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7-Nitro-4-(phenylthio)benzofurazan is a potent generator of superoxide and hydrogen peroxide

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Abstract

Here, we report on 7-nitro-4-(phenylthio) benzofurazan (NBF-SPh), the most potent derivative among a set of patented anticancer 7-nitrobenzofurazans (NBFs), which have been suggested to function by perturbing protein–protein interactions. We demonstrate that NBF-SPh participates in toxic redox-cycling, rapidly generating reactive oxygen species (ROS) in the presence of molecular oxygen, and this is the first report to detail ROS production for any of the anticancer NBFs. Oxygraph studies showed that NBF-SPh consumes molecular oxygen at a substantial rate, rivaling even plumbagin, menadione, and juglone. Biochemical and enzymatic assays identified superoxide and hydrogen peroxide as products of its redox-cycling activity, and the rapid rate of ROS production appears to be sufficient to account for some of the toxicity of NBF-SPh ($LC_{50} = 12.1 \mu M$), possibly explaining why tumor cells exhibit a sharp threshold for tolerating the compound. In cell cultures, lipid peroxidation was enhanced after treatment with NBF-SPh, as measured by 2-thiobarbituric acid-reactive substances, indicating a significant accumulation of ROS. Thioglycerol rescued cell death and increased survival by 15-fold to 20-fold, but pyruvate and uric acid were ineffective protectants. We also observed that the redox-cycling activity of NBF-SPh became exhausted after an average of approximately 19 cycles per NBF-SPh molecule.

Electrochemical and computational analyses suggest that partial reduction of NBF-SPh enhances electrophilicity, which appears to encourage scavenging activity and contribute to electrophilic toxicity.

Keywords

Benzofurazan; Reactive oxygen species; Oxidative stress; Electrochemistry; Electrophilic stress

Introduction

Benzofurazan (BFZ) derivatives are widely employed for their scavenging activity and their ability to target and label specific functional groups, including amines, thiols, hydroxyls, carbonyls, and carboxyls (Andrews et al. 1982; Birkett et al. 1970; Imai et al. 1993; Santa et al. 1999; Toyo'oka and Imai 1983; Toyo'oka et al. 1991; Uchiyama et al. 2001; Watanabe and Imai 1981). Researchers have also used specific BFZs to detect superoxide and peroxyl radicals, and these include 7-nitro-4-(2-diphenylphosphinoethylamino)benzofurazan and three derivatives of 7-nitro-4-[*N*-methyl-*N*-(4-hydroxyphenyl)amino]benzofurazan, which fluoresce after oxidation by reactive oxygen species (ROS) (Heyne 2007; Heyne et al. 2008; Onoda et al. 2003). In contrast to BFZs that react with specific functional groups, exposure to broadly reactive BFZs often prompts toxicity, as evidenced by the interruption of DNA, RNA, and peptide syntheses in cultured tumor cells. The toxicities of broadly reactive BFZs are thought to correlate with the characteristics of electron withdrawing groups (Ghosh et al. 1972, 1981; Ghosh and Whitehouse 1968, 1969; Whitehouse and Ghosh 1968).

In addition to targeting and labeling functional groups, we have found that select BFZs can readily participate in redox-cycling, transferring electrons from cellular reductants to electrophiles. Redox-cycling BFZs can reduce O₂ and generate ROS, which cause cellular damage and promote cell death, sometimes through apoptosis. To date, only a few BFZs have been shown to promote oxidative stress, including 4,7-dicyanobenzofurazan and 4-bromo-6-cyanobenzofurazan, and little is known about their ability to generate ROS (Takabatake et al. 1990, 1991, 1992a, b), but toxic redox-cycling has been widely demonstrated for other nitroaromatic and quinoid compounds (Castro et al. 2008; Cenas et al. 1995; Giulivi and Cadenas 1994; Inbaraj and Chignell 2004; Juchau et al. 1986; Kappus and Sies 1981; Moreno and Docampo 1985).

In this paper, we report on one 7-nitrobenzofurazan (NBF) derivative, which is among several that have garnered attention for their potential use as anticancer agents (Caccuri and Ricci 2006; Federici et al. 2009; Ricci et al. 2005; Turella et al. 2005), and for this study, we chose the most potent derivative among this set of patented anti-cancer NBFs: 7-nitro-4-(phenylthio)benzofurazan (NBF-SPh or 1). Previous studies suggest the antitumor activity of NBF-SPh is a consequence of its glutathione conjugate, which is formed in the GST active site and is thought to perturb protein–protein interactions (Caccuri and Ricci 2006). Our investigation confirms that the compound is toxic in the micromolar range, and our mechanistic approach demonstrates for the first time that NBF-SPh readily accepts electrons and participates in redox-cycling. The compound's nitro group is initially reduced in a manner similar to that of other nitroaromatic compounds (Fig. 1), but subsequent to reduction, the compound consumes molecular oxygen and rapidly generates ROS.

Materials and methods

7-Nitro-4-(phenylthio)benzofurazan and piperidinium 7-sulfo-4-(phenylthio)benzofurazan (SBF-SPh) were synthesized according to published procedures (Andrews et al. 1982;

Belton 1974; Ghosh 1968), and the 4-chloro-benzofurazans, L-adrenaline, sodium pyruvate, and 2-thio-barbituric acid were purchased from Alfa Aesar (Ward Hill, MA, USA) or TCI America (Portland, OR, USA). Dulbecco's Modified Eagle's Medium (DMEM) and GSH were purchased from Invitrogen (Carlsbad, CA, USA) or USB Corporation (Cleveland, OH, USA). Juglone, paraquat, plumbagin, menadione, glucose-6-phosphate (G6P), butylated hydroxytoluene, uric acid, bovine superoxide dismutase (SOD), and bovine catalase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), ACROS (Morris Plains, NJ, USA), MP Biomedicals (Santa Ana, CA, USA), or EMD Biosciences (Gibbstown, NJ). The remaining materials were purchased from Sigma (St. Louis, MO, USA), including glucose oxidase from *Aspergillus niger*, glucose-6-phosphate dehydrogenase (G6PDH) from *Saccharomyces cerevisiae*, and human NADPH: cytochrome P450 reductase (P450Red).

