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Bioorganic & Medicinal Chemistry Letters 15 (2005) 4790–4793
Bioorganic & Medicinal Chemistry Letters

Synthesis and evaluation of 3-anilino-quinoxalinones as glycogen phosphorylase inhibitors

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Received 31 March 2005; revised 18 July 2005; accepted 20 July 2005
Available online 6 September 2005

Abstract—A series of 3-anilino-quinoxalinones has been identified as a new class of glycogen phosphorylase inhibitors. The lead compound **1** was identified through high throughput screening as well as through pharmacophore-based electronic screening. Modifications were made to the scaffold of **1** to produce novel analogues, some of which are 25 times more potent than the lead compound.

Hepatic glucose output is abnormally high in diabetic patients and contributes to hyperglycemia in both the fasted and fed states.[1] Hepatic glucose production is the net sum of two metabolic processes: glycogenolysis and gluconeogenesis. Some evidence suggests that 30–60% of total hepatic glucose production can be affected by blocking glycogenolysis.[2] Glycogen phosphorylase is the enzyme responsible for glycogenolysis by catalyzing the breakdown of glycogen to glucose-1-phosphate in the liver and skeletal muscle. Thus, compounds that specifically inhibit glycogen phosphorylase should reduce blood glucose levels in the post-absorptive state, thereby providing adjunctive therapy for Type II diabetes. Several classes of glycogen phosphorylase inhibitors have been reported, including dihydropyridine diacids,[3] *N*-benzoylureas,[4] and indole carboxamides.[5] In the initial series of indole carboxamides, select analogues were reported to inhibit glycogenolysis in vitro with an IC_{50} of ~200 nM. Our goal was to identify an inhibitor with activity in this same range.

In our efforts to identify a series of novel, non-indole carboxamide-containing glycogen phosphorylase inhibitors, high throughput screening and pharmacophore-based electronic database searching[6] were carried out in parallel. The 3-anilino-quinoxalinone **1** appeared as a potential lead using both methodologies. For the ligand-based pharmacophore searching protocol, selected three-point pharmacophore hypotheses derived from the SAR of known GPA inhibitors were electronically screened against our corporate database, and the resulting electronic hits were manually tested in a directed assay. Compound **1** (IC_{50} = 2 μM) was one of the hits identified from the pharmacophore searches. In the high throughput screening assay, compound **1** inhibited GPA with an IC_{50} of 2.5 μM.[7] The synthesis and biological activity of a series of compounds modified from **1** are reported here.

Our goal was to synthesize compounds that would address the importance of three regions of compound **1**: the imidazole portion, the aromatic ring of the quinoxalinone, and the substituent on N-1 of the quinoxalinone. Analogues that explored the SAR of the aniline moiety were prepared according to Scheme 1.[8] Commercially available *N*-methyl-1,2-diaminobenzene was treated with ethyl chlorooxoacetate to give the dione **2**, which was reacted with phosphorous oxychloride to yield the chloroimidate **3**. Addition of the desired aniline proceeded to give the substituted intermediates **4**. These intermediates could then be elaborated to various analogues. In the case of R^2 = I, palladium catalyzed cross-coupling conditions[9] with heteroaryl boronic acids gave the requisite covalent-bonded heterocycles **5**. When $R^2 = NH_2$, treatment with the appropriate acid chloride resulted in the amide-linked heterocycles **6**. Finally, when $R^2 = CO_2Me$, trimethylaluminum mediated coupling[10] of the amino heterocycle to the ester gave the desired reverse amide-linked analogues **7**.

