Published in final edited form as:

Bioorg Chem. 2020 October ; 103: 104128. doi:10.1016/j.bioorg.2020.104128.

Derivatives of 1,2,4-triazole imines acting as dual iNOS and tumor cell growth inhibitors

Christophe Guillon^a, Anna M. Vetrano^b, Jaya Saxena^a, Angela Hunter^a, Geraldine Verderone^a, Thomas M. Finetti^a, Jeffrey Wisnoski^a, Peter W. DeMatteo^a, Robert D. Rapp^a, Ned D. Heindel^a, Laurie B. Joseph^c, Diane E. Heck^d, Jeffrey D. Laskin^b

^aDepartment of Chemistry, Lehigh University, Bethlehem, PA 18015, USA

^bDepartment of Environmental and Occupational Health, Rutgers University School of Public Health, Piscataway, NJ 08854, USA

^cErnest Mario School of Pharmacy, Rutgers University School of Public Health, Piscataway, NJ 08854, USA

^dDepartment of Environmental Science, New York Medical College, Valhalla, NY 10595, USA

Abstract

A set of 4-(R₂-imino)-3-mercapto-5-(R₁)-4*H*-1,2,4-triazoles derivatives were synthesized, characterized and evaluated for their ability to inhibit nitric oxide (NO) production in PAM212 mouse keratinocytes, which led to the discovery and the subsequent evaluation of their growth inhibitory cytotoxic potency toward that same mouse cell line together with a number of human cells lines (PC3, HT-29 and HeLa). Some limited SAR could be established for both NO production inhibition potency and growth inhibition cytotoxicity. Noticeably, the compounds designed to be nitrofurantoin mimics were the most potent anti-neoplastic agents.

Graphical Abstract

The authors listed on the submitted article entitled, have no conflict of interest, financial or otherwise, to declare.

All authors are academics and no one is in the employment of a corporation. These candidate compounds are not under consideration for commercial development.

Christophe Guillon, on behalf of the other authors.

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Introduction

In our studies on interventional pharmaceuticals to treat sulfur mustard-induced injury, inhibitors of inducible nitric oxide synthase (iNOS) have shown promise.⁽¹⁾ A number of five-membered ring nitrogen heterocyclics have been found to function as anti-inflammatory agents (Figure 1) and a NOS-inhibitory mechanism has been invoked.^(1–3) Imidazoles such as itraconazole, ketoconazole, and miconazole are known inhibitors of iNOS in intact cells.⁽⁴⁾ In addition, itraconazole has recently been reported to be an effective eNOS inhibitor although its exact mechanism of action was not elucidated.⁽⁵⁾ Aryl-substituted imidazoles in particular SB203580 have been established as inhibitors of iNOS at high concentrations⁽⁶⁾ while certain indazoles and triazoles (of both the 1,2,3- and the 1,2,4- isomeric types) have also displayed impressive iNOS inhibition.^(7–9)

The ability of cells to synthesize NO is part of their natural immune defense mechanism. However, excessive NO production can lead to tissue damage and, therefore, drugs that suppress NO production are helpful in those situations. For host cells, exposure to many bacterial pathogens can lead to over-production of NO. This may also explain the fact that some successful antibacterial agents possess simultaneous iNOS inhibition as part of their antibacterial activity.^(10,11) The nitrofurantoin antibacterials, *e.g.*, nitrofurantoin (NF) and nitrofurazone, have been classically employed against urinary tract infections and there are reports of their concurrent anti-inflammatory activity.^(12–16)

In our earlier studies of 1,2,4-triazole imines we observed significant anti-bacterial activity against urogenital pathogens such as *G. vaginalis* and *E. coli* and also made note of a structural similarity of our system to nitrofurantoin.^(17,18) The question then arises that if these imines possess activity against urinary tract infectious organisms do they also manifest an ability to inhibit NO production. The focus of this work was therefore centered on evaluating a small library of such compounds for NO inhibition. It is important to note that although the compounds presented are designed as partial mimics of Nitrofurantoin and Nitrofurazone, known for their adverse side effects and toxicity; we do not anticipate such problems because we envision a different route of administration, namely topical, which should lead to reduced toxicity.

Discussion and results

Synthesis

The starting 4-amino-5- R_1 -2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (general structure **2**) were prepared according to the procedure by Reid (Scheme 1).⁽¹⁹⁾ The synthetic route employed to obtain the target 1,2,4-triazole imines, compounds **3(a-z)**, is outlined in Scheme 2. Structures of R_1 and R_2 for **3(a-z)** are given in Table 1. Compounds **3(a-z)** were prepared by reacting 4-amino-2,4-dihydro-5- R_1 -3*H*-1,2,4-triazole-3-thiones with various aldehydes at reflux in absolute ethanol (EtOH) or tetrahydrofuran (THF) to give the expected corresponding imine in moderate to excellent yields (45 – 97%). Usually the compounds were obtained by precipitation upon cooling, and purified by recrystallization in some cases. However, when a nitro-aldehyde derivative was utilized, the purification was not so straightforward. Most of the time, an excess of aldehyde had to be used to drive the reaction

to completion. At the end of the reaction, upon cooling, both excess of aldehyde and expected compound precipitated. In those cases, the crude solid had to be purified by flash silica gel column chromatography followed by recrystallization and in these conditions, pure material was obtained in lower yields (22 – 60%).

