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INDQ/NO, a Bioreductively Activated Nitric Oxide Prodrug

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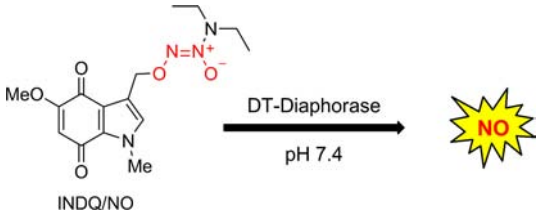
ABSTRACT



The design, synthesis, and development of INDQ/NO, a novel nitric oxide (NO) prodrug targeted by a bioreductive trigger, are described. INDQ/NO, an indolequinone-diazeniumdiolate is found to be metabolized to produce NO by DT-diaphorase, a bioreductive enzyme that is overexpressed in certain cancers and hypoxic tumors. Cell-based assays revealed that INDQ/NO induces DNA damage and is a potent inhibitor of cancer cell proliferation.

Numerous studies demonstrate the efficacy of nitric oxide (NO) as a potent tumoristatic agent.[1] However, due to its multifarious biological effects, controlled and localized generation of therapeutic NO using prodrugs is necessary.[2] Among various available methods for generation of NO under physiological conditions, diazeniumdiolates are highly suited for site-directed delivery of therapeutic NO.[3] These NO donors can be derivatized into stable protected forms; activation of these prodrugs by a suitable metabolic trigger, typically an enzyme that is overexpressed in certain tissues, produces NO in a localized manner.

NAD(P)H:quinone oxidoreductase (DT-diaphorase; DT-D) has received considerable attention for prodrug development, as this enzyme is overexpressed in numerous cancers as compared with the paired normal tissue as well as hypoxic (low oxygen tension) regions of solid tumors.[4] Although prodrug methodologies for numerous cytotoxic species are known,[5] a candidate for DT-diaphorase-activated

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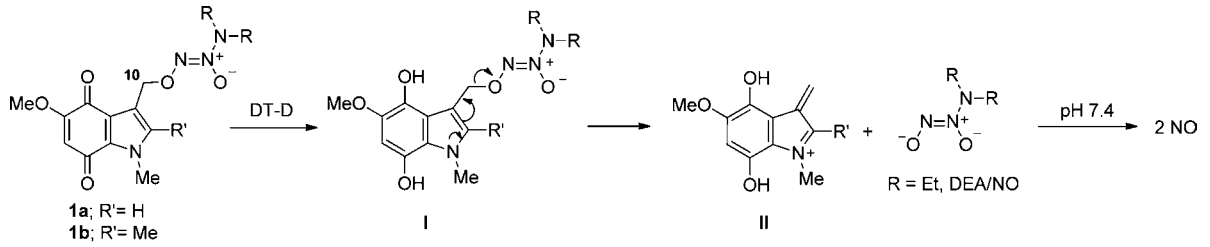
Scheme 1. Design of a DT-Diaphorase (DT-D)-Activated Nitric Oxide (NO) Prodrug



delivery of NO is not yet available. Here, we proposed to develop a new nitric oxide prodrug that is selective toward activation by DT-D to produce NO.

DT-D is an obligate two-electron reductase, and bioreductively activated prodrug candidates by placing a suitable leaving group on an appropriate indolequinone scaffold are in development.[5] It was envisaged that a diazeniumdiolate placed in the 10-position of a 4,7-dioxoindole might be well suited for nitric oxide delivery (Scheme 1). Bioreduction of this indolequinone-based diazeniumdiolate by DT-D produces a 4,7-dihydroxyindole intermediate (**I**, Scheme 1). As a consequence of this transformation, the nitrogen which was previously under the influence of an electron-withdrawing quinone group is now conjugated with two electron-donating hydroxy groups. Thus, the lone pair on the indole nitrogen becomes available for a rearrangement to release a diazeniumdiolate anion, which is expected to dissociate in physiological pH to form NO and **II**, which is capable of alkylating DNA (Scheme 1).[5]

In order to test this hypothesis, INDQ/NO, **1a**, was synthesized in eight steps starting from 5-methoxyindole **2a** (Scheme 2). Briefly, formylation of **2a** produced **3a** in quantitative yield; methylation of **3a** using methyl iodide in the presence of sodium hydride gave **4a** in 100% yield. Nitration of **4a** using conc. HNO_3 in acetic acid gave **5a** in 90% yield. Reduction of the nitro group of **5a** using Sn/HCl produced the amine **6a** followed by oxidation using Fremy's salt gave **7a**, which was subsequently reduced using $NaBH_4$ to afford **8a** in 55% yield. In order to install a diazeniumdiolate at the 10-position, we converted the alcohol **8a** into the bromide **9a** using PBr_3 in DCM. The bromide **9a** (crude) was reacted soon after preparation with sodium *N*,*N*-(diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) in THF in the presence of 15-crown-5 to give INDQ/NO, **1a**, in 10% yield.