Cell culture

EMT6 murine mammary carcinoma cells were maintained in 75 cm² plastic flasks as monolayers, in the presence of DMEM supplemented with 10 % FBS in humidified air/5 % CO₂ at 37 °C. The cells were subcultured every 2–3 days.

Treatment of EMT6 cells with NBF-SPh and SBF-SPh

Cell survival (clonogenic) assays were conducted using a previously described method (Baumann et al. 2005). Plastic flasks (25 cm²) were each seeded with 7–10 × 10⁴ cells, and when confluent, rinsed with medium and then treated with either NBF-SPh (0–100 μM) or SBF-SPh (0–200 μM), dissolved in 3 mL of fresh culture medium for 2 h in humidified air/5 % CO₂ at 37 °C. Both reagents were dissolved in DMSO, and the final concentration of DMSO was 0.1 % (v/v). For the rescue experiments, cells were pretreated for 1 h with sodium pyruvate (5 mM) or uric acid (500 μM), in the presence or absence of thioglycerol (5 mM); cells were then exposed to 50 μM NBF-SPh for 30 min at 37 °C. Following each experiment, monolayers were rinsed and detached with 0.25 % trypsin-EDTA, suspended in culture medium, and counted. Serial dilutions were carried out in triplicate with culture medium, and for each of several tenfold dilutions, 1 mL aliquots were pipetted into 3 wells, mixed with 1 mL of DMEM and 10 % FBS, and incubated for colony formation. After seven to ten days of growth, colonies were fixed and quantified, following staining with crystal violet (0.25 %) in 80 % methanol. All analyses were corrected for plating efficiency using DMSO concentrations of 0.1 % (v/v), which were non-toxic.

Oxygen consumption by redox-cycling

The consumption of molecular oxygen (O₂) was monitored using a temperature-controlled Gilson oxygraph chamber fitted with a magnetic stirrer and a Clark electrode, attached to a YSI 5300 Biological Oxygen Monitor (Yellow Springs Instruments).

Oxygen consumption was supported by NADPH as the initial reductant, and a set of redox-cycling agents coupled electron transfer from NADPH/P450Red to O₂. Reactions were conducted at 37 °C in 100 mM potassium phosphate (pH 7.4). Just before use, stock solutions for each redox-cycling agent were prepared in DMSO, except for paraquat which was dissolved in buffer. In each assay, the redox-cycling agent was 25 μM nitrofurazone, paraquat, metronidazole, juglone, menadione, plumbagin, or NBF-SPh. A solution of 25 μM SBF-SPh was also prepared to test its ability to redox-cycle. After thermal equilibration of the assay mixture, electron transfer from 2 mM NADPH was initiated by addition of 0.26 U/mL of P450Red. The initial linear slope of the progress curve was reported as the apparent initial velocity. For the purpose of determining the point of complete oxygen depletion, O₂ consumption was also followed in a reaction with 10 mM glucose, 2 U/mL of glucose oxidase, and 120 U/mL of catalase. Air-saturated buffer was assumed to contain 212 μM O₂ (Weiss 1970). Non-enzymatic reactions represented <1 % of each enzymatic reaction.

Additional experiments measured the effects of NBF-SPh concentration, following the reaction progress to either oxygen depletion or the cessation of oxygen consumption. O₂ consumption was followed in the presence of various concentrations of NBF-SPh (0, 2, 5, 10, or 25 µM), and electron transfer from 100 µM NADPH was initiated by addition of 0.26 U/mL of P450Red.

Determination of reactive oxygen species (ROS)

The consumption of O₂ by redox-cycling suggested the production of ROS, so additional experiments were conducted to identify the reaction products. The disproportionation of hydrogen peroxide into water and O₂ was monitored using the Gilson oxygraph chamber as above, while superoxide was detected using a Beckman DU 640 spectrophotometer to monitor the colorimetric adrenochrome assay (Baez and Segura-Aguilar 1995; Bindoli et al. 1999; Sun and Zigman 1978).

In experiments designed to detect the generation of O₂ from the disproportionation of hydrogen peroxide, oxygen consumption was supported with a coupled assay. The combination of 1 mM G6P and 5 U/mL of G6PDH served to maintain the reduced form of NADP⁺, and P450Red then coupled electron transfer to SBF-SPh (50 µM) or NBF-SPh (50 µM). Just before use, stocks of SBF-SPh or NBF-SPh were composed in DMSO, and each reaction was conducted in phosphate buffer (pH 7.4) at 37 °C. Here, electron transfer from 100 µM NADPH was initiated by the addition of 0.26 U/mL of P450Red. After O₂ was depleted, addition of 10,000 U/mL of catalase confirmed the production of hydrogen peroxide.