Functional assays

NO production inhibition in PAM212 cells—The ability of the compounds synthesized to inhibit nitric oxide production was assessed *in vitro*. Nitric oxide production by IFN- γ stimulated PAM212 mouse keratinocytes was quantified by the accumulation of nitrite in the cell culture medium using the Greiss reaction, with sodium nitrite as the standard as previously described.⁽²⁰⁾ In brief, 24 hours after treatment, the culture medium was collected, 100 μ L was incubated with 100 μ L of Griess reagent (0.1% naphthaethylenediamine dihydrochloride, 1% sulfanilamide, 2.5% H₃PO₄) and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.⁽²¹⁾

Cellular Growth Inhibition—The primary *in vitro* screen for the anti-proliferative activity was based on the ability of the beneficial therapeutic compound to inhibit the growth of tumor cells grown in culture. This type of growth assay can be used with mammalian cancer cells as well as with pathogenic and non-pathogenic microbes including, but not limited to, yeasts and bacteria. In our case, to assay the candidate triazoles for anti-proliferative activity, PAM212 keratinocytes grown *in vitro* in monolayer culture were used as previously described.^(20, 22–25) Briefly, cells were plated at low density (10⁴ cells /well) in 6 well tissue culture dishes in 2 mL of growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, and allowed to adhere overnight. The medium was then changed to growth medium supplemented with increasing concentrations of the triazoles (compounds **3(a-z)**). After 5 days, the medium was drained from the culture dishes and the cells washed with phosphate buffered saline. The cells from each well were removed by trypsin treatment and the number of cells in each culture dish was determined using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL). The effects of compound **3d** was evaluated in HT-29, PC3 and HeLa human cell lines using the same technique as described above.

Structure-activity Relationships

Nitrite inhibition—While low μ M IC₅₀s were observed for NO production inhibition for some of the compounds, it was generally not in the nitro-containing analogs (Table 1). Compounds (**3d**, **3f-g**, **3j**, **3l-p**, **3t-z**) proved to be too toxic to the PAM212 cells used to generate the iNOS inhibition data and, as a result, their inhibition could not be measured. However, in compounds (**3a**, **3c**, **3e**, **3h-i**, **3k**, **3q-3s**) wherein cytotoxicity was less manifest, clear evidence of NO production inhibition was observed. All compounds except **3c** inhibited NO production with IC₅₀'s ranging from 14 to 34 μ M while possessing an R₂ moiety (see Scheme 1) derived from a simple cinnamaldehyde or the α -substituted-cinnamaldehyde. The most active iNOS inhibitor was **3c**, derived from *o*-nitrocinnamaldehyde and an electronic structural contrast to **3b** (from *o*-methoxycinnamaldehyde). The latter manifested neither growth inhibition – over the

maximum concentration range through which it could be tested – nor NOS inhibition. In view of the cytotoxicity of some of these compounds toward PAM212 cells, we decided to investigate further the potential utility of these triazole imines as anti-neoplastic agents.

Growth inhibition—Results obtained are summarized in Table 1. From the data collected and displayed in Table 1, we could establish the following structure-activity relationships. Keeping the R₁ group unchanged, modification of the structure of R₂ had a significant impact on cytotoxicity. Compounds bearing a substituent on the cinnamyl chain display increased cytotoxicity, as can be seen for examples with R₁ = 2-thienyl. In this series, the addition of a halogen (Br or Cl) resulted in an increase of cytotoxicity greater than when a methyl or methoxy group were present instead; as exemplified by compounds **3d** (Br, IC₅₀ = 4.6 μM) < **3f** (Cl, IC₅₀ = 8.1 μM) < **3e** (Me, IC₅₀ = 93 μM) < **3a** (H, IC₅₀ > 100 μM). Similar trends could be observed when comparing **3o** (Br, IC₅₀ = 5.9 μM) < **3q** (H, IC₅₀ > 100 μM); **3j** (Br, IC₅₀ = 6.2 μM) < **3m** (Me, IC₅₀ = 12 μM) < **3h** (H, IC₅₀ > 100 μM); **3p** (Br, IC₅₀ = 5.2 μM) < **3s** (H, IC₅₀ = 61 μM); **3l** (Br, IC₅₀ = 2.9 μM) < **3k** (H, IC₅₀ = 97 μM) and **3g** (Br, IC₅₀ = 4.2 μM) < **3i** (H, IC₅₀ > 100 μM). While keeping R₂ constant as in compounds (**3d**, **3g**, **3j**, **3l**, **3n**, **3o**, **3p**), where R₂ is an α-bromocinnamyl group, it is interesting to note that R₁ seemed to have little impact on the potency of the compounds, with IC₅₀s very narrowly falling between 2 and 7 μM. Independent from R₁, the presence of a nitro group on R₂ also increased the cytotoxicity, as exemplified by **3c** (NO₂, IC₅₀ = 59 μM) < **3a** (H, IC₅₀ > 100 μM). Of special interest, are compounds (**3t-3z**), designed as nitrofurantoin mimics, which displayed the most enhanced activity with IC₅₀s as low as 1.5 to 3.5 μM (**3v-w** and **3y-z**). Lastly for the same R₂ group (cinnamyl), there is no significant change for the IC₅₀ values when one modifies R₁ as exemplified by compounds **3a**, **3h**, **3i**, **3k**, **3q** and **3r**.

Following the discovery of the cytotoxicity of the triazole imines against the murine keratinocyte cell line PAM212, we selected compound (**3d**) for additional *in vitro* testing against human cancer cell lines HT-29 (colorectal cancer), PC3 (prostate cancer) and HeLa (cervical cancer). The results, displayed in Figure 2, demonstrated the marked potency of **3d** to inhibit *in vitro* the growth of all three human cell lines with IC₅₀'s ranging from 0.5 μM for the HT-29 (colon), 1.6 μM for PC3 (prostate), and 2.8 μM for HeLa (cervix). For comparison, the IC₅₀ for **3d** in mouse keratinocytes was 4.6 μM.

Experimental

Synthesis—Unless otherwise indicated, all materials were purchased from commercial suppliers and used without further purification.

