

Supporting Material

Chemistry

General Methods. Melting points were determined on a Büchi B-540 and are uncorrected. ¹H NMR spectra were recorded on a Varian Unity Inova 300 MHz instrument, or a Varian Unity Inova 400 MHz instrument. Chemical shifts were recorded as δ values in parts per million (ppm) and were directly referenced to tetramethylsilane (TMS) at 0.0 ppm. Mass spectra were measured on a Hewlett Packard 1100 MSD.

General Procedure for the preparation of 2-aminobenzamidines¹⁵. A suspension of 2-aminobenzonitrile (10 g, 0.084 mol), sodium methoxide (4.65 g, 0.084 mol) and hydroxylamine hydrochloride (5.88 g, 0.048 mol) in methanol was heated under reflux for 18 hours. The mixture was concentrated to an oil which was partitioned between ethyl acetate and 10% sodium hydroxide solution. The basic phase was separated and extracted three times with ethyl acetate. The combined organic solution was washed three times with saturated brine and dried over magnesium sulphate. Evaporation of the solvent and purification of the residue by flash column chromatography on silica gel, eluting with dichloromethane/ethanol to afford the intermediate **2-aminobenzamidoxime** as an oil (7.3 g., 58%), m/z (FAB+) 152 (M⁺+1), ¹H NMR (CDCl₃) δ 7.3-6.7 (4H, m), 5.1-4.7 (4H, br.s).

A suspension of 2-aminobenzamidoxime (4.0 g, 0.026 mol) and wet Raney nickel (ca. 2 g) in ethanol was stirred under 3 atmospheres of hydrogen at 60 °C for 16 hours. The catalyst was removed by filtration and the solvent evaporated to give an oil that was dissolved in a small amount of ethanol. 1N HCl in ether (60 ml) was added with stirring and the solid produced was collected by filtration to give **2-aminobenzamidine dihydrochloride¹⁵** as an off-white powder (4.5 g., 82%), m.p. 222-225 °C.

The following were prepared similarly:

2-amino-6-fluorobenzamidine dihydrochloride m.p. 215-217°C. NMR (300MHz, DMSO) δ 6.45 (1H, t, Ar-5H), 6.61 (1H,d, Ar-3H), 7.22 (1H, q, Ar-H4), ca. 6.7-7.6 (3H, v. broad, NH₃⁺), 9.27 (2H, s, NH₂), 9.42 (2H, s, NH₂). Found C 37.33, H 4.56, N 18.23, Cl 30.84. C₇H₈FN₃ .2HCl. requires C37.19, H 4.46, N 18.59, Cl 31.36.

2-amino-6-chlorobenzamidine dihydrochloride hygroscopic yellow powder, m.p. 287-289 °C (decomp). NMR (300MHz, DMSO) δ 6.72 (2H, m, Ar-3 & 5H), 7.19 (1H, t, Ar-4H), 9.29, (2H,s), 9.44 (2H,s). (The signal for the NH₃⁺ group was too broad to quantify precisely). m/z (CI+) 170/172.

2-amino-3,6-difluorobenzamidine dihydrochloride, amorphous powder. NMR (300MHz, DMSO) δ ca. 4.5 -5.2 (3H, v. broad, NH₃⁺), 6.46 (1H, complex, m, Ar-H5), 7.24 (1H, complex m, Ar-H4), 9.35(2H,s, NH₂), 9.78 (2H, s, NH₂). Found C 34.63, H 3.66, N 17.26, Cl 28.66. C₇H₇F₂N₃ .2HCl requires C 34.45, H 3.72, N. 17.22, Cl 29.05. m/z (CI+) 172 (M⁺+1).

General procedures for the preparation of 1,2-dihydro-4-quinazolinamines.

