Design and synthesis of benzopyran-based inhibitors of the hypoxia-inducible factor-1 pathway with improved water solubility

Jalisa H. Ferguson, Georgia State University
Zeus De Los Santos, Georgia State University
Saroja N. Devi, Emory University
Stefan Kaluz, Emory University
Erwin Van Meir, Emory University
Sarah K. Zingales, Georgia State University
Binghe Wang, Georgia State University

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Jalisa H. Ferguson, Zeus De Los Santos, Saroja N. Devi, Stefan Kaluz, Erwin G. Van Meir, and Binghe Wang

ABSTRACT

While progress has been made in treating cancer, cytotoxic chemotherapeutic agents are still the most widely used drugs and are associated with severe side-effects. Drugs that target unique molecular signalling pathways are needed for treating cancer with low or no intrinsic toxicity to normal cells. Our goal is to target hypoxic tumours and specifically the hypoxia inducible factor (HIF) pathway for the development of new cancer therapies. To this end, we have previously developed benzopyran-based HIF-1 inhibitors such as arylsulfonamide KCN1. However, KCN1 and its earlier analogs have poor water solubility, which hamper their applications. Herein, we describe a series of KCN1 analogs that incorporate a morpholine moiety at various positions. We found that replacing the benzopyran group of KCN1 with a phenyl group has been developed, which led to the discovery of 64b showing potent inhibition activity (IC\textsubscript{50} of \approx 0.6 \mu M) in a HIF-dependent bioassay.

Further in vivo studies demonstrated 1’s very pronounced inhibitory activity against brain, and pancreatic cancers. In addition, 1 was well tolerated in mice; daily treatments with 60 mg/kg for up to 12 weeks had minimal side effects. Neither did 1 nor its analogs demonstrate cytotoxicity, indicating the selective inhibitory effects being based on pathways unique to cancer. Such results strongly suggest that this is a very promising class of compounds and warrant further studies. In fact, a previously synthesised and analysed class of analogs has been developed, which led to the discovery of 64b showing potent inhibition activity (IC\textsubscript{50} of \approx 0.6 \mu M). However, 1 and its analogs possess poor solubility in water (0.009 mg/mL). Therefore, dissolution in DMSO is necessary for in vitro assays and cremophor:ethanol-based formulations are needed for in vivo models. Such a formulation introduces undesirable properties. It is well known that the successful development of potential therapeutics relies on more parameters than potency alone. Other properties, including solubility, can play a critical role. Therefore, we are interested in designing water-soluble analogs of 1 and 2 to address this critical aspect of drug development.

Introduction

Cancer is one of the leading causes of death, second only to heart disease. One of the hallmarks of cancer is the formation of hypoxic areas inside of solid tumours. This hypoxic tumour microenvironment leads to many changes such as the upregulation of pro-angiogenic and pro-glycolytic pathways, as well as increases in cell proliferation, genetic instability, and metastatic potential. A major mediator of the hypoxic response is the hypoxia inducible factor (HIF) pathway. HIF is a heterodimeric transcription factor consisting of two subunits, HIF-\(\alpha\), the stability of which is regulated by oxygen, and HIF-1\(\beta\), which is constitutively expressed. There are three known isoforms of HIF-\(\alpha\), HIF-1\(\alpha\), HIF-2\(\alpha\), and HIF-3\(\alpha\), with HIF-1\(\alpha\) being the most commonly expressed and most extensively studied. Under normoxic conditions, HIF-\(\alpha\) subunits are hydroxylated by a prolyl hydroxylase (PHD2) using molecular oxygen and then degraded via a VHL-dependent ubiquitination pathway. Under hypoxic conditions, however, HIF-\(\alpha\) subunits are stabilised, heterodimerise with HIF-1\(\beta\) and recruit co-activators such as p300 and CBP, to form active transcription complexes that bind to \(S^\prime-HREs\) (hypoxia response elements) in promoter regions of hypoxia-inducible genes. Increased levels of HIF-1\(\alpha\) are linked to cancer progression and poor patient outcome. Therefore, HIF is an attractive target for developing anticancer therapeutics.