IR spectra were recorded using a Mattson Polaris FT-IR spectrophotometer. Solids compounds were analyzed using the KBr disk methods, or solubilized in nujol. Liquids were analyzed as thin film between two NaCl blocks.

¹H spectra were recorded at 360 MHz on a Bruker AMX 360 spectrometer. Chemical shifts were measured relative to CDCl₃ (δ 7.24 ppm), CD₃OD (δ 3.30 ppm), acetone (d₆) (δ 2.04 ppm) and DMSO (d₆) (δ 2.49 ppm). The following abbreviations are used to describe the signal multiplicities: s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet).

Chemical shifts are expressed in ppm and listed as follows: shift in ppm (multiplicity, integration, and coupling).

Thin-layer chromatography (TLC) was performed on plates (0.25 mm) precoated with fluorescent silica gel GF (Analtech). Reaction components were then visualized under UV light and/or following staining with iodine and/or with a saturated solution of KMnO_4 in methanolic NaOH (1N). Silica gel (230–400 mesh, EM Sciences) was used for flash chromatography separations. Uncorrected melting points (mp) were determined with a Thomas Hoover capillary melting point apparatus. Elemental analyses were provided by Intertek.

Synthesis of 4-(R_2 -imino)-3-mercapto-5-(R_1)-4*H*-1,2,4-triazoles, 3(a-y)

General Procedure—The corresponding 4-amino-3-mercapto-5-(R_1)-4*H*-1,2,4-triazole (general structure **2**) and aldehyde were solubilized in absolute ethanol or anhydrous tetrahydrofuran. The α -bromo and α -chlorocinnamaldehydes used were the (*E*)-isomers whereas the (*Z*)-isomer was used for α -methylcinnamaldehyde. The mixture was stirred at reflux until completion was shown by TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 96/4, v/v), usually 6 hours, and a solid precipitated out of solution. The flask was cooled down to room temperature, and then put in the freezer (-20°C) overnight. The precipitate was filtered and rinsed with cold ethanol or tetrahydrofuran to give the expected 4(R_2 -imino)-3-mercapto-5-(R_1)-4*H*-1,2,4-triazole. Variations of this general procedure are described for each individual compound.

4-(((1*Z*,2*E*)-3-phenylallylidene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3thione **3a**—

Compound **3a** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.1 g, 0.50 mmol), *trans*-cinnamaldehyde (0.146 g, 1.16 mmol) and 4 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 101 mg (0.323 mmol, 64 %) of **3a** as a yellow solid. ^1H NMR ($\text{DMSO}-d_6$): 7.22 (t, 1H, $J = 4.9$ Hz); 7.30 (dd, 1H, $J = 9.5$ Hz and $J = 16.0$ Hz); 7.43–7.52 (m, 4H); 7.767.84 (m, 4H); 9.58 (d, 1H, $J = 9.5$ Hz). IR (nujol): 1457 cm^{-1} . m.p. = $206\text{--}207^\circ\text{C}$. Analysis Calculated for $\text{C}_{15}\text{H}_{12}\text{N}_4\text{S}_2$: C, 57.67; H, 3.87; N, 17.93. Found: C, 57.76; H, 3.65; N, 17.56.

4-(((1*Z*,2*E*)-3-(2-methoxyphenyl)allylidene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione **3b**—

Compound **3b** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (0.1 g, 0.50 mmol), *trans*-2-methoxycinnamaldehyde (0.123 g, 0.76 mmol) and 4 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 129 mg (0.378 mmol, 75 %) of **3b** as a yellow solid. ¹H NMR (DMSO-d₆): 3.89 (s, 3H); 7.02 (t, 1H, *J* = 7.51 Hz); 7.11 (d, 1H, *J* = 8.4 Hz); 7.22 (t, 1H, *J* = 4.50 Hz); 7.28 (dd, 1H, *J* = 9.6 Hz and *J* = 16.0 Hz); 7.43 (t, 1H, *J* = 7.2 Hz); 7.63 (d, 1H, *J* = 16.0 Hz); 7.79–7.83 (m, 3H); 9.50 (d, 1H, *J* = 9.5 Hz). IR (nujol): 1459 cm⁻¹. m.p. = 227–228°C. Analysis Calculated for C₁₆H₁₄N₄OS₂·0.5 H₂O: C, 54.71; H, 4.30; N, 15.95. Found: C, 54.70; H, 3.94; N, 16.01.

4-(((1Z,2E)-3-(2-nitrophenyl)allylidene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3c—

Compound **3c** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (0.1 g, 0.50 mmol), *trans*-2-nitrocinnamaldehyde (0.268 g, 1.51 mmol) and 4 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 150 mg (0.42 mmol, 83 %) of **3c** as a yellow solid. ¹H NMR (DMSO-d₆): 7.21 (t, 1H, *J* = 3.9 Hz); 7.31 (dd, 1H, *J* = 9.0 Hz and *J* = 16.0 Hz); 7.68 (t, 1H, *J* = 7.9 Hz); 7.74–7.85 (m, 5H); 8.09 (dd, 2H, *J* = 7.9 Hz and *J* = 18.0 Hz); 9.76 (d, 1H, *J* = 9.0 Hz). IR (nujol): 1457 cm⁻¹. m.p. = 214–215°C. Analysis Calculated for C₁₅H₁₁N₅O₂S₂: C, 50.41; H, 3.10; N, 19.60. Found: C, 50.50; H, 2.77; N, 19.29.

