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Nitroreductase-activated nitric oxide (NO) prodrugs

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ABSTRACT

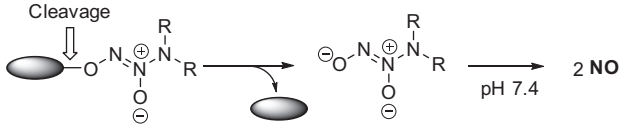
Due to the involvement of nitric oxide (NO) in numerous and diverse physiological processes, site-directed delivery of therapeutic NO in order to minimize unwanted side-effects is necessary. O^2-(4-Nitrobenzyl) diazeniumdiolates are designed as substrates for *Escherichia coli* nitroreductase (NTR), an enzyme that is frequently used to facilitate directed delivery of cytotoxic species to cancers. O^2-(4-Nitrobenzyl) diazeniumdiolates are found to be stable in aqueous buffer but are metabolized by NTR to produce NO. A cell viability assay revealed that cytotoxic effects of O^2-(4-nitrobenzyl)1-(2-methylpiperidin-1-yl)diazen-1-ium-1,2-diolate (**4b**) towards two cancer cell lines is significantly enhanced in the presence of NTR suggesting the potential for use of this compound in nitric oxide-based directed prodrug therapy.

Nitric oxide (NO) mediates numerous physiological processes including vasodilation, neurotransmission and immune response.[1] At elevated concentrations NO can damage biomacromolecules such as proteins, DNA and lipids and can cause cell death by triggering apoptosis.[2] Nitric oxide has also been considered as a potential cancer therapeutic agent.[3–5] However, due to its involvement in a myriad of biological processes, delivery of NO must be localized at the tumour site.[3]

Due to the limited utility of NO gas, typically, nitric oxide 'donors' are used as surrogates for NO in biological studies.[6] NO donors are compounds that are otherwise stable but when exposed to physiological conditions dissociate to produce NO. Among the available NO donors, diazeniumdiolates are routinely used in biological studies as reliable sources of NO (Scheme 1).[5,7] These NO donors are also highly suited for site-directed delivery of NO. Diazeniumdiolate anions can be derivatized using appropriate functional groups into stable compounds.[7] The protective group is chosen so that the stable diazeniumdiolate derivative is cleaved in the presence of an enzyme to produce a diazeniumdiolate anion, which dissociates at pH 7.4 to produce NO (Scheme 1). Depending on the choice of the enzyme and its distribution in tissues, these protected diazeniumdiolates can be optimized for localized delivery of NO. Some notable examples of triggers are glutathione/glutathione *S*-transferases, glycosidases, esterases, DT-diaphorase[8] and cytochromes P450.[7,9–11] Together, these compounds form a cohort of metabolically-activated NO prodrugs with potential for application as therapeutic agents against numerous diseases including cancer.[4,5,10]

Escherichia coli nitroreductase (NTR) is an enzyme that is frequently used as a metabolic trigger for 'directed' prodrugs including gene-directed enzyme prodrug therapy (GDEPT) and antibody-directed enzyme prodrug therapy (ADEPT).[12–14] As NTR is not usually found in human cells, this enzyme is introduced either by transfection methodologies (for GDEPT)[15] or by the use of tumour-specific antigens conjugated to the enzyme (for ADEPT). Upon exposure to the exogenous enzyme, the inactive prodrug, which is a substrate for the enzyme is metabolized to produce the cytotoxic species either intracellularly or in the proximity of tumours. As normal cells do not express this enzyme, potential deleterious side-effects can be minimized.[16] NTR-activated cancer drugs and cytotoxic species including DNA alkylating agents are previously reported.[17] However, a NTR-activated nitric oxide prodrug is yet unavailable. Here, we report our results of design, synthesis and evaluation of NTR-activated NO donor candidates which have potential for applications in directed or targeted prodrug therapy.

A typical NTR-activated prodrug consists of a 4-nitrobenzyl group attached to a leaving group such as a phosphoramide or a



Scheme 1. Diazeniumdiolate-based prodrugs of nitric oxide that can be used for enzyme-activated delivery of NO.