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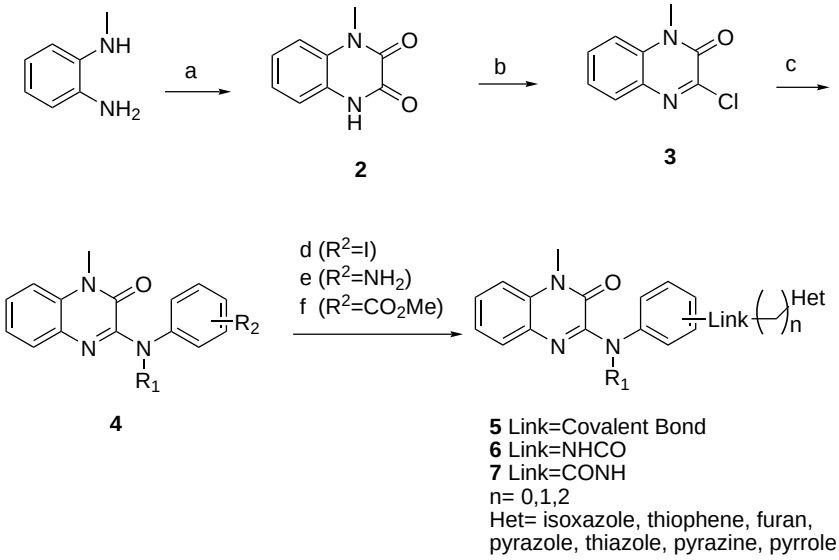
doi:10.1016/j.bmcl.2005.07.021

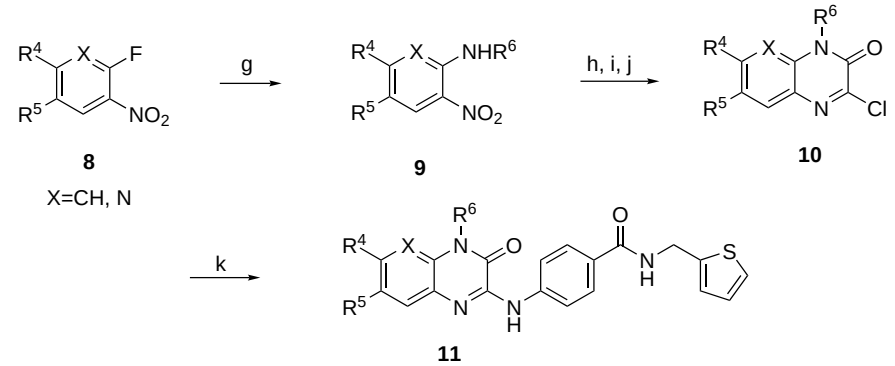


Scheme 1. Reagents and conditions: (a) $ClC(O)CO_2Et$, Et_3N, CH_2Cl_2, rt, 71%; (b) $POCl_3$, Huning's base, toluene 110 °C, 83%; (c) $R^1N(H)$-Ph-R^2, CH_3CN, 80 °C, 75–84%; (d) Het-$B(OH)_2$, $Pd(PPh_3)_4$, 2N Na_2CO_3, DME, 90 °C, 45–60%; (e) Het-COCl, Et_3N, CH_2Cl_2, rt, 80–90%; (f) $Het(CH_2)_nNH_2$, $AlMe_3$, toluene, 120 °C, 30–50%.

For analogues that explored aromatic ring substituents or the N-1 substituent of the quinoxalinone, the synthetic route outlined in Scheme 2 was employed. In certain instances, the desired *ortho*-nitro aniline **9** was commercially available. Otherwise, a substituted amine was added to the corresponding 2-fluoronitrobenzene **8** to give **9** in good yield. Hydrogenation of the nitro group followed by cyclization to the dione and reaction with phosphorous oxychloride gave the chloroimidate **10**.[11] Reaction of **10** with the 4-amino benzamide moiety (obtained by coupling of 4-nitrobenzoyl chloride and 2-aminomethyl thiophene followed by tin-mediated reduction of the nitro group) gave the desired analogues **11a**–**l**.