A library of 10,000 products containing the 2,2-dimethyl-2H-chromene moiety was screened for compounds with HIF inhibitory activity. This led to the identification of a compound designated KCN1 (Figure 1, 1, N-(2,2-dimethyl-2H-chromen-6-yl)methyl)-3,4-dimethoxy-N-phenylbenzenesulfonamide) showing potent inhibition activity (IC\textsubscript{50} of \approx 0.6 \mu M) in a HIF-dependent bioassay.

Further in vivo studies demonstrated 1’s very pronounced inhibitory activity against brain, and pancreatic cancers. In addition, 1 was well tolerated in mice; daily treatments with 60 mg/kg for up to 12 weeks had minimal side effects. Neither did 1 nor its analogs demonstrate cytotoxicity, indicating the selective inhibitory effects being based on pathways unique to cancer. Such results strongly suggest that this is a very promising class of compounds and warrant further studies. In fact, a previously synthesised and analysed class of analogs has been developed, which led to the discovery of 64b (Figure 1, 2, N-cyclobutyl-N-((2,2-dimethyl-2H-pyrano[3,2-b]pyridin-6-yl)methyl)-3,4-dimethoxybenzenesulfonamide) with an IC\textsubscript{50} value of \approx 0.3 \mu M. However, 1 and its analogs possess poor solubility in water (0.009 mg/mL). Therefore, dissolution in DMSO is necessary for in vitro assays and cremophor:ethanol-based formulations are needed for in vivo models. Such a formulation introduces undesirable properties. It is well known that the successful development of potential therapeutics relies on more parameters than potency alone. Other properties, including solubility, can play a critical role. Therefore, we are interested in designing water-soluble analogs of 1 and 2 to address this critical aspect of drug development.
Materials and methods

Synthesis

General methods and materials

All commercial chemicals were of reagent grade from VWR (Radnor, PA), Aldrich (St. Louis, MO), or Oakwood Chemicals (Estill, SC), and were used without further purification unless otherwise indicated. 

1H and 13C spectra were obtained on a Bruker 400 MHz spectrometer at 400 and 100 MHz, respectively, in deuterated solvent with TMS (δ = 0.00 ppm) or deuterated solvent as internal reference. For all reactions, analytical grade solvent was used. Anhydrous solvents were used for all moisture-sensitive reactions. The Mass Spectrometry Facilities at Georgia State University obtained high-resolution mass spectra on a Waters Micromass Q-TOF (ESI) instrument.

Typical procedure for morpholine substitution (8a–c)

Benzyl bromide (1 equivalent) was dissolved in acetonitrile. Morpholine (1.1 equivalents) and K2CO3 (2 equivalents) were added and the reaction was stirred overnight at room temperature. The reaction was filtered through Celite and concentrated to give the product in quantitative yield.

4-(4-Bromobenzyl)morpholine (8a).

1H NMR (CDCl3): δ 7.41 (d, J = 8 Hz, 2H), 7.19 (d, J = 8 Hz, 2H), 3.67 (t, J = 4 Hz, 4H), 3.41 (s, 2H), 2.40 (s, 4H) ppm. 13C NMR (CDCl3): δ 137.0, 131.4, 130.8, 120.9, 66.9, 62.6, 53.6 ppm. HRMS (ESI) m/z calculated for C11H15NOBr [(M + H)+] 256.0337, found 256.0333.

4-(3-Bromobenzyl)morpholine (8b).

1H NMR (CDCl3): δ 7.46 (s, 1H), 7.32 (d, J = 8 Hz, 1H), 7.20 (d, J = 7 Hz, 1H), 7.12 (t, J = 8 Hz, 1H), 3.64 (d, J = 4 Hz, 4H), 3.39 (s, 2H), 2.37 (s, 4H) ppm. 13C NMR (CDCl3): δ 140.4, 131.9, 130.2, 129.8, 127.6, 122.5, 66.9, 62.7, 53.6 ppm. HRMS (ESI) m/z calculated for C11H15NOBr [(M + H)+] 256.0337, found 256.0348.

4-(2-Bromobenzyl)morpholine (8c).

