Metabolic Activation of the Antitumor Drug 5-(Aziridin-1-yl)-2,4-Dinitrobenzamide (CB1954) by NO Synthases

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Nitric oxide synthases (NOSs) are flavohemeproteins that catalyze the oxidation of L-arginine to L-citrulline with formation of the signaling molecule nitric oxide (NO). In addition to their fundamental role in NO biosynthesis, NOSs are also involved in the formation of reactive oxygen and nitrogen species (RONS) and in the interactions with some drugs. 5-(Aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) is a dinitroaromatic compound tested as an antitumor prodrug that requires reduction to the 2- and 4-hydroxylamines to be cytotoxic. Here, we studied the interaction of neuronal, inducible, and endothelial NOSs with CB1954. Our results showed that the three purified recombinant NOSs selectively reduced the 4-nitro group of CB1954 to the corresponding 4-hydroxylamine with minimal 2-nitroreduction. Little further two-electron reduction of the hydroxylamines to the corresponding 2- and 4-amines was observed. The reduction of CB1954 catalyzed by the neuronal NOS (nNOS) was inhibited by O_2 and a flavin/NADPH binding inhibitor, diphenyliodonium (DPI), but insensitive to the addition of the heme ligands imidazole and carbon monoxide and of L-arginine analogues. This reduction proceeded with intermediate formation of a nitro-anion free radical observed by EPR. Involvement of the reductase domain of nNOS in the reduction of CB1954 was confirmed by the ability of the isolated reductase domain of nNOS to catalyze the reaction and by the stimulating effect of Ca^{2+}/calmodulin on the accumulation of 4- and 2-hydroxylamines. The recombinant inducible and endothelial NOS isoforms reduced CB1954 with lower activity but higher selectivity for the cytotoxic 4-hydroxylamine compared with nNOS. Finally, CB1954 did not modify the formation of L-citrulline and RONS catalyzed by nNOS. Our results show that all three NOS isoforms are involved in the nitroreduction of CB1954, with predominant formation of the cytotoxic 4-hydroxylamine derivative. This nitroreduction could be of interest for the selective activation of prodrugs by NOSs overexpressed in tumor cells.

Introduction

Nitric oxide, NO, is an important mediator in mammals that plays key roles in a variety of physiological processes such as neurotransmission, vasorelaxation, platelet aggregation, and immune responses (*1, 2*). It is generated by hemeproteins called NO synthases (NOSs),[1] which catalyze a two-step, NADPH- and O_2-dependent oxidation of L-arginine to citrulline and NO. Three NOS isozymes have been characterized in mammals: neuronal NOS (nNOS), cytokine-inducible NOS (iNOS), and endothelial NOS (eNOS). NOSs are homodimeric enzymes and each NOS subunit contains an NH_2-terminal oxygenase domain, which bears binding sites for the heme prosthetic group and the cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4), and a CO_2H-terminal reductase domain, which contains binding sites for the flavins, FMN and FAD, and the cofactor NADPH. These two domains are fused by a Ca^{2+}-dependent calmodulin (CaM)-binding sequence (*3–5*). CaM-binding to NOS activates both intradomain and interdomain electron transfers and is required for maximal NO-forming activity (*6, 7*). The reductase domain has a close homology to NADPH−cytochrome P450 reductase (*8*) and is able to catalyze the reduction of exogenous electron acceptors such as K_3FeCN_6, ferric cytochrome *c* (cyt *c*), and the azo-dye methyl-red (*6, 9*). NOSs also reduce some quinones including the antitumor drugs adriamycin, daunorubicin, and mitomycin C with the concomitant generation of superoxide anion (*10–14*). Strong electron acceptors, such as isomeric dinitrobenzenes (DNBs), also interact with NOSs and enhance electron flow from the reductase to the heme domain and increase both NO and superoxide formation by these enzymes (*15*). The rapid reaction of NO with superoxide anion results in formation of peroxynitrite (*16*). Recently, we have shown that the reductase domain of NOSs is also able to selectively reduce a nitroaromatic drug, nilutamide, to its hydroxylamine

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[1] Abbreviations: BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; CaM, calmodulin; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; cyt *c*, cytochrome *c*; DHR 123, dihydrorhodamine 123; DNBs, isomeric dinitrobenzenes; DPI, diphenyliodonium chloride; EGTA, ethylene glycol bis(aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GDEPT, gene-directed enzyme−prodrug therapy; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum coherence; ImH, imidazole; NOSs, nitric oxide synthases; nNOS, neuronal NOS; $nNOS_{oxy}$, oxygenase domain of nNOS; $nNOS_{red}$, reductase domain of nNOS; eNOS, endothelial NOS; iNOS, inducible NOS; NMMA, N^{ω}-methyl-L-arginine; NO2-Arg, N^{ω}-nitro-L-arginine; NQO-2, NAD(P)H quinone oxidoreductase; NTR, nitroreductase; RONS, reactive oxygen and nitrogen species; SEITU, *S*-ethyl-*iso*-thiourea.

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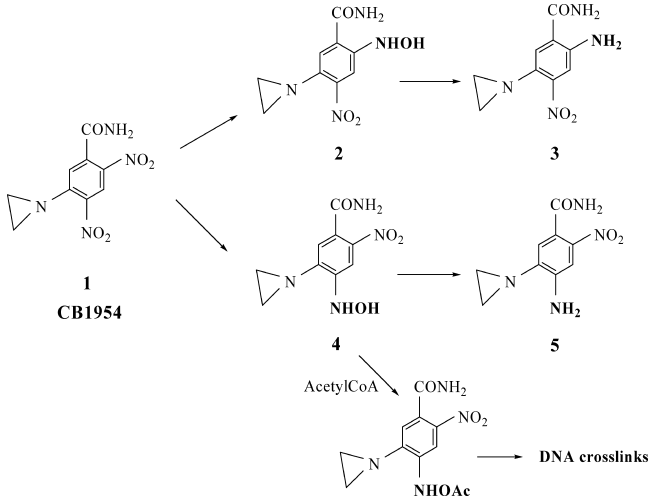
Scheme 1. Metabolic and bioactivation pathways of CB1954, 1[a]



[a] The parent compound **1** can undergo reduction at the 2-nitro position to generate the 2-hydroxylamine **2** and 2-amine **3**. Alternatively, it can undergo reduction at the 4-nitro position to generate the 4-hydroxylamine **4**, which may react with acetyl-CoA to form a DNA cross-linking species or undergo further reduction to the 4-amine **5**.

derivative (*17*). Nilutamide is a nonsteroidal drug that behaves as a competitive antagonist of the androgen receptors. However, its use in prostate carcinoma treatments has been hampered by toxic side effects that remain poorly understood (*18, 19*), but the hydroxylamine derivative of nilutamide is suspected to be responsible for the toxic effects of the drug. Formation of this metabolite by NOSs highlights the putative roles of these hemeproteins in the metabolism and toxicity of xenobiotics, including drugs.