In order to detect superoxide production, a colorimetric coupled assay was conducted at 25 °C in Buffer A: sodium carbonate buffer (pH 6.5) with 300 µM diethylenetriaminepentaacetic acid. Just before use, a stock of NBF-SPh was dissolved in DMSO, and L-adrenaline was composed in 1:1 DMSO: Buffer A. The combination of 1 mM G6P and 5 U/mL of G6PDH served to maintain the reduced form of 25 µM NADP⁺, and 0.26 U/mL of P450Red then coupled electron transfer to NBF-SPh (25 µM) with 250 µM L-adrenaline. The oxidation of L-adrenaline to its chromophoric product, adrenochrome, was monitored at 451 nm. In separate control reactions, addition of 10,000 U/mL of SOD abrogated the oxidation of L-adrenaline, and addition of 10,000 U/mL of catalase verified that L-adrenaline oxidation was dependent on superoxide rather than hydrogen peroxide.

Lipid peroxidation studies

The protocol to detect 2-thiobarbituric acid-reactive substances (TBARS) was adapted from published methods (Johnson et al. 1998; Uchiyama and Mihara 1978). Plastic flasks (25 cm²) were each seeded with 7–10 × 10⁴ cells, and when near confluent, they were rinsed twice with fresh DMEM (no FBS), and left in 1 mL of fresh DMEM (no FBS). Cultures were treated with NBF-SPh (50, 25, and 10 µM) or FeCl₃-EDTA (50 µM), placed for 1 h in humidified air/5 % CO₂ at 37 °C, and gently mixed at 20 and 40 min. Prior to treatment, NBF-SPh was dissolved in DMSO, and the final concentration of DMSO was 0.1 % (v/v). The stock solution of FeCl₃-EDTA was composed in DMEM (no FBS).

As discussed below, the oxidative processes yielding TBARS were halted at 1 h with a stop solution: 100 mM butylated hydroxytoluene and 1 mM deferoxamine mesylate, composed in methanol with 15 % TCA (v/v). After treatments with NBF-SPh or FeCl₃-EDTA, 100 µL of 10 % Triton X-100 was added to each cell culture, and the remaining cells were scraped into solution. An aliquot (0.667 µL) of each lysed sample was mixed with 0.333 µL of the stop solution, while the remainder of each sample was flash frozen for protein determination using the Bradford protein assay. To each “stopped” solution, which appeared milky and

opaque, was added 6 mL of 150 mM potassium phosphate (pH 2.15) and 2 mL of 0.67 % (w/v) 2-thiobarbituric acid, composed in 0.5 M NaOH. The reaction mixture was vortexed and heated at 95 °C for 1 h.

After heat treatment, the solution was cooled on ice, and 2 mL of *n*-butanol was added for extraction of the fluorescent analyte. The solution was vigorously shaken and then centrifuged for 10 min at 1,000 rpm. An aliquot of the *n*-butanol layer was re-centrifuged at 14,000 rpm (Eppendorf 5145 C), and its fluorescence was quantified ($\lambda_{\text{ex}} = 532 \text{ nm}$, $\lambda_{\text{em}} = 550 \text{ nm}$) within 20 min of heat treatment.

All fluorescence values were normalized to protein concentration, and the adjusted values were presented with respect to controls that were incubated with just DMEM (no FBS). Data and standard error represent 10 replicate experiments.

Differential pulse (DP) polarography and cyclic voltammetry (CV)

The electrochemical reactivities of NBF-SPh and SBF-SPh were investigated using a Model 394 Princeton Applied Research Electrochemical Trace Analyzer (Princeton Applied Research), linked to a Model 303A Static Mercury Electrode (Princeton Applied Research). Prior to use, the compounds were dissolved in DMSO and diluted to 25 μM in 50 mM phosphate buffer, 100 mM potassium chloride (pH 7.0). In CV experiments, 15 % dimethylformamide effectively attenuated adsorption of NBF-SPh to the chamber surface. Prior to analyses, oxygen was removed from each sample by purging with N_2 for 2 min. DP polarograms were collected at a scan rate of 2 mV/s (1 drop/s), with a pulse height of 50 mV. Cyclic voltammograms were collected at increasing scan rates (pH 7.0), and for voltammograms collected over pH 4–9.5, the scan rate was held constant at 60 mV/s (0.33 step/s). In all cases, the mercury drop surface area was calculated as 0.01454 cm^2 .

Construction of electrochemical H-cell

Electrochemical reductions of 25 μM NBF-SPh or SBF-SPh with glassy carbon or gold planar electrodes yielded attenuated currents, so a custom apparatus with mercury electrodes was constructed for bulk electrolysis studies. Centricon[®] concentrators (Millipore) were modified to form a simple H-cell, and Vycor[®] frits (Ametek) served as the electrochemical bridge. Copper wires, used to complete the circuit, were submerged in approximately 8 mL of mercury on both sides of the cell. An adjustable, voltage-regulated DC power supply was used as a power source. Current and voltage were followed with multimeters and a Combination (Saturated) Ag/AgCl Reference Electrode (Beckman Coulter).

Bulk electrolysis and mass spectrometry

The products resulting from bulk electrochemical reduction of NBF-SPh and SBF-SPh were analyzed by mass spectrometry. Compounds were dissolved in DMSO and diluted to final concentrations of 50 μM NBF-SPh or 400 μM SBF-SPh, using 50 mM phosphate buffer, 100 mM sodium or potassium chloride (pH 7.0). In the case of NBF-SPh, 15 % dimethylformamide and 30 % acetonitrile were used in efforts to attenuate its adsorption. The oxidation of reduced glutathione (40 mM) was used as the half-cell reaction at the anode. During the electrochemical processes, a voltage regulator effectively controlled the applied potential.