Next, HPLC analysis was used to monitor the decomposition of **1a** in the presence of DT-D, NADPH in pH 7.4 buffer. We found that, within 5 min, a significant amount of **1a** was decomposed in the presence of DT-D and, in 150 min, nearly 80% of the compound was consumed (Figure 1a). During the same time period, in the absence of DT-D, **1a** remained stable in pH 7.4 buffer.[6]

Nitric oxide produced during incubation of **1a** in pH 7.4 was assayed using a chemiluminescence-based assay for NO (Figure 1b).[7] During incubation of **1a** (50 μM) in the presence of DT-D, a rapid increase in NO within 5 min was observed and the yield of NO was nearly 30 μM for 3 h.[8] In the absence of DT-D, negligible NO was produced during this time period (Figure 1b). Taken together, our data are indicative of **1a** undergoing selective decomposition under bioreductive conditions in the presence of DT-D to produce NO.

DT-D is overexpressed in several colon cancers including DLD-1 human adenocarcinoma cells.[9] In order to test whether **1a** was a source of NO upon incubation with DLD-1 cells, extracellular nitrite produced during incubation of DLD-1 cells with **1a** during 5 h was evaluated. We found that, in the absence of **1a**, negligible amounts of nitrite were produced during this time period (Figure 1c), but upon incubation of DLD-1 cells with **1a** (50 μM), we found a significant increase in the amount of nitrite produced, implying that NO was liberated (Figure 1c).

Reactive nitrogen species including NO are known to damage biomacromolecules such as DNA leading to double strand breaks; the inability of the cell to completely repair the damage is partially responsible for cell death.[12] An early specific cellular response to double strand breaks includes phosphorylation of the histone protein H2AX at Ser139 (γ-H2AX).[1f] The formation of foci is an essential step for signaling the recruitment of appropriate repair enzymes. We next examined whether INDQ/NO treatment induced DNA double strand breaks, as detected by γ-H2AX foci formation in HeLa cervical cancer cells. Immunofluorescence analysis (Figure 2a) of HeLa cells treated with **1a** during 1.5 h showed that INDQ/NO induces significant and dose-dependent γ-H2AX foci formation as early as 1.5 h after INDQ/NO exposure (Figure 2b). These observations are consistent with a previous report on the nitric oxide prodrug JS-K, which induced significant H2AX phosphorylation at early time points.[1f]

(6) The decomposition pattern of **1a** in pH 7.4 was nearly identical in the presence of gluathione suggesting that **1a** was not highly reactive with thiols.

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(8) Under the assay conditions any nitrite that is produced during decomposition is also measured.

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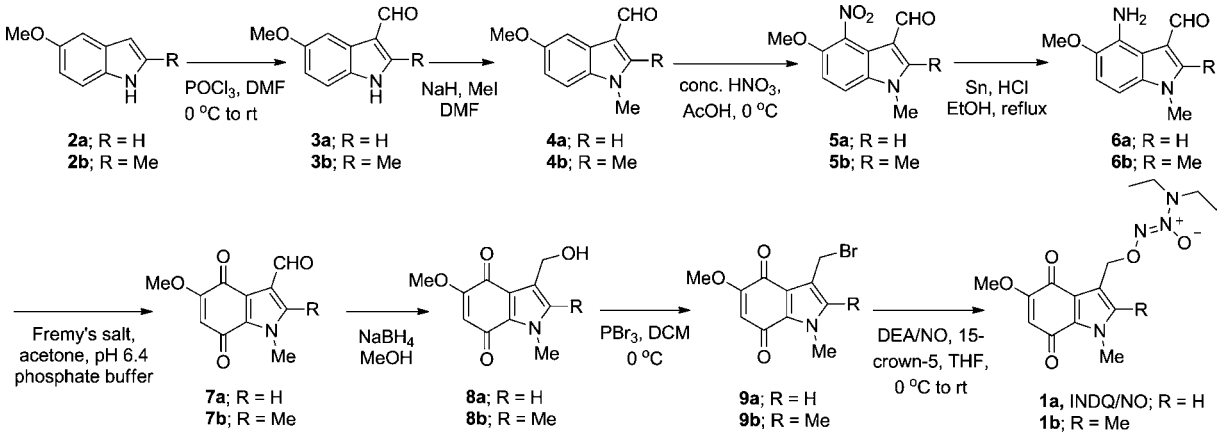
Scheme 2. Synthesis of Nitric Oxide Prodrug Candidates **1a** and **1b**