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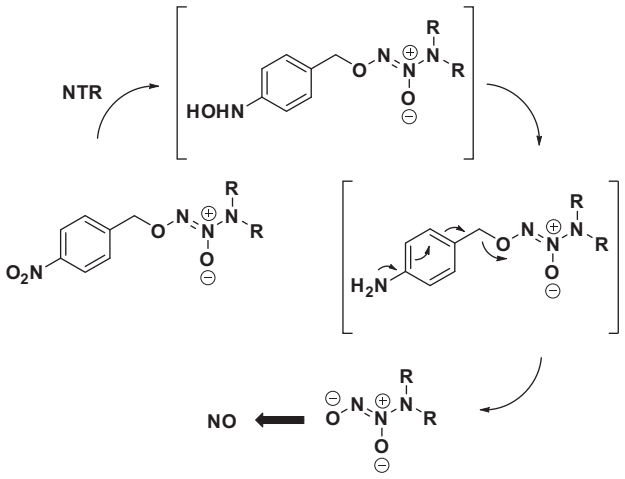

Scheme 2. Design of O^2-(4-nitrobenzyl)diazeniumdiolates as NTR-activated prodrugs of nitric oxide.

Table 1
Reported half-lives of nitric oxide release of **1a–4a** and synthesis of O^2-(4-nitrobenzyl) diazeniumdiolates **1b–4b**[19]

R^1NR^2	Diazeniumdiolate	$t_{1/2}$[a]	Product	Yield[b]
Me_2N	DMA/NO, **1a**	24 s	**1b**	9
Et_2N	DEA/NO, **2a**	2 min	**2b**	15
Pyrrolidine	PYRRO/NO, **3a**	2 s	**3b**	16
2-Me-piperidine	**4a**	8.3 min	**4b**	25

[a] Reported half-lives of nitric oxide release upon decomposition of the anion in pH 7.4 buffer (See Ref. 19).
[b] Isolated yield in %.

carboxylate. As diazeniumdiolate anions resemble carboxylate ions in their leaving group ability, we proposed O^2-(4-nitrobenzyl) diazeniumdiolates as candidates for NTR activation (Scheme 2).[18] Reduction of the nitro group by NTR produces a hydroxylamine/amine; this transformation converts an electron-withdrawing nitro group into an electron donating group. Rearrangement of electrons would be expected to produce a NO-releasing diazeniumdiolate anion.

O^2-(4-Nitrobenzyl) diazeniumdiolates **1b–4b** were prepared by treatment of the corresponding diazeniumdiolate anion **1a–4a**[19] with 4-nitrobenzyl bromide (Table 1).[20] The NO donors **1a–4a** were chosen in part due to a good range in half-lives of NO release from 2 s to 8.3 min (Table 1).

In order to assess the suitability of the nitro group for reduction, cyclic voltammetric (CV) analysis of **1b–4b** was conducted to determine reduction potentials. 4-Nitrotoluene was used as a reference for nitro-group reduction and a $1-e^-$ reduction potential (E_{red}) of −0.11 V was recorded (Table 2). E_{red} of **1b–4b** was found to be nearly identical suggesting that the diazeniumdiolate group did not significantly affect the propensity for the nitro group to undergo reduction (Table 2). Next, in order to test whether O^2-(4-nitrobenzyl) diazeniumdiolates were substrates for NTR, **1b–4b** were independently treated with NTR in the presence of NADPH and decomposition was studied using HPLC.[21] During 1 h, we found nearly 60% of **1b** and **2b** remained while in the cases of

Table 2
Cyclic voltammetry analysis and NTR-mediated decomposition and nitric oxide release profiles

Entry	Compd	E_{red} (V)[a]	Remaining[b] (%)	NO yield[c] (μM)
1	**1b**	−0.09	60	13.6
2	**2b**	−0.11	60	6.8
3	**3b**	−0.11	28	18.3
4	**4b**	−0.11	31	19.7
5	**5b**	−0.14	68	1.1

[a] One electron reduction potential measured by cyclic voltammetry. Conditions: Pt disc working electrode; Pt wire auxiliary electrode; $Ag/AgNO_3$ reference electrode; scan rate = 25 mV/s; NBu_4PF_6 = 100 mM as the background electrolyte in MeCN. E_{red} for 4-nitrotoluene was −0.11 V.
[b] Decomposition of **1b–4b** (50 μM) in the presence of nitroreductase (NTR) in pH 7.0 buffer after 1 h was estimated by HPLC.
[c] Nitric oxide released during decomposition of **1b–5b** (50 μM) in the presence of nitroreductase (NTR) in pH 7.0 buffer after 1 h measured using a chemiluminescence assay.