Bulk electrolysis of 50 μM NBF-SPh was carried out by increasing the applied voltage every 10 min: -0.3 , -0.6 , -0.9 , and -1.2 V versus a reference electrode (saturated Ag/AgCl). The electrochemical reduction was sampled at 5-min intervals, and 20 μL aliquots were prepared for FT-ICR mass spectrometry, using C18-ZipTips[®] (Millipore) for buffer exchange.

Bulk electrolysis of 400 μM SBF-SPh was carried out by maintaining an applied potential at -1.2V versus a reference electrode (saturated Ag/AgCl). The electrochemical reduction was sampled at 5-min intervals, and 20 μL aliquots were prepared for FT-ICR mass spectrometry, using C18-ZipTips[®] (Millipore) for buffer exchange.

Theoretical calculations

Geometry optimizations of NBF-SPh (**1**), **2–6**, SBF-SPh, and the corresponding one-electron reduced species were performed using Becke's three-parameter hybrid DFT method B3LYP (Becke 1993) in conjunction with the 6-311+G(2d,2p) basis set. Bulk solvation was modeled by C-PCM formalism (Barone and Cossi 1998; Cossi 2003). This is the implementation of the conductor-like screening model COSMO (Klamt and Schuurmann 1993) in the PCM framework, with atomic radii optimized for COSMO-RS (Klamt 1998). Frequency calculations were performed at the same level of theory to verify that the stationary points were true energy minima. In evaluation of data, the correction to the Gibbs energy at 298 K was added to the minimum energies. All calculations were carried out in the Gaussian09 program (Frisch et al. 2009).

Adiabatic electron affinities were calculated by comparing the Gibbs energies of the optimized neutral molecules to the corresponding anions, and in the case of SBF-SPh, the optimized anion species to the dianion species.

Results

Cytotoxicity of 7-nitro-4-(phenylthio)benzofurazan (NBF-SPh)

In order to assess the toxicity of NBFs in cell cultures, EMT6 mouse mammary tumor cells were treated with NBF-SPh (Fig. 2a). The LC_{50} value (12.1 μM) was comparable to that reported after 48 h of exposure in other cell lines (Caccuri and Ricci 2006; Ricci et al. 2005), and the marginal difference between the values suggested that the cytotoxic effects of NBF-SPh were expended within the first 2 h. Notably, cell blebbing was widely visible 20 min after treatment with 25 μM NBF-SPh. Cell debris accumulated after 1 h with 50 μM NBF-SPh, indicating that moderate concentrations of NBF-SPh promptly induced acute cell death.

In contrast to NBF-SPh, the SBF-SPh exhibited no apparent cytotoxic effects after treatments lasting 2 h and with up to 200 μM SBF-SPh (Fig. 2a), indicating the acute toxicity of NBF-SPh was dependent on the 7-nitro group.

Pretreatment with thioglycerol partially rescues cell death

Experiments were conducted to gauge the ability of anti-oxidants (pyruvate, uric acid, and thioglycerol) to rescue EMT6 cells from oxidative or electrophilic damage caused by ROS and reactive intermediates of NBF-SPh, but pyruvate and uric acid offered little protection. In contrast, thioglycerol attenuated the toxicity of NBF-SPh, increasing percent survival by 15- to 20-fold for each of the matched reaction conditions (Fig. 2b).

Redox-cycling of NBF-SPh consumes molecular oxygen

To examine whether acute cell death could be attributed to redox-cycling by NBF-SPh, we characterized the initial rates of O_2 consumption for an array of redox-cycling compounds, employing P450Red to initiate one-electron transfers from NADPH (Fig. 3). No significant reaction occurred until P450Red was introduced, whereupon NBF-SPh exhibited the fastest initial rate of O_2 consumption ($91 \pm 4.4 \mu\text{M}/\text{min}$), rivaling even plumbagin ($86 \pm 5.0 \mu\text{M}/\text{min}$), menadione ($83 \pm 2.3 \mu\text{M}/\text{min}$), and juglone ($68 \pm 2.5 \mu\text{M}/\text{min}$), while the SBF-SPh did not redox-cycle. The rates of O_2 consumption did not correlate with redox potentials of the compounds.

In previous studies, cultured tumor cells were exposed to 7-nitro-4-(hexylthio)benzofurazan (NBDHEX), which was employed at two concentrations (2 μ M and 10 μ M), and two modes of cell death were reported (Caccuri and Ricci 2006; Turella et al. 2005); however, concentration effects for the NBDHEX compound have not yet been explored. To determine whether the concentration of NBF-SPh affected its ability to consume O₂, the redox-cycling activity of NBF-SPh was studied over a range of concentrations. The rate of O₂ consumption increased as the concentration of NBF-SPh was increased from 2 to 25 μ M, exhibiting an initial burst of activity that increased linearly with the square root of the concentration (Fig. 4a). The time course showed that the original compound was depleted after approximately 19 cycles, such that 2 μ M NBF-SPh ceased redox-cycling after consuming 38 μ M O₂ over nearly 10 min, and it took longer to deplete greater concentrations of NBF-SPh.

Generation of reactive oxygen species by NBF-SPh

To determine whether hydrogen peroxide was a product of redox-cycling with molecular oxygen, we employed P450Red to couple one-electron transfers from NADPH to NBF-SPh (Fig. 4b). Once the oxygen had been depleted, catalase was added and the disproportionation of hydrogen peroxide yielded additional oxygen that was measured by the oxygraph.

The colorimetric adrenochrome assay was employed to determine whether superoxide was a product of redox-cycling with molecular oxygen. The oxidation of L-adrenaline produced a broad spectrum that is typical of adrenochrome, indicating that redox-cycling of NBF-SPh supported super-oxide production, and in the absence of NBF-SPh, the oxidation of L-adrenaline was negligible (Fig. 4c). Therefore, superoxide was a primary product of redox-cycling by NBF-SPh, and this was further confirmed by the inclusion of superoxide dismutase, which abrogated the formation of adrenochrome.