1H NMR (CDCl3): δ 7.52 (d, J = 8 Hz, 1H), 7.46 (d, J = 8 Hz, 1H), 7.26 (t, J = 7 Hz, 1H), 7.08 (t, J = 7 Hz, 1H), 3.71–3.68 (m, 4H), 3.57 (s, 2H), 2.49–2.48 (m, 4H) ppm. 13C NMR (CDCl3): δ 137.2, 132.8, 130.8, 128.5, 127.2, 124.7, 67.0, 62.2, 53.6 ppm. HRMS (ESI) m/z calculated for C11H15NOBr [(M + H)+] 256.0337, found 256.0348.

Typical procedure for lithium halogen exchange to form aldehydes (9a–c)

Arylbromide (1 equivalent) was dissolved in anhydrous THF under N2 and cooled in a dry ice and acetone bath for 30 min before treatment with n-buLi (1.4 equivalents). After 30 additional minutes, anhydrous DMF (1.4 equivalents) was added and stirring continued 1 h. The reaction was quenched with saturated NH4Cl, taken up in ethyl acetate, washed with brine, dried over MgSO4, and concentrated in vacuo. Purification by column chromatography was performed in 4:1 hexanes/ethyl acetate.

4-(Morpholinomethyl)benzaldehyde (9a). Yield: 74%. 1H NMR (CDCl3): δ 9.96 (s, 1H), 7.81 (d, J = 8 Hz, 2H), 7.49 (d, J = 8 Hz, 2H), 3.68–3.68 (m, 4H), 3.54 (s, 2H), 2.43 (m, 4H) ppm. 13C NMR (CDCl3): δ 191.9, 145.3, 135.6, 129.8, 129.5, 66.9, 63.0, 53.6 ppm. HRMS m/z calculated for C12H16NO2 [(M + H)+] 206.1181, found 206.1182.

3-(Morpholinomethyl)benzaldehyde (9b). Yield: 88%. 1H NMR (CDCl3): δ 9.92 (s, 1H), 7.77 (s, 1H), 7.69 (d, J = 8 Hz, 1H), 7.54 (d, J = 8 Hz, 2H), 7.41 (t, J = 8 Hz, 1H), 3.63 (m, 4H), 3.50 (s, 2H), 2.39 (m, 4H) ppm. 13C NMR (CDCl3): δ 192.2, 138.8, 136.5, 135.2, 130.2, 129.0, 128.7, 66.7, 62.6, 53.4 ppm. HRMS m/z calculated for C12H16NO2 [(M + H)+] 206.1181, found 206.1183.

2-(Morpholinomethyl)benzaldehyde (9c). Yield: 85%. 1H NMR (CDCl3): δ 10.37 (s, 1H), 7.81 (d, J = 8 Hz, 1H), 7.44 (d, J = 8 Hz, 1H), 7.37–7.33 (m, 2H), 3.76 (s, 2H), 3.58–3.57 (m, 4H), 2.40–2.39 (m, 4H) ppm. 13C NMR (CDCl3): δ 192.0, 140.4, 135.0, 133.2, 130.6, 129.4, 127.9, 67.0, 66.9, 60.0, 53.5, 53.3 ppm. HRMS m/z calculated for C12H16NO2 [(M + H)+] 206.1181, found 206.1186.

Procedure for 2,2-dimethyl-2H-chromene-6-carbaldehyde (12)

Synthesised and purified as described in previous examples13. Yield: 37% over two steps. 

1H NMR (CDCl3): δ 9.83 (s, 1H), 7.64 (d, J = 8 Hz, 1H), 7.52 (s, 1H), 6.87 (d, J = 8 Hz, 2H), 6.37 (d, J = 10 Hz, 1H), 5.70 (d, J = 10 Hz, 1H), 1.47 (s, 6H) ppm.

Typical procedure for reductive amination with aniline (10a–d, 14a)

Aldehyde (1 equivalent), NaNH2 (1.5 equivalents), and InCl3 (0.15 equivalents) were dissolved in anhydrous ACN under inert gas. Aniline (1.5 equivalents) was added and the reaction was stirred until completion as monitored by TLC (typically ~20 min). The reaction was quenched with saturated NH4Cl, taken up in ethyl acetate, washed with brine, dried over MgSO4, and concentrated. Column chromatography (1:1 hexane/ethyl acetate) was used to yield the final pure product.