(i) A solution of 2-aminobenzamidine dihydrochloride (0.416g., 2 mmol) and ethyl 4-oxopiperidine-1-carboxylate (0.342g., 2mmol) in dry ethanol (10 ml) was heated at reflux for 4 hr. when TLC (silica; methanol:dichloromethane (1:9)) showed no amidine remaining and a single bright blue fluorescent spot formed. The solution was cooled and concentrated to dryness under vacuum and the residual gum was triturated with an ethanol/ether mixture to give the **Ethyl 4'aminospiro[piperidine-4,2'(1'H)-quinazoline]-1-carboxylate (11) hydrochloride** as a bright yellow powder (0.55g., 85%) m.p. 192 - 194°C. Found C 54.48, H 6.73, N 16.61, Cl 10.58. C₁₅H₂₀N₄O₂.HCl.0.25H₂O requires C 54.66, H 6.53, N 17.00, Cl 10.77, H₂O 1.22%. m/z (FAB+), 289 (M⁺+1, 100%).

(ii) A solution of 2-Amino-3,6-difluorobenzamidine hydrochloride (0.57g., 2mmol) and 2-furanyl (0.2 g., 2.1 mmol) in ethanol (10ml) were heated at reflux for 6hr. The solution was cooled and ether added to precipitate **5,8-difluoro-2-(2-furanyl)-1,2-dihydroquinazolin-4-amine (17) hydrochloride** as a pale yellow powder (0.4 g., 70%) m.p. 237-238°C. NMR (300MHz, DMSO) δ 6.20 (1H, d, furan-5H), 6.41 (1H,d, furan-3H), 6.45 (1H,dd, furan-4H), 6.71 (1H, complex m, quinazoline-7H), 7.53 (1H, complex m, quinazoline-6H), 7.68 (1H,s, quinazoline-2H), 8.60 (1H, bs, NH), 8.81 (1h, bd, NH), 9.55 (1H,bs, NH), 10.53 (1H,bs, NH). m/z (CI+) 250 (M⁺+1, 100%)

(iii) A solution of 1-(6-cyanonicotinoyl)-4-piperidone ethylene ketal (5.47g., 20mmol) and 2-Amino-3,6-difluorobenzamidine hydrochloride (4.16g., 20mmol) in 2-propanol (100ml) was heated at reflux for 18 hr. On cooling **1-(6-Cyano-3-pyridylcarbonyl)-5',8'-difluorospiro[piperidine-4,2'(1'H)-quinazoline]-4'-amine hydrochloride (25, AR-C102222)** separated as a pale yellow crystalline solid which was collected and washed with cold 2-propanol (7.0g., 84%) m.p. 304-305 °C: NMR (300MHz, DMSO) δ 1.85-2.10 (3H, m), 2.20 (1H, bd), 3.42-3.62 (3H, m), 4.25 (1H, bd), 6.71 (1H, m, quinazoline-7H), 7.54 (1H, m, quinazoline-

6H), 7.84 (1H, br, pyridine-2H), 8.10 (1H, dd, pyridine-4H), 8.17 (1H, d, pyridine-5H), 8.75 (1H, bd, NH), 8.78 (1H, bs, NH), 9.21 (1H, s, NH), 11.07 (1H, s, NH). m/z (CI+) 383 (M⁺+1, 100%).

As we were unable to obtain samples of 3-aminothiophene-2-carboxamide of adequate purity by using the general procedure outlined above the following alternative approach was used to prepare **ethyl 7'-aminospiro[piperidine-4,5'(4'H)-(thieno[3,2-d]pyrimidine)]-1-carboxylate hydrochloride (26)**.

A solution of 3-aminothiophene-2-thiocarboxamide (0.5 g, 3.16 mmol), methyl iodide (0.2 ml, 3.16 mmol) and N-carbethoxy-4-piperidone (0.47 ml, 3.16 mmol) in acetonitrile was stirred at r.t. for 18 hr. The product was collected by filtration, washed with acetonitrile and dried to leave crude **ethyl 7'-(methylthio)spiro[piperidine-4,5'(4'H)-(thieno[3,2-d]pyrimidine)]-1-carboxylate hydroiodide** as a bright yellow solid (0.88g., 62%). m/z (FAB+) 326 (M⁺+1). A solution of this intermediate (0.88g., 1.94 mmol) and ammonium iodide (0.28g., 1.94 mmol) in the minimum of acetonitrile was saturated with ammonia gas and then heated at reflux for 6hr. The solvent was evaporated and the residue purified by column chromatography on neutral alumina using dichloromethane : methanol mixtures as eluant. Evaporation of product-containing fractions gave a gummy solid which was dissolved in a small amount of ethanol and treated with excess ethereal HCl to give **(26) hydrochloride salt monohydrate** as a bright yellow hygroscopic powder (0.15g., 25%) m.p. 239°C. m/z (FAB+) 295 (M⁺+1)

