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Bridged tetrahydroisoquinolines as selective NADPH oxidase 2 (Nox2) inhibitors†

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Eugenia Cifuentes-Pagano,[a] Jaideep Saha,[ab] Gábor Csányi,[a] Imad Al Ghouleh,[a] Sanghamitra Sahoo,[a] Andrés Rodríguez,[a] Peter Wipf,[bc] Patrick J. Pagano*[a] and Erin M. Skoda*[b]

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(1*SR*,4*RS*)-3,3-Dimethyl-1,2,3,4-tetrahydro-1,4-(epiminomethano)naphthalenes were synthesized in 2–3 steps from commercially available materials and assessed for specificity and effectiveness across a range of Nox isoforms. The *N*-pentyl and *N*-methylenethiophene substituted analogs **11g** and **11h** emerged as selective Nox2 inhibitors with cellular IC_{50} values of 20 and 32 μM, respectively.

Introduction

Reactive oxygen species (ROS) play a pivotal role in the development of cardiovascular diseases, cancer, neurological disorders, and other pathologies.[1–4] A major source of ROS is a family of enzymes, NADPH oxidases (Nox), that catalyze electron transfer from NADPH to molecular oxygen to give superoxide ($O_2^{\cdot-}$) and/or hydrogen peroxide (H_2O_2). Nox plays a crucial role in signaling cascades initiated by pro-inflammatory stimuli including hormones, vasoactive agents, and cytokines as well as mechanical stress.[5–9] Members of this family include Nox1–5 as well as Duox1 and 2; in the human cardiovascular system, Nox1, 2, 4, and 5 isoforms are prevalent.

The major catalytic subunit of these Nox isozymes possesses six transmembrane domains with a cytosolic C-terminus containing NADPH- and FAD-binding domains. Specifically, Nox1, 2, and 4 are constitutively associated with membrane-bound $p22^{phox}$, the complex of which forms cytochrome b558.[10–13] On the other hand, Nox5 does not require $p22^{phox}$ or cytosolic subunits but uniquely contains calcium-activating EF domains at its *N*-terminus.[14–18] Furthermore, the Nox isozymes differ in requirements for specific cytosolic subunits for activation and organization.[19,20] Nox1 associates with GTPase Rac1, cytosolic activator NoxA1, and cytosolic organizer NoxO1. Nox2 (Fig. 1) associates with Rac1 or Rac2 as well as cytosolic activator $p67^{phox}$ and cytosolic organizer $p47^{phox}$ while Nox4 requires no classical cytosolic subunits but is regulated by Poldip2.[21,22] The result of activation of these enzymes is the generation of ROS in the form of $O_2^{\cdot-}$ (Nox1, 2, 5) and H_2O_2 (Nox4).[23,24] ROS production is mediated by electron transfer from NADPH in the cytosol to FAD to form $FADH_2$. Single electron transfer to heme groups on the transmembrane domains and subsequent transfer to molecular oxygen on the opposite side of the membrane forms $O_2^{\cdot-}$, which can be converted to H_2O_2 by superoxide dismutase (SOD).[19,25] Downstream effects of this ROS generation include changes in gene expression, cellular signaling, host defense and inflammation, and cell growth regulation.[19] The inability of currently available agents to specifically inhibit a particular NADPH oxidase along with the combinative and varied expression of these isozymes in cells and tissue has made it difficult to assess their individual contributions to disease.[26]

Among the isoforms, Nox2 (aka $gp91^{phox}$, the first Nox isoform discovered) has been implicated in cardiovascular disease (CVD) processes including atherosclerosis,[27] hypertension,[28,29] ischemia reperfusion,[30] cardiac hypertrophy,[31] stroke,[32] and restenosis.[33,34] In addition to CVDs, Nox2 has more recently

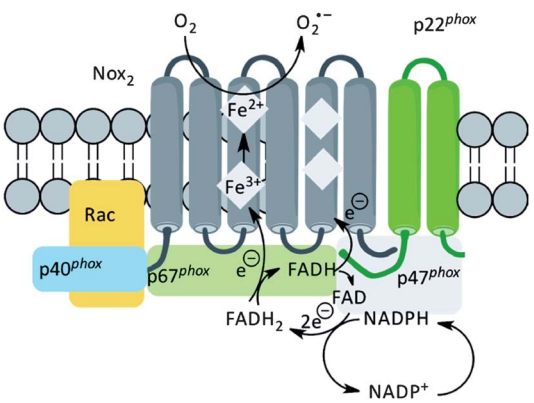


Fig. 1 Representation of Nox2 structure and activation.