In the present study, we have investigated the reductive metabolism of another antitumor agent, 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954, Scheme 1), by purified recombinant NOSs. CB1954 is a prodrug that is not directly toxic but requires reduction of one of its nitro groups (nitroreduction) for activity. Both nitro groups of CB1954 can be reduced to form the hydroxylamine metabolites **2** and **4**, and the amines **3** and **5**, which are products of further two-electron reduction. It has been shown that the most cytotoxic metabolite of CB1954 is the 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide **4** (Scheme 1) (*20–25*). The potent cytotoxicity of this metabolite is related to the further reaction with acetyl CoA and the putative formation of a highly reactive nitrenium intermediate. This reactive moiety can then act in concert with the aziridine group as a bifunctional DNA cross-linking agent (*22–24*). Although hydroxylamine **4** is the most cytotoxic metabolite in DNA cross-link repair defective cells, metabolite **3** is as active as **2** in repair-competent human tumor cell lines (*25*). Moreover, metabolite **3** is relatively stable and may contribute significantly to the cytotoxic activity of the CB1954 prodrug in tumors (*25*).

Presently, there is considerable interest in the development of prodrugs that can be activated selectively within tumors by enzymatic reduction. These prodrugs have the potential for killing radioresistant hypoxic cells in tumors, and for exploiting hypoxia as a basis for selective intratumoral activation (*26–28*). Activation of bioreducible drugs could have therapeutic applications in cancer chemotherapy if reductases either are overexpressed in tumors or can be selectively up-regulated or delivered to tumors. Gene-directed enzyme−prodrug therapy (GDEPT) involves the specific expression of a prodrug-activating enzyme in tumor cells, with utilization of this enzyme to selectively activate a prodrug within the tumor (*28, 29*). An enzyme that has been explored for GDEPT is an oxygen-insensitive nitroreductase (NTR) from *E. coli* that uses either NADPH or NADH as cofactors to reduce CB1954 to an equimolar mixture of metabolites **2** and **4** (*30, 31*). Another strategy to selectively activate CB1954 to metabolite **4** involves its reduction by the endogenous NAD(P)H quinone oxidoreductase-2 (NQO-2) and a synthetic cofactor (caricotamide) (*32*). Both these approaches are in current clinical trials (*33*, www.protherics.com).

We have recently demonstrated that human, rat and mouse livers can bioactivate CB1954 to cytotoxic metabolites (*34, 35*). However, the enzyme(s) involved in the nitroreduction of CB1954 by these liver subcellular fractions were not identified, even though a role for multiple endogenous reductases, both oxygen-sensitive and -insensitive, was hypothesized (*34*). A family of enzymes that may contribute to the metabolic reduction of the nitro groups of CB1954 is NOSs. Moreover, NOS isoforms, particularly inducible NOS, are often found overexpressed in tumor cells and may be a potential activating enzyme system for prodrugs that require reduction to be active (*1–3, 36, 37*). In the present study, we have found evidence that neuronal, inducible, and endothelial NOSs reduce CB1954 to the hydroxylamines **2** and **4**, with minor formation of the amines **3** and **5**. Hydroxylamine **4** was the predominant metabolite detected, and iNOS was particularly regioselective in the formation of this metabolite.

Reduction of CB1954 to the corresponding hydroxylamines is the second example of metabolic transformation of a xenobiotic by NOSs, with complete identification of the metabolites. These data confirm the possible involvement of NOSs in the metabolism of xenobiotics such as drugs and in the formation of cytotoxic metabolites. They also illustrate the possible pharmacological interest of NOSs in the activation of antitumor compounds and prodrug therapy.

Materials and Methods

Chemicals. L-Arginine, CB1954, N^{ω}-methyl-L-arginine (NMMA), N^{ω}-nitro-L-arginine (NO2-Arg), *S*-ethyl-*iso*-thiourea hydrobromide (SEITU), dihydrorhodamine 123 (DHR 123), diphenyliodonium hydrochloride (DPI), and superoxide dismutase, catalase, and nitroreductase from *Escherichia coli* (NTR) came from Sigma. BH_4 came from Alexis (Coger, France). NADPH and NADH were purchased from Boehringer (Mannheim, Germany). All other chemicals were purchased from Aldrich or Acros and were of the highest grade commercially available. [2,3,4-^{3}H]L-Arginine hydrochloride (specific activity 58 Ci/mmol) came from Perkin-Elmer. Reactions were monitored by TLC using Merck precoated silica gel $60F_{254}$ (0.25 mm thickness) on aluminum plates. Millipore SA silica gel 60 (35–70MY) was used for flash chromatography. All NMR experiments (^{1}H, ^{13}C, HMQC, and HMBC) were carried out at room temperature on a Bruker ARX 250 MHz spectrometer. Chemical shifts are reported in ppm (δ) relative to TMS, and coupling constants (J) are reported in Hz.

Synthesis of Hydroxylamines 2 and 4 and Amines 3 and 5. CB1954 was selectively reduced to hydroxylamines **2** and **4** by hydrogen using platinum oxide poisoned with triethylphosphite as a catalyst (*38*). CB1954 (50 mg, 0.2 mmol) was dissolved in 5.0 mL of ethyl acetate containing 20 μL of $P(OEt)_3$ and 5 mg of PtO_2. The reaction mixture was placed under H_2 atmosphere at room temperature until the H_2 uptake had ceased (about 5 h). The solution was filtered and concentrated under vacuum to give a mixture of 2- and 4-hydroxylamines, **2** and **4**, in a 3:1 ratio. These two compounds displayed identical UV, MS, and ^{1}H NMR spectra as previously described (*20*).

5-(Aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide, **2**: R_f 0.43 (SiO_2, CH_2Cl_2/ethanol 5:1); UV (H_2O/CH_3CN 1:1) λ_{max} 263 and

308 nm; ^{1}H NMR (d_6 acetone) 2.18 (m, 4H$_{aziridine}$), 7.41 (s, 1H, H$_{C6}$), 7.61 (s, 1H, H$_{C3}$), 7.66 (s, 1H, H$_{CONH_2}$), 8.19 (s, 1H, H$_{CONH_2}$), 8.81 (s, 1H, NHOH), 9.11 (s,1H, NHOH).