Table 1: Microanalytical data for compounds 16-26:

Compound	Molecular Formula	Analysis found	Analysis required
16	C ₁₂ H ₁₄ FN ₃ .HCl	C 56.09, H 6.06, N 16.28, F 7.30, Cl 13.90.	C 56.36, H 5.91, N 16.43, F 7.43, Cl 13.86.
17	C ₁₂ H ₉ F ₂ N ₃ O.HCl	C 50.45, H 3.48, N 14.51, Cl 12.40.	C 50.45, H 3.48, N 14.71, Cl 12.41.
18	C ₁₄ H ₁₁ F ₂ N ₃ .HCl	C 56.72, H 4.23, N 14.30, Cl 11.99.	C 56.86, H 4.09, N 14.21, Cl 11.99.
19	C ₁₅ H ₁₉ FN ₄ O ₂ .HCl.0.25 H ₂ O	C 51.47, H 6.09, N 16.25, Cl 10.54.	C 51.82, H 5.91, N 16.13, Cl 10.21.
20	C ₁₅ H ₁₉ ClN ₄ O ₂ .HCl.	C 50.20, H 5.68, N 15.60.	C 50.15, H 5.61, N 15.60.
21	C ₁₆ H ₂₁ FN ₄ O ₂ S.HCl.0.5H ₂ O.	C 48.55, H 5.87, N 14.02, S 8.12	C48.29, H 5.78, N 14.08, S 8.04.
22	C ₁₇ H ₁₇ FN ₄ OS.HCl	C 53.67, H 5.03, N14.79 Cl 9.00, S 8.34.	C 53.61, H 4.76, N14.71, Cl 9.31, S 8.34.
23	C ₂₀ H ₁₈ FN ₅ O.HCl	C 59.93, H 4.85, N 17.67, F 4.63, Cl 8.78.	C 60.08, H 4.79, N 17.52, F 4.75, Cl 8.87.
24	C ₂₀ H ₁₇ F ₂ N ₅ O.HCl	C 57.06, H 4.47, N 16.45, F 8.92, Cl 8.55.	C 57.49, H 4.34, 16.76, F 9.09, Cl 8.48.
25	C ₁₉ H ₁₆ N ₆ F ₂ O.HCl	C 54.60, H 3.99, N 20.22, Cl 8.24.	C 54.49, H 4.09, N 20.07, Cl 8.46.
26	C ₁₃ H ₁₈ N ₄ O ₂ S.HClH ₂ O	C 45.47, H 6.06, N 15.76, Cl 10.20, S 8.88, H ₂ O, 5.56.	C 44.76, H 6.07, N 16.06, Cl 10.16, S 9.19.H ₂ O 5.17.

Representative Procedures for Parallel Synthesis: Preparation of a dihydroquinazoline library.

Parallel synthetic chemistry was carried out in 1.2ml polypropylene tubes in 96-well microtube racks (Qiagen Ltd., UK). Tubes in rows 2 – 11 only were used for reactions. A diverse selection of 237 aldehydes and 158 ketones from commercial suppliers was used. (Benzaldehyde was also included as a random reactant in each plate to act as a standard). Each tube was charged with a portion of an aldehyde or ketone (~2mg) and dimethylsulphoxide (0.5ml) was added to each. After shaking for 10 mins a portion (0.1ml) was removed for biochemical assay of the reagents as iNOS inhibitors as a control. Non showed significant activity at a concentration of 10µM. A 0.4ml aliquot of a stock solution of 2-aminobenzamidine dihydrochloride in dimethylsulphoxide (~5mg/ml) was added to the remaining content of each tube. The tubes were sealed and shaken in a heating bath at 65°C for 6hr. At this point MS indicated formation of the desired dihydroquinazoline product in >90% of the tubes. After cooling, portions of the contents of each tube were used directly, without purification by dilution to appropriate concentrations with assay buffer. Their i-NOS inhibitory activity was determined using the method described below except that % inhibition of enzyme was determined for a single concentration of the parallel synthesis compounds (~10 µM).