[a]*Vascular Medicine Institute, Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15260, USA. E-mail: pagano@pitt.edu*

[b]*Center for Chemical Methodologies and Library Development, Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15261, USA. E-mail: emf53@pitt.edu*

[c]*Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA*

† Electronic supplementary information (ESI) available: Experimental procedures and complete spectroscopic data for new compounds, protocols for cell and cell-free assays. See DOI: 10.1039/c3md00061c

been implicated in neurodegenerative diseases such as Huntington's,[35] Alzheimer's,[36] and Parkinson's diseases.[37] Many of the insights into the involvement of Nox2 in these processes have until now been obtained by using a peptidic isoform-specific inhibitor of Nox2 developed in our laboratory.[38–40] Nevertheless, small molecules are still the preferred therapeutic strategy for clinical use and thus the quest for such isoform-specific inhibitors of Nox has intensified in recent years. However, due to the complex assembly and the high degree of homology among the various members of the Nox family, the development of isoform-specific inhibitors has proven challenging.

To date, several small molecule dual or multi-target Nox inhibitors have been identified (Fig. 2) and have been used as positive controls in phenotypic assays.[41–44] Diphenylene iodonium (DPI, **1**) is a commonly used positive control for Nox testing; however, it is also an irreversible and non-selective inhibitor of flavin-dependent enzymes such as xanthine oxidase (XO) and nitric oxide synthase.[19,42] Apocynin (**2**) was originally thought to inhibit Nox; however, recent reports attribute activity solely to its role as an nonspecific inhibitor and antioxidant.[45,46] Similarly, S17834 is an inhibitor of $O_2^{\cdot-}$ formation by a number of enzymes;[47] and AEBSF (**3**) is a serine protease inhibitor that also inhibits binding of cytochrome b558 to $p47^{phox}$.[48] Ebselen (**4**), a known ROS scavenger and mimic of glutathione peroxidase, and its analogs inhibit both Nox1 and Nox2 activity as well as other Nox isoforms to a lesser extent.[49] Pan-inhibitors of Nox include VAS2870 (ref. 50) and its derivative VAS3947 (ref. 51) shown to inhibit Nox1, Nox2, and Nox4 but not XO. Dual Nox inhibitors include fulvene-5 (**5**) that inhibits Nox2 and Nox4,[52] GKT136901 that inhibits Nox1 and Nox4,[53] and celastrol (**6**), which preferentially inhibits Nox1 and Nox2 over Nox4.[54] Perhaps the only inhibitor to date reported to display isoform specificity is ML171, which inhibits Nox1.[55] Due to the wide distribution of the Nox enzymes in a variety of cells in the body as well as their beneficial role in signalling, nonspecific Nox inhibitors are likely to cause undesired effects *in vivo*. A selective inhibitor of Nox2 would help to differentiate the Nox isoforms in a complex whole cell or *in vivo* environment. Heterologous Nox1, 2, 4, and 5 cell systems were used as a testing paradigm for these studies.

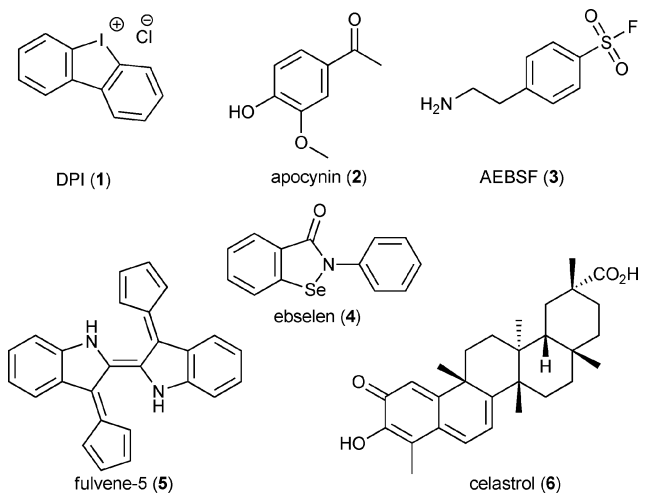


Fig. 2 Nonspecific or indirect inhibitors of Nox2 activity.

Results and discussion

In order to accelerate the understanding of the role of Nox2 in disease etiology, we set out to identify a probe molecule that inhibits Nox2 selectively over Nox1, 4, and 5. An acceptable probe would also show a lack of activity against XO, which also produces ROS. The latter screen would serve two purposes: (1) to eliminate compounds that inhibit an oxidase of distinct composition; and (2) to preclude agents that directly scavenge ROS. Accordingly, we optimized a cell-based primary assay using stable Nox2-transfected COS cells[56] with L-012 chemiluminescence[57] as a detection system for $O_2^{\cdot-}$ generation. A screen of a subset of small organic molecules from the University of Pittsburgh Center for Chemical Methodologies and Library Development (UPCMLD) at concentrations of 100, 50, 25, and 12.5 μM led to the identification of hit compound **7** (Fig. 3). Structurally related compounds **8** and **9** were also present in the screening library, which allowed for some initial structure–activity relationship (SAR) information to be gained. Compound **8** was inactive, suggesting that substitution at the 5-position of the heterocycle was required for activity. In contrast, **9** was active, suggesting that substitution could also be tolerated at C-8 of the bridged tetrahydroisoquinoline motif.

