Evaluation of analogues of furan-amidines as inhibitors of NQO2

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NRH: quinone oxidoreductase 2 (NQO2) is a cytosolic flavoprotein enzyme [1] widely distributed in human heart, brain, lung, liver and skeletal muscle [2]. NQO2 is a potential target for cancer chemotherapy as its inhibition has therapeutic and/or preventative potential. In our laboratory, non-symmetrical furan-amidine 1 (Figure 1) and para-substituted analogues were identified as novel lead inhibitors of NQO2 with both anti-cancer and anti-malarial activities [3]. Here, further modifications to these non-symmetrical furan-amidines have been evaluated. Some of the non-symmetrical furan-amidines [3] showed poor water solubility, therefore the furan ring of 1 was replaced by more water-soluble isosteric heterocycles, including imidazole and oxazole. The lead NQO2 furan inhibitor possesses the highly basic amidine group, which will potentially decrease its passive diffusion and oral bioavailability [4, 5]. Here, analogues of the non-symmetrical furan-amidine 1 were synthesized in which the amidine group was isosterically replaced with less basic groups: imidate, reversed amidine, N-arylamide and amidoxime to probe NQO2 activity, improve solubility and decrease basicity of the lead furan amidine. All compounds were fully characterised spectroscopically and the structure of the unexpected product N-hydroxy-4-(5-methyl-4-phenylfuran-2-yl)benzamidine was established by X-ray crystallography. The analogues were evaluated for inhibition of NQO2, which showed lower activity than the lead furan amidine. The observed structure-activity relationship for the furan-amidine series with NQO2 was rationalized by preliminary molecular docking and binding mode analysis. In addition, the oxazole-amidine analogue inhibited the growth of Plasmodium falciparum with an IC$_{50}$ value of 0.3 μM.

Keywords: NQO2 inhibitors, Furan-amidines, Isosteres, Anti-cancer, Malaria, SAR

Figure 1. Structures of the non-symmetrical furan-amidine 1, the symmetrical 3,4-dimethylfuran-amidine 2 and the proposed 4-methylfuran-amidine 3.
and 2, it is of interest to assess the activity of non-symmetrical 4-phenylfuran-amidine analogue 3 (Figure 1) as an NQO2 inhibitor, the synthesis of which is attempted in this study.

In order to enhance the aqueous solubility of furan amine 1 (clogS -1.81, 4.0 mg/ml [7]), the furan ring was first replaced with an imidazole group to give 4 (clogS -1.27, 13.9 mg/ml [7]). The synthesis of imidazole-amidine 4 is shown in Scheme 1. 4-(4-Phenyl-1H-imidazol-2-yl)benzonitrile 7 was synthesized by the reaction of 4-cyanobenzaldehyde [8] with phenylglyoxal [5], which was reduced to the amidoxime intermediate 8 [5], which was reduced to the amidine 4 using ammonium formate [9] (Scheme 1) [10].

The N-ethylimidazole analogue 9 was synthesized from the reaction of nitrile 7 with methyl iodide (Scheme 2) giving the amidoxime intermediate 8 [5], which was reduced to the amidine 4 using ammonium formate [9] (Scheme 1) [10].

The 4-cyanobenzaldehyde 5 was synthesized as shown in Scheme 3. The key precursor 4-cyanobenzaldehyde 5 was obtained via the Pinner synthesis (Scheme 1, steps iv and v) giving the amidoxime intermediate 8 [5], which was reduced to the amidine 4 using ammonium formate [9] (Scheme 1) [10].

The oxazole-amidine 13 (clogS -1.30, 13.3 mg/ml [7]) was synthesized as shown in Scheme 3. The key precursor 4-cyanobenzaldehyde 5 was obtained via the Pinner synthesis (Scheme 1, steps iv and v) giving the amidoxime intermediate 8 [5], which was reduced to the amidine 4 using ammonium formate [9] (Scheme 1) [10].

The oxazole-amidine 13 (clogS -1.30, 13.3 mg/ml [7]) was synthesized as shown in Scheme 3. The key precursor 4-cyanobenzaldehyde 5 was obtained via the Pinner synthesis (Scheme 1, steps iv and v) giving the amidoxime intermediate 8 [5], which was reduced to the amidine 4 using ammonium formate [9] (Scheme 1) [10].