Compounds which showed inhibition greater than or equal to that shown by the tubes containing benzaldehyde were re-prepared using conventional synthesis on a 1 mmol scale for purification and accurate IC₅₀ measurement.

Note: In almost all cases where reaction was observed, no other product could be detected in the mixture; in a few instances small amounts (<5%) of the corresponding fully aromatized quinazoline could be detected in the lc-ms when the reactant was an aldehyde. As the purified dihydro compounds (**8**, R=H) are stable to these conditions it was postulated that these by-products arose from reaction with (**9**) of acidic impurities, either initially present in the aldehydes or formed during the heating process.

Although the required product could be readily detected by lc and ms, it was impossible to exact purity as many of the aldehyde and ketone reactants in particular gave poor responses. It was appreciated that the products of these reactions would probably not be pure (they would at least be likely to be contaminated with one or other of the starting materials). For this reason all the carbonyl components and the o-aminobenzamidine were assayed for iNOS inhibitory activity at the concentration used in this study. No significant activity was observed; it was therefore assumed that any activity observed in the reaction wells was due to reaction product (uncyclised intermediate products were never observed under these conditions). The objective of these experiments, however, was not to generate quantitative data directly, but rather to guide conventional synthetic effort toward the most profitable areas for investigation. All IC₅₀ data reported were generated from individually synthesised samples that were of confirmed purity (Elemental Analyses, MS, NMR, HPLC).

Preparation of spiroquinazoline libraries.

Spiro[piperidine-4,2'(1'H)-quinazoline]-4'-amine dihydrochloride (12**).** A solution of 2-aminobenzamidine dihydrochloride (1.04 g., 5mmol) and 4-piperidone ethylene ketal (0.72g ., 5mmol) in ethanol (50ml) containing 1M ethereal hydrogen chloride (5ml., 5mmol) was heated at reflux for 24hr. The solid which separated on cooling was filtered and recrystallised from 2-propanol giving the title compound as pale yellow hygroscopic crystals (1.3g., 90%). m.p. 271-272°C (decompn). m/z (FAB) 217 (M+1) 100%. ¹H-NMR showed that this material was a solvate containing ~1 equiv. of 2-propanol. In the iNOS inhibition assay described below it had an IC₅₀ of ~60 μM.

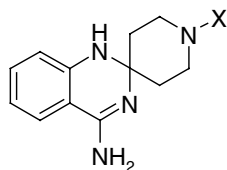
Box Chemistry. Three boxes were made up containing ~2mg each of (i) 79 acyl halides, (ii) 79 sulphonyl halides and (iii) 65 chloroformates + 14 carbamoyl chlorides in 0.5ml of DMSO. Ethyl chloroformate in DMSO was added to the 80th tube in each box. A 0.1ml portion of each was withdrawn and assayed in the iNOS screen described below. None showed significant inhibitory activity at 10 μM. An aliquot (0.4ml) of a stock solution of (**12**) in DMSO (~5mg/ml) containing di-isopropylethylamine (~5mg/ml) was added to each tube. The tubes were sealed and heated at 70°C for 18 hr. The wells were assayed as described previously using a test compound concentration of ~3 μM. Products in wells that showed inhibitory activity greater than or equal to that of the wells which had contained ethyl chloroformate were re-synthesised individually for purification and accurate IC₅₀ determination.

