Supporting Information

Synthesis, biological evaluation and docking studies of N-substituted acetamidines as selective inhibitors of inducible nitric oxide synthase

Cristina Maccallini,[†,•] Antonia Patruno,[†,•] Neva Bešker,[†,•] Jamila Isabella Alì,[†] Alessandra Ammazzalorso,[†] Barbara De Filippis,[†] Sara Franceschelli,[‡] Letizia Giampietro,[†] Mirko Pesce,[‡] Marcella Reale,[§] Maria L. Tricca,[†] Nazzareno Re,[†] Mario Felaco,[‡] Rosa Amoroso[†,*]

[†] Dipartimento di Scienze del Farmaco, Università "G. d'Annunzio", Chieti, Italy

[‡] Dipartimento di Scienze del Movimento Umano, Università "G. d'Annunzio", Chieti, Italy

[§] Dipartimento di Oncologia e Neuroscienze, Università "G. d'Annunzio", Chieti, Italy

Contents:

General information on instrumentation	S2
Spectral data for **2**, (*S*)-**3**, ***rac*-4**, **7**, **8**, **9**	S2,S3
Measurement of cellular NOS activity	S3
NOS activity assay	S4
Docking analysis	S4, S5, S6
Scheme 4	S6
Figure 2	S6
Figure 5	S7
Figure 6	S7
References	S8

General information on instrumentation. All chemicals were purchased from commercial sources and used without further purification. Flash chromatography was performed on silica gel 60 (Merk) and TLC on silica gel 60, F_{254}. Melting points were determined on a Buchi apparatus and given uncorrected. Infrared spectra were recorded on a FT-IR 1600 Perkin-Elmer spectrometer. NMR spectra were run at 300 MHz on a Varian instrument; chemical shifts are (δ) are reported in ppm. HPLC separations were performed on a 600W Waters apparatus equipped with a 2487 dual band UV spectrometer, using a column Polarity (Waters), 5μm, 4.6 x 250 mm or 10μm, 10 x 250 mm. Mass spectra were obtained on a Thermofinnigan LCQ Advantage spectrometer (ESI). Elemental analyses were carried out with an Eurovector Euro EA 3000 model analyzer and purity of all compounds was ≥ 95%. Spectroscopic data are reported only for iNOS-selective compounds.

1-(Benzylamino)ethaniminium bromide (2): hygroscopic white solid, 82% yield; mp 121-122 °C; IR (KBr) 3235, 1673, 1631 cm^{-1}; ^{1}H NMR (DMSO) δ 2.22 (s, 3H, C*H*$_3$C), 4.45 (s, 2H, C*H*$_2$Ar*)*, 7.22-7-7.40 (m, 5H, Ar); ^{13}C NMR (DMSO) δ 19.37 (*C*H$_3$C) 45.78 (*C*H$_2$Ar) 126.90, 128.53, 129.33 and 135.98 (*C* Ar), 164.85 (*C*NH). MS 149.1 (MH^+); anal. ($C_9H_{13}BrN_2$) C, H, N.

1-[(1-Phenylethyl)amino]ethaniminium bromide [(*S*)-3]: hygroscopic white solid, 77% yield; $[\alpha]_D^{20}$ -91° (c 1.15, H_2O); mp 122-123 °C; IR (KBr) 3218, 1673, 1623 cm^{-1}; ^{1}H NMR (DMSO) δ 1.44 (d, 3H, C*H*$_3$CH), 2.19 (s, 3H, C*H*$_3$C), 4.97 (q, 1H, C*H*CH$_3$), 7.31-7.41 (m, 5H, Ar); ^{13}C NMR (DMSO) δ 19.4 (*C*H$_3$C), 22.60 (*C*H$_3$CH), 51.75 (*C*HCH$_3$), 126.90, 128.35, 129.25 and 141.68 (*C*H Ar), 163.70 (*C*NH). MS 163.1 (MH^+); anal. ($C_{10}H_{15}BrN_2$) C, H, N.

