Supporting Information

Nitric Oxide Synthase Inhibitors that Interact with both Heme Propionate and H₄B show High Isoform Selectivity

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1. Synthesis of 28

**Scheme S1. Synthesis of 28.**

2-Bromo-6-(2,5-dimethyl-1H-pyrrol-1-yl)-4-methylpyridine (S2). To a dry 20 mL microwave vial equipped with a magnetic stir bar was added 6-bromo-4-methyl-pyridin-2-ylamine (935 mg, 5.0 mmol) dissolved in toluene (15 mL). 2,5-Hexadione (687 mg, 6.0 mmol) and p-toluenesulfonic acid (0.20 g) were then added, and the vial was capped with a rubber septum. The vial was heated in the microwave irradiator for 60 min at 150 °C. The reaction mixture was cooled, concentrated in vacuo, and purified by flash column chromatography to give the title product (1.09 g, 82%).

1H NMR (500 MHz, CDCl₃) δ 7.35 (s, 1H), 7.01 (s, 1H), 5.90 (s, 2H), 2.44 (s, 3H), 2.17 (s, 6H); 13C NMR (126 MHz, CDCl₃) δ 151.85, 151.67, 140.60, 128.63, 127.19, 121.43, 107.34, 20.81, 13.29. MS (ESI) m/z 265.5 [M + H]+.

2-(2,5-Dimethyl-1H-pyrrol-1-yl)-6-ethynyl-4-methylpyridine (28). The reaction mixture of S2 (1.0 g, 3.77 mmol), Pd(PPh₃)₂Cl₂ (134 mg, 0.2 mmol), CuI (37 mg, 0.2 mmol), PPh₃ (200 mg, 0.75 mmol), trimethylsilylacetylene (0.59 mL, 4.2 mmol), diethylamine (7 mL), and DMF (7 mL) were heated at 120 °C for 20 min in the microwave cavity. Then the reaction mixture was treated with diethyl ether, filtered, and concentrated in vacuo. After being stirred with 1N NaOH (10 mL) and MeOH (30 mL) for 30 min, the reaction mixture was diluted with water (50 mL) and ethyl acetate. The organic layer was partitioned, dried over MgSO₄, concentrated in vacuo, and then purified using silica gel column chromatography to give 28 (756 mg, 95%) as a pale brown solid. 1H NMR (500 MHz, CDCl₃) δ 7.46 – 7.31 (m, 1H), 7.04 (dd, J = 1.4, 0.8 Hz, 1H), 5.89 (s, 2H), 3.16 (s, 1H), 2.44 (d, J = 0.8 Hz, 3H), 2.15 (s, 7H); 13C NMR (126 MHz, CDCl₃) δ 152.21, 149.87, 141.51, 128.55, 127.06, 122.89, 106.93, 82.30, 20.90, 13.22; MS (ESI) m/z 443.08 [2M + Na].
2. Inhibitor Complex Crystal Preparation. The rat nNOS or bovine eNOS heme domain proteins used for crystallographic studies were produced by limited trypsin digest from the corresponding full length enzymes and further purified through a Superdex 200 gel filtration column (GE Healthcare) as described previously. The nNOS heme domain (at 9 mg/mL containing 20 mM histidine), or the eNOS heme domain (at 12 mg/mL containing 2 mM imidazole) was used for the sitting drop vapor diffusion crystallization setup under conditions previously reported. Fresh crystals (1-2 days old) were first passed stepwise through cryoprotectant solutions and then soaked with 10 mM inhibitor for 4-6 h at 4 °C before being flash cooled with liquid nitrogen.

3. X-ray Diffraction Data Collection, Data Processing, and Structural Refinement. The cryogenic (100 K) X-ray diffraction data were collected remotely at the Stanford Synchrotron Radiation Lightsource (SSRL) or Advanced Light Source (ALS) through the data collection control software Blu-Ice and a crystal mounting robot. When a Q315r CCD detector was used, 90-100° of data were typically collected with 0.5° per frame. If a Pilatus pixel array detector was used, 140-160° of fine-sliced data were collected with 0.2° per frame. Raw CCD data frames were indexed, integrated, and scaled using HKL2000, but the pixel array data were processed with XDS and scaled with Aimless. The binding of inhibitors was detected by the initial difference Fourier maps calculated with REFMAC. The inhibitor molecules were then modeled in COOT and refined using REFMAC. Disordering in portions of inhibitors bound in the NOS active sites was often observed, sometimes resulting in poor density quality. However, partial structural features usually could still be visible if the contour level of the sigmaA weighted 2m|Fo| – D|Fc| map dropped to 0.5 σ, which afforded the building of reasonable models into the disordered regions. Water molecules were added in REFMAC and checked by COOT. The TLS protocol was implemented in the final stage of refinements with each subunit as one TLS group. The omit Fo – Fc density maps were calculated by repeating the last round of TLS refinement with inhibitor coordinate removed from the input PDB file to generate the map coefficients DELFWT and PHDELWT. The refined structures were validated in COOT before deposition in the RCSB protein data bank. The crystallographic data collection and structure refinement statistics are summarized in Table S1 of the Supporting Information, with the PDB accession codes included.
4. Table S1. Crystallographic data collection and refinement statistics