5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, **4**: R_f 0.38 (SiO_2, CH_2Cl_2/ethanol 5:1); UV/vis (H_2O/CH_3CN 1:1) λ_{max} 270 and 314 nm; ^{1}H NMR (d_6 acetone) 2.13 (m, 4H$_{aziridine}$), 6.92 (s, 1H, H$_{C6}$), 7.41 (s, 1H, H$_{C3}$), 7.45 (s, 1H, H$_{CONH_2}$), 7.84 (s, 1H, H$_{CONH_2}$), 8.50 (s,1H, NHOH), 8.81 (s, 1H, NHOH).

Reduction of CB1954 by H_2 using platinum oxide led to a mixture of amines **3** and **5**. CB1954 (50 mg, 0.2 mmol) was dissolved in 5.0 mL of ethyl acetate containing 5 mg PtO_2 and placed under H_2 atmosphere at room temperature until H_2 uptake had ceased (about 16 h). The reaction mixture was then filtered and concentrated under vacuum. As indicated by the ^{1}H NMR spectra of the mixture, 2- and 4-amines, **3** and **5**, were formed in a 5:1 ratio. Their properties were identical to those previously described (*20*).

5-(Aziridin-1-yl)-2-amino-4-nitrobenzamide, **3**: UV/vis (H_2O/CH_3CN 1:1) λ_{max} 262 and 310 nm; ^{1}H NMR (d_6 acetone) 2.18 (m, 4H$_{aziridine}$), 6.22 (s, 2H, H$_{CONH_2}$), 7.26 (s, 1H, H$_{C6}$), 7.41 (s, 1H, H$_{C3}$), 7.45 (s, 1H, H$_{CONH_2}$).

5-(Aziridin-1-yl)-4-amino-2-nitrobenzamide, **5**: UV/vis (H_2O/CH_3CN 1:1) λ_{max} 264 nm; ^{1}H NMR (d_6 acetone) 2.14 (m, 4H$_{aziridine}$), 5,29 (s, 1H, H$_{CONH_2}$), 5,35 (s, 1H, H$_{CONH_2}$), 6.97 (s, 1H, H$_{C6}$), 7.21 (s, 1H, H$_{C3}$).

Preparation of Recombinant NOSs. Recombinant full-length rat neuronal NOS was isolated and purified from BL21(DE3) *E. coli* strain transformed with plasmid pCWNOS I containing rat brain NOS. Growing of transformed *E. coli* and purification of full-length nNOS were performed following previously described protocols (*39*). Recombinant full-length murine inducible NOS, bovine endothelial NOS, the oxygenase domain of nNOS (nNOS$_{oxy}$), and the reductase domain of nNOS (nNOS$_{red}$) were overexpressed in *E. coli* and purified as previously described (*40–42*). The heme concentrations of the purified n-, i-, and eNOS were determined from the 444 nm absorbance of their ferrous-CO complexes using an extinction coefficient of 76 mM^{-1}·cm^{-1} (*40*). Their protein contents were measured using the Bradford reagent from Biorad and bovine serum albumin as a standard (*43*). They were more than 95% pure as judged by SDS−PAGE.

Anaerobic Incubations of CB1954 in the Presence of NOSs. Anaerobic incubations were performed in glass tubes previously purged with argon and stopped with a rubber septum. Protein samples were gently degassed by passing a slow flow of argon for 30 min at 4 °C at the surface of the samples. All the other compounds were bubbled with argon for 30 min at 4 °C. Typical incubation mixtures (final volume 500 μL) contained 500 μM CB1954, 1 mM $CaCl_2$, 100 μM BH_4, 10 μg/mL CaM, and 0.3–1.2 μM NOS in 50 mM Hepes buffer, pH 7.4, containing 1 mM DTT. The reactions were started by the addition of 1 mM NADPH, shaken at 37 °C for 30 min, and quenched by the addition of 100 μL of cold CH_3CN containing 100 μM phenol red (internal standard) and 1 mM ascorbic acid. The tubes were flushed with argon and centrifuged for 5 min at 10 000 rpm at 4 °C, and 25 μL aliquots of the supernatants were injected onto the HPLC system. Incubations in the presence of iNOS were performed similarly but $CaCl_2$ and CaM were omitted.

Reverse-Phase HPLC Separation of CB1954 Metabolites. Separations of CB1954 and metabolites were performed at room temperature on a 250 mm × 4.6 mm Hypersil MOS column (AIT, France) using a Thermo-Finnigan HPLC system. The mobile phase comprised a gradient of CH_3OH in 10 mM NaH_2PO_4, pH 7.0: 0–30% CH_3OH for 0–30 min, 30–100% CH_3OH for 30–40 min, followed by reequilibration in NaH_2PO_4 for 10 min. Flow rate was 0.8 mL/min. The absorbance was monitored at 330 and 270 nm and recorded with the Borwin data acquisition system. Calibration curves were made from mixtures containing various concentrations of hydroxylamines **2** and **4** and amines **3** and **5** in the presence of phenol red.

In some experiments, metabolites were further identified by online HPLC−mass spectrometry using a Surveyor ThermoQuest system coupled to a LCQ Advantage mass spectrometer fitted with an ESI source. Separations were performed at 35 °C on a 100 mm × 2 mm Nucleosil 100C18. The mobile phase comprised a mixture of solvent A (H_2O/CH_3CN/HCO_2H 97:2:1) and solvent B (CH_3CN/HCO_2H 98:2), and flow rate was 150 μL/min The ratios between solvents A and B were the following: 100% A for 15 min, 100–80% A in 10 min, 80–10% A in 5 min, then 10–100% A in 5 min, and reequilibration for 5 min. MS detection was done using the negative and positive modes and scanning in full scan mode (mass/charge, *m/z*, ratio from 100 to 500). Data were recorded and analyzed with the XCalibur acquisition system.

Aerobic Incubations of CB1954 in the Presence of *E. coli* Nitroreductase. Incubation mixtures (final volume 100 μL) contained 500 μM CB1954 and 10–25 μg of NTR in 50 mM Hepes, pH 7.4. The reactions were started by the addition of 1 mM NADPH, shaken at 37 °C for 10 min, and quenched by the addition of 100 μL of cold CH_3CN containing 100 μM phenol red and 1 mM ascorbic acid. The tubes were flushed with argon and centrifuged, and 25 μL aliquots of the supernatants were injected onto the HPLC system as described above.