To address the high basicity of the amidine group, several less basic isosteres of 1 were synthesized in which the amidine group was replaced with methyl imidate 23, amidoxime 24, N-aryl amides (reversed amidines) 25-26 and N-aryl amide 27-29. pK\textsubscript{a} and clogS are given in Table 1 and clogP and solubilities (mg/ml) are given in SI for the key compounds, with the non-amidine analogues being less basic, potentially enhancing passive permeability. The syntheses of these analogues are illustrated in Schemes 5 and 6. It was anticipated that heating of ethyl benzimidate hydrochloride 30 (prepared by reaction of nitrile 31 with ethanol) [3] at reflux with ammonium chloride methanol/water would give the furan-amidine 1, however the isolated product was the methyl imidate 23 [15] (Scheme 5). The methyl imidate group is a much less basic isostere (pK\textsubscript{a} 6.2) [15] than the highly basic amidine group (pK\textsubscript{a} 11.8) [16].

**Scheme 1.** Synthesis of 4-(4-phenyl-1H-imidazol-2-yl)benzamidine acetate 4. Reagents and conditions: (i) NH\textsubscript{4}OAc, MeOH, rt. (ii) NH\textsubscript{2}OH.HCl, t-BuOK, dry DMSO, 0 °C - rt; (iii) HCO\textsubscript{2}NH\textsubscript{2}, Pd/C, AcOH, reflux; (iv) HCl\textsubscript{et}, abs. EtOH, CHCl\textsubscript{3}, 0 °C - rt; (v) NH\textsubscript{4}OAc, Abs. EtOH, rt, 12 hr.

**Scheme 2.** Synthetic pathway for 4-((1-methyl-4-phenyl-1H-imidazol-2-yl)benzamidine 9. Reagents and conditions: (i) CH\textsubscript{3}I, KOH, acetone, rt. (ii) NH\textsubscript{2}OH.HCl, t-BuOK, dry DMSO, 0 °C - rt; (iii) HCO\textsubscript{2}NH\textsubscript{2}, Pd/C, AcOH, reflux.

**Scheme 3.** Synthesis of 4-(5-phenoxazol-2-yl)benzamidine acetate 13. Reagents and conditions: (i) NaHCO\textsubscript{3}, DCM, 0 °C – rt; (ii) Ac\textsubscript{2}O, conc. H\textsubscript{2}SO\textsubscript{4}, rt; (iii) NH\textsubscript{2}OH.HCl, t-BuOK, dry DMSO, 0 °C - rt; (iv) HCO\textsubscript{2}NH\textsubscript{2}, Pd/C, AcOH, reflux.
Scheme 4. Synthesis of the non-symmetrical 4-(5-phenylthiophen-2-yl)benzamidine acetate 19: Reagents and conditions: (i) 60 °C, Lawesson’s reagent; (ii) NH$_2$OH.HCl, t-BuOK, dry DMSO, 0 °C - rt; (iii) (Et)$_3$SiH, PdCl$_2$, AcOH, Ac$_2$O, rt - reflux.

Scheme 5. Syntheses of methyl 4-(5-phenylfuran-2-yl)benzimidate hydrochloride 23 and N-hydroxy-4-(5-phenylfuran-2-yl)benzamidine 24 from nitrile 31: Reagents and conditions: i-EtOH, ii- NH$_4$Cl, MeOH/H$_2$O, reflux.; iii - NH$_2$OH. HCl, t-BuOK, dry DMSO, 0 °C - rt.

Scheme 6. Synthesis of N-aryl amidines (reversed amidines) 25-26 and N-aryl amides 27-29; Reagents and conditions: i- HCl(g), CHCl$_3$, 0 °C – rt; (ii) Lawesson’s reagent, THF, 55 °C; (iii) NaBH$_4$, CuSO$_4$, EtOH, 0 °C – rt; (iv) AcCl, dry CH$_3$CN, rt; (v) S-2-naphthylmethyl thioacetimidate hydrobromide, AcOH, CHCl$_3$, rt.

An isosteric analogue of the asymmetric furan-amidine 1 with an amidoxime group 24 was synthesized as a less basic isostere (pKa 5-6) for the furan amidine [17]. In addition, the amidoxime group is a known prodrug for the amidine group and can enhance oral bioavailability of amidine-containing drugs [4, 5] which is activated through reduction of the amidoxime group by human liver microsomes [18]. N-Hydroxy-4-(5-phenylfuran-2-yl)benzamidine 24 was synthesized by the reaction of nitrile 31.

The first step in the syntheses of the reverse amidine and amide analogues 25-29 was the preparation of the key 1,4-diketones intermediates 32 and 33 [3] (Scheme 6). The cyclization of the 1,4-diketones 32, 33 into furans 34, 35 and thiophenes 36, 37 were catalysed by dry hydrogen chloride gas and Lawesson’s reagent, respectively. The nitro-groups in the intermediates 34-37 were reduced to amines 38-41 using sodium borohydride in the presence of catalytic copper sulfate [19]. The reduction of the nitro-groups into amines was confirmed by upfield shift of the protons of the aromatic ring: The peaks of the H-2', H-4', H-5' and H-6' protons of 34 were shifted up-field from 8.57, 8.12, 7.59 and 8.05 ppm to 6.87, 6.52, 7.08 and 6.94 ppm in 38, respectively (Figure S2). The N-aryl amides 25 and 26 were synthesized from the reaction of the amines 39 and 41, respectively, with S-2-naphthylmethyl thioacetimidate hydrobromide (Scheme 6) [20-22]. The furan N-aryl amides 27 and 28 and N-(3-(5-phenylthiophen-2-yl)phenyl)acetamide 29 were synthesized from the reaction of acetyl chloride with amines 38, 39 and 40, respectively (Scheme 6).