A series of secondary assays was used to confirm activity and rule out undesirable mechanisms of action. First, the concentration-dependent effect of **7** was studied using the same conditions as the primary screen (L-012 chemiluminescence in COS-Nox2 cells), revealing that **7** had an IC_{50} of 45 μM (Fig. 4A, closed squares). Cell-free activity in lysed COS-Nox2 cells was promising, since **7** was also able to inhibit $O_2^{\cdot-}$ production in a system in which assembly of active Nox2 subunits is achieved by treatment with the anionic amphiphile LiDS and the reaction is initiated by cofactor NADPH.[58] Under these conditions, **7** appeared to be effective at inhibiting Nox2 activity since 6.3 μM ameliorated LiDS-stimulated $O_2^{\cdot-}$ generation to almost non-stimulated levels (Fig. 4B). Next, because selectivity over the closely related isoform Nox4 was one of our most stringent benchmarks, the concentration–response activity of **7** against this isoform was tested. Since Nox4 is generally accepted to directly form H_2O_2 rather than $O_2^{\cdot-}$, the more appropriate Amplex® Red fluorescence was used as the detection reporter in Nox4 transfected COS cells;[13] and it was compared to similar assay conditions using the COS-Nox2 cells with addition of SOD

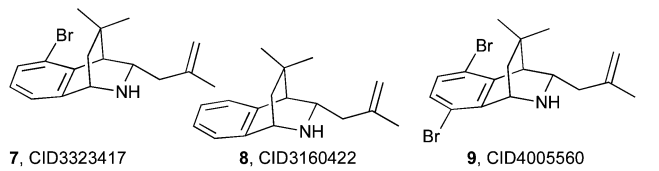


Fig. 3 Tetrahydroisoquinoline hit **7** (CID3323417) and related analogs **8** (CID3160422) and **9** (CID4005560) from a screen of a subset of compounds from the UPCMLD library.

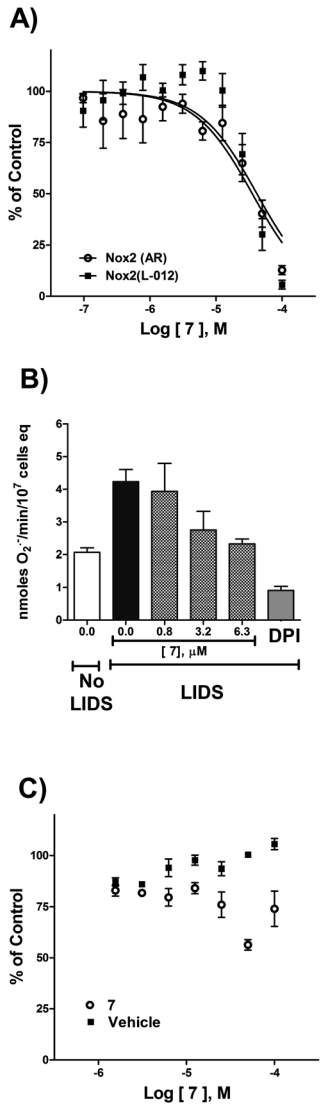
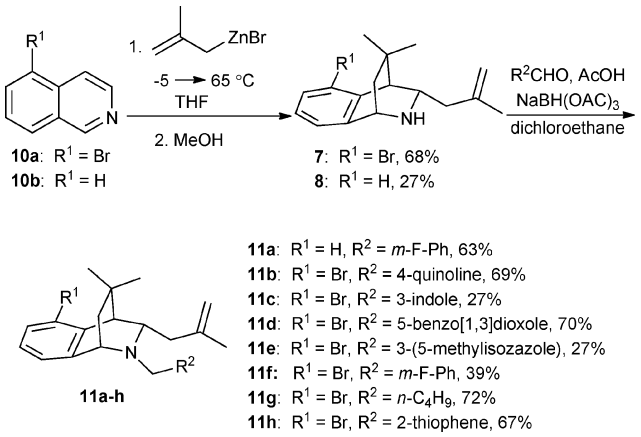