Effects of CB1954 on the Activities of NOSs. [^{3}H]L-Citrulline formation by NOSs. NOS-dependent oxidation of [2,3,4-^{3}H]L-arginine to [2,3,4-^{3}H]L-citrulline was determined according to a previously described protocol (*44*). Briefly, enzymatic reactions were conducted at 37 °C for 5 min in 50 mM Hepes buffer, pH 7.4, containing 1 mM DTT, 1 mM NADPH, 1 mM $CaCl_2$, 10 μg/mL CaM, 20 μM BH_4, 4 μM FAD, 4 μM FMN, ∼500 000 cpm of [2,3,4-^{3}H]L-arginine, and 10 μM L-arginine. CB1954 was added to the incubation mixtures (final volumes 100 μL) as 5 μL portions dissolved in DMSO. Control incubations contained similar amounts (5%) of DMSO without CB1954. The reactions were started by the addition of NOS and terminated by the addition of 500 μL of cold stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA). Samples (500 μL) were applied to glass columns containing 1 mL of Dowex AG 50W-X8 (Na^+ form) pre-equilibrated with stop buffer, and a total of 1.5 mL of stop buffer was added to elute [2,3,4-^{3}H]L-citrulline. Aliquots were mixed with Pico-Fluor 40 and counted on a Packard Tri-Carb 2300 liquid scintillation spectrometer. Control samples without NOS or NADPH were included for background determinations. Incubations in the presence of iNOS were performed similarly, but $CaCl_2$ and CaM were omitted.

NADPH Oxidation by nNOS. The initial rates of NADPH oxidation by nNOS were quantitated by monitoring the decrease in absorbance at 340 nm on a Kontron 942 spectrophotometer using an extinction coefficient of 6.2 mM^{-1} cm^{-1}, as previously described (*6*). Cuvettes (total volume 150 μL) contained 1 mM DTT, 200 μM NADPH, 20 μM BH_4, 1 mM $CaCl_2$, 10 μg/mL CaM, and various concentrations of CB1954 in 50 mM Hepes, pH 7.4. Incubations were run for 4 min at 25 °C and were initiated by the addition of nNOS.

Cytochrome *c* Reduction by nNOS. The initial rates of flavin-dependent reduction of cyt *c* by nNOS were quantitated spectrophotometrically at 550 nm using an extinction coefficient of 21 mM^{-1} cm^{-1} (*6*). Cuvettes (total volume 150 μL) contained 50 mM Hepes buffer, pH 7.4 (without DTT), 100 μM NADPH, and 50 μM cyt *c*. When required, assays contained 1 mM $CaCl_2$, 10 μg/mL CaM, and various concentrations of CB1954.

Peroxynitrite Formation by nNOS. The effects of CB1954 on the generation of peroxynitrite by nNOS were evaluated spectrophometrically by using the oxidation of dihydrorhodamine 123 to rhodamine 123 as previously described (*45*). Assay mixtures contained 100 μM L-arginine, 2.5 μM BH_4, 100 μM NADPH, 100 μM DHR 123, and 10 U/mL catalase in 50 mM Hepes, pH 7.4 (final volume 150 μL). When required, 1 mM $CaCl_2$, 10 μg/mL CaM, and 0–500 μM CB1954 were added. The reactions were started by the addition of 0.2–0.5 μM nNOS to the sample cuvette, and the changes in absorbance at 500 nm were followed against control incubations performed without nNOS. Reactions were conducted at 25 °C for 10 min, and peroxynitrite formation was quantitated using an extinction coefficient of 78.8 mM^{-1}·cm^{-1} (*45*).

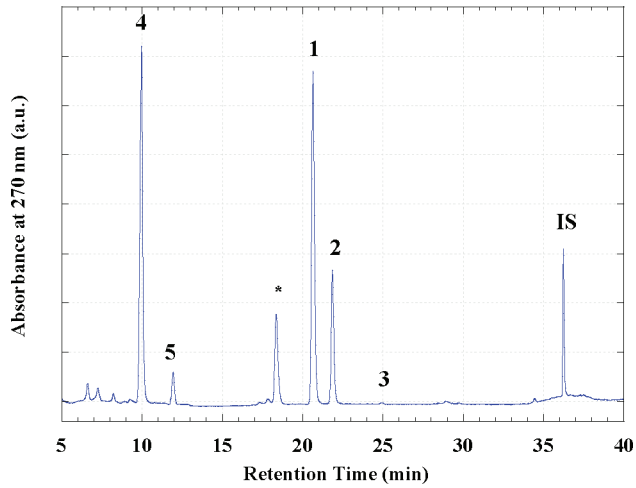


Figure 1. Products generated by the anaerobic reduction of CB1954, **1**, catalyzed by nNOS. The RP-HPLC profile was obtained from a typical anaerobic incubation of 500 μM CB1954 in the presence of 50 μg of nNOS, 1 mM $CaCl_2$, 100 μM BH_4, 1 mM DTT, and 5 μg of CaM in 50 mM deoxygenated Hepes buffer, pH 7.4. Following 2 min of preincubation, the reaction was started by the addition of 1 mM NADPH and stopped after 10 min by the addition of 100 μL of cold methanol containing 100 μM phenol red (IS) and 1 mM ascorbic acid. The resulting mixture was treated and analyzed on the RP-HPLC system as described under Materials and Methods. Elution times of the authentic compounds were CB1954 (**1**), 20.8 min; 4-hydroxylamine (**4**), 10.1 min; 4-amine (**5**), 12.0 min; 2-hydroxylamine (**2**), 22.0 min; 2-amine (**3**), 25.0 min; phenol red (IS), 36.4 min. The asterisk indicates an unidentified compound.

EPR Experiments. Room-temperature EPR experiments were performed on mixtures containing 0.8–1.5 μM nNOS, 500 μM CB1954, 1 mM $CaCl_2$, 10 μg/mL CaM, 100 μM BH_4, 1 mM NADPH, and 1 mM DTT in argon-purged 50 mM Hepes buffer, pH 7.4. The anaerobic reaction mixtures were transferred via a Teflon capillary tube to a Bruker Aqua-X cell inserted in a shq 0011 cavity (Bruker). EPR spectra were recorded on a Elexsys E 500 EPR spectrometer (Bruker) using the following instrument settings: microwave frequency, 9.82 GHz; field modulation frequency, 100 kHz; sampling time, 20 ms; field sweep, 10 mT; field modulation amplitude, 0.32 mT; microwave power, 10 mW. Thirty scans were recorded between 0 and 620 s.