Scheme 3. Synthesis of **5b**. EDC: *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; DMAP: 4-Dimethylaminopyridine.

the cyclic derivatives **3b** and **4b**, 28 and 31% of compound remained during 1 h (Table 2, entries 1–4). It appears that the cyclic compounds **3b** and **4b** were better substrates for NTR in comparison with **1b** and **2b**. During this time period, no significant decomposition of **1b–4b** was observed in the absence of NTR suggesting that these compounds were not candidates for hydrolysis in pH 7.0 buffer and the decomposition must be due to metabolism by NTR.[22]

Nitric oxide produced during decomposition of **1b–4b** was measured using a chemiluminescence-based assay for NO (Table 2, entries 1–4).[21] Compounds **1b–4b** were reacted with NTR in the presence of NADPH as a co-factor in pH 7.0 buffer. An aliquot was taken after 1 h for nitric oxide analysis. Among O^2-(4-nitrobenzyl) diazeniumdiolates tested, **3b** and **4b** were found to be the best sources of NO with nearly 20 μM generated in 1 h followed by **1b** and then **2b** (Table 2). In the absence of NTR, we found negligible amounts of NO, again, indicating the selectivity of O^2-(4-nitrobenzyl) diazeniumdiolates towards activation by NTR.

Next, in order to study if the nitro group on the aryl group was a source of NO, 4-nitrobenzyl benzoate (**5b**)[23] was prepared by reaction of 4-nitrobenzyl alcohol with benzoic acid (Scheme 3). This compound is expected to undergo decomposition in the presence of NTR but without generating NO. Indeed, **5b** was metabolized by NTR with 68% remaining after 1 h and analysis of reaction mixtures of **5b** in the presence and absence of NTR showed negligible NO formation during decomposition of this compound suggesting that the nitro group on the aromatic ring itself was not was not a significant source of NO (Table 2, entry 5).[24,25]

Thus, amongst O^2-(4-nitrobenzyl)diazeniumdiolates synthesized in this study, **3b** and **4b** were the best sources of NO (Table 2, entries 3–4). This observation is also consistent with HPLC data on decomposition of **3b** and **4b** that these compounds were also the best substrates for NTR amongst **1b–4b** (Table 2, entries 1–4). Next, a cell viability assay was conducted to study the selectivity of **1b–4b** in inhibiting proliferation of DLD-1 human colon adenocarcinoma cells. Our initial assessment was conducted at an elevated concentration of 75 μM and we found moderate inhibitory activity

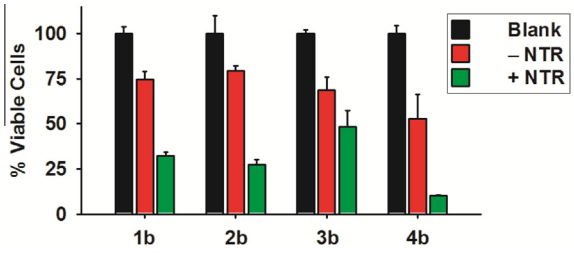

Figure 1. Cell viability assay to assess anti-proliferative activity of **1b**–**4b** at 75 μM in the presence and absence of NTR conducted with DLD-1 human adenocarcinoma cells. Blank is untreated cells.

for all compounds in the absence of NTR (Fig. 1).[26] However, in the presence of NTR, we found significant inhibition of proliferation in the case of **4b** with only 10% of cells being viable with respect to control. Although **3b** produced similar amounts of NO in comparison with **4b**, the enhancement in cytotoxicity in the presence of NTR was higher in the case of **4b** (Fig. 1). The origin of this difference is unclear. However, a recent study on NTR-activated DNA alkylating agents indicated that the anti-proliferative activity of the NTR substrate did not always correlate with propensity for its metabolism by NTR.[17f] Based on these results, **4b** was identified as a NTR-activated nitric oxide donor with enhanced cytotoxicity in the presence of exogenously added NTR and was chosen for further evaluation.