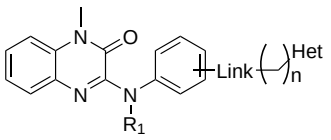
The SAR of the aniline portion of the series was explored first (Table 1). Changing from the directly attached imidazole ring of **1** to other heterocycles led to similar potency (**5a**–**b**). While introducing an amide linkage between the two rings initially led to poor results, the substitution of pyrrole (**6a**) for furan (**6b**) and then isoxazole (**6c**–**d**) led to significant increases in potency. Moving the amide linkage from the 4-position to the 3-position led to a complete loss of activity (**6e**). Potency was further improved, however, when the amide linkage was reversed to give compounds **7a**–**g**. This reverse amide gave consistent results for 5-membered ring heterocycles (**7a**–**c**). Inserting a methylene group (**7d**–**e**) between the amide nitrogen and the heterocycle maintained the potency and simplified synthetic methods. This potency did not extend to a 2-carbon spacer (**7f**). Finally, the hydrogen donor ability of the aniline nitrogen was shown to be imperative for activity, as illustrated by compound **7g** when compared to **7a**.

With the important structural features of the aniline portion well established, attention was next turned to the quinoxalinone moiety (Table 2). As compared to compound **7d**, substitution of the C-6 and C-7 positions with lower alkyl groups or halogens (**11a**–**b**) and replacement of C-8 with nitrogen (**11c**) did not have a dramatic effect on potency. Altering the substituent on N-1 led to



Scheme 2. Reagents and conditions: (g) R^6NH_2, NaOAc, 80 °C, 40–52%; (h) H_2 (50 psi), 10% Pd/C, EtOH; (i) diethyl oxalate, 68–76% over 2 steps; (j) $POCl_3$, DMF, 95 °C 60–74%; (k) 4-amino-*N*-thiophen-2yl-methyl-benzamide, CH_3CN, 80 °C, 77–94%.

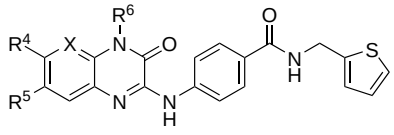
Table 1. SAR of the aniline moiety



5 Link=Covalent Bond
6 Link=NHCO
7 Link=CONH

Entry	R^1	Link position	*n*	Het	IC_{50} (μM ± 10%)
1					2.5
5a	H	4	0	2-Pyrrole	2.5
5b	H	4	0	3-Furan	5
6a	H	4	0	2-Pyrrole	>10
6b	H	4	0	2-Furan	2.5
6c	H	4	0	3-Isoxazole	0.73
6d	H	4	0	5-Isoxazole	0.71
6e	H	3	0	5-Isoxazole	>10
7a	H	4	0	3-Isoxazole	0.11
7b	H	4	0	3-Pyrazole	0.2
7c	H	4	0	2-Thiazole	0.48
7d	H	4	1	2-Thiophene	0.11
7e	H	4	1	2-Furan	0.12
7f	H	4	2	2-Thiophene	>10
7g	CH_3	4	0	3-Isoxazole	>10

Table 2. SAR of the quinoxalinone moiety



11

Entry	X	R^4	R^5	R^6	IC_{50} (μM ± 10%)
11a	CH	H	F	CH_3	0.14
11b	CH	CH_3	H	CH_3	0.28
11c	N	H	H	CH_3	0.19
11d	CH	H	H	H	0.16
11e	CH	H	H	CH_2CH_3	0.19
11f	CH	H	H	$CH(CH_3)_2$	>10
11g	CH	H	H	CH_2CH_2OH	0.32
11h	CH	H	H	CH_2CO_2H	5.7
11i	CH	H	H	$CH_2CH_2N(CH_3)_2$	0.71
11j	CH	H	H	CH_2CH_2-2-pyridine	0.58
11k	CH	H	H	CH_2CH_2-*N*-piperidine	0.42
11l	CH	H	H	CH_2CH_2-*N*-morpholine	0.35

varied results. While hydrogen and ethyl groups (**11d–e**) gave results similar to **7d**, the branched alkyl analogue (**11f**) showed a sharp decrease in potency. Substitution of the ethyl side chain with hydrophilic groups was also explored. While the hydroxyl-substituted analogue (**11g**) showed potency similar to that of **11e**, the corresponding carboxylic acid (**11h**) was not well tolerated. Amino-substituted compounds were also prepared (**11i–l**), with the morpholine moiety retaining the most potency.