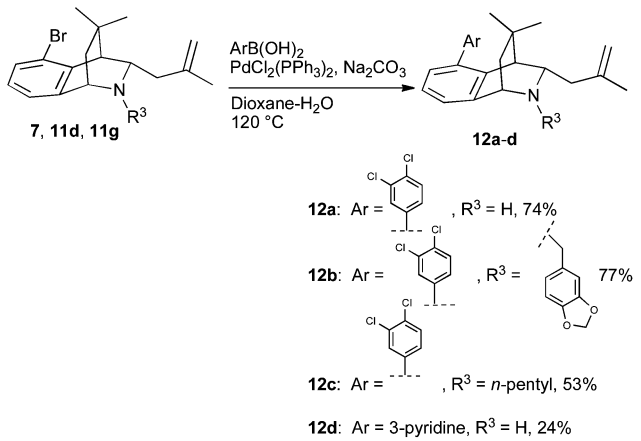
Fig. 4 Concentration–response testing of **7** in (A) COS-Nox2 cells, (B) lysed COS-Nox2 cells (cell-free preparation), and (C) COS-Nox4 cells. (A) Effect of **7** on Nox2 activity was measured in whole COS-Nox2 cells stimulated by 5 μM PMA using L-012 and Amplex® Red (AR). Data are expressed as % of vehicle control and represent the mean ± SEM of 6 independent experiments. (B) COS-Nox2 cell lysate was preincubated with various concentrations of **7** for 15 min at 25 °C. After the addition of 130 μM LiDS, $O_2^{\cdot-}$ production was initiated by the addition of 180 μM NADPH and measured by the initial linear rate of SOD-inhibitable cytochrome C reduction. $O_2^{\cdot-}$ production is expressed as nmol $O_2^{\cdot-}$ per min per 10^7 cell equivalents. (C) COS-Nox4 cells were pretreated with various concentrations of **7** for 15 min. Initial rate of H_2O_2 production was measured using Amplex® Red. Data are expressed as the mean ± SEM of **7** independent experiments.

to convert $O_2^{\cdot-}$ to H_2O_2. Compound 7 had an IC_{50} of 40 μM against Nox2-expressing cells when ROS inhibition was assessed using Amplex® Red (Fig. 4A, open circles), thus validating our results using two distinct ROS-detection assays. However, 7 was found to be inactive against Nox4 (Fig. 4C). Finally, the structure of compound 7 was confirmed by resynthesis, and the freshly prepared sample retained its biological activity.

After the activity and selectivity of 7 for Nox2 was confirmed in both whole cell and cell-free assays, analogs of 7 were

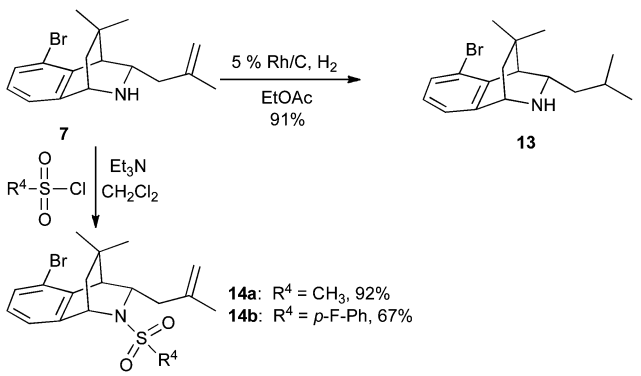


Scheme 1 Synthesis of hit **7** and related analog **8**, and reductive amination of these building blocks to form tertiary amines **11a–h**.



Scheme 2 Synthesis of biaryl analogs **12a–d** *via* Suzuki cross-coupling with aryl bromides.

synthesized to explore the SAR of this observed selectivity for Nox2 over Nox4. Bridged tetrahydroisoquinoline derivatives were synthesized in 2–3 steps from commercially available isoquinolines. Compound **8** was accessed through a double allylation and carbometallation–cyclization sequence from isoquinoline as reported previously,[59,60] and an analogous method



Scheme 3 Reduction of alkene **7** to alkane **13** and sulfonamide formation to form analogs **14a** and **b**.

Table 1 IC_{50} values of inhibitors of ROS generation in Nox enzymes

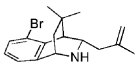
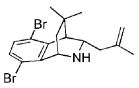
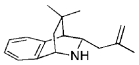
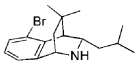
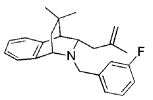
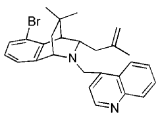
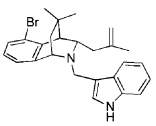
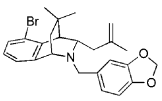
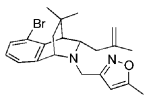
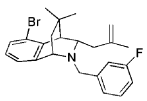
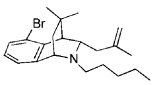
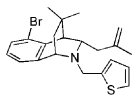
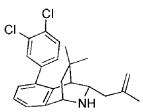
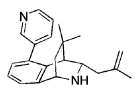
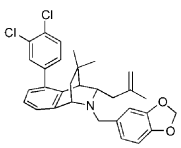
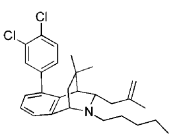
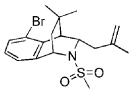