N-(4-(Morpholinomethyl)benzyl)aniline (10a). Yield: 60%. 1H NMR (CDCl3): δ 7.23–7.17 (m, 4H), 6.79–6.66 (m, 5H), 4.34 (s, 2H), 3.74 (m, 4H), 3.52 (s, 2H), 2.74 (m, 4H) ppm. 13C NMR (CDCl3): δ 148.2, 138.4, 136.8, 129.5, 129.3, 127.5, 118.6, 117.6, 115.1, 112.9, 67.0, 63.2, 53.6, 48.1 ppm. HRMS m/z calculated for C18H23N2O [(M + H)+] 283.1810, found 283.1805.

N-(3-(Morpholinomethyl)benzyl)aniline (10b). Yield: 60%. 1H NMR (CDCl3): δ 7.36–7.17 (m, 6H), 6.76–6.65 (m, 3H), 4.35 (s, 2H).
N-(2-(Morpholinomethyl)benzyl)aniline (10c). Yield: 54%. \(^1^H\) NMR (CDCl\(_3\)): \(\delta 7.44\) (d, \(J = 7\) Hz, 1H), 7.32–7.22 (m, 3H), 6.75 (d, \(J = 7\) Hz, 3H), 5.37 (bs, 1H), 4.39 (s, 2H), 3.75 (m, 4H), 3.57 (s, 2H), 2.51 (m, 4H) ppm. 13C NMR (CDCl\(_3\)): 148.6, 138.9, 135.8, 131.5, 130.0, 129.3, 128.2, 127.2, 117.4, 67.1, 61.7, 53.5, 46.9 ppm. HRMS (ESI) \(m/z\) calculated for C\(_{18}\)H\(_{20}\)NO \([\text{M} + \text{H}]^+\) 283.1810, found 283.1809.

N-(4-Morpholinobenzyl)cyclobutanamine (11d). Crude yield: 90%. \(^1^H\) NMR (CDCl\(_3\)): \(\delta 7.22\) (d, \(J = 8\) Hz, 2H), 6.87 (d, \(J = 8\) Hz, 2H), 3.86–3.84 (m, 4H), 3.62 (s, 2H), 3.28 (quintet, \(J = 6.8\) Hz, 1H), 3.14–3.11 (m, 4H), 2.22–2.19 (m, 2H), 1.70–1.62 (m, 4H) ppm. 13C NMR (CDCl\(_3\)): \(\delta 150.3, 131.9, 129.1, 115.7, 66.9, 53.5, 50.4, 49.5, 31.1, 14.8\) ppm. HRMS (ESI) \(m/z\) calculated for C\(_{13}\)H\(_{23}\)N\(_2\)O \([\text{M} + \text{H}]^+\) 247.1810, found 247.1819.

Typical procedure for reductive amination with cyclobutyl and alkylmorphino amines (11a–d, 13a–b, 14b)

Aldehyde (1 equivalent) and amine (1 equivalent) were dissolved in anhydrous MeOH under inert gas and the reaction was stirred overnight at room temperature. NaBH\(_4\) (1.6 equiv.) was added and the reaction stirred for an additional hour. The reaction was quenched with NaOH (1 M), stirred for an hour, then taken up in ethyl acetate, washed with brine, dried over MgSO\(_4\), and concentrated. The product was purified by column chromatography on silica gel in 4:1 or 1:1 hexane/ethyl acetate.

Typical procedure for sulfonation with 3,4-dimethoxybenzenesulfonyl chloride (3a–d, 4a–d, 5a–b)

Amine (1 equivalent) was dissolved in DCM. K\(_2\)CO\(_3\) (2 equivalents) and 3,4-dimethoxybenzenesulfonyl chloride (2 equivalents) were added. The reaction was stirred overnight at room temperature, then washed with brine, dried over MgSO\(_4\), and concentrated. The product was purified by column chromatography on silica gel in 4:1 or 1:1 hexane/ethyl acetate.
3.48 (s, 2H), 3.09 (t, 4H), 3.46 (m, 4H), 3.15–110.5, 67.2, 61.1, 56.3, 56.2, 53.6, 51.3, 31.0, 30.8, 13.6 ppm. **HRMS (ESI) m/z** calculated for C_{26}H_{35}N_{2}O_{6}S [(M + H)^+] 469.1797, found 469.1796.

