B) BQ-106 without HX/XO (continuous line), BQ-106 and 2 mol of HX per mol of BQ-106, and 20 mU XO/mL (broken line).

Figure 3.

Uric acid formation after quenching with 100 μM allopurinol a N_2 -saturated reaction containing 500 μM HX, 50 mU XO/mL, and 100 μM NBQ-91 (squares) or BQ-106 (circles) in the presence (black symbols) or absence (open symbols) of 2.0 mM DNA in 30 mM phosphate buffer, pH 7.4.

Figure 4.

Absorption spectra before (continuous line) and after (broken line) dialysis of N_2 -saturated solutions containing 30 mM phosphate buffer (pH 7.4) and (A) 100 μ M NBQ-91, 200 μ M HX, 20 mU XO/mL, and 2.0 mM DNA and (B) 100 μ M BQ-106, 200 μ M HX, 20 mU XO/mL, and 2.0 mM DNA. DNA was added first in both samples A and B.

Figure 5.

Change in absorbance ($\Delta A = A_{\text{with HX}} - A_{\text{wo HX}}$) of N₂-saturated (black symbols) and air-saturated (open symbols) solutions containing 30 mM phosphate buffer (pH 7.4), 0–200 μM HX, 2.0 mM DNA, 0–20 mU XO/mL, and 100 μM NBQ-91 (□) or 100 μM BQ-106 (Δ).

Figure 6.

Chromatographic analysis for the reaction of NBQ-91 with 2'-dG with the highest HX:NBQ-91 molar ratio (6:1). The presences of XO (2.10 min), HX (2.48 min), NBQ-91 (7.47 min), and 2'-dG (12.20 min) are clearly observed. Small unknown peaks were also present. HPLC analysis was monitored at a wavelength range of 254–370 nm. The stationary phase used was a Supelcosil LC-18-S 15 cm × 3 mm, 5 μ M (Supelco). From each reaction, 20 μ L injections were eluted using a 30 min nonlinear gradient from 98:2 ($\text{H}_2\text{O}/\text{MeOH}$) to 100% B in 25 min at a flow rate of 350 $\mu\text{L}/\text{min}$. The adduct detection region refers to the area where the $[\text{M} + \text{H}]^{2+}$ molecular ion reported in Figure 8 was observed upon LC-ESI-MS analysis. A region rather than a specific RT is mentioned given the slight variations in the exact retention time observed within reactions with different HX:NBQ-91 molar ratios for the detection of the monitored molecular ion.

Figure 7.

Full scan positive ESI-MS analysis of the parent compound NBQ-91 through direct infusion through capillary tubing at a flow rate of 5 μ L/min. The MW of the parent NBQ-91 containing the Cl⁻ anion (330.7 Da) was not observed. Instead, a molecular ion at m/z 295.4 consistent with the [M]⁺ ion of the NBQ-91 (exact mass, 295.3 Da) after the loss on the chloride anion was observed. Fragments of the parent were also observed (not presented) as the capillary and cone voltages changed. The ESI-MS conditions used were as follows: drying gas, N₂; flow rate, 125 L/h; capillary voltage, 6.08 kV; cone voltage, 70 V; extractor, 2 V; source temperature, 100 °C; and desolvation temperature, 200 °C.

Figure 8.

Full scan positive ESI-MS analysis of the reaction of NBQ-91 with 2'-dG after HPLC separation. The molecular ion at m/z 266.0 represents the $[M + 1]^{2+}$ ion of the proposed 2'-dG adduct MW 530.6 obtained upon chromatographic analysis as in Figure 6. The monitored adduct was detected in the reaction where the HX:NBQ-91 molar ratio was the highest (6:1). ESI-MS conditions used were as follows: drying gas, N_2 ; flow rate, 200 L/h; capillary voltage, 3.08 kV; cone voltage, 70 V; extractor, 5 V; source temperature, 100 °C; and desolvation temperature, 200 °C.

Figure 9.

Chromatographic analysis for the reactions of NBQ-91 with DNA with the highest HX:NBQ-91 molar ratio (6:1). Samples were enzymatically digested prior to the HPLC analysis. The chromatographic analysis presents the separation of the digestion enzymes, NBQ-91 and nucleosides. Small unknown peaks (potential adducts) were also observed. HPLC analysis was monitored at a wavelength range of 254–370 nm. The adduct-detected region refers to the area where the molecular ions reported in Figure 10 were obtained upon LC-ESI-MS analysis. A region rather than a specific RT is mentioned given the slight variations in the exact retention time observed within reactions with different HX:NBQ-91 molar ratios for the detection of the monitored molecular ion. The stationary phase used was a Supelcosil LC-18-S 15 cm × 3 mm, 5 μM (Supelco). From each reaction, 20 μL injections were eluted using a 30 min nonlinear gradient from 98:2% (H₂O/MeOH) to 100% B in 25 min at a flow rate of 350 μL/min.

Figure 10.

Full scan positive ESI-MS analysis of the reaction mixture of NBQ-91 with DNA after enzymatic digestion and HPLC separation. As with the 2'-dG reactions, ESI-MS analysis was applied to detect the formation of a 2'-dG-NBQ-91 adduct at similar mass. A prominent ion at m/z 266.0 corresponding to the $[M + 1]^{2+}$ molecular ion of 2'-dG-NBQ-91 adduct was clearly detected. The molecular ion at m/z 266.0 represents the ion of the proposed 2'-dG adduct after chromatographic analysis presented in Figure 9. The molecular ion $[M]^+$ at 530.7 m/z but could represent the unprotonated adduct carrying its natural positive charge. In this reaction, the HX:NBQ-91 molar ratio was also 6:1 as in the 2'-dG-NBQ-91 reaction presented above. ESI-MS conditions used were as follows: drying gas, N_2 ; flow rate, 200 L/h; capillary voltage, 3.08 kV; cone voltage, 70 V; extractor, 5 V; source temperature, 100 °C; and desolvation temperature, 200 °C.

Figure 11.

Some possible structures of 2'-dG-NBQ-91 adducts with molecular mass of 530.58 Da, which could be produced after reduction of NBQ-91 to the corresponding hydroxylamine followed by reaction with 2'-dG.

Scheme 1.

Redox Cycling of NBQ-91 in the Presence of HX/XO

Table 1
CT-DNA Binding Constants and Redox Potentials of the Quinolinium Derivatives under Study

quinolinium	$E_{1/2}$ (mV) ^a	CT-DNA binding constant, K_Q (M ⁻¹)
BQ-106	-616	$(2.4 \pm 2.8) \times 10^4$
NBQ-91	-324	$(1.7 \pm 2.0) \times 10^4$

^a Determined by DPV against an Ag/AgCl reference electrode at 25 °C in 1:1 DMSO:50 mM phosphate (v/v) solutions.

Table 2Ratios of Plate Efficiency under Aerobic to Hypoxic Conditions (CTR Values) Obtained after Clonogenic Assays^a

compound	air	hypoxia	CTR
MC (1 μ M)	73 $\pm$ 6	53 $\pm$ 5	0.7
<i>m</i> -AMSA (250 nM)	46 $\pm$ 6	51 $\pm$ 4	1.1
NBQ-91 (200 μ M)	65 $\pm$ 11	26 $\pm$ 7	0.4
BQ-106 (200 μ M)	60 $\pm$ 8	59 $\pm$ 4	1.0

^aValues are the average $\pm$ SEM of four independent experiments, each in duplicate.