Results

Metabolism of CB1954 Catalyzed by nNOS. When nNOS containing all its cofactors was incubated anaerobically with CB1954, three new peaks were clearly detected by HPLC (Figure 1). The first, predominant peak displayed the same retention time (10.2 min) and UV spectrum (λ_{max} = 270 nm) as authentic 4-hydroxylamine, **4**. LC/MS studies showed that this compound could be detected in the positive mode with a molecular ion $[M + H]^+$ at m/z = 239, indicating a molecular mass of 238 and confirming that it was indeed the 4-hydroxylamine, **4**. The second metabolite had the same characteristics as the authentic 4-amine, **5**, with retention time 12.0 min, λ_{max} = 265 nm, and $[M + H]^+$ at m/z = 223. The third metabolite displayed retention time 22.0 min, λ_{max} = 263 nm, and $[M + H]^+$ at m/z = 239, identical to those of an authentic sample of the hydroxylamine **2**. Finally, very minor amounts of the amine **3** were also identified by comparison with an authentic standard (retention time 25.0 min, λ_{max} = 262 and 310 nm, and $[M + H]^+$ at m/z = 223) but in too low amounts for quantitative measurements.

Comparison of the HPLC profiles obtained after anaerobic incubations of CB1954 in the presence of nNOS to those obtained after aerobic incubation of CB1954 in the presence of NTR, an enzyme that reduces CB1954 to equivalent amounts of **2** and **4** (*31*), confirmed that nNOS reduced CB1954 to **2** and **4** with predominant formation of the 4-hydroxylamine **4** (data not shown).

Table 1. Effects of Incubation Conditions on the Anaerobic Reduction of CB1954 Catalyzed by nNOS

conditions[a]	metabolites (**2** + **4**)[b] (% CS)
CS	100
−NOS	<0.1
−NADPH	1.9 ± 1.4
−NADPH + NADH	2.3 ± 0.8
+FAD + FMN (5 μM)	107.6 ± 37.4
−BH_4	116.0 ± 22.8
−CaM	40.1 ± 13.9
+O_2[c]	7.0 ± 5.0
+DPI (100 μM)	<0.1
+NMMA (1 mM)	91.0 ± 10.0
+NO2-Arg (1 mM)	129.8 ± 24.4
+SEITU (1 mM)	124.0 ± 14.8
+imidazole (10 mM)	98.2 ± 15.5
+CO[d]	115.5 ± 12.5

[a] The complete system (CS) contained 500 μM CB1954, 2 μM nNOS, 1 mM $CaCl_2$, 100 μM BH_4, and 10 μg of CaM in 50 mM deoxygenated Hepes buffer containing 1 mM DTT. The reactions were started by the addition of 1 mM NADPH and stopped after 10 min by the addition of 100 μL of cold stop mixture of deoxygenated acetonitrile/water 1:1 containing 1 mM ascorbic acid and 100 μM phenol red. The resulting mixtures were treated and analyzed by RP-HPLC as described under Materials and Methods. [b] Results are expressed as % of the maximal activity of reduction of CB1954 to the hydroxylamines **2** and **4** catalyzed by nNOS (4.0 ± 1.8 and 11.5 ± 3.5 nmol·min^{-1}·(mg protein)$^{-1}$, respectively) and are means ± SD from three independent experiments. Amines **3** and **5** were always formed in very low amounts and were not quantitated in these experiments. [c] Experiments were performed in aerated 50 mM Hepes buffer, pH 7.4. [d] Experiments were performed in 50 mM Hepes buffer, pH 7.4, previously deoxygenated by bubbling argon and then saturated by bubbling CO.

Because of possible further chemical oxidations of hydroxylamines **2** and **4**, such as the formation of nitrosoarenes (*23*), that could occur in incubates left for some time in the presence of air, incubation mixtures were stored under argon after addition of ascorbate. Assays for stability of authentic hydroxylamines **2** and **4** and amines **3** and **5** confirmed that these compounds were unaltered when stored in the presence of ascorbate and under an argon atmosphere (data not shown). Under these conditions, reproducible HPLC profiles such as those mentioned above were obtained.

Aerobic incubations of CB1954 with nNOS under conditions identical to those used previously for anaerobic incubations, except for the use of an aerated buffer, led to identical HPLC profiles with the major formation of **4** and **2** and minor formation of **5** and **3**. However, the rates of formation of these metabolites were much lower (7% of the rates observed under anaerobic conditions, Table 1), indicating that O_2 is an inhibitor of these reductions.

These data showed that nNOS can selectively reduce CB1954 to hydroxylamines **2** and **4** with minor formation of amines **3** and **5**, both under anaerobic and aerobic conditions, even though this activity is greatly decreased in the presence of dioxygen. Under both conditions, reduction of the 4-nitro group was predominant with a ratio of metabolites **4** + **5** to metabolites **2** + **3** (derived from reduction at position 2) close to 3.

Effects of Incubation Conditions on the Reduction of CB1954 Catalyzed by nNOS. The reduction of CB1954 to the hydroxylamines **2** and **4** was linearly dependent upon time (0–10 min) and upon the amounts of purified nNOS (up to 2.0 μM) (data not shown). Longer incubation times or higher amounts of proteins resulted in higher amounts of amines **3** and **5**,

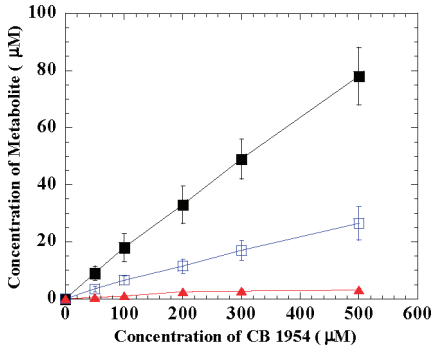


Figure 2. Effects of increasing concentration of CB1954 on the formation of 4-hydroxylamine, **4** (■), 2-hydroxylamine, **2** (□), and 4-amine **5** (▲) during the anaerobic reduction of CB1954 catalyzed by nNOS. The incubation mixtures contained 50–500 μM CB1954, 50 μg of nNOS, 1 mM $CaCl_2$, 100 μM BH_4, 1 mM DTT, and 5 μg of CaM in 50 mM deoxygenated Hepes buffer, pH 7.4. Following a 2 min preincubation, the reactions were started by the addition of 1 mM NADPH and stopped after 10 min by the addition of 100 μL of cold methanol containing 100 μM phenol red and 1 mM ascorbic acid. The reaction mixtures were treated and analyzed by RP-HPLC as described under Materials and Methods. Data represent means ± SD of three separate experiments.

although they remained minor products. Formation of metabolites **2** and **4** was dependent upon the concentration of CB1954 (up to 500 μM, due to the low solubility of CB1954 in buffer, Figure 2). The rates of formation of hydroxylamines **2** and **4** increased linearly with increasing CB1954 concentration, whereas formation of amine **5** was maximal at 250 μM. The **2**/**4** ratio remained unchanged as CB1954 concentrations changed, and very minor amounts of **3** could be detected. As observed previously (*20, 23, 24*), formation of amines **3** and **5** seems to arise from nonenzymatic reduction of hydroxylamines **2** and **4**.