Formation of lipid peroxidation end products

The TBARS assay was used to probe for lipid peroxidation products resulting from intracellular redox-cycling by NBF-SPh, and FeCl₃-EDTA was employed for comparison. After 1-h treatment of EMT6 mouse mammary tumor cells, lipid peroxidation was substantially enhanced with NBF-SPh (Fig. 5); treatment with 50 μ M NBF-SPh produced 25 percent more TBARS (1.56 ± 0.08 -fold) than did 50 μ M FeCl₃-EDTA (1.24 ± 0.08 -fold), and the level of lipid peroxidation was also comparable to treatments with 200 μ M FeCl₃-EDTA (1.85 ± 0.08 -fold).

Differential pulse (DP) polarography

Through reductive processes, nitroaromatic compounds are often converted to the corresponding amines (Heimbrook and Sartorelli 1986; Kennedy et al. 1980; Knox et al. 1983), whereas the reduction of benzofurazans can involve a ring-opening event (Stradyn et al. 1974; Tsveniashvili et al. 1966). In the case of NBF-SPh, redox-cycling may involve either of the two reductive pathways, and delineation of this mechanism would contribute to the design of future BFZs. To identify the reductive processes for both NBF-SPh and SBF-SPh, DP polarography was employed using a static dropping mercury electrode. DP polarograms were recorded in 50 mM potassium phosphate buffer with 0.2 M ionic strength (pH = 7.0), which was N₂-saturated to avoid the diffusion of atmospheric oxygen into solution. Several redox transitions were identified for NBF-SPh and one was found for SBF-SPh (Fig. 6).

Only one peak was sufficiently electropositive for redox-cycling with NBF-SPh (*I*_a), with a midpoint potential of -96 mV versus the standard hydrogen electrode (SHE). The half-width (*W*_{1/2}) of *I*_a was 54 mV, indicating the first reduction step for NBF-SPh occurs with a two-electron transfer as the rate-determining step. The latter peaks (*I*_b and *I*_c), which were too

electronegative for biological reduction, exhibited midpoint potentials of (1_b) -604 mV and (1_c) -798 mV (SHE), suggesting the rate-limiting steps occur with one ($W_{1/2}=107$ mV) and three ($W_{1/2}=31$ mV) electrons, respectively. In the case of SBF-SPh, peak 2_a had a midpoint potential of -696 mV, which was also too electronegative for biological reduction, and its half-width was $W_{1/2}=98$ mV, correlating to a one-electron reduction as the rate-limiting step (Bard and Faulkner 2001).

Cyclic voltammetry (CV)

To further detail the electron transfer steps, cyclic voltammetry was employed to examine the reversibility of each peak detected by DP polarography. With N_2 -saturated phosphate buffer and scan speeds of 25 – 400 mV/s, cyclic voltammograms were collected in the range of peak 1_a (Fig. 7a), and they revealed both cathodic ($1_{cath.}$) and anodic ($1_{anod.}$) peaks, indicating that 1_a is a reversible electron transfer step. Cyclic voltammograms in range of the remaining peaks (1_b , 1_c , and 2_a) found only cathodic peaks with no anodic peaks, signifying they were all irreversible steps (Fig. 7b, c) (Bard and Faulkner 2001).

To scrutinize the reversibility of each step, CV was conducted over a broad pH range between 4 and 9 , using N_2 -saturated buffers and a sweep rate of 60 mV/s. Each electron transfer step observed with NBF-SPh and SBF-SPh became less favorable with increasing pH, and their currents were maximal near pH 4.0 . Plotting the shifting peak potentials of NBF-SPh versus pH revealed a pK_a at pH $=5.5$, indicated by the two intersecting slopes for each of $1_{cath.}$, $1_{anod.}$, 1_b , and 1_c (Fig. 8a). A similar plot for SBF-SPh revealed a pK_a at pH $=6.5$, seen by the intersecting slopes for 2_a (Fig. 8b).

In the case of NBF-SPh, each component of peak 1_a exhibited two linear ranges that were described by the following equations.

NBF-SPh ($1_{cath.}$):

$$E_p = -31.4\text{pH} + 61.0 \quad R^2 = 0.9876 \quad (1)$$

$$E_p = -42.9\text{pH} - 2.41 \quad R^2 = 0.9965 \quad (2)$$

NBF-SPh ($1_{anod.}$):

$$E_p = -57.6\text{pH} - 265 \quad R^2 = 0.9968 \quad (3)$$

$$E_q = -48.5\text{pH} - 217 \quad R^2 = 0.9856 \quad (4)$$

The effects of pH on the *cathodic* peak potential had slopes of -31.4 and -42.9 mV/pH, while the effects of pH on the *anodic* peak potential had slopes of -57.6 and -48.5 mV/pH. These data are consistent with a reversible two-electron transfer, where the reactions involve 1.5 protons at pH > 5.5 , or 1 and 2 protons at pH < 5.5 for the cathodic and anodic peaks, respectively (Bard and Faulkner 2001).

For the two irreversible reductions of NBF-SPh (peaks 1_b and 1_c), the following equations describe the linear ranges for each plot.