4-(((1Z,2Z)-2-bromo-3-phenylallylidene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3d—

Compound **3d** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)2,4-dihydro-3H-1,2,4-triazole-3-thione (0.1 g, 0.50 mmol), α -bromocinnamaldehyde (0.32 g, 1.51 mmol) and 4 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 174 mg (0.44 mmol, 88 %) of **3d** as a yellow solid. ^1H NMR (DMSO- d_6): 7.25 (t, 1H, $J = 4.1$ Hz); 7.51–7.54 (m, 3H); 7.86 (d, 1H, $J = 4.9$ Hz); 7.96–8.02 (m, 3H); 8.11 (s, 1H); 10.11 (s, 1H). IR (nujol): 1457 cm^{-1} . m.p.= 197–198°C. Analysis Calculated for $\text{C}_{15}\text{H}_{11}\text{BrN}_4\text{S}_2$: C, 46.06; H, 2.83; N, 14.32. Found: C, 46.14; H, 2.83; N, 14.26.

4-(((1Z,2E)-2-methyl-3-phenylallylidene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3e—

Compound **3e** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)2,4-dihydro-3H-1,2,4-triazole-3-thione (0.1 g, 0.50 mmol), α -methylcinnamaldehyde (0.221 g, 1.51 mmol) and 4 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 74 mg (0.23 mmol, 45 %) of **3e** as a yellow solid. ^1H NMR (DMSO- d_6): 2.28 (s, 3H); 7.22–7.30 (m, 2H); 7.38–7.50 (m, 3H); 7.59 (d, 2H, $J = 7.6$ Hz); 7.827.86 (m, 2H); 9.62 (s, 1H). IR (nujol): 1457 cm^{-1} . m.p.= 200–201°C. Analysis Calculated for $\text{C}_{16}\text{H}_{14}\text{N}_4\text{S}_2$: C, 58.87; H, 4.32; N, 17.16. Found: C, 58.51; H, 4.47; N, 17.14.

4-(((1Z,2Z)-2-chloro-3-phenylallylidene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3f—

Compound **3f** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.1 g, 0.50 mmol), α -chlorocinnamaldehyde (0.252 g, 1.51 mmol) and 4 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 114 mg (0.33 mmol, 65 %) of **3f** as a yellow solid. ¹H NMR (DMSO-*d*₆): 7.24 (t, 1H, *J* = 3.8 Hz); 7.53 (t, 3H, *J* = 8.6 Hz); 7.81–8.00 (m, 5H); 10.18 (s, 1H). IR (nujol): 1457 cm⁻¹. m.p. = 223–224°C. Analysis Calculated for C₁₅H₁₁ClN₄S₂: C, 51.94; H, 3.20; N, 16.15. Found: C, 51.66; H, 2.86; N, 15.85.

4-(((1*Z*,2*Z*)-2-bromo-3-phenylallylidene)amino)-5-(furan-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione **3g—**

Compound **3g** was prepared according to the above procedure using 4-amino-5-(furan-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.141 g, 0.77 mmol), α -bromocinnamaldehyde (0.216 g, 1.02 mmol) and 6 mL of absolute ethanol. After filtration, the product was purified by recrystallization in CHCl₃/Hexanes to give 244 mg (0.65 mmol, 84%) of **3g** as a yellow solid. ¹H NMR (Acetone-*d*₆): 6.71 (dd, 1H, *J* = 3.4 Hz and *J* = 1.8 Hz); 7.47–7.56 (m, 3H); 7.61 (dd, 1H, *J* = 3.7 Hz and *J* = 0.7 Hz); 7.83 (dd, 1H, *J* = 1.7 Hz and *J* = 0.7 Hz); 8.00 (s, 1H); 8.04–8.07 (m, 2H); 10.59 (s, 1H). m.p. = 184–185°C. Analysis Calculated for C₁₅H₁₁BrN₄OS: C, 48.01; H, 2.96; N, 14.93. Found: C, 48.07; H, 3.02; N, 14.80.

5-methyl-4-(((1*Z*,2*E*)-3-phenylallylidene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione **3h—**

Compound **3h** was prepared according to the above procedure using 4-amino-5-methyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.13 g, 0.99 mmol), *trans*-cinnamaldehyde (0.396 g, 2.99 mmol) and 6 mL of absolute ethanol. After filtration, the product was purified by column chromatography (CHCl₃) on silica gel to give 144 mg (0.59 mmol, 59 %) of **3h** as a yellow solid. ¹H NMR (Acetone-d₆): 2.36 (s, 3H); 7.09 (dd, 1H, *J* = 16.0 Hz and *J* = 9.5 Hz); 7.39 (d, 1H, *J* = 15.8 Hz); 7.42–7.48 (m, 3H); 7.72 (dd, 1H, *J* = 6.3 Hz and *J* = 1.6 Hz); 10.14 (d, 1H, *J* = 9.4 Hz). m.p. = 193–194°C. Analysis Calculated for C₁₂H₁₂N₄S·0.2 H₂O: C, 58.13; H, 5.04; N, 22.60. Found: C, 58.08; H, 5.05; N, 22.83.

4-(((1*Z*,2*E*)-3-phenylallylidene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3thione **3i—**

Compound **3i** was prepared according to the general procedure using 4-amino-5-(furan-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.192 g, 1.05 mmol), *trans*-cinnamaldehyde (0.545 g, 4.12 mmol) and 8 mL of absolute ethanol. The reaction mixture was dry evaporated, the residue solubilized in CHCl₃ and the orange solid obtained by precipitation with hexanes was recrystallized in acetone/hexanes to give 262 mg (0.64 mmol, 84 %) of **3i** as an orange solid. ¹H NMR (CD₃OD): 6.63 (dd, 1H, *J* = 3.4 Hz and *J* = 1.8 Hz); 7.15–7.23 (m, 2H); 7.37–7.45 (m, 4H); 7.64–7.73 (m, 3H); 9.70 (d, 1H, *J* = 9.4 Hz). m.p. = 181–182°C. Analysis Calculated for C₁₅H₁₂N₄OS: C, 60.79; H, 4.08; N, 18.91. Found: C, 60.91; H, 4.33; N, 18.50.