A concentration-dependent cell viability profile of **4b** with DLD-1 cells showed that the potency of **4b** was enhanced in the presence of NTR at concentrations below 25 μM as well (Fig. 2). Compound **5b** also did not show significant inhibitory activity against DLD-1 cells even at elevated concentrations tested suggesting that the metabolites formed during decomposition of **4b** may not have significant cytotoxicity (Table S4, Supporting Information). A similar cell viability assay was conducted with **4b** using HeLa human cervical cancer cells and again, a dose-dependent enhancement in toxicity in the presence of NTR was observed (See Supporting Information, Fig. S1).

Taken together, our data indicates that the nitric oxide prodrug **4b** is a potential candidate for nitroreductase-based directed prodrug therapy. The use of directed prodrug therapy using NTR has been reported to be effective in inhibiting tumour growth.[27–29] Some examples of NTR-mediated therapeutics include generation of alkylating agents, ene-diynes, pyrazolidines, and 2-fluoroadenines.[17] However, the efficiency of gene transfer or antibody conjugation determines the efficiency of generation and transfer of toxic metabolites. Hence, the success of such targeted therapy is in part dependent on the ability of the toxic species to diffuse and kill non-transfected neighboring cells as well. Also known as bystander effect, this collateral killing of neighboring cells aids in tumour regression.[16] By the use of nitric oxide-based therapeutics,

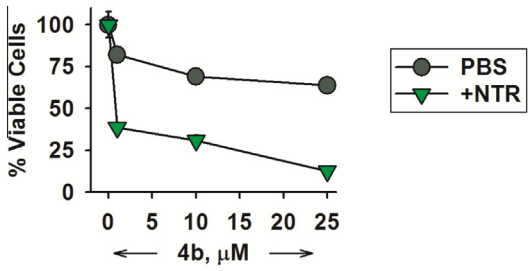

Figure 2. Cell viability assay to assess anti-proliferative activity of **4b** in the presence and absence of NTR was conducted with DLD-1 human colon adenocarcinoma cells.

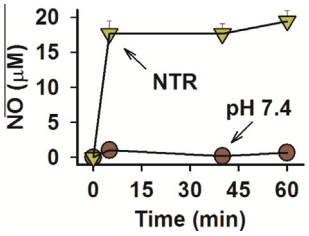

Figure 3. Nitric oxide released during decomposition of **4b** (50 μM) in the presence of NTR in pH 7.4 phosphate buffered saline.

an enhanced bystander effect is predicted as a freely diffusible reactive gas NO is produced.[2,3] However, for this to occur, NO must be released rapidly in the proximity of the tumour. A time course of NO release from **4b** (50 μM) in the presence of NTR showed an increase in levels of NO (17 μM in 5 min) suggesting that a burst of NO would be generated upon exposure to NTR (Fig. 3). Previous studies have indicated that NO by itself and in conjunction with radiation inhibits cancer cell growth under hypoxic conditions.[28–31] As hypoxic tumours are known to be acidic, we recorded NO release from **4b** in pH 6.5. NO (6 μM) was produced in 5 min in pH 6.5 (Fig. S2, Supporting Information) suggesting that **4b** might be useful for NO delivery to hypoxic tumours.[32] The sensitivity of *Mycobacterium tuberculosis* to reactive nitrogen species has been demonstrated and the use of such NTR-activated NO prodrugs to target this bacterium might be possible.[25]

In conclusion, we report 1-(2-methylpiperidin-1-yl)diazen-1-ium-1,2-diolate (**4b**) as a nitroreductase activated nitric oxide prodrug with enhanced cytotoxicity towards two cancer cell lines in the presence of NTR suggesting potential applications for this compound in nitric oxide-based directed prodrug therapy.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.08.066.