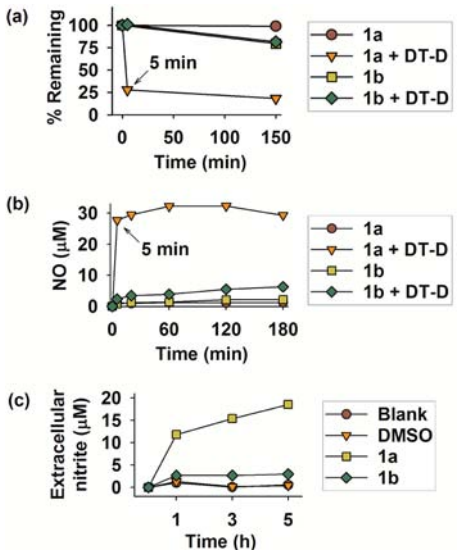


Figure 1. (a) Decomposition profiles of **1a** and **1b** in pH 7.4 in the presence and absence of DT-D were determined by HPLC analysis of reaction mixtures. (b) Nitric oxide release profiles of **1a** and **1b** in pH 7.4 in the presence and absence of DT-D were analyzed by chemiluminescence-based assay for NO.[10] (c) Nitric oxide released during independent incubation of **1a** and **1b** with cultured DLD-1 human adenocarcinoma cells for 5 h was estimated by analysis of an aliquot of extracellular medium using a chemiluminescence-based assay.[11]

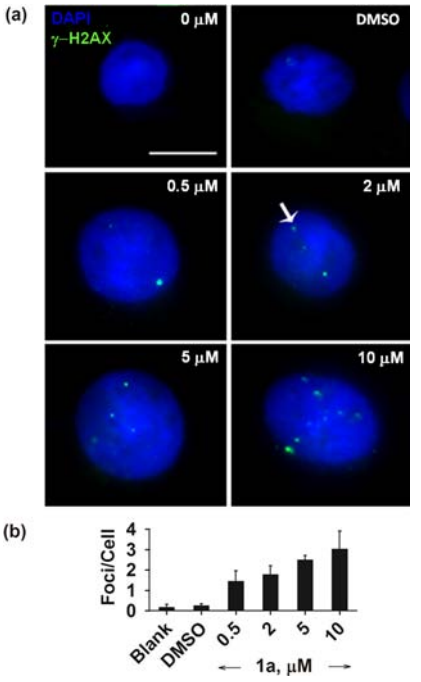


Figure 2. (a) Representative images of Hela cells labeled with anti-γ-H2AX antibody in immunofluorescence assays after treatement with **1a** for 1.5 h. Nuclei were counterstained with DAPI. Arrow indicates γ-H2AX foci (Scale bar: $\sim$5 μm). (b) Average foci per HeLa nucleus upon treatment with increasing concentrations of **1a**.

The efficacy of **1a** to inhibit proliferation of DLD-1 cells was evaluated using a standard cell viability assay after incubation for 72 h. We found **1a** to be a potent inhibitor of proliferation of DLD-1 cells (Figure 3a), and its IC_{50} was found as 0.25 μM (Figure 3b). A similar potent antiproliferative activity of INDQ/NO was found when tested against human urinary bladder carcinoma T-24 (IC_{50} = 0.56 μM) and HeLa human cervical cancer (IC_{50} = 0.96 μM) cells both known to overexpress DT-D.[13]

Next, in order to study the effect of introduction of a methyl group on the stability of 4,7-dioxoindole-based

(10) The yield of NO was 30% (assuming DEA/NO produces 2 mol NO/mol) suggesting that NO was trapped before it could be detected.