**N-Cyclobutyl-3,4-dimethoxy-N-(4-morpholinobenzyl)benzenesulfonamide (6a).**

**Yield:** 15%. **1H NMR** (CDCl_3): δ 7.43 (dd, J = 8 Hz, 2H), 7.34–7.25 (m, 5H), 6.94 (d, J = 9 Hz, 1H), 4.39 (s, 2H), 4.27 (quintet, J = 9 Hz, 1H), 3.96 (s, 3H), 3.92 (s, 3H), 3.75–3.72 (m, 4H), 3.51 (s, 2H), 2.46 (s, 4H), 1.99–1.94 (m, 4H), 1.57–1.52 (m, 2H) ppm. **13C NMR** (CDCl_3): δ 152.4, 149.0, 137.8, 132.0, 129.4, 127.0, 129.0, 119.9, 110.9, 69.9, 63.5, 60.2, 56.2, 53.5, 52.9, 48.2, 29.2, 15.0 ppm. **HRMS (ESI) m/z** calculated for C_{27}H_{37}N_{2}O_{6}S [(M + H)^+] 517.2367, found 517.2366.

**Typical procedure for sulfonylation with 4-morpholinosulfonyl chloride (6a–b)**

Amine (1 equivalent) was dissolved in dichloroethane. Pyridine (3 equivalents) and 4-morpholinosulfonyl chloride (1.3 equivalents) were added. The reaction was refluxed for 2 days, then concentrated, taken up in ethyl acetate, washed with saturated NH_4Cl and brine, then dried over MgSO_4 and concentrated. The residue was then purified by column chromatography in 4:1 hexane/ethyl acetate.

**N-(2,2-Dimethyl-2-chromen-6-yl)methyl-3,4-dimethoxy-N-(morpholinomethyl)benzenesulfonamide (5a).**

**Yield:** 49%. **1H NMR** (CDCl_3): δ 7.45 (dd, J = 8 Hz, 1H), 6.93 (t, J = 9 Hz, 2H), 6.88–6.85 (m, 1H), 6.67 (d, J = 8 Hz, 1H), 6.22 (d, J = 10 Hz, 1H), 5.59 (d, J = 7 Hz, 1H), 4.23 (s, 2H), 3.93 (s, 3H), 3.89 (s, 3H), 3.59–3.56 (m, 4H), 3.19 (t, J = 7 Hz, 2H), 2.30 (t, J = 7 Hz, 2H), 2.25 (s, 4H), 1.23 (s, 6H) ppm. **13C NMR** (CDCl_3): δ 152.7, 152.5, 149.1, 131.8, 131.3, 129.1, 128.3, 126.5, 122.0, 121.4, 121.0, 116.3, 110.6, 109.8, 66.8, 57.3, 57.3, 56.2, 53.6, 52.2, 44.4, 27.9 ppm. **HRMS (ESI) m/z** calculated for C_{28}H_{35}N_{2}O_{6}S [(M + H)^+] 503.2210, found 503.2204.