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20. *General procedure for synthesis of O^2-(4-nitrobenzyl) diazeniumdiolates.* To an ice cold solution of the diazeniumdiolate salt (1.041 mmol) in THF (10 mL), 15-crown-5 (0.025 mL) was added. After 15 min of stirring at 0 °C, 4-nitrobenzyl bromide (0.694 mmol) was added. The reaction mixture was stirred at room temperature for 3–5 h. Reaction mixture was concentrated under reduced pressure. The resulting crude was chromatographed using a silica gel support to isolate pure material.
Analytical Data: O^2-(4-Nitrobenzyl) 1-(*N*,*N*-dimethyl)diazen-1-ium-1,2-diolate (**1b**). Pale yellow solid (15 mg, 9 %); FT-IR (ν_{max}, cm^{-1}): 2933, 1605, 1518, 1495, 1383, 1349, 1271, 1071; ^1H NMR (400 MHz, $CDCl_3$) δ: 8.21 (d, J = 8.7 Hz, 2H), 7.53 (d, J = 8.8 Hz, 2 H), 5.28 (s, 2H), 2.98 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 147.9, 143.2, 128.7, 123.8, 73.7, 42.3; HRMS (ESI) for $[C_9H_{12}N_4O_4+Na]^+$: Calcd, 263.0756. Found: 263.0779. O^2-(4-Nitrobenzyl) 1-(*N*,*N*-diethyl)diazen-1-ium-1,2-diolate (**2b**). Light brown solid (27 mg, 15 %); FT-IR (ν_{max}, cm^{-1}): 2359, 2341, 1606, 1520, 1384, 1355, 1018; ^1H NMR (400 MHz, $CDCl_3$) δ: 8.20 (d, J = 8.7 Hz, 2H), 7.53 (d, J = 8.6 Hz, 2H), 5.34 (s, 2H), 3.08 (q, J = 7.0 Hz, 4H), 1.00 (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 147.9, 143.1, 128.6, 123.8, 73.9, 48.7, 11.5; HRMS (ESI) for $[C_{11}H_{16}N_4O_4+Na]^+$: Calcd, 291.1069. Found: 291.1069. O^2-(4-Nitrobenzyl) 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**3b**). Pale yellow solid (15 mg, 16 %); FT-IR (ν_{max}, cm^{-1}): 2360, 1652, 1540, 1521, 1507, 1343; ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.20 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 8.7 Hz, 2H), 5.24 (s, 2H), 3.50–3.47 (m, 4H), 1.92–1.89 (m, 4H), 1.65–1.79 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 147.8, 143.6, 128.6, 123.8, 73.5, 50.9, 22.9; HRMS (ESI) for $[C_{11}H_{14}N_4O_4+Na]^+$: Calcd, 289.0912. Found: 289.0922. O^2-(4-Nitrobenzyl) 1-(2-methylpiperidin-1-yl)diazen-1-ium-1,2-diolate (**4b**). Pale yellow solid (50 mg, 25 %); FT-IR (ν_{max}, cm^{-1}): 2934, 2857, 1521, 1505, 1383, 1344, 1020; ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.19 (d, J = 8.6 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H), 5.34 (s, 2H), 3.13–3.23 (m, 3H), 1.65–1.79 (m, 4H), 1.31–1.42 (m, 2H), 0.91 (d, J = 6.1 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 147.9, 143.0, 128.5, 123.8, 56.7, 54.1, 25.1, 23.1, 18.2; HRMS (ESI) for $[C_{13}H_{18}N_4O_4+Na]^+$: Calcd, 317.1225. Found: 317.1245.
21. Enzyme stock solution was prepared by dissolving 1.5 mg of nitroreductase (NTR) in phosphate buffer pH 7.0 (100 μL). A 10 mM stock solution of the compound was prepared in DMSO. 50 μL of the freshly prepared enzyme stock was added to a solution consisting of 10 μL of the test compound (10 mM stock in DMSO) and 400 μL NADPH (1 mM stock in phosphate buffer pH 7.0) and 1540 μL of phosphate buffer pH 7.0. The blank consisted of 10 μL of the test compound (10 mM stock in DMSO) and 1990 μL of phosphate buffer pH 7.0. Data reported are averages of two independent runs. These reaction mixtures were stirred at 37 °C and after 1 h an aliquot was analyzed for decomposition and nitric oxide generation. Decomposition: HPLC (Eclipse Plus C-18, 5 μm, 4.6 × 250 mM; flow rate: 1 mL/min; eluant 70% MeCN:H_2O) analysis was used to determine amount of compound remaining. For quantifying amount of NO release from enzymatic decomposition of **1b**–**5b**, the reaction mixtures were prepared in pH 6.5, pH 7.0, and 7.4. 