The formation of hydroxylamines **2** and **4** required the presence of active nNOS and NADPH, whereas NADH was unable to support the reduction (Table 1). The reaction rates and the **4** to **2** ratios were affected neither by the addition of FMN, FAD, or both, all 5 μM, nor by the addition of usual NOS inhibitors such as L-arginine analogues (NMMA, NO2-Arg, or SEITU) and of the heme ligands imidazole and CO (Table 1). The reduction of a nitro group did not require the presence of BH_4, an obligatory cofactor for optimal NO synthesis from L-arginine but was stimulated 2- to 3-fold by the addition of CaM (Table 1). Finally, the reduction of CB1954 by nNOS was strongly inhibited by DPI, an inhibitor of flavin-dependent enzymes (Table 1). These results suggested that the reduction of CB1954 mainly occurred at the level of the reductase domain of nNOS.

Reduction of CB1954 Catalyzed by the Reductase Domain of nNOS. In order to clearly identify the site of nNOS responsible for the reduction of CB1954, anaerobic incubations were performed in the presence of either the purified oxygenase ($nNOS_{oxy}$) or the reductase domains of nNOS ($nNOS_{red}$). Neuronal NOS_{oxy} was inactive, whereas $nNOS_{red}$ catalyzed the reduction of CB1954 by NADPH to hydroxylamines **2** and **4** with an activity and a regioselectivity that matched those of full-length nNOS (14.2 ± 3.5 nmol•min^{-1}•(mg protein)$^{-1}$, **4**/**2** ratio = 3.3 ± 0.5, Table 2). As previously observed with full-length nNOS, the reduction of CB1954 by NADPH catalyzed by $nNOS_{red}$ was slown in the absence of CaM and insensitive to SEITU or NO2-Arg but inhibited by O_2 and DPI (data not shown). These results demonstrated that the nNOS-catalyzed reduction of CB1954 was dependent upon the flavin-containing reductase domain of nNOS.

Table 2. Anaerobic Reductions of CB1954 by NADPH Catalyzed by the Three NOSs and by $nNOS_{red}$ and $nNOS_{oxy}$

enzyme[a]	hydroxylamine **2**[b]	hydroxylamine **4**[b]	amine **5**[b]
nNOS	4.0 ± 1.8	11.5 ± 3.5	0.5 ± 0.08
iNOS	0.9 ± 0.3	8.7 ± 2.0	0.9 ± 0.05
eNOS	0.3 ± 0.1	2.1 ± 0.8	<0.05
$nNOS_{red}$	3.5 ± 1.2	10.7 ± 2.5	<0.05
$nNOS_{oxy}$	<0.05	<0.05	<0.05

[a] Conditions are as in Table 1, except that $CaCl_2$ and CaM were omitted with iNOS and $nNOS_{oxy}$. [b] Results are expressed in nmol•min^{-1}•(mg protein)$^{-1}$ and are means ± SD from three independent experiments.

Reduction of CB1954 Catalyzed by Inducible and Endothelial NOS. Under identical anaerobic conditions, iNOS and eNOS also reduced CB1954 to a mixture of hydroxylamines **2** and **4**, and very low amounts of amines **3** and **5**. Reductions of CB1954 catalyzed by i- and eNOSs were 1.5- and 7-fold less efficient than its reduction by nNOS (9.6 ± 2.5 and 2.4 ± 1.0 nmol•min^{-1}•(mg protein)$^{-1}$, respectively, Table 2). However, iNOS and eNOS were more regioselective toward the formation of metabolite **4** ((**4** + **5**)/**2** = 10 and 7, respectively, instead of 3 in the case of nNOS, Table 2). As previously observed with nNOS, reduction of CB1954 catalyzed by iNOS and eNOS required NADPH, was inhibited by O_2 or DPI, and was almost insensitive to the addition of the usual NOS inhibitors SEITU, NMMA, or NO2-Arg (data not shown).

Effect of CB1954 on the Activities of nNOS. We investigated the effects of increasing concentrations of CB1954 on the formation of citrulline and NO catalyzed by nNOS. In the absence of CB1954, the oxidation of radiolabeled L-arginine to citrulline catalyzed by nNOS occurred with initial rates of 280 ± 50 nmol•min^{-1}•(mg protein)$^{-1}$. The addition of increasing concentrations of CB1954 (up to 100 μM) had no significant effect on the formation of radiolabeled citrulline (data not shown). Then, we investigated whether CB1954 could modify electron transfer from NADPH to the heme. First, the rate of reduction of ferric cyt *c* to ferrous cyt *c* catalyzed by nNOS was not significantly modified by the addition of CB1954 (up to 100 μM, data not shown). Second, CB1954 (up to 100 μM) did not significantly increase the rate of NADPH consumption catalyzed by nNOS containing 100 μM L-arginine (Figure 3) or in the absence of L-arginine (data not shown). Finally, we investigated the effects of increasing concentrations of CB1954 on peroxynitrite ($ONOO^-$) formation by nNOS. Using the oxidation of DHR 123 to rhodamine 123 as a test for $ONOO^-$ (*45*), CB1954 (up to 100 μM) did not significantly affect the rates of rhodamine 123 formation (Figure 4). Under identical conditions, the addition of increasing amounts of 1,4-DNB to nNOS strongly increased NADPH oxidation rates (3-fold) and $ONOO^-$ formation (4-fold) catalyzed by nNOS, as previously described (Figures 3 and 4) (*15*). These activities were abolished by the addition of NO2-Arg, a potent inhibitor that blocks electron transfer from the reductase to the heme domain of NOS (*2, 3, 7*).

These experiments suggested that CB1954 was without significant effects on the electron transfer between the reductase and the oxygenase domains of nNOS. Further UV/visible spectroscopic studies showed that stepwise addition of CB1954 to either native $nNOS_{oxy}$ or $nNOS_{oxy}$ containing imidazole (*46*) did not change their absorption spectra, indicating that CB1954 interacted neither with the L-arginine binding site nor directly with the Fe^{III} atom of the heme (data not shown).