The synthesis of the 3-methylfuran-amidine analogue 3 was attempted as shown in Scheme 7, however coupling of 4-cyanophenyl methyl ketone 42 and α-bromomethyl phenyl ketone 43 failed to give the diaryl mono-methyl 1,4-diketone 44. Diketone 44 would have cyclised to give furan 45, a precursor for amidine 3. Instead, the condensation of 42 and 43 led to the formation of 5-methyl-2,4-diaryluran nitrile 46. The structure was confirmed by X-ray crystallography of its amidoxime derivative 47, the ORTEP diagram of which is shown in Figure 2, annotated with the numbering scheme adopted. Further
Scheme 7. Synthetic pathway for the preparation of furan-amidine 48. Reagents and conditions: (i) EtMgBr, Et₂NH, dry THF, 0 °C – rt; (ii) HCl, abs. EtOH, CHCl₃, 0 °C- rt; (iii) NH₄OAc, Abs. EtOH, rt; (iv) NH₂OH·HCl, t-BuOK, dry DMSO, 0 °C – rt.; (v) NH₄formate, Pd/C.

Figure 2. ORTEP diagram for the amidoxime derivative 47.

Scheme 8. Proposed mechanism for the formation of furan nitrile 46.

detailed crystallographic discussion of compound 47 can be found in Supplementary Material. A proposed mechanism for the formation of nitrile 46 is shown in Scheme 8. The amidoxime 47 was then converted to the amidine 48 for evaluation as an NQO2 inhibitor.

The ability of the synthesized compounds to inhibit the enzymatic activity of NQO2 was determined by a spectrophotometric method that monitored the decolouration of the blue redox dye 2,6-dichlorophenolindophenol (DCPIP) (pH 7.4). The rate of decolouration of DCPIP is indicative of NQO2 activity [3].

The isosteric replacement of furan ring 1 into imidazole 4, N-methylimidazole 9, oxazole 13 and thiophene 19 led to an increase of the IC₅₀ values when compared with 1 (Table 1).

The isosteric replacement of amidine group 1 by methyl imidate 23, amidoxime 24, N-aryl amidines (reversed amidines) 25-26 and para-N-aryl amide 28 all led to the loss of NQO2 inhibitory activity (Table 1). The meta-N-aryl amides 27 and 29 showed moderate NQO2 inhibitory activity with IC₅₀ values of approximately 1 and 2 μM, respectively. It should be noted that as the assay is performed at physiological pH, the amidine group is protonated. On the other hand, imidate 23 will be present as both the protonated and neutral forms since the pKₐ of the conjugate acid is estimated to be 6.7.

In order to provide insight into the observed SAR, the synthesized NQO2 inhibitors were then computationally docked into the NQO2 active site using the X-ray crystal structure of human NQO2 with bound FAD (PDB code 1QR2; resolution of 2.1 Å) [23]. This was performed via the GOLD 5.1 docking software (CCDC, Cambridge, UK) with the ChemScore scoring function [24]. The ten top-scoring poses were saved and those with a steric clash term exceeding 6 kJ/mol were omitted.

Our prior in silico prediction of the NQO2-bound poses of a set of asymmetric furan-amidine compounds, including compound 1,[3] found that π–π stacking interactions between ligands and NQO2 were a common feature, formed by the ligand aromatic rings with the isoalloxazine ring of FAD in particular, but also with other aromatic residues such as Phe178’ and Phe126’. Secondly, the bound ligand poses were found to fall into three basic types [3], depending on the hydrogen bonding interaction of their amidine group with rather distinct regions of the active site: with Gln122 (pose I), with Asn161’ (pose II) or with Thr71 (pose III).