4-(((1*Z*,2*Z*)-2-bromo-3-phenylallylidene)amino)-5-methyl-2,4-dihydro-3*H*-1,2,4-triazole-3thione **3j—**

Compound **3j** was prepared according to the general procedure using 4-amino-5-methyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.13 g, 0.99 mmol), *α*-bromocinnamaldehyde (0.634 g, 3.00 mmol) and 5 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 174 mg (0.54 mmol, 54 %) of **3j** as an orange solid. ¹H NMR (Acetone-*d*₆): 2.42 (s, 3H); 7–46–7.52 (m, 3H); 7.86 (s, 1H); 7.99–8.02 (m, 2H); 10.49 (s, 1H). m.p.= 194–195°C. Analysis Calculated for C₁₂H₁₁BrN₄S·0.3H₂O: C, 43.86; H, 3.56; N, 17.05. Found: C, 43.83; H, 3.38; N, 17.05.

5-(4-fluorophenyl)-4-(((1Z,2E)-3-phenylallylidene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione **3k—**

Compound **3k** was prepared according to the general procedure using 4-amino-5-(4-fluorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.105 g, 0.49 mmol), *trans*-cinnamaldehyde (0.198 g, 1.49 mmol) and 5 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 100 mg (0.31 mmol, 620 %) of **3k** as a yellow solid. ¹H NMR (Acetone-*d*₆): 7.16 (dd, 1H, *J* = 16.1 Hz and *J* = 9.5 Hz); 7.30–7.38 (m, 2H); 7.43–7.48 (m, 4H); 7.22–7.45 (m, 2H); 8.02–8.07 (m, 2H); 9.81 (d, 1H, *J* = 9.5 Hz). m.p.= 216–217°C. Analysis Calculated for C₁₇H₁₃FN₄S·0.3H₂O: C, 61.92; H, 4.16; N, 16.99. Found: C, 61.83; H, 4.05; N, 16.64.

4-(((1Z,2Z)-2-bromo-3-phenylallylidene)amino)-5-(4-fluorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione **3l—**

Compound **3l** was prepared according to the above procedure using 4-amino-5-(4-fluorophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (0.106 g, 0.5 mmol), α -bromocinnamaldehyde (0.32 g, 1.51 mmol) and 5 mL of absolute ethanol. After filtration, the crude product obtained was purified by recrystallization in CHCl_3 /Hexanes to give 104 mg (0.25 mmol, 51%) of **3l** as a yellow solid. ^1H NMR (Acetone- d_6): 7.32 (t, 2H, $J = 8.9$ Hz); 7.48–7.54 (m, 3H); 7.98 (s, 1H); 8.00–8.06 (m, 2H); 8.18 (dd, 2H, $J = 9.0$ Hz and $J = 5.2$ Hz); 10.38 (s, 1H). m.p. = 212–213°C. Analysis Calculated for $\text{C}_{17}\text{H}_{12}\text{FN}_4\text{S} \cdot 0.55\text{H}_2\text{O}$: C, 49.42; H, 3.20; N, 13.56. Found: C, 49.44; H, 3.09; N, 13.23.

4-(((1Z,2Z)-2-chloro-3-phenylallylidene)amino)-5-methyl-2,4-dihydro-3H-1,2,4-triazole-3-thione 3m—

Compound **3m** was prepared according to the above procedure using 4-amino-5-methyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (0.15 g, 1.15 mmol), α -chlorocinnamaldehyde (0.384 g, 2.30 mmol) and 5 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 283 mg (1.01 mmol, 88 %) of **3m** as an off-white solid. ^1H NMR (CD_3OD): 2.41 (d, 3H, $J = 3.5$ Hz); 7.41–7.51 (m, 4H); 7.91–7.97 (m, 3H); 10.46 (s, 1H). m.p. = 221–222°C. Analysis Calculated for $\text{C}_{17}\text{H}_{19}\text{ClN}_4\text{S}$: C, 51.70; H, 3.98; N, 20.10. Found: C, 51.88; H, 3.93; N, 19.97.

4-(((1Z,2Z)-2-bromo-3-phenylallylidene)amino)-5-cyclohexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione 3n—

Compound **3n** was prepared according to the above procedure using 4-amino-5-cyclohexyl-2,4dihydro-3*H*-1,2,4-triazole-3-thione (0.146 g, 0.74 mmol), α -bromocinnamaldehyde (0.39 g, 1.84 mmol) and 5 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 279 mg (0.71 mmol, 97 %) of **3n** as a pale yellow solid. ^1H NMR (Acetone- d_6): 1.28–1.50 (m, 3H); 1.51–1.65 (m, 2H); 1.70–1.80 (m, 1H); 1.81–1.90 (m, 2H); 2.01–2.10 (m, 2H); 2.98 (tt, 1H, J = 3.4 Hz and J = 11.5 Hz); 7.42–7.49 (m, 1H); 7.77 (s, 1H); 7.93–8.00 (m, 1H); 10.46 (s, 1H). m.p. = 199–200°C. Analysis Calculated for $\text{C}_{17}\text{H}_{19}\text{BrN}_4\text{S}$: C, 52.18; H, 4.89; N, 14.32. Found: C, 52.36; H, 4.82; N, 14.14.