| Data set | nNOS-3(3S) | nNOS-3R | nNOS-4 | nNOS-5(5S) |
|----------|------------|---------|--------|------------|
| **Data collection** | | | | |
| PDB code | 4CTP | 4CTQ | 4CTR | 4CTT |
| Space group | P2₁P₂₁ | P2₁P₂₁ | P2₁P₂₁ | P2₁P₂₁ |
| Cell dimensions | 51.9 111.0 163.4 | 52.0 111.2 164.2 | 51.5 111.4 164.3 | 51.7 111.3 164.3 |
| a, b, c (Å) | | | | |
| Detector | CCD | CCD | CCD | CCD |
| Resolution (Å) | 2.05 (2.09-2.05) | 2.00 (2.03-2.00) | 2.20 (2.24-2.20) | 2.30 (2.34-2.30) |
| Rmerge | 0.065 (0.708) | 0.065 (0.721) | 0.070 (0.728) | 0.065 (0.754) |
| Rpim | n/c | n/c | n/c | n/c |
| CC 1/2 | n/c | n/c | n/c | n/c |
| I / σI | 24.7 (2.1) | 25.7 (2.0) | 23.0 (1.9) | 21.3 (1.5) |
| No. unique reflections | 59,681 | 64,775 | 48,715 | 43,742 |
| Completeness (%) | 99.9 (99.9) | 99.4 (99.1) | 99.5 (100.0) | 99.9 (100.0) |
| Redundancy | 4.1 (4.0) | 4.0 (3.6) | 4.0 (3.8) | 3.6 (3.6) |
| **Refinement** | | | | |
| Resolution (Å) | 2.05 | 2.00 | 2.20 | 2.30 |
| No. reflections used | 56,652 | 61,524 | 46,205 | 40,927 |
| R_work / R_free² | 0.184/0.224 | 0.186/0.225 | 0.198/0.262 | 0.195/0.249 |
| No. atoms | | | | |
| Protein | 6665 | 6669 | 6662 | 6654 |
| Ligand/ion | 183 | 183 | 183 | 181 |
| Water | 315 | 261 | 124 | 124 |
| R.m.s. deviations | | | | |
| Bond lengths (Å) | 0.010 | 0.012 | 0.012 | 0.011 |
| Bond angles (deg) | 1.93 | 1.49 | 2.05 | 2.00 |
| Data set | nNOS-6 | nNOS-7 | nNOS-8(8R) | nNOS-8S |
|----------|--------|--------|------------|--------|
| **Data collection** | | | | |
| PDB code | 4CTU | 4CTV | 4CTW | 4CTX |
| Space group | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) |
| Cell dimensions (Å) | 51.8 110.5 164.0 | 51.9 111.1 164.5 | 52.2 110.8 164.1 | 51.9 111.0 164.0 |
| Detector | CCD | Pixel array | CCD | CCD |
| Resolution (Å) | 2.16 (2.20-2.216) | 1.78 (1.88-1.78) | 1.90 (1.96-1.90) | 1.83 (1.86-1.83) |
| Rmerge | 0.064 (0.736) | 0.053 (1.412) | 0.057 (0.366) | 0.075 (>1.000) |
| Rpim | n/c | 0.025 (0.664) | 0.045 (0.310) | 0.040 (0.669) |
| CC 1/2 | n/c | n/c (74.2) | n/c (50.0) | n/c (61.0) |
| I / σI | 21.5 (2.1) | 17.8 (1.3) | 10.8 (2.3) | 18.3 (0.6) |
| No. unique reflections | 50,182 | 91,044 | 74,707 | 83,379 |
| Completeness (%) | 97.2 (97.3) | 99.8 (99.8) | 99.9 (99.6) | 98.0 (84.8) |
| Redundancy | 3.4 (3.3) | 5.5 (5.4) | 3.5 (3.4) | 4.7 (2.9) |
| **Refinement** | | | | |
| Resolution (Å) | 2.16 | 1.78 | 1.90 | 1.83 |
| No. reflections used | 47,507 | 86,616 | 70,939 | 79,050 |
| R\(_{work}\) / R\(_{free}\) | 0.185/0.234 | 0.200/0.239 | 0.189/0.228 | 0.186/0.226 |
| No. atoms | | | | |
| Protein | 6655 | 6681 | 6682 | 6683 |
| Ligand/ion | 185 | 185 | 185 | 185 |
| Water | 233 | 316 | 443 | 341 |
| R.m.s. deviations | | | | |
| Bond lengths (Å) | 0.010 | 0.015 | 0.010 | 0.010 |
| Bond angles (deg) | 1.93 | 1.79 | 1.37 | 1.38 |
### Data set