With several examples of potent analogues in hand, we turned our attention to whole cell and in vivo efficacy experiments. Compounds **7a**, **7b**, **7d**, and **7e** were tested in the rat hepatocyte glycogenolysis assay.[12] None of these compounds inhibited glycogen breakdown at a concentration of 30 μM. The surprising lack of cellular activity could be attributed to one of three reasons: (1) the series is isozyme specific (muscle vs. liver), (2) the series is species specific (rabbit vs. rat), or (3) physicochemical properties of the compounds themselves does not allow for cellular penetration.

To determine whether the lack of activity in the rat hepatocyte assay is due to species or isozyme differences, we measured inhibitory activity against rat liver glycogen phosphorylase. Although the compounds were not as potent against the rat liver enzyme, the level of inhibition was similar to that of the standard used as a positive control in the hepatocyte assay. Therefore, it is unlikely that species or isozyme differences contribute to the lack of activity in the cell-based assay.

The physicochemical properties of selected analogues were then addressed. Permeability and absorption properties were assessed by the CaCO-2 layer assay.[13] A diverse set of compounds, including **7b**, **7d**, **11a**, **11b**, and **11c**, were all found to have high potential for permeability with mean A-B Papp <15. Select compounds were also tested for human liver microsomal stability, indicative of a compound's first pass liability.[14] While **7d** showed marginal stability at 27 min, **7a**, **7b**, **11b**, and **11c** all showed stabilities in the 40–60 min range. Compounds with amine substitution in R^6 (**11k**, **l**) showed very poor stability (<5 min). Finally, solubility of the compounds was measured.[15] All analogues were shown to be insoluble in aqueous media at neutral pH, with only modest solubility observed at pH 2 for analogues (**11k**, **l**), which contain a basic amine. We concluded that the lack of activity in the cell-based assay is most likely due to poor solubility in the assay media. Unfortunately, increasing the DMSO concentration within the assay led to cell lysis.

In a final attempt to assess this series as a candidate for further development, we chose to test compounds **7d**, **11c**, and **11e** in the ob/ob mouse efficacy model.[16] The summary of results in Table 3 shows that no difference in fed blood glucose levels was observed for our test compounds versus the vehicle. In separate experiments, these compounds showed no bioavailability in standard rat PK studies. Efforts to successfully modify the formulation of the compounds for in vivo dosing were not realized.

In summary, a series of 3-anilino-quinoxalinones has been established as a new class of glycogen phosphorylase inhibitors in vitro. Structural features of the aniline moiety were most important for potent inhibitory activity, while the quinoxalinone ring is more tolerant to substitution. Entries **7a** and **7d** were found to be 25 times more potent than the original lead **1**. The in vitro activity did not translate to inhibition of glycogen phosphorylase in vivo, and this may be due to the poor aqueous solubility of the compounds, which will ultimately affect their bioavailability potential.

Table 3. Fed blood glucose levels in *ob/ob* mice after compound administration

Vehicle	Hours after compounds dosing:				
	0	1	2	4	8
	339.5 ± 37.4	319.3 ± 21.5	375.3 ± 15.8	341.1 ± 28.8	222.9 ± 17.3
7d	334.0 ± 35.9	306.4 ± 12.1	351.3 ± 36.3	313.3 ± 29.1	224.9 ± 22.6
11c	357.4 ± 37.5	305.4 ± 23.8	356.0 ± 28.9	362.5 ± 38.7	328.4 ± 50.7
11e	320.3 ± 36.5	349.1 ± 40.6	338.4 ± 31.5	383.3 ± 33.5	325.9 ± 47.4

$^{*}P < 0.05$, compared with vehicle treated group.