4-(((1Z,2Z)-2-bromo-3-phenylallylidene)amino)-5-phenethyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione 3o—

Compound **3o** was prepared according to the above procedure using 4-amino-5-phenethyl-2,4dihydro-3*H*-1,2,4-triazole-3-thione (0.15 g, 0.68 mmol), α -bromocinnamaldehyde (0.287 g, 1.36 mmol) and 5 mL of absolute ethanol. A pale yellow solid was isolated after filtration and was purified by a column chromatography on silica (CHCl_3) followed by recrystallization in CHCl_3 /Hexanes to give 189 mg (0.47 mmol, 67 %) of **3o** as a pale yellow solid. ^1H NMR (CD_3OD): 3.03–3.13 (m, 4H); 7.15–7.25 (m, 5H); 7.25–7.30 (m, 4H); 7.43–7.52 (m, 3H); 7.74 (s, 1H); 7.93–8.00 (m, 1H); 10.30 (s, 1H). m.p. = 175–176°C. Analysis Calculated for $\text{C}_{19}\text{H}_{17}\text{BrN}_4\text{S}$: C, 55.21; H, 4.15; N, 13.55. Found: C, 54.96; H, 3.99; N, 13.15.

4-(((1Z,2Z)-2-bromo-3-phenylallylidene)amino)-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3thione 3p—

Compound **3p** was prepared according to the above procedure using 4-amino-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.151 g, 0.78 mmol), *α*-bromocinnamaldehyde (0.333 g, 1.56 mmol) and 5 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 269 mg (0.70 mmol, 89 %) of **3p** as a pale yellow solid. ¹H NMR (Acetone-*d*₆): 1.28–1.50 (m, 3H); 1.51–1.65 (m, 2H); 1.70–1.80 (m, 1H); 1.81–1.90 (m, 2H); 2.01–2.10 (m, 2H); 2.98 (tt, 1H, *J* = 3.4 Hz and *J* = 11.5 Hz); 7.42–7.49 (m, 1H); 7.77 (s, 1H); 7.93–8.00 (m, 1H); 10.46 (s, 1H). m.p. = 195–196°C. Analysis Calculated for C₁₇H₁₃BrN₄S: C, 53.00; H, 3.40; N, 14.54. Found: C, 52.94; H, 3.46; N, 14.28.

5-phenethyl-4-(((1Z,2E)-3-phenylallylidene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione 3q—

Compound **3q** was prepared according to the general procedure using 4-amino-5-phenethyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.15 g, 0.68 mmol), *trans*-cinnamaldehyde (0.18 g, 1.34 mmol) and 5 mL of absolute ethanol. After filtration, the product was recrystallized with acetone/hexanes to give 0.128 g (0.383 mmol, 56 %) of **3q** as a pale yellow solid. ¹H NMR (Acetone-*d*₆): 3.03–3.06 (m, 4H); 7.10 (dd, 1H, *J* = 16.1 Hz and *J* = 9.4 Hz); 7.16–7.22 (m, 1H); 7.24–7.29 (m, 4H); 7.37 (d, 1H, *J* = 16.1 Hz); 7.40–7.47 (m, 3H); 7.70–7.74 (m, 2H); 10.05 (d, 1H, *J* = 9.4 Hz). m.p. = 150–151°C. Analysis Calculated for C₁₉H₁₈N₄S·0.25 H₂O: C, 67.33; H, 5.50; N, 16.53. Found: C, 67.43; H, 5.50; N, 16.37.

4-(((1Z,2E)-3-phenylallylidene)amino)-5-(pyridin-3-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3thione 3r—

Compound **3r** was prepared according to the general procedure using 4-amino-5-(pyridin-3-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.122 g, 0.63 mmol), *trans*-cinnamaldehyde (0.168 g, 1.27 mmol) and 5 ml of absolute ethanol. The reaction mixture was dry evaporated, and the yellow residue was purified by column chromatography (CHCl₃/MeOH, 95/5, v/v) on silica gel to give 0.117 g (0.38 mmol, 60 %) of **3r** as a yellow solid. ¹H NMR (CD₃OD): 7.14 (dd, 1H, *J* = 15.9 Hz and *J* = 9.5 Hz); 7.38–7.44 (m, 4H); 7.57–7.59 (m, 1H); 7.60–7.68 (m, 2H); 8.39 (dt, 1H, *J* = 1.1 Hz and *J* = 8.1 Hz); 8.66 (dd, 1H, *J* = 1.6 Hz and *J* = 4.9 Hz); 9.08 (d, 1H, *J* = 1.6 Hz); 9.77 (d, 1H, *J* = 9.5 Hz). m.p. = 198–199°C. Analysis Calculated for C₁₆H₁₃N₅S: C, 62.52; H, 4.26; N, 22.78. Found: C, 62.28; H, 4.16; N, 22.60.

5-phenyl-4-(((1*Z*,2*E*)-3-phenylallylidene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione 3s—

Compound **3s** was prepared according to the general procedure using 4-amino-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.179 g, 0.93 mmol), *trans*-cinnamaldehyde (0.188 g, 1.43 mmol) and 5 mL of absolute ethanol. The product was isolated via suction filtration and washed with cold ethanol to give 0.191 mg (0.62 mmol, 67 %) of **3s** as a yellow solid. ¹H NMR (Acetone-d₆): 7.17 (dd, 1H, *J* = 16.0 Hz and *J* = 9.5 Hz); 7.42–7.55 (m, 7H); 7.73–7.76 (m, 2H); 7.94–7.98 (m, 2H); 9.76 (d, 1H, *J* = 9.4 Hz). m.p. = 191–193°C. Analysis Calculated for C₁₇H₁₄N₄S: C, 66.64; H, 4.61; N, 18.29. Found: C, 66.56; H, 4.68; N, 17.93.