***N*-(1-Phenylpropyl)ethanimidamide hydrobromide [(*rac*-4)]**: white solid, 63% yield; mp 57-62 °C; IR (KBr) 3219, 3071, 1675, 1494 cm^{-1}; ^{1}H NMR (CD_3OD) δ 0.96 (t, 3H, C*H*$_3$CH$_2$), 1.85-1.98 (m, 2H, C*H*$_2$CH$_3$), 2.28 (s, 3H, C*H*$_3$CN), 4.57 (t, 1H, C*H*CH$_2$), 7.30-7.44 (m, 5H, Ar); ^{13}C NMR (CD_3OD) δ 9.92 (*C*H$_3$CH$_2$), 17.93 (*C*H$_3$CN), 29.39 (*C*H$_2$CH$_3$), 58.62 (*C*HCH$_2$), 126.42, (*C* Ar), 128.12 and 128.81 (*C*H Ar), 164.45 (*C*NH). MS 177.1 (MH^+); anal. ($C_{11}H_{17}BrN_2$) C, H, N.

1-[(1-Propionylpiperidin-4-yl)amino]ethaniminium bromide (9): white solid, 90% yield; mp 169-172 °C; IR (KBr) 3285, 1682, 1648 cm^{-1}; ^1H NMR (CD_3OD) δ 1.25 (t, 3H, C*H*$_3$CH$_2$), 1.39-1.52 (m, 2H, *H*HCCHCH*H*), 1.93-1.99 (m, 2H, H*H*CCHC*H*H), 2.22 (s, 3H, C*H*$_3$CN), 2.88-3.29 (m, 2H, *H*HCNCH*H*), 3.67-3.74 (m, 1H, CH$_2$C*H*CH$_2$), 4.08-4.16 (m, 4H, H*H*CNC*H*H and CH$_3$C*H*$_2$); ^{13}C NMR (CD_3OD) δ 13.74 (*C*H$_3$CH$_2$), 17.98 (*C*H$_3$NH), 30.19 (*C*H$_2$CH*C*H$_2$), 42.19 (*C*H$_2$N*C*H$_2$), 49.86 (CH$_2$*C*HCH$_2$), 61.70 (CH$_3$*C*H$_2$), 155.86 (*C*NH), 164.04 (*C*O). MS 214.1 (MH^+); anal. ($C_{10}H_{20}BrN_3O_2$) C, H, N.

1-[(3-{[(Pyridin-4-ylmethyl)amino]methyl}benzyl)amino]ethaniminium bromide (7): hygroscopic brown solid, 90% yield. IR (nujol) 3327, 1676, 1411 cm^{-1}; ^1H NMR (DMSO) δ 2.17 (s, 3H, C*H*$_3$C), 3.31 (s, 6H, C*H*$_2$Ar and C*H*$_2$Pyr), 7.2-7.32 (4H, m, Ar), 7.35 (d, 2H, Pyr), 8.49 (d, 2H, Pyr); ^{13}C NMR δ 19.44 (*C*H$_3$C), 45.90 and 52.56 (*C*H$_2$Ar), 51.48 (*C*H$_2$Pyr), 123.75 and 150.10 (*C*H Pyr), 126.95, 128.16, 129.24 and 135.76 (*C*H Ar), 141.82 and 149.05 (*C*Ar), 164.91 (*C*NH); MS: 269.2 (MH^+); anal. ($C_{16}H_{21}BrN_4$) C, H, N.

1-{[3-({[(4-Methylphenyl)sulfonyl]amino}methyl)benzyl]amino}ethaniminium chloride (8): oil, 30% yield. IR (neat): 3370, 1657, 1316, 1154 cm^{-1}; ^1H NMR (DMSO) δ 2.18 (s, 3H, C*H*$_3$Ar), 2.37 (s, 3H, C*H*$_3$C), 3.91 (s, 2H, C*H*$_2$Ar), 4.41 (s, 2H, C*H*$_2$Ar), 7.09-7.28 (m, 4H, Ar), 7.37 (d, 2H, Ar), 7.68 (d, 2H, Ar); ^{13}C NMR δ 19.93 (*C*H$_3$C), 21.8 (*C*H$_3$Ar), 45.78 (*C*H$_2$Ar), 47.56 (*C*H$_2$Ar), 126.65, 127.98, 129.32 and 131.03 (*C*H Ar), 137,42, 138.21, 139.75 and 143.87 (*C* Ar), 164.05 (*C*NH). MS 332.1 (MH^+); anal. ($C_{17}H_{22}ClN_3O_2S$) C, H, N.