| Data set       | eNOS-3R | eNOS-5(S) | eNOS-8(8R) | eNOS-8S |
|----------------|---------|-----------|------------|---------|
| **Data collection** |         |           |            |         |
| PDB code       | 4CTY    | 4CTZ      | 4CU0       | 4CU1    |
| Space group    | P2₁,2₁,2₁ | P2₁,2₁,2₁ | P2₁,2₁,2₁  | P2₁,2₁,2₁ |
| Cell dimensions| a, b, c (Å) | 57.8 106.5 156.8 | 58.0 106.7 158.4 | 57.9 106.4 156.9 | 57.3 105.2 155.0 |
| Detector       | CCD     | CCD       | CCD        | CCD     |
| Resolution (Å) | 2.30 (2.34-2.30) | 2.00 (2.03-2.00) | 2.09 (2.13-2.09) | 1.90 (1.93-1.90) |
| Rmerge         | 0.090 (0.761) | 0.058 (0.566) | 0.063 (0.703) | 0.085 (>1.000) |
| Rpim           | n/c     | n/c       | n/c        | 0.046 (0.724) |
| CC 1/2         | n/c     | n/c       | n/c        | n/c (53.8)   |
| I / σI         | 15.7 (1.9) | 34.8 (2.2) | 26.1 (2.1) | 28.5 (1.2)   |
| No. unique reflections | 43,670 | 65,590 | 57,825 | 74,481 |
| Completeness (%)| 98.9 (99.8) | 98.5 (100.0) | 99.3 (100.0) | 99.0 (99.8) |
| Redundancy     | 3.7 (3.7) | 4.1 (4.3) | 3.6 (3.6) | 4.7 (4.2)   |
| **Refinement** |         |           |            |         |
| Resolution (Å) | 2.30    | 2.01      | 2.09       | 1.90    |
| No. reflections used | 41,276 | 62,115 | 55,687 | 70,704 |
| R<sub>work</sub> / R<sub>free</sub> | 0.161/0.219 | 0.165/0.205 | 0.166/0.214 | 0.170/0.205 |
| No. atoms      |         |           |            |         |
| Protein        | 6454    | 6446      | 6438       | 6438    |
| Ligand/ion     | 207     | 197       | 193        | 200     |
| Water          | 333     | 271       | 291        | 295     |
| R.m.s. deviations |         |           |            |         |
| Bond lengths (Å) | 0.015 | 0.019 | 0.019 | 0.011 |
| Bond angles (deg) | 1.72  | 1.97 | 1.96 | 1.55 |

¹ See Figure1 for the inhibitor chemical formulas.
² R<sub>free</sub> was calculated with the 5% of reflections set aside throughout the refinement. The set of reflections for the R<sub>free</sub> calculation were kept the same for all data sets of each isoform according to those used in the data of the starting model.
5. $^1$H and $^{13}$C spectrum of 6-(2-Amino-2-(3-(2-(6-amino-4-methylpyridin-2-yl)ethyl)phenyl)ethyl)-4-methylpyridin-2-amine (3S and 3R)
6. $^1$H and $^{13}$C spectrum of 2-(6-(2,5-dimethyl-1H-pyrrol-1-yl)-4-methylpyridin-2-yl)-1-(3-(2-(4-methylpyridin-2-yl)ethyl)phenyl)ethan-1-amine (5S, 5R)
7. $^1$H and $^{13}$C spectrum of 6-(3-Amino-2-(2-(6-amino-4-methylpyridin-2-yl)ethyl)phenyl)propyl)-4-methylpyridin-2-amine (6)
8. $^1$H and $^{13}$C spectrum of 6-(3-amino-2-(6-(2-(6-amino-4-methylpyridin-2-yl)ethyl)pyridin-2-yl)propyl)-4-methylpyridin-2-amine (7)
9. $^1$H and $^{13}$C spectrum of (S)-6-(3-amino-2-(5-(2-(6-amino-4-methylpyridin-2-yl)ethyl)pyridin-3-yl)propyl)-4-methylpyridin-2-amine (8S, and 8R)
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