4-(((1*Z*,2*E*)-3-(5-nitrofur-2-yl)allylidene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione 3t—

Compound **3t** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)2,4-dihydro-3H-1,2,4-triazole-3-thione (0.14 g, 0.71 mmol), 5-nitro-2-furanacrolein (0.26 g, 1.54 mmol) and 6 mL of absolute ethanol. After filtration, the crude product was purified by recrystallization in acetone to give 92 mg (0.26 mmol, 37%) of **3t** as a yellow solid. ^1H NMR (Acetone- d_6): 7.10–7.35 (m, 4H); 7.44 (d, 1H, $J = 15.9$ Hz); 7.62 (d, 1H, $J = 3.9$ Hz); 7.77 (d, 1H, $J = 5.0$ Hz); 7.96 (d, 1H, $J = 2.8$ Hz); 10.29 (d, 1H, $J = 9.4$ Hz). IR (KBr): 1369 cm^{-1} . m.p. = 205–207°C. Analysis Calculated for $\text{C}_{13}\text{H}_9\text{N}_5\text{O}_3\text{S}_2 \cdot 0.5 \text{H}_2\text{O}$: C, 43.81; H, 2.82; N, 19.65. Found: C, 43.29; H, 2.56; N, 19.36.

(Z)-4-(((5-nitrothiophen-2-yl)methylene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3u—

Compound **3u** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)2,4-dihydro-3H-1,2,4-triazole-3-thione (0.138 g, 0.696 mmol), 5-nitro-2thiophenecarboxaldehyde (0.218 g, 1.39 mmol) and 6 mL of anhydrous THF. The reaction mixture was dry evaporated and the crude product was recrystallized in acetone to give 0.142 g (0.42 mmol, 60%) of **3u** as an orange solid. ^1H NMR (Acetone- d_6): 7.13–7.28 (m, 1H); 7.73 (dd, 2H, $J = 14.9$ Hz and $J = 4.2$ Hz); 7.90 (d, 1H, $J = 3.6$ Hz); 8.07 (d, 1H, $J = 4.3$ Hz); 10.95 (s, 1H). IR (KBr): 1368 cm^{-1} . m.p. = 241–243°C. Analysis Calculated for $\text{C}_{11}\text{H}_7\text{N}_5\text{O}_2\text{S}_3$: C, 39.16; H, 2.09; N, 20.75. Found: C, 39.38; H, 1.82; N, 20.43.

5-(furan-2-yl)-4-(((1Z,2E)-3-(5-nitrofuran-2-yl)allylidene)amino)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3v—

Compound **3v** was prepared according to the above procedure using 4-amino-5-(furan-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (0.234 g, 1.28 mmol), 5-nitro-2-furanacrolein (0.322 g, 1.92 mmol) and 8 mL of anhydrous THF. The reaction mixture was dry evaporated and the crude product was recrystallized in acetone to give 0.184 g (0.56 mmol, 44%) of **3v** as an orange solid. ^1H NMR (Acetone- d_6): 6.69 (m, 1H); 7.19 (d, 1H, $J = 3.8$ Hz); 7.26–7.46 (m, 3H); 7.62 (d, 1H, $J = 8.6$ Hz); 7.82 (d, 1H, $J = 1.5$ Hz); 10.23 (d, 1H, $J = 10.2$ Hz). IR (KBr): 1369 cm^{-1} . m.p. = $217\text{--}218^\circ\text{C}$. Analysis Calculated for $\text{C}_{13}\text{H}_9\text{N}_5\text{O}_4\text{S} \cdot 0.2$ acetone: C, 47.67; H, 3.00; N, 20.42. Found: C, 48.08; H, 3.16; N, 20.50. HRMS: ESI, (M+H) formula: $\text{C}_{13}\text{H}_{10}\text{N}_5\text{O}_4\text{S}$, Calculated 332.0448, Found 332.0457.

(Z)-4-(((5-nitrofuran-2-yl)methylene)amino)-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3w—

Compound **3w** was prepared according to the above procedure using 4-amino-5-(thiophen-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (0.196 g, 1.00 mmol), 5-nitro-2-furaldehyde (0.278 g, 1.97 mmol) and 6 mL of anhydrous THF. The reaction mixture was dry evaporated and the crude product was purified by a column chromatography on silica (CHCl_3) and then recrystallized in CHCl_3 /Hexanes to give 72 mg (0.224 mmol, 23%) of **3w** as a light orange solid. ^1H NMR (Acetone- d_6): 7.24 (m, 1H); 7.62 (d, 1H, $J = 3.8$ Hz); 7.71 (d, 1H, $J = 3.9$ Hz); 7.79 (d, 1H, $J = 5.0$ Hz); 8.00 (d, 1H, $J = 4.0$ Hz); 10.83 (s, 1H). IR (KBr): 1363 cm^{-1} . m.p. = $190\text{--}191^\circ\text{C}$. Analysis Calculated for $\text{C}_{11}\text{H}_7\text{N}_5\text{O}_3\text{S}_2$: C, 41.12; H, 2.19; N, 21.79. Found: C, 41.00; H, 2.34; N, 21.48.

(Z)-5-(furan-2-yl)-4-(((5-nitrothiophen-2-yl)methylene)amino)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3x—

Compound **3x** was prepared according to the above procedure using 4-amino-5-(furan-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (0.2 g, 1.09 mmol), 5-nitro-2-thiophenecarboxaldehyde (0.256 g, 1.63 mmol) and 8 mL of anhydrous THF. The reaction mixture was dry evaporated and the crude product was recrystallized in acetone to give 80 mg (0.25 mmol, 22%) of **3x** as an orange solid. ^1H NMR (Acetone- d_6): 6.70–6.80 (m, 1H); 7.28 (d, 1H, $J = 3.7$ Hz); 7.80–7.95 (m, 2H); 8.14 (d, 1H, $J = 4.2$ Hz); 10.84 (s, 1H). IR (KBr): 1365 cm^{-1} . m.p. = 244–245°C. Analysis Calculated for $\text{C}_{11}\text{H}_7\text{N}_5\text{O}_3\text{S}_2$: C, 41.12; H, 2.19; N, 21.79. Found: C, 40.79; H, 2.06; N, 21.58.