NBF-SPh (1_b):

$$E_p = -63.6\text{pH} - 465 \quad R^2=0.9803 \quad (5)$$

$$E_p = -2.67\text{pH} - 804 \quad R^2=0.9106 \quad (6)$$

NBF-SPh (1_c):

$$E_p = -46.1\text{pH} - 623 \quad R^2=0.9836 \quad (7)$$

$$E_p = -76.0\text{pH} - 455 \quad R^2=0.9986 \quad (8)$$

For 1_b , the dependence of the peak potential on pH had slopes of -63.6 and -2.67 mV/pH, suggesting that the one-electron step involved 1 proton, but at $\text{pH} > 5.5$, electron transfer was independent of pH. In the case of 1_c , the effects of pH on peak potential exhibited slopes of -46.1 and -76.0 mV/pH, suggesting that the rate-limiting, three-electron transfer step involved 2 protons at $\text{pH} < 5.5$ or 4 protons at $\text{pH} > 5.5$ (Bard and Faulkner 2001).

The irreversible one-electron transfer step associated with SBF-SPh (peak 2_a) is described by the following equations.

SBF-SPh (2_a):

$$E_p = -44.0\text{pH} - 475 \quad R^2=0.9962 \quad (9)$$

$$E_p = -66.1\text{pH} - 332 \quad R^2=0.9979 \quad (10)$$

Here, the dependence of peak potential on pH exhibited slopes of -44.0 and -66.1 mV/pH, suggesting that the rate-limiting, one-electron transfer involved 1 proton at $\text{pH} > 6.5$ or 0.75 protons at $\text{pH} < 6.5$ (Bard and Faulkner 2001).

Products formed by electrochemical reduction

The chemical products that formed during electroreduction of NBF-SPh or SBF-SPh were used to infer the total number of electrons transferred during reduction. Bulk electrolysis reactions were conducted as described in “Materials and methods” and aliquots were analyzed by mass spectrometry.

In the case of NBF-SPh, mass spectrometry yielded monoisotopic peaks of 274.028 (m/z) at 0 min and 232.090 (m/z) at 20 min, corresponding to NBF-SPh and 4-(phenylthio)benzene-1,2,3-triamine, respectively, indicating that the benzofurazan ring had opened and the nitro group was reduced to the amine during electrochemical reduction. The presence of numerous isotopic peaks suggested there were several reaction products for NBF-SPh, preventing resolution of intermediate NBF species over the course of reduction.

With SBF-SPh, mass spectrometry yielded monoisotopic peaks of 306.984 (m/z) at 0 min and 295.021 (m/z) at 20 min, corresponding to SBF-SPh and 2,3-diamino-4-(phenylthio)benzenesulfonic acid, respectively, indicating that the benzofurazan ring had opened during electrochemical reduction. The only other monoisotopic peak to develop during reduction of SBF-SPh was at 308.999 (m/z), supporting a protonated intermediate of a ring-opening event.

Electron localization by computational theory

In an effort to detail a mechanism for the redox-cycling of NBF derivatives, compounds **1** (NBF-SPh), **2–6**, and SBF-SPh were computationally investigated, and their electron affinities, charge densities, and N–O bond lengths were determined. As seen in Table 1, the electron affinity of SBF-SPh (77.9 kcal/mol) was substantially lower than those for the NBF derivatives **1**, **2**, and **4**, which had affinities of 95.6, 92.2, and 97.9 kcal/mol, respectively. For each of **1**, **2**, and **4**, the added electron localized to the 7-nitro substituent, while that for SBF-SPh localized to the furazan ring. The affinities for the remaining NBF species (**3**, **5**, and **6**) were in the range of SBF-SPh, and in these cases, the electron localized to the furazan ring. The changes in N–O bond lengths for each compound were consistent with the changes in the Mulliken charge distributions (Table 1), supporting a ring-opening event when the electron localized to the furazan ring.

Discussion

Numerous benzofurazans exhibit electrophilic scavenging activity, but little is known about their reactivities with molecular oxygen, and this includes the anticancer 7-nitrobenzofurazan (NBF) series, which are thought to disrupt protein–protein interactions (Caccuri and Ricci 2006; Federici et al. 2009; Ricci et al. 2005; Turella et al. 2005). Here, we investigated the ability for the patented anticancer agent NBF-SPh to redox-cycle and generate ROS, and this is the first report to confirm redox-cycling activity and ROS production for any anticancer NBF, placing NBF-SPh among the few redox-cycling benzofurazans (Takabatake et al. 1990, 1991, 1992a, b).

Our study demonstrates that NBF-SPh rapidly converts oxygen to ROS at a rate that rivals even the most toxic redox-cycling quinones, including plumbagin, menadione, and juglone (Castro et al. 2008; Giulivi and Cadenas 1994; Inbaraj and Chignell 2004). We confirm previous reports that NBF-SPh is highly toxic in the sub-micromolar range, and we show that EMT6 murine mammary tumor cells have a sharp threshold for tolerating the compound (Fig. 2a); thus, exposure to 6.25 μ M NBF-SPh presents no discernible toxicity, while the LC₅₀ value is just twice this concentration (12.1 μ M). Given that NBF-SPh rapidly consumes molecular oxygen (Fig. 3) and converts it to hydrogen peroxide (Fig. 4b) and superoxide (Fig. 4c), the steep drop in survival may indicate that EMT6 cells are able to address oxidative or electrophilic stress until the production of ROS becomes overwhelming at NBF-SPh concentrations above 6.25 μ M. This is supported by our TBARS analysis (Fig. 5), which demonstrates that the level of lipid peroxidation by NBF-SPh is physiologically relevant and comparable to treatments with FeCl₃-EDTA. This conclusion is also consistent with the increased duration of redox-cycling activity observed at increased concentrations of NBF-SPh (Fig. 4a).