Generation of Free Radicals during the Reduction of CB1954 Catalyzed by nNOS. Introduction of an anaerobic

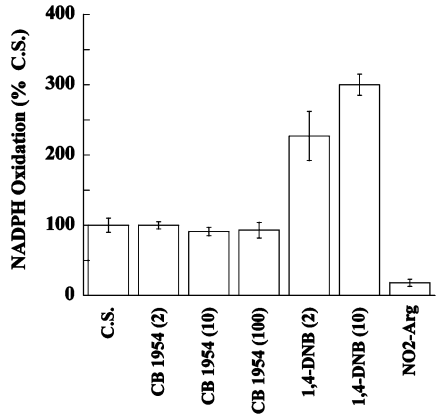


Figure 3. Effects of increasing concentrations of CB1954 and 1,4-DNB on the oxidation of NADPH catalyzed by nNOS. Complete system (CS) contained 1 mM DTT, 200 μM NADPH, 20 μM BH_4, 1 mM $CaCl_2$, 10 μg/mL CaM, and CB1954 (2, 10, or 100 μM), 1,4-DNB (2 or 10 μM), or NO2-Arg (100 μM) in 50 mM Hepes buffer, pH 7.4. The initial rates of NADPH oxidation were measured after the addition of 50–80 nM nNOS to the sample cuvette as described under Materials and Methods. Data are expressed as % of the CS (350 $\pm$ 80 nmol•min^{-1}•(mg protein)$^{-1}$) and are the mean $\pm$ SE of three independent experiments.

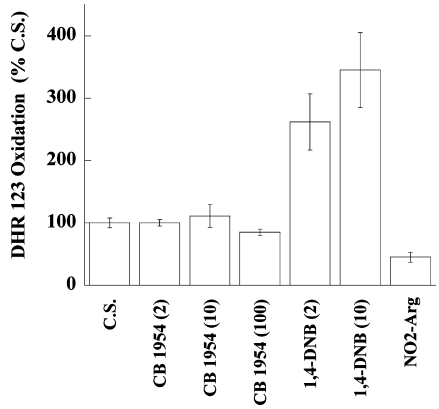


Figure 4. Effects of increasing concentrations of CB1954 and 1,4-DNB on the oxidation of DHR 123 catalyzed by nNOS. Complete system (CS) contained 100 μM L-arginine, 2.5 μM BH_4, 100 μM NADPH, 100 μM DHR 123, 10 U/mL catalase, and CB1954 (2, 10, or 100 μM), 1,4-DNB (2 or 10 μM), or NO2-Arg (100 μM) in 50 mM Hepes buffer, pH 7.4. The reactions were started by the addition of 100 nM nNOS to the sample cuvette, and the changes in absorbance at 500 nm were followed as described under Materials and Methods. Data are expressed as % of the CS (4.8 $\pm$ 1.5 nmol•min^{-1}•(mg protein)$^{-1}$) and are the mean $\pm$ SE of three independent experiments.

mixture containing purified nNOS, $CaCl_2$, BH_4, CaM, NADPH, and CB1954 into the Aqua-X cell fitted into the EPR-spectrometer cavity caused the appearance of a free radical species at *g* value close to 2.0, with a coupling constant of 1.33 mT and a line width 0.3 mT, consistent with spectra of a nitro-anion radical (Figure 5). Maximal intensity of the radical signal was reached about 5 min after the introduction of the mixture in the EPR cell, and a steady-state radical concentration was observed for about 10 min. The intensity of the signals decreased thereafter and became almost undetectable after 30 min (data not shown). Introduction of dioxygen in the incubation mixture abolished the EPR signals (data not shown). Anaerobic reduction of CB1954 catalyzed by nNOS in the absence of CaM resulted in the detection of the same signals but with slower rate and lower maximal intensity by comparison to incubations containing CaM. The CB1954-derived radical is stable enough to be detected without the use of a spin-trap, but it is not detected

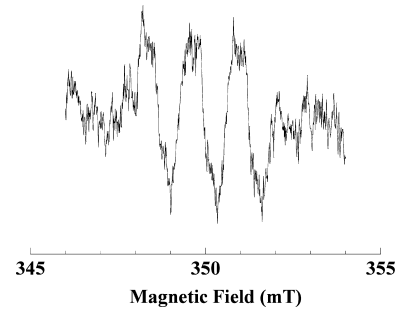
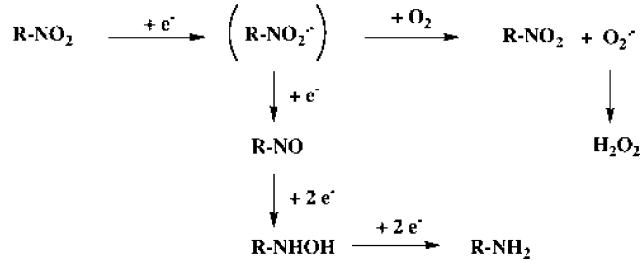


Figure 5. EPR spectra recorded during the anaerobic incubation of CB1954. Room-temperature EPR experiments were performed on anaerobic mixtures containing 500 μM CB1954, 1 mM NADPH, 10 μg/mL CaM, 1 mM $CaCl_2$, 100 μM BH_4, and 300 μg of nNOS in 50 mM Hepes buffer, pH 7.4. The anaerobic mixture was transferred to an Aqua-X cell, and the EPR spectra were recorded using the instrument settings indicated in Materials and Methods. The spectrum is the sum of 30 scans recorded between 0 and 620 s after the introduction of the mixture in the cell.

Scheme 2. Possible Reactions Occurring during the Reduction of Nitroaromatic Compounds



under aerobic conditions, a result attributed to the fast oxidation of the nitro-anion radical by O_2 that regenerates the parent nitro compound **1** with concomitant formation of the superoxide anion (Scheme 2). The presence of two nitro groups that could lead to distinct radical species could explain the complex features and low intensity of the EPR spectra. HPLC analysis of the incubation mixtures following the EPR experiments showed that there was a direct relationship between the detection of the EPR radical signal and the formation of metabolites **2** and **4**.