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6. A three dimensional pharmacophore was derived from a known GPa inhibitor, CP-91149. 3D searches were carried out against a multiconformer version of the corporate database using the Catalyst software available from Accelrys, Inc., (www.accelrys.com). Compounds with desirable ADME (absorption, distribution, metabolism, and excretion) properties were subsequently selected from the original Catalyst electronic hit list by calculating ADME descriptors with the MOE software (available from The Chemical Computing Group, 1010 Sherbrooke Street West, Suite 910, Montreal, Canada H#A 2R7). The final list of compounds was tested and afforded compound **1** as one of the confirmed hits.
7. Enzyme inhibitory activity was determined using glycogen phosphorylase from rabbit muscle (Sigma P1261) by the method of Martin et al. Martin, W. H.; Hoover, D. J.; Armento, S. J.; Stock, I. A.; McPherson, R. K.; Danley, D. F.; Stevenson, R. W.; Barrett, E. J.; Treadway, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1776.
8. All compounds provided satisfactory spectral data (^{1}H NMR, LCMS) and were homogeneous by TLC.
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12. The rat hepatocyte glycogenolysis assay was adapted from the method of Ciaraldi, G. et al. Diabetes 1992, 41, 975. Briefly, hepatocytes are obtained by in vivo collagenase perfusion followed by ex vivo dispersion of rat liver. Resultant cells are plated in 6 well plates and allowed to attach for several hours, followed by addition of [^{14}C]glucose for overnight incorporation into glycogen. The next day, unincorporated radioactivity is removed and cells are treated with test compound with or without .1 nM glucagon for 2 h. Cellular glycogen is collected by potassium hydroxide solubilization of the cells followed by ethanol extraction and the radioactivity determined. Glycogen breakdown is determined by comparing reduction of radioactivity from cells at time 0 versus time 2 h and inhibition of this breakdown by test compounds assessed.
13. Tests were conducted at Absorption Systems, Exton, PA. Caco-2 monolayers were grown to confluence, dosed at 10 μM on the apical side (A-to-B) or basolateral side (B-to-A), and incubated at 37 °C. The permeability assay buffer was Hanks' balanced salt solution containing 10 mM HEPES and 15 mM glucose at a pH of 7.0–7.2. Samples were assayed by LC/MS using electrospray ionization. The apparent permeability, Papp, and percent recovery were calculated using $\text{Papp} = (\mathrm{d}C_r/\mathrm{d}t) \times V_r/(A \times C_0)$ and Percent Recovery $= 100 \times ((V_r \times C_r^{\text{final}}) + (V_d \times C_d^{\text{final}}))/(V_d \times C_0)$, where $\mathrm{d}C_r/\mathrm{d}t$ is the cumulative concentration in the receiver compartment versus time in M s^{-1}. V_r is the volume of the receiver compartment in cm^3. V_d is the volume of the donor compartment in cm^3. A is the area of the cell monolayer. C_0 is the concentration of the dosing solution in M. C_r^{final} is the cumulative receiver concentration in M at the end of the incubation period. C_d^{final} is the concentration of the donor in Mat the end of the incubation period.
14. Tests were conducted at Absorption Systems, Exton, PA. Incubation at 37 °C with test compound at 5 μM and 1 mg protein/mL human liver microsomal prep. Half life was determined by measuring the percent parent compound remaining. In this system, values above 30 min were considered acceptable.
15. Tests were conducted at Absorption Systems, Exton, PA. Equilibrium solubility was measured in pH 2.0 and pH 7.4 aqueous buffers. At least 1 mg of powder was combined with 1 mL of buffer to make $\geqslant$1 mg/mL mixture. These samples were shaken for $\geqslant$2 h and left to stand overnight at room temperature. The samples were then filtered through a 0.45-μm Nylon syringe filter that was first saturated with the sample. The filtrate was sampled twice, consecutively. All samples were assayed by LC/MS using electrospray ionization.
16. Female C57BL/6J *ob/ob* mice (C57BL/KsJ-*Lep*$^{ob/ob}$ Jackson Labs, Bar Harbor, ME) were at 6–7 weeks of age when received. Upon arrival, they were quarantined for 5 days, housed two mice per cage and given access to water and food ad libitum. Next, these mice were grouped based on their blood glucose levels. They were then given either vehicle (0.5% methycellulose), or compound (suspended in 0.5% methycellulose) at 30 mg/kg body weight via oral gavage. Tail blood samples were collected at 0, 1, 2, 4, and 8 h after dosing to measure the blood glucose using a glucometer (One Touch Ultra, Lifescan, Milpitas, CA).