As for this previous work, the binding geometries of asymmetric furan-, imidazole-, N-methylimidazole- and thiophene-amidines predicted here fit well in the deep NQO2 pocket and form a range of π–π stacking interactions. Furan-amidine 1, the experimentally most potent NQO2 inhibitor, is
predicted to adopt only pose I (magenta, Figure 3), forming a strong hydrogen bond between the amidine group and Glu122 sidechain and nearby sidechain of Glu193. For imidazole-amidine compound 4 (gold, Figure 3) and N-methylimidazole-amidine 9 (cyan, Figure 3), pose I structures are preferred and superimpose well with 1. The N-methyl group of compound 9 points into the active site, slightly displacing the ligand outwards from the bracing Trp105 (Figure S8). For thiophene-amidine 19, pose I (Figure 3) and pose III orientations (Figure S9) are predicted. For the latter, the amidine group hydrogen bonds to the sidechain OH of Thr71 and the backbone carbonyl of Asp117, although this pose possesses a relatively high active site clash energy, of 5.2 kJ/mol, as compared with pose I (1.9 kJ/mol). Therefore despite predicting a preference for pose I for compounds 1, 4, 9 and 19, these results are unlikely to explain the observed differences in their inhibitory activity. Nevertheless, subtle changes in electrostatic and steric interactions associated with the O to S substitution may be at play in determining the overall difference in experimental potency for compounds 1 and 19.

The malaria parasite Plasmodium falciparum has an enzyme that has a similar activity to NQO2, PpNQDH2 [25], therefore some of the analogues were also tested against Plasmodium (Table 1). The oxazole-amidine 13 (IC50 0.3 μM) was more active than the furan 1, imidazole 4 and thiophene 19 analogues. The reverse amidine analogues 25 and 26, which were not active as NQO2 inhibitors, both showed sub-micromolar IC50 activities in the Plasmodium parasite assay, indicating the intrinsic difference between the actual targets in human and in parasites. In conclusion, novel heterocyclic derivatives (e.g. imidazole, oxazole and thiophene) with a range of side chains (e.g. imidate, N-aryl amide, amidoxime) were designed to enhance the drug-like properties (improved aqueous solubility and decreased basicity) of the lead furan-amidine 1. Most of the synthesized analogues showed decreased or loss of activity as NQO2 inhibitors, when compared with 1, however these results provide an insight into the SAR. The inactive amidoxime 24 is of interest as a potential pro-drug for the non-symmetric furan-amidine 1. The oxazole-amidine 13 is the most active in the preliminary Plasmodium falciparum screen, showing a different SAR, promising therapeutic index against human cells and with mechanism of action studies on-going.

Table 1. cLogS, pKa, IC50 (± SE) for the inhibition of NQO2 and IC50 (± SE) for the inhibition of growth of Plasmodium falciparum by the non-symmetrical analogues of the lead furan-amidine 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>cLogS</th>
<th>pKa</th>
<th>IC50 NQO2 (μM) ± SE</th>
<th>IC50 Pf (μM) ± SE</th>
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<tr>
<td>1</td>
<td>-1.81</td>
<td>11.1</td>
<td>0.068 ± 0.009</td>
<td>1.5 ± 0.002</td>
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<tr>
<td>4</td>
<td>-1.27</td>
<td>11.2</td>
<td>1.111 ± 0.005</td>
<td>5.3 ± 0.001</td>
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<td>9</td>
<td>-0.75</td>
<td>11.2</td>
<td>Inactive</td>
<td>NT</td>
</tr>
<tr>
<td>13</td>
<td>-1.30</td>
<td>10.8</td>
<td>Inactive</td>
<td>0.31 ± 0.001</td>
</tr>
<tr>
<td>19</td>
<td>-2.25</td>
<td>11.3</td>
<td>0.773 ± 0.004</td>
<td>18.9 ± 0.002</td>
</tr>
<tr>
<td>23</td>
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<td>6.7</td>
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</tr>
<tr>
<td>24</td>
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<td>NT</td>
</tr>
<tr>
<td>25</td>
<td>-3.42</td>
<td>9.5</td>
<td>Inactive</td>
<td>0.77 ± 0.002</td>
</tr>
<tr>
<td>26</td>
<td>-3.87</td>
<td>9.6</td>
<td>Inactive</td>
<td>0.97 ± 0.001</td>
</tr>
<tr>
<td>27</td>
<td>-5.34</td>
<td>-</td>
<td>1.14 (n=1)</td>
<td>NT</td>
</tr>
<tr>
<td>28</td>
<td>-5.34</td>
<td>-</td>
<td>Inactive</td>
<td>NT</td>
</tr>
<tr>
<td>29</td>
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<td>-</td>
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<td>-</td>
<td>0.913 ± 0.023</td>
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DB75 [3,26] 0.00 11.42 0.035±0.0008 0.0005±0.0000 2

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Supplementary Material

Supplementary data related to this article can be found, in the online version, at…

References


Figure 3. (A) Docked pose I of compounds 1 (magenta), 4 (gold), 9 (cyan) and 19 (pink) in NQO2 active site for. Distances in Å; in parentheses for distances involving 19.
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