(Z)-5-(4-hydroxyphenyl)-4-(((5-nitrothiophen-2-yl)methylene)amino)-2,4-dihydro-3H-1,2,4-triazole-3-thione 3y—

Compound **3y** was prepared according to the above procedure using 4-amino-5-(4hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (0.1 g, 0.48 mmol), 5-nitro-2thiophenecarboxaldehyde (0.204 g, 1.29 mmol) and 4 mL of absolute ethanol. After filtration, the crude product was purified by column chromatography on silica (CH_2Cl_2) to give 89 mg (0.26 mmol, 53%) of **3y** as an orange solid. ^1H NMR (Acetone- d_6): 6.99–7.03 (m, 2H); 7.80–7.85 (m, 2H); 7.86 (d, 1H, $J = 4.3$ Hz); 8.12 (d, 1H, $J = 2.2$ Hz). m.p. = 275–276°C. Analysis Calculated for $\text{C}_{13}\text{H}_9\text{N}_5\text{O}_3\text{S}_2$: C, 44.95; H, 2.61; N, 20.16. Found: C, 45.57; H, 2.76; N, 19.46.

(Z)-5-(furan-2-yl)-4-(((5-nitrofuran-2-yl)methylene)amino)-2,4-dihydro-3H-1,2,4-triazole-3thione 3z—

Compound **3z** was prepared according to the above procedure using 4-amino-5-(furan-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (0.2 g, 1.09 mmol), 5-nitro-2-furaldehyde (0.385 g, 2.72 mmol) and 8 mL of anhydrous THF. The reaction mixture was dry evaporated and the crude product was recrystallized in acetone to give 82 mg (0.27 mmol, 25%) of **3z** as an orange solid. ¹H NMR (Acetone-*d*₆): 6.73 (s, 1H); 7.43 (d, 1H, *J* = 3.6 Hz); 7.62 (d, 1H, *J* = 3.9 Hz); 7.71 (d, 1H, *J* = 3.9 Hz); 7.84 (s, 1H); 10.77 (s, 1H). m.p. = 177–179°C. Analysis Calculated for C₁₁H₇N₅O₄S: C, 43.28; H, 2.31; N, 22.94. Found: C, 43.36; H, 2.44; N, 22.30.

Conclusion

While investigating a class 4-(R₂-imino)-5-(R₁)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones, potentially useful as inhibitors of NO production in PAM212 cells, we also established their potential usefulness as anti-neoplastic agents. The initial findings were obtained with a murine keratinocyte cell line (PAM212) but were also extended to human cancer cell lines (HT-29, PC3 and HeLa), demonstrating the potency of such compounds to efficiently inhibit the growth of such malignant cell lines. Amongst some of the most active compounds were the nitrofuran and the isosteric nitrothiophenes, designed to structurally mimic nitrofurantoin. It should be noted that the mechanism of iNOS inhibition by our compounds is not known. Further studies are needed to determine if the mechanism involves direct interaction with the iNOS protein (e.g., competitive inhibition, inhibition of active iNOS dimerization) or inhibition of iNOS expression.⁽²⁶⁾

Acknowledgments

This work was funded in part by the National Institutes of Health CounterACT Program through the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIH Grant number U54AR055073). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the federal government.

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- A set of synthesized and fully characterized 1,2,4-triazole imines were evaluated as iNOS inhibitors
- A sub-set, within that group, also showed promising inhibitory growth properties against a range of human cancer cell lines (PC3, HeLa, HT-29).
- Within that subset, the compounds designed to be nitrofurantoin mimics showed the best anti-neoplastic activity.

Figure 1:
Some examples of imidazole, triazole and Nitrofurantoin derivatives with demonstrated biological activity

Growth Inhibition for compound 3d

Figure 2:
Cellular growth inhibition curves for compound **3d** in various cancer cell lines such as (HT-29: human colon), (PC3: human prostate), (HeLa: human cervical) and (PAM212: mouse keratinocytes)

METHOD 1 **METHOD 2** **Scheme 1:**Synthesis of 4-amino-5- R_1 -2,4-dihydro-3H-1,2,4-triazole-3-thione

Table 1:iNOS & growth inhibition data obtained with PAM 212 keratinocytes for compounds **3(a-z)**.

Compound	R ₁	R ₂	Nitrite inhibition IC ₅₀ (μM)	Growth inhibition IC ₅₀ (μM)
3a			22	> 100 ^(*)
3b			> 100 ^(*)	> 100 ^(*)
3c			14	59
3d			NA	4.6
3e			26	93
3f			NA	8.1
3g			NA	4.2
3h	Me-		15	> 100 ^(*)
3i			15	> 100 ^(*)

Compound	R ₁	R ₂	Nitrite inhibition IC ₅₀ (μM)	Growth inhibition IC ₅₀ (μM)
3j	Me-		NA	6.2
3k			26	97
3l			NA	2.9
3m	Me-		NA	12
3n			NA	5.1
3o			NA	5.9
3p			NA	5.2
3q			23	> 100 ^(*)
3r			34	102
3s			28	61

Compound	R ₁	R ₂	Nitrite inhibition IC ₅₀ (μM)	Growth inhibition IC ₅₀ (μM)
3t			NA	13
3u			NA	15
3v			NA	2.9
3w			NA	3.2
3x			NA	15
3y			NA	3.1
3z			NA	1.5

NA: Not Available (compound was too cytotoxic to cell line);

(*) Maximum [] tested and for which 50% nitrite inhibition was not reached and 50% growth inhibition was not attained.