It is intriguing that the initial burst of oxygen consumption by NBF-SPh is dependent upon the square root of its concentration; there are several factors which could account for this finding. Although speculative, one possibility is that while redox-cycling, two molecules of compound **2** disproportionate to yield oxidized NBF-SPh and compound **3**, which may be less prone to oxidation by molecular oxygen; in this case, the generation of super-oxide would be dependent on the square root of NBF-SPh concentration. Additional factors contributing to this dependence could include other events that suppress redox-cycling activity, substrate-enzyme interactions, or the availability of either reductant or molecular oxygen.

While it is clear that NBF-SPh is a potent inducer of ROS (Fig. 4b, c), its rapid depletion over the course of redox-cycling (Fig. 4a, b) is significant and distinguishes it from other redox-cycling agents like plumbagin, menadione, and juglone. Perhaps the most

straightforward explanation for this behavior is that the nitro group of NBF-SPh undergoes rapid reduction to the amine as depicted in Scheme 1 (Fig. 1), which involves the common stepwise reduction pathway for other nitroaromatic compounds. A second possibility is that the reduction of NBF-SPh to compound **2** or **3** may induce scavenging activity, and this would be consistent with our discovery of numerous isotopic peaks after bulk reduction of NBF-SPh, as well as the broad use of reactive NBFs in scientific research for the fluorescent labeling of specific functional groups. A third explanation is that reduction promotes the conversion of NBF-SPh directly to the α -phenylenediamine product we identified by mass spectrometry, 4-(phenylthio)benzene-1,2,3-triamine. While each explanation is reasonable, they would all introduce products likely to exhibit their own unique toxicities.

There is some evidence that compound **2** or **3** exhibits scavenging activity, so experiments intended to attenuate ROS production may not be sufficient to rescue cell death; efforts to protect against electrophilic stress induced by NBF-SPh must also address reactive intermediates of the redox-cycler. The antioxidant and nucleophile thioglycerol provides strong protection against general electrophilic stress, and offered significant protection, while pyruvate (a permeable and relatively specific hydrogen peroxide scavenger) and uric acid (a peroxynitrite scavenger) appeared ineffective (Fig. 2b). The lack of protection by pyruvate may be a consequence of its inadequate ability to augment the protection afforded by endogenous cellular protectants, such as catalase and glutathione peroxidase, during acute peroxide exposure. Since it is not a substrate for GSTs, thioglycerol should not interfere with specific interactions between GSTs and NBF-SPh, so the substantial protection afforded by thioglycerol supports a non-specific mechanism of electrophilic toxicity for NBF-SPh.

DP polarograms (Fig. 6) and cyclic voltammograms (Fig. 7a) clearly show a reversible electron process that could generate ROS, and in order to detail the more electronegative processes for NBF-SPh, we first characterized the electrochemical process for SBF-SPh, which did not redox-cycle and exhibited only one reductive peak in DP polarograms. Below pH 6.5, irreversible reduction of SBF-SPh involves a single one-electron/one-proton step centered around -696 mV (SHE), and the reductive step becomes less favorable at increasing pH (Figs. 7c, 8b). Since this is the only peak for SBF-SPh, it seems this one-electron transfer initiates the ring-opening event that yields the 4-(phenylthio)benzene-1,2-diamine-3-sulfonic acid, identified by mass spectrometry. After the initial rate-limiting step, it is reasonable to expect that regaining aromaticity in the benzene ring would spontaneously propel the downstream reductions. In the case of NBF-SPh, the one-electron/one-proton electron transfer step indicated by peak 1_b ($E_p = -604$ mV, SHE) seems to be the most likely candidate for initiating a similar ring-opening event (Fig. 6). After reduction beyond compound **3**, electron density in the furazan ring would make it susceptible to additional electrophilic attack (Table 1), which could explain observed reactivities. It should be said that the redox potentials for the irreversible electron transfers are too electronegative for biological reduction, so while in vitro reduction of NBF-SPh appears to proceed via Scheme 2 (Fig. 9), reduction of NBF-SPh in vivo would likely halt at compound **3**.

Our electrochemical analyses and computational studies afforded us a possible conclusion for the depletion of NBF-SPh during redox-cycling supported by enzymatic reduction; it seems that compounds **2** and **3** may be reactive intermediates with electrophilic scavenging activity, which could freely react with cellular components, as do many other reactive NBFs. This conclusion is supported by thioglycerol's ability to rescue cell death, while pyruvate and uric acid are ineffective protectants. Together, with the observation that ROS production and lipid peroxidation are dependent on NBF-SPh concentration, our findings suggest the electrophilicity of NBF-SPh contributes to its toxicity.

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Glossary

BFZ	Benzofurazan
NBF	7-Nitrobenzofurazan
ROS	Reactive oxygen species
NBF-SPh	7-Nitro-4-(phenylthio)benzofurazan
GST	Glutathione S-transferases
SBF-SPh	7-Sulfo-4-(phenylthio)benzofurazan
DMEM	Dulbecco's modified Eagle's medium
G6P	Glucose-6-phosphate
SOD	Superoxide dismutase
G6PDH	Glucose-6-phosphate dehydrogenase
P450Red	NADPH:cytochrome P450 reductase
TBARS	2-Thiobarbituric acid-reactive substances
DP	Differential pulse
CV	Cyclic voltammetry
NBDHEX	7-Nitro-4-(hexylthio)benzofurazan

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Fig. 1.
Scheme 1. Predicted reduction of NBF-SPh from the 7-nitro to a 7-amine

Fig. 2.