10 μL of the reaction mixture and blank solution were injected at the specified time points into a Sievers Nitric Oxide Analyzer (NOA 280i) using argon as the carrier gas. The reservoir contained NaI and acetic acid, which converts nitrite to NO. A calibration curve was generated using $NaNO_2$ standards, which under the assay conditions would get converted to NO. The so formed NO is quantified using the chemiluminescence detector. Under the enzymatic assay conditions any nitrite that is produced during decomposition is also measured.
22. We also found no reaction in the presence of glutathione during 1 h suggesting that these compounds were not candidates for nucleophilic substitution by a biologically relevant thiol.
23. To an ice cold solution of 4-nitrobenzyl alcohol (0.652 mmol), benzoic acid (0.979 mmol) and 4-dimethylaminopyridine (DMAP, 1.30 mmol) in dry DCM (10 mL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC.HCl, 1.30 mmol) was added. The reaction was stirred at room temperature for 3 h.The reaction mixture was diluted with water (25 mL) and the resulting aqueous mixture was extracted with DCM (3 × 10 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and the filtrate was evaporated to give crude product. Silica gel chromatography (10% ethyl acetate: pet ether) of the resulting crude gave **5b** (33%) as a white solid. Analytical data for **5b**: ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.23 (d, J = 8.7 Hz, 2H), 8.07 (d, J = 8.6 Hz, 2H), 7.57–7.60 (m, 3H), 7.45 (t, J = 7.4 Hz, 2H), 5.44 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 166.0, 147.6, 143.3, 133.4, 129.7, 128.5, 128.3, 123.8, 65.1. See: Ohno, O.; Ye, M.; Koyama, T.; Yazawa, K.; Mura, E.; Matsumoto, H.; Ichino, T.; Yamada, K.; Nakamura, K.; Ohno, T.; Yamaguchi, K.; Ishida, J.; Fukamizu, A.; Uemura, D. *Bioorg. Med. Chem.***2008**, *16*, 7843.
24. Some possible mechanisms of NO generation from nitroaromatic compounds are discussed in Ref. 25. The levels of NO produced by 5b is ~1% and hence might be a minor component of NO generating pathways from **1b**–**4b**. The precise mechanism for NO generation from **5b** is not clear at this time and requires further investigations.
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26. DLD-1 human adenocarcinoma cells were seeded at 3×10^{-4} cells/well overnight in a 96-well plate in complete RPMI 1640 media. *E. Coli* nitroreductase (lyophilized powder, Sigma) was used to prepare a stock solution containing 6 mg in 2 mL of Dulbecco's phosphate-buffered saline (DPBS). NADPH stock solution (0.5 mM) was prepared in DPBS. Cells were exposed to varying concentrations of the test compound prepared as a DMSO stock solution so that the final concentration of DMS was 1%. The cells were incubated for 72 h at 37 °C. A stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was prepared 3.5 mg in 700 μL DPBS. This stock was diluted with 6.3 mL DPBS and 100 μL of the resulting solution was added to each well after aspiration of media. After 4 h incubation, the MTT solution was removed carefully and 100 μL of DMSO was added. Spectrophotometric analysis of each well using a microplate reader (Thermo Scientific Varioscan) at 570 nm was carried out to estimate cell viability. A similar assay was conducted in the presence of NTR (5 μL stock) and NADPH (10 μL stock) to analyze the effect of addition of NTR on cell viability.
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