**N-(2,2-Dimethyl-2-chromen-6-yl)methyl-3,4-dimethoxy-N-(3-morpholinopropyl)benzenesulfonamide (5b).**

**Yield:** 15%. **1H NMR** (CDCl_3): δ 7.43 (dd, J = 8 Hz, 1H), 6.97 (d, J = 8 Hz, 1H), 6.94 (d, J = 8 Hz, 1H), 6.87 (s, 1H), 6.68 (d, J = 8 Hz, 1H), 6.21 (s, J = 10 Hz, 1H), 4.19 (s, 2H), 3.94 (s, 3H), 3.90 (s, 3H), 3.60 (s, 4H), 3.12 (t, J = 8 Hz, 2H), 2.22 (s, 4H), 2.18 (t, J = 7 Hz, 2H), 1.59–1.52 (m, 2H), 1.40 (s, 6H) ppm. **13C NMR** (CDCl_3): δ 152.7, 152.4, 149.1, 131.6, 131.3, 129.3, 128.4, 126.5, 121.9, 121.3, 121.0, 116.3, 110.6, 109.8, 66.9, 56.3, 56.2, 55.9, 53.4, 52.0, 46.2, 28.0, 25.4 ppm. **HRMS (ESI) m/z** calculated for C_{29}H_{39}N_{2}O_{6}S [(M + H)^+] 517.2362, found 517.2362.
Table 1. Structures, HRE-luciferase reporter inhibitory activity, cLog $D$, and cLog $S$ of analogs.

<table>
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<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC$_{50}$ (µM)</th>
<th>cLog $D$</th>
<th>cLog $S$</th>
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<td><img src="image1.png" alt="Structure 1" /></td>
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</tr>
<tr>
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<td><img src="image2.png" alt="Structure 2" /></td>
<td>~0.3</td>
<td>3.34</td>
<td>-4.53</td>
</tr>
<tr>
<td>3a</td>
<td><img src="image3.png" alt="Structure 3a" /></td>
<td>0.9</td>
<td>3.69</td>
<td>-4.39</td>
</tr>
<tr>
<td>3b</td>
<td><img src="image4.png" alt="Structure 3b" /></td>
<td>&gt;5</td>
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</tr>
<tr>
<td>3c</td>
<td><img src="image5.png" alt="Structure 3c" /></td>
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</tr>
<tr>
<td>3d</td>
<td><img src="image6.png" alt="Structure 3d" /></td>
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<tr>
<td>4c</td>
<td><img src="image9.png" alt="Structure 4c" /></td>
<td>&gt;5</td>
<td>3.05</td>
<td>-3.47</td>
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</table>

(continued)
Lipophilicity and solubility prediction

The in silico log D and log S values of all analogs were predicted using Calculator Plugins from MarvinSketch 4.3.0, 2017, ChemAxon (http://www.chemaxon.com), with results detailed in Table 1. Graphical representations of the log D and log S from pH 0 to 14 are provided in the Supplemental Information.

Luciferase assay

These analogs were first evaluated for their ability to inhibit hypoxia-induced HIF transcriptional activity in LN229-HRE-luciferase glioma cells as described previously10–12. Their inhibitory activities are presented as IC50 in Table 1.

Solubility studies using dynamic light scattering

To further investigate the true enhancement of solubility, particle aggregation was examined using dynamic light scattering (DLS).

Selected compounds were treated according to the following procedure:

1. All centrifuge tubes and cuvettes were rinsed with either DCM or water and then vacuum dried before use to remove dust and any particulates.
2. Stock solutions (10 mM) of each compound of interest were prepared in filtered DMSO.
3. Six dilutions (0, 10, 20, 30, 50, and 100 μM) were prepared in filtered de-ionised water with 1% DMSO and allowed to rest at room temperature for 24 h after vortex.
4. DLS analysis was performed for each concentration on the Brookhaven Instrument Corporation, NanoBrook 90Plus Particle Size Analyzer, Version 5.20 (Holtsville, NY).
5. Additional experiments were performed at specific concentrations for each compound as follows: 0, 1, 3, 10, and 20 μM concentrations of 1; 0, 10, 12, and 20 μM of 2; 0, 5, 7, and 10 μM of 3a; and 0, 10, 20, 30, and 50 μM concentrations of 4a.
6. Additional experiments were repeated in filtered PBS with 1% DMSO as follows: 0, 0.5, 1, 2, 3, and 5 μM concentrations of 1; 0, 5, 7, 10, 12, and 15 μM of 2; 0, 10, 12, 15, and 20 μM of 3a; and 0, 10, 20, 30, and 50 μM concentrations of 4a.