Effects of BFZ derivatives and protectants on the survival of EMT6 cells. **a** Cultured EMT6 tumor cells were incubated with increasing concentrations of NBF-SPh (*filled circles*) and SBF-SPh (*open circles*) for 2 h. **b** Prior to a 30-min treatment with 50 μM NBF-SPh, cultured EMT6 tumor cells were first incubated for 1 h with various antioxidant protectants (*light bars*): sodium pyruvate (5 mM), uric acid (500 μM), and thioglycerol (5 mM). Parallel studies were also conducted without thioglycerol (*dark bars*). For both experiments, the treated cells were then grown for 7–10 days as described in the “Materials and methods”. Percent survival was measured by colony formation, using cells treated with solvent vehicle

as a reference. Each *point* represents the average of four separate experiments, and the *associated* bars represent SE. For rescue experiments, one-way analysis of variance (ANOVA) confirmed the significance between the two sets of data ($P < 0.0001$)

Fig. 3.

Initial rates of O₂ consumption for redox-cycling compounds. The redox-cycling activity exhibited by 25 μM of each of six quinones or nitroaromatic redox-cycling compounds was compared against NBF-SPh. O₂ consumption was monitored using 0.26 U/mL of P450Red to couple electron transfer from 2 mM NADPH. Each *point* represents the average of three separate experiments, and the associated *bars* represent standard error. According to one-way analysis of variance (ANOVA), the mean values are significantly different ($P < 0.0001$). As indicated by (*double asterisks*) for $P < 0.01$ and (*triple asterisks*) for $P < 0.001$, Dunnett's post-test shows O₂ consumption is statistically different between NBF-SPh and juglone ($P < 0.01$), and also between NBF-SPh and each of metronidazole, paraquat, and nitrofurazone ($P < 0.001$)

Fig. 4.

Redox-cycling of NBF-SPh and production of reactive oxygen species. **a** O_2 consumption was monitored with increasing concentrations of NBF-SPh (0, 2, 5, 10, and 25 μM), using 0.26 U/mL of P450Red to couple electron transfer from 100 μM NADPH. **b** O_2 consumption by 50 μM NBF-SPh (black line) or 50 μM SBF-SPh (gray line) was monitored, using a coupled assay with 1 mM G6P and 5 U/mL of G6PDH to maintain reduced NADPH (100 μM). Addition of 0.26 U/mL of P450Red initiated the reaction. The control assay with 10 mM glucose, 2 U/mL of glucose oxidase, and 10,000 U/mL of catalase is also presented (dotted line). Arrows indicate injection of P450Red or catalase. **c** A coupled assay with 1

mM G6P, 5 U/mL of G6PDH, 25 μ M NADP⁺, 0.26 U/mL of P450Red, and 25 μ M NBF-SPh was used to oxidize 250 μ M L-adrenaline. The accumulation of adrenochrome was followed for 5 min at 451 nm (*thick line*), and control experiments were conducted with 10,000 U/mL of superoxide dismutase (*thin line*) or without 25 μ M NBF-SPh (*dotted line*)

Fig. 5.

Lipid peroxidation by NBF-SPh in EMT6 cells. The 2-thio-barbituric acid-reactive substances (TBARS) were quantified with fluorescence ($\lambda_{\text{ex}} = 532 \text{ nm}$, $\lambda_{\text{em}} = 550 \text{ nm}$), after 1 h treatment with NBF-SPh (10, 25, and 50 μM) or FeCl_3 -EDTA (50 and 200 μM). Values are normalized to the sample protein content and expressed as the increase of TBARS from control samples. Data are mean $\pm$ SE of 10 replicates. According to one-way analysis of variance (ANOVA), the mean values are significantly different ($P = 0.0002$). As indicated by (*double asterisks*) for $P < 0.01$ and (*triple asterisks*) for $P < 0.001$, Dunnett's post-test shows TBARS were statistically different between treatments of 50 and 200 μM FeCl_3 -EDTA ($P < 0.001$) and between treatments of 10 and 50 μM NBF-SPh ($P < 0.01$).

Fig. 6. DP polarography of NBF-SPh and SBF-SPh. The electroactive species of 25 μM NBF-SPh (black) or SBF-SPh (gray) were examined at a scan rate of 2 mV/s. NBF-SPh exhibited 3 peaks (1_a , 1_b , and 1_c), while SBF-SPh exhibited only 1 peak (2_a). The predicted products are shown. Arrows indicate the observed reversible or irreversible electrochemical transitions. SHE standard hydrogen electrode

Fig. 7. Cyclic voltammetry of BFZ derivatives. Scan rates of 10–400 mV/s were used to investigate 25 μ M NBF-SPh (peaks I_a , I_b , and I_c from Fig. 6) or 25 μ M SBF-SPh (peak 2_a from Fig. 6). **a** Voltammetric analysis of I_a revealed a reversible electron transfer step with cathodic ($I_{\text{cath.}}$) and anodic ($I_{\text{anod.}}$) peaks. **b** Analysis of I_b and I_c revealed irreversible electron transfer steps. **c** Analysis of 2_a revealed an irreversible electron transfer step. Ag/AgCl was a saturated Ag/AgCl reference electrode

Fig. 8. Investigating the proton/electron ratios. Plotting peak potentials versus pH revealed pK_a values at **a** pH = 5.5 for 25 μ M NBF-SPh ($I_{anod.}$, $I_{cath.}$, I_b , I_c) or **b** pH = 6.5 for 25 μ M SBF-SPh (2_a). All *peaks* became more electronegative at increasing pH values, and the slopes are described in the text. Ag/AgCl was a saturated Ag/AgCl reference electrode

Fig. 9.
Scheme 2. Observed conversion of NBF-SPh to the 1,2,3-triamine