Measurement of Cellular NOS Activity. THP-1, a human myelomonocytic leukemia cell line, was cultured at a density of 10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 ng/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine in a 5% CO_2- air humidified atmosphere at 37°C and passaged every 4 to 5 days. Cells were induced to express iNOS with 10 μg/mL of bacterial LPS (Sigma) for 24 h after a 1 h preincubation with

inhibitors (150 μM). THP-1 cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 lg/ml of leupeptin, 3 mM NaF and 1 mM DTT at 4 °C for 30 min. iNOS and eNOS activity was detected in the 11,000xg supernatant of cell homogenates, measured by monitoring the conversion of L-[2,3-3H]arginine to L-[2,3-3H]citrulline. Cell lysates were used as the enzyme source after treating with Dowex-50 (Na^+ form) ion-exchange resin to remove endogenous arginine. 1 μl of radioactive arginine, L-(2,3,4,5)-[3H]Arginine Monohydrochloride 64 Ci/mM, 1μCi/μl (Amersham, Arlington Heigths, Illinois, USA), 5 μl NADPH 10 mM, 5 μl $CaCl_2$ 6 mM, (Calbiochem, CA USA) were added to each sample and incubated for 30 min at room temperature. After incubation, the reactions were stopped with 400 μl of stop-buffer (50 mM HEPES, pH 5.5, 5 mM EDTA) and equilibrated resin was added to each sample to bind unreacted arginine. After centrifugation, the radioactivity corresponding to L-[3H]-citrulline was measured with liquid scintillation spectrometry. Calcium was omitted from these incubations to favour the determination of the calcium independent iNOS isoform.

Cytotoxicity experiments were performed using Cytotox 96 (Promega, Southampton, UK) for all inhibitors.

NOS Activity Assay. NOS activity was measured spectrophotometrically using the oxyhemoglobin assay as described by Heavel and Marletta.[1] iNOS activity was detected in the supernatant of THP-1 cell homogenates spun at 11,000xg. Cells were induced to express iNOS with 10 μg/mL of bacterial LPS (Sigma) for 24 h. The eNOS isoform used was bovine recombinant enzyme, isolated from a Baculovirus overexpression system in Sf9 cells.

Docking Analysis. Docking calculation were performed using Glide 3.5 program[2] on a E6600 processor running Linux. The protein structures used in the docking studies were human iNOS and eNOS complexed with W1400 (pdb codes 1QWS and 1F01, respectively).

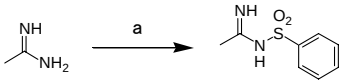
All crystal structures were prepared according to the protein preparation procedure recommended. The ligands and solvent molecules were removed, but the cofactors, heme and H_4B for iNOS or GOL for eNOS were retained near the active site. For each protein structure, polar hydrogen atoms were added, and Kollman united atom charges were assigned. Hydrogens were also added to the heme and H_4B, and charges were calculated by the Gasteiger-Marsili method. The Fe atom of heme was assigned a charge of +3. Docking was carried out in standard docking mode with the receptor after removal of the substrate. Potential steric clashes were alleviated via energy minimization with the OPLS-AA force field.[3]

The binding region was defined by a 14 Å ×14 Å ×14 Å box centered on the mass center of the crystallographic ligand. Default input parameters were used in all computations (no scaling factor for the vdW radii of nonpolar protein atoms, 0.8 scaling factor for nonpolar ligand atoms). All compounds were docked and scored using the Glide standard-precision (SP) mode.[2a] Upon completion of each docking calculation, ten poses per ligand were saved. The best-docked structures were ranked using a model energy score (Emodel) derived from a combination of the Glide Score (Gscore, a modified and extended version of the empirically based ChemScore function), Coulombic and the van der Waals energies, and the strain energy of the ligands.[2a] The top-ranked compounds obtained in this way were redocked and rescored using the Glide extra-precision (XP)[13b] mode and the best-docked structure was chosen using Emodel.

Conformational searches on all ligands were performed using the MCMM with the OPLS-AA force field as implemented in the MacroModel package.[4] Aqueous solution conditions were simulated using the continuum dielectric water solvent model (GB/SA).[5] For each MCMM search, up to 1000 starting structures were generated and minimized until the gradient was less than 0.05 kJ/molÅ, or continued until a limit of 5000 iterations was reached, using the PR Conjugant Gradient (PRCG) method implemented in MacroModel. Extended cutoff distances were defined at 8 Å for van der

Waals, 20 Å for electrostatics, and 4 Å for H-bonds. MCMM torsional variations and low mode parameters were set up automatically within Maestro graphical user interface.