(11) An aliquot of the reaction mixture is treated with an aqueous solution of NaI and acetic acid, which converts nitrite to NO (which is quantified by a chemiluminescence detector).

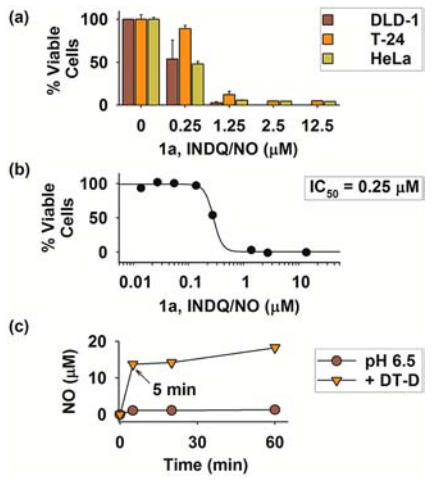


Figure 3. (a) Cancer cell proliferation inhibitory activity of **1a** was determined using a standard cell viability assay against DLD-1 human adenocarcinoma, T-24 human renal carcinoma, and HeLa human cervical carcinoma cells. (b) IC_{50} of **1a** against DLD-1 cells was obtained by curve fitting (R^2 = 0.996, $P < 0.0001$) as 0.25 μM. (c) Nitric oxide release profile of **1a** in pH 6.5 in the presence and absence of DT-D was analyzed by a chemiluminescence-based assay for NO.

diazeniumdiolate, we synthesized **1b** from **2b** (Scheme 2). In comparison with **1a**, the analogue **1b** was a poor substrate for metabolism by DT-D (Figure 1a); did not produce significant amounts of NO in the presence of DT-D (Figure 1b); and generated diminished amounts of nitrite during incubation with DLD-1 cells (Figure 1c). Diminished decomposition of **1b** by DT-D in comparison with **1a** is consistent with previous reports which indicate that introduction of a substituent at the 2-position of the 4,7-dioxoindole scaffold resulted in a slower DT-D metabolism rate.[14] A cell viability assay showed that **1b** was a good inhibitor of proliferation of DLD-1 cells with an IC_{50} of 3.0 μM, but this was 10-fold higher than the IC_{50} for **1a** (0.25 μM, Figure 3b). When tested against T-24 renal carcinoma cells, the IC_{50} of **1b** was 9.4 μM, again, diminished in comparison with **1a**. Together, these results suggest that the propensity to undergo metabolism by DT-D to produce NO played a role in the observed inhibitory potency of **1a**. Further evidence for NO-mediated cytotoxic effects was obtained when indolequinones **8a** and **8b**, which were not sources of NO, were found to have diminished inhibitory potency against DLD-1 cells (see Supporting Information, Table S4).[15]

DT-D forms the basis for targeted delivery of a number of bioreductive prodrugs of cytotoxic species, including DNA alkylating agents, which are in development for use in targeting hypoxic tumors.[4] Approximately one-third of human tumors contain areas with hypoxia in comparison with normal cells.[4] As oxygen is a potent radiosensitizer, hypoxic tumors are often less responsive to radiation treatment.[16] Due to a diminished and chaotic blood supply, hypoxic areas are poorly accessible to cancer drugs and hence chemotherapeutics also have diminished efficacy in shrinking hypoxic tumors.[4e] Together, these effects lead to higher levels of treatment failures in tumors with significant areas of hypoxia. NO is also a chemical radiosensitizer and has been shown to synergize with radiation to promote apoptosis in hypoxic cells.[17] Furthermore, due to its highly diffusible nature, NO can penetrate cells leading to enhancement in cytotoxic effects.[18] As hypoxic regions are acidic in nature, we evaluated the ability of **1a** to produce NO in pH 6.5. We found that **1a** was capable of producing NO in the presence of DT-D in pH 6.5 suggesting that INDQ/NO may have potential in targeting hypoxic tumors as well (Figure 3c).

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Supporting Information Available. Preparative procedures, spectral characterization data, and assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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(15) 3-(Acetoxymethyl)-5-methoxy-1,2-dimethyl-1*H*-indole-4,7-dione has been previously reported to inhibit proliferation of colon cancer cells suggesting that the indole-methide intermediate II (Scheme 1), which is produced upon production of NO, might contribute to the cytotoxicity of **1a**. See ref 5g.

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