Table 1. Continued

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC50 (μM)</th>
<th>cLog D</th>
<th>cLog S</th>
</tr>
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<tr>
<td>4d</td>
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<td>2.6</td>
<td>3.24</td>
<td>−4.15</td>
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<tr>
<td>5a</td>
<td></td>
<td>≥5</td>
<td>3.13</td>
<td>−4.23</td>
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<tr>
<td>5b</td>
<td></td>
<td>≥5</td>
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<tr>
<td>6b</td>
<td></td>
<td>≥5</td>
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<td>−4.12</td>
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</table>

28.1, 14.8 ppm. HRMS (ESI) m/z calculated for C20H29N2O4S [(M + H)⁺] 393.1843, found 393.1834.
of the 2,2-dimethyl-2H-morpholinomethylphenyl or morpholinophenyl moiety instead of the 2,2-dimethyl-2H-pyrano[3,2-b]pyridin-6-yl moiety and substitutes the 2,2-dimethyl-2H-chromene moiety for an N-phenyl group; Class C incorporates either a 2,2-dimethyl-2H-phenyl group replacing the benzopyran ring in 1, which allow for the preservation of HIF inhibition activity. Indeed, 9a−c, which have exchanged the benzopyran ring for an N-phenyl group, and Class D has the 2,2-dimethyl-2H-chromene moiety with a N-phenyl-morpholine-4-sulfonamide.

Chemistry

Synthesis of Class A compounds (Scheme 1) was accomplished in four steps from 2-, 3-, or 4-bromomethylbenzylbromide 7a−c or in two steps from 4-morpholinobenzaldehyde 9d. Intermediates 7a−c were substituted with morpholine to yield morpholinomethylbenzylbromides 8a−c in quantitative yield. Next, the phenyl bromides 8a−c were converted to benzaldehydes 9a−c via lithium-halogen exchange at −78°C under inert gas. The aryllithium intermediate was treated with DMF as the electrophile in situ to generate the final benzaldehydes 9a−c. The aldehydes 9a−d underwent reductive amination with aniline to afford the secondary amines 10a−d. Finally, 10a−d were reacted with 3,4-dimethoxybenzenesulfonyl chloride to afford sulfonamides 2a−d. Class B compounds (Scheme 1(C)) were synthesised in almost the same fashion as Class A, except that reductive amination of 9a was with cyclobutylamine and was not catalysed by any Lewis acid.

Class C compounds were synthesised (Scheme 2) from 2,2-dimethyl-2H-chromene-6-carbaldehyde 12, which was readily synthesised from published procedures. The aldehyde 12 underwent reductive amination with either ethylaminomorpholine or propylaminomorpholine to give secondary amines 13a−b, which were then reacted with 3,4-dimethoxybenzenesulfonyl chloride to afford sulfonamides 5a−b.

Class D compounds were synthesised (Scheme 3) from 12 in two steps. First, 12 underwent reductive amination with either aniline or cyclobutylamine to give secondary amines 14a−b. Next, the amines 14a−b were reacted with 4-morpholinosulfonyl chloride to afford sulfonamides 6a−b.

Biology

All the analogs were assessed for their ability to inhibit the HIF-1 pathway using a luciferase reporter assay described previously. This assay reports the ability for a compound to inhibit HIF transcriptional activity. However, it does not specifically reveal the mode of action at the biochemical level. As can be seen from Table 1, introduction of a morpholino unit on the sulfonamide nitrogen led to compounds 5 with substantially diminished activity. The same is true if the morpholino unit is directly attached to the sulfonyle nitrogan. In the two series of compounds (3, 4), with either phenyl or cyclobutyl ring in place of the benzopyran ring in 1, only introduction of the morpholino moieties at the para positions (3) allowed for the preservation of HIF inhibition activity. Indeed, compounds 3a and 3d, which have exchanged the benzopyran ring for a para-morpholinomethylphenyl and para-morpholinophenyl, respectively, exhibit IC_{50} values of 0.9 and 3.8 μM. Similarly, analogs 4a and 4d, which replace the N-phenyl with a N-cyclobutyl, but are otherwise structurally the same as 3a and 3d, have IC_{50} values of 1.0 and ~2.6 μM, respectively. No other
Scheme 1. Synthesis of Class A & B compounds. (A) Synthesis of precursors. (B) Synthesis of Class A. (C) Synthesis of Class B. Reagents and conditions: (a) morpholine, K$_2$CO$_3$, ACN, room temperature, overnight; (b) BuLi, DMF, THF, $-78^\circ$C, 1 h; (c) aniline, InCl$_3$, NaBH$_4$, ACN, 20 min; (d) 3,4-dimethoxybenzenesulfonyl chloride, K$_2$CO$_3$, DCM, overnight; (e) cyclobutylamine, NaBH$_4$, MeOH, overnight.