A total of 1000 search steps were performed, and the conformations with energy difference of 50 kJ/mol from the global minimum were saved.



11

Scheme 4. Reagents and conditions: (a) $PhSO_2Cl$, NaOH 50% w/w, CH_2Cl_2, reflux to rt, 17 h.

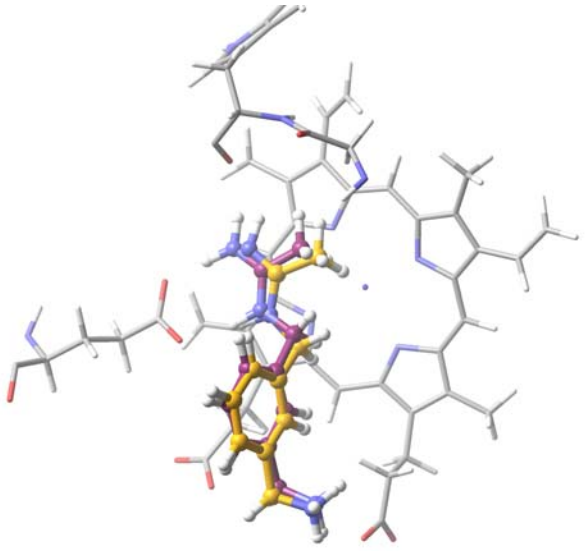


Figure 2. Superimposition of the crystallographic structure and docked conformation of **1**.

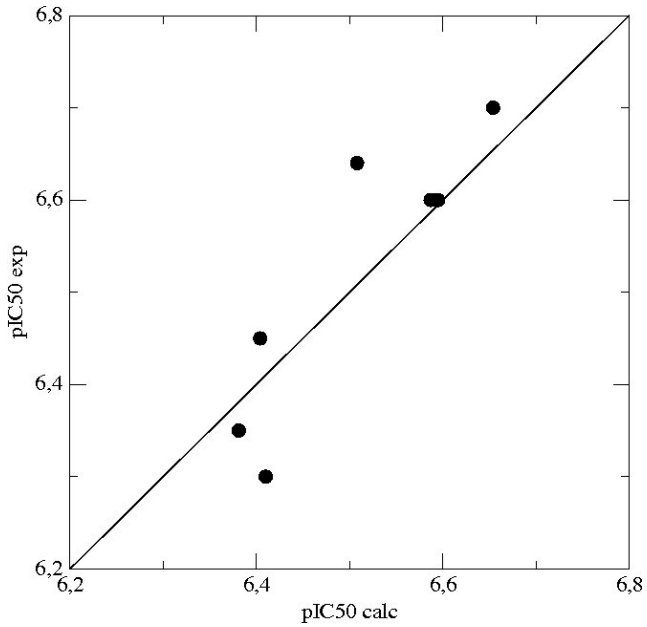


Figure 5. Correlation between predicted and experimental activities, expressed as pIC_{50} values.

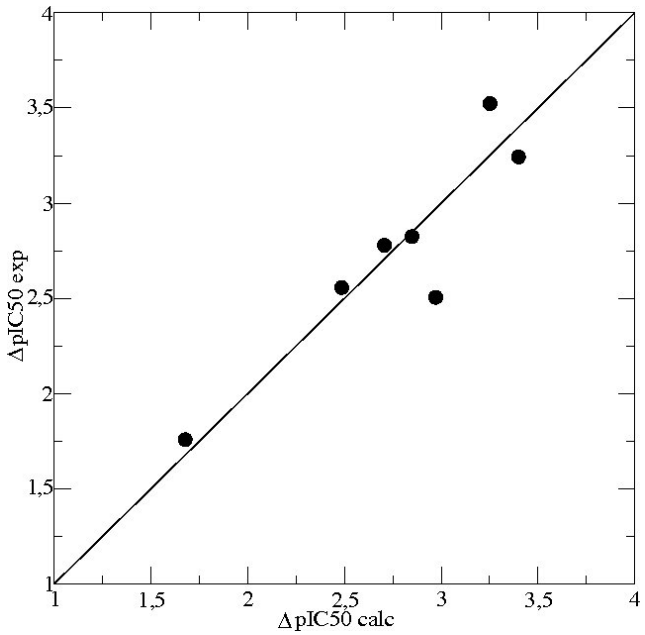


Figure 6. Correlation between predicted and experimental selectivities, expressed as ΔpIC_{50} values.

References of Supporting Informations

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