Discussion

Many proteins such as NQO, cytochrome P450, cytochrome P450 reductase, xanthine oxidoreductase, or cytochrome b_5 reductase are able to catalyze the reduction of nitroaromatic compounds (*47*). The present study shows that purified n-, i-, and eNOS catalyze the reduction of the antitumor agent CB1954 to hydroxylamines **2** and **4** with intermediate formation of a nitro-anion radical (*47, 48*). This reduction requires NADPH and is strongly inhibited by O_2 and DPI, a NADPH−cytochrome P450 reductase inhibitor. It thus displays similarities with previously known dioxygen-sensitive flavin-dependent class II nitroreductases (*47, 48*). The reduction of CB1954 catalyzed by NOSs involves the reductase domain of these proteins as shown by experiments using purified $nNOS_{red}$ and by the stimulating effects of CaM, a coenzyme of nNOS that increases electron transfer rates within the reductase domain itself (Tables 1 and 2) (*6, 7*). By contrast, the heme ligands, imidazole and CO, and the L-arginine analogues, NMMA, NO2-Arg, and SEITU, that are usual inhibitors of NO formation are unable to inhibit the reaction (Table 1). Differences in the rates of reduction of CB1954 by n-, i-, and eNOSs may reflect the rates of electron transfers within the reductase domains themselves, the $nNOS_{red}$ and $eNOS_{red}$ being the most and least active ones, respectively (*3, 4, 49*).

Our results show that full-length n-, i-, and eNOS and the reductase domain $nNOS_{red}$ catalyze the reduction of CB1954 by NADPH to hydroxylamines **2** and **4** with very minor formation of amines **3** and **5**. Reduction of CB1954 by the three NOS isoforms occurs with a regioselectivity largely in favor of hydroxylamine **4**. Differences in the relative amounts of reduction at position 2 or 4 by the three isoforms could be linked to differences in the structures of their reductase domains (*50, 51*). Distinct regulatory loops are identified in the reductase domains of NOSs and could affect the selectivity of the reduction, either by steric constraints leading to differences in binding of CB1954 close to the FAD site as previously observed with NTR (*52*) or by modulating the rate of electron transfers and the relative reactivity of each nitro group.

The ability of NOSs to transfer electrons to xenobiotics such as quinones (*10–14*) or nitroaromatics (*15, 17*) via their reductase domain has been previously reported. A feature common to such compounds undergoing reduction with formation of relatively stable anion radical is their ability to shuttle back and forth between the reduced (anion radical, $R\text{-}NO_2^{\bullet -}$) and native oxidized state ($R\text{-}NO_2$) (*47, 48, 53*). During this futile cycle, a flow of electrons can be transferred to dioxygen thus generating superoxide anion (Scheme 2). Previous studies have shown that when certain redox-active compounds such as quinones (*10–14*) and DNBs (*15*) are incubated in the presence of NOS containing NADPH and L-arginine, $O_2^{\bullet -}$ could be produced at the same time as L-citrulline and NO. NO and $O_2^{\bullet -}$ can then combine to produce $ONOO^-$ at a near-diffusion-limited rate (*16*). As previously shown in stopped-flow kinetic studies, the rate of electron flux through the nNOS reductase is more than sufficient to support both NO and L-citrulline production, as well as simultaneous electron transfer to some exogenous electron acceptors (*54*). Low concentrations of 1,4-DNB induce an increase in nNOS activity with a shift in product formation from L-citrulline and NO to L-citrulline and $ONOO^-$ (*15*). This originates from a redox-cycling by the nitro compound that leads to superoxide anion and also from the particular ability of 1,4-DNB to stimulate NADPH oxidation by yet unknown mechanism(s). 1,4-DNB increases the electron flux through the reductase domain of nNOS to its oxygenase domain and thus increases the rate of formation of L-citrulline and NO. Superoxide and NO then combine and form high amounts of $ONOO^-$. Our results show that CB1954 behaves in a manner distinct from that observed for 1,4-DNB. CB1954 has no significant effect on the rates of L-citrulline formation, NADPH oxidation, and $ONOO^-$ generation catalyzed by nNOS. This could originate from differences in the redox potentials of CB1954 and 1,4-DNB or in their mode(s) of interaction(s) with the protein. The electronic properties and the position of the substituents on the benzene ring also greatly influence the reactivity and product formation from nitroaromatic compounds. In the case of nNOS, it has been observed that 1,4-DNB more efficiently stimulated NADPH consumption than 1,2- and 1,3-DNB (*15*).

Actually, this article reports the second example of metabolic reduction of a xenobiotic by NOSs with a complete identification of the resulting metabolites. Thus, two different drugs bearing nitroaromatic groups, nilutamide (*17*) and CB1954 (this work), are efficiently reduced by NOSs with formation of the corresponding hydroxylamines. The data concerning nilutamide reduction (*17*) suggested a possible role of NOSs in toxicology, via the formation of an arylhydroxylamine as reactive and toxic metabolite, whereas the present data about CB1954 suggest a possible beneficial role of NOSs in the activation of antitumor drugs to reactive metabolites highly cytotoxic for tumor cells.

The relative importance of the contribution of NOSs in the metabolism of CB1954 *in vivo* remains to be determined. Metabolism of CB1954 by human liver subcellular fractions involves the reduction of its nitro substituents on positions 4 and 2, with a regioselectivity largely in favor of the reduction of the $4\text{-}NO_2$ group ($4\text{-}NO_2/2\text{-}NO_2$ reduction close to 8) (*34*). Reduction of CB1954 to amines **3** and **5** by mouse liver microsomes also occurs with a very similar regioselectivity (*35*). Oxygen-insensitive reductases should play an important role in the metabolism of CB1954 in human normoxic tissues. Under these aerobic conditions, the contribution of NOSs to CB1954 metabolic reduction should be weak as O_2 inhibits NOS-catalyzed CB1954 reduction (Table 1), even though reduction of CB1954 by NOSs under aerobic conditions leads to a metabolic pattern almost identical to that observed under anaerobic conditions. However, the relative contributions of NOSs in metabolic reduction of CB1954 could be more important in physiologically hypoxic zones, such as the centrolobular regions of human liver (*34*). Inducible NOS, which produces a regioselectivity of CB1954 reduction ($4\text{-}NO_2/2\text{-}NO_2$ reduction around 10, Table 2) close to that observed in human liver subcellular fractions ($4\text{-}NO_2/2\text{-}NO_2$ reduction around 8 (*34*)), could be the main isoform involved in this case. The relative contribution of NOSs could also be much more important in pathological situations resulting in severe hypoxia, as in tumors. In hypoxic tumoral tissues, iNOS, which is often overexpressed in tumors, could play a role in the bioactivation of CB1954 to cytotoxic hydroxylamines. This hypothesis is currently under study with tumor cell lines in which active NOS are highly expressed.

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