Scheme 2. Synthesis of Class C compounds. Reagents and conditions: (a) amine, NaBH$_4$, MeOH, overnight; (b) 3,4-dimethoxybenzenesulfonyl chloride, K$_2$CO$_3$, DCM, overnight.

Scheme 3. Synthesis of Class D compounds. Reagents and conditions: (a) aniline, InCl$_3$, NaBH$_4$, ACN, 20 min or cyclobutylamine, NaBH$_4$, MeOH, overnight; (b) 4-morpholinosulfonyl chloride, pyridine, DCE, reflux 2 days.
solubility studies

To investigate the true enhancement of aqueous solubility, particle aggregation was examined using DLS. DLS can detect particle sizes in solution by measuring changes in scattered light in relation to the Brownian motion of particles. It is commonly used to detect the particle sizes of various chemical and biological molecules, including small molecule inhibitors. Though there are several methods for detecting solubility, we chose the DLS method due to its ease, reproducibility, minimal sample requirement, and relative sensitivity to small particles.

The active compounds 3a and 4a, were compared to their non-morpholine containing counterparts, 1 and 2, respectively (Figure 3). Solutions of varying concentrations of each compound were made in either water or PBS with 1% DMSO. Each solution was measured in the particle size analyser to identify which samples showed formation of aggregates in solution. DLS measurements, summarised in Table 2, reveal that 3a forms aggregates at approximately 10 μM, an order of magnitude higher than 1, which is insoluble at just 1 μM in water. The N-cyclobutyl analog 4a, forms aggregates in excess of 100 μM, significantly higher than its counterpart 2, which forms particles at a mere 10 μM. In PBS, the solubilities parallel those seen in the water solution, where 1 and 3a exhibit comparable particle formation at 1 μM and 15 μM, respectively. 2 shows particle formation at 10 μM, while 4a shows none at this concentration, as expected. Indeed, with a log D of 2.94 log S of -3.36 (Table 1), 4a is predicted to be quite soluble in aqueous solutions.

The described results clearly indicate that (1) the benzopyran ring can be modified with minimal loss of activity and (2) the para position of the phenyl ring can tolerate substantial changes and can be used for improvement of water solubility. Such results will help future optimisation work.

Conclusion

Of the 12 new morpholine-containing analogs developed in this work, four demonstrate HIF inhibition in the low or sub-micromolar range. In particular, 3a and 4a both exhibit inhibition of HIF transcriptional activity with IC₅₀ values of 0.9 and 1.0 μM, respectively. As expected, the in silico log P and log S values of these analogs are considered more favourable than lead compound 1 or its more potent analog 2, and are therefore likely to be more bioavailable. Following these indications, solubility as measured by particle detection with DLS reveal the exceptional solubility of analogs 3a and 4a over their non-morpholine containing predecessors 1 and 2. Particle formation of 4a is undetected in excess of 100 μM in water and 50 μM in PBS, while still displaying HIF inhibition in the same order of magnitude as lead 1. These results encourage exploration and use of more soluble moieties to further probe the SAR (structure–activity relationship) and SSR (structure–solubility relationship) of potential analogs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration of particle appearance in water (μM)</th>
<th>Concentration of particle appearance in PBS (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>3a</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>4a</td>
<td>&gt;100</td>
<td>&gt;50</td>
</tr>
</tbody>
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Figure 3. Structures of compounds used for dynamic light scattering.
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Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Jalisa H. Ferguson http://orcid.org/0000-0002-4095-4357
Erwin G. Van Meir http://orcid.org/0000-0003-2444-7707
Sarah K. Zingales http://orcid.org/0000-0002-4908-6226
Binghe Wang http://orcid.org/0000-0002-2200-5270

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