Biology

Recombinant enzyme inhibition assays. cDNA encoding the various human isoforms of NOS were supplied by Prof. P.A. Marsden, University of Toronto, Ontario. Recombinant human NO synthases were expressed in E. coli and lysates were prepared in Hepes buffer (pH 7.4) containing co-factors (FAD, FMN, H4B), protease inhibitors, lysozyme and CHAPS. These preparations were used, at suitable dilution, to assess inhibition of the various isoforms. Inhibition of NOS was determined by measuring the formation of L-[³H]citrulline from L-[³H]arginine using an adaptation of the method of Förstermann et al.¹⁶ Enzyme assays were performed in the presence of 3μM [³H]arginine, 1mM NADPH and other co-factors required to support NOS activity (FAD, FMN, H4B, calmodulin, Ca²⁺). Since various NOS inhibitors have been reported to exhibit slow binding kinetics, or to inactivate the enzyme in a time dependent manner, enzyme and inhibitor were pre-incubated for 60 minutes in the presence of NADPH before addition of arginine to initiate the reaction. Incubations continued for a further 60 minutes before the assays were quenched and [³H]citrulline separated from unreacted substrate by chromatography on Dowex-50W resin in a 96-well format. IC₅₀ values were calculated from data for at least 5 different concentrations of each test substance and are quoted as the mean of at least two separate determinations. For compounds quoted in this communication the range between highest and lowest individual pIC₅₀ values measured was <0.5 log unit.

Inhibition of NO production in stimulated DLD-1 cells. Human colorectal carcinoma cell line (DLD-1) was grown in RPMI 1640 (medium) supplemented with fetal bovine serum (10 vol.%) and L-glutamine (2 mM) at 37 °C in CO₂ (5%). Nitric oxide synthase was induced in the cells by adding a medium containing human recombinant gamma-interferon (1000 units/ml), TNF-alpha (200 units/ml), IL-6 (interleukin) (200 units/ml) and IL-1 beta (250 units/ml). After incubation for 18 hours at 37°C, the medium was removed and the cells were washed with warm phosphate buffered saline. Cells were then incubated for an additional 5 hours at 37 °C in CO₂ (5%), RPMI 1640 containing L-arginine (100 µM). The quantity of nitrite produced in the culture medium in the presence and absence of test compounds was measured using the Griess reagent, and IC₅₀ for the compounds calculated in the usual way.

Inhibition of lipopolysaccharide (LPS)-induced nitric oxide production in conscious rats. Male CR/CD rats (Charles River, 250-500 g.) were warmed in a hot box at 42°C for 4 mins and a 0.35ml blood sample taken from a lateral tail vein (0h sample). LPS (E. coli, 0.03mg/kg) in 0.9% saline was then injected into the same lateral vein. Test compounds were given either orally or intravenously into the contralateral tail vein. Further blood samples were taken after 2hr., 4hr. and 6hr. Total plasma [nitrite + nitrate] concentration was then determined for each sample by reduction of the nitrate with nitrate reductase followed by assaying total nitrite using the Griess reagent. The IC₅₀ was the concentration of compound producing a 50% reduction in increase in plasma nitrite concentration at the 4hr time point.

Table 2. iNOS inhibition potency of representative spirocyclic parallel synthesis compounds

Compd.	X	iNOS IC50 (μM)
12	H	50
11	CO ₂ Et	0.7
13b	CO ₂ Me	2.0
13c	CO ₂ iPr	3.5
13d	CO ₂ Ph	n.s.
13e	COPh	8.8
13f	CO-4-ClPh	0.9
13g	CO-4-CNPh	0.4
13h	CO-2-thienyl	0.7
13i	COMe	100
13j	COPr	24
14a	SO ₂ Me	53
14b,c,d	SO ₂ Ar (Ar=Ph, p-NO ₂ Ph, p-OMePh)	n.s.
15a	CONH ₂	90

Inhibition of isolated human enzyme determined with pure, individually prepared compounds. Mean of at least n=2. n.s. = no significant activity at 100 μM.

Table 3: Representative pharmacokinetic data for compounds 23 – 25.

Compound No	LogD	Cl (ml/min/kg)	Vs (l/kg)	Half-life (oral, hr.)	Bioavailability %
23	0.66	20	2.5	3.3	50
24	0.82	68	3.3	1.6	24
25	0.51	57	4.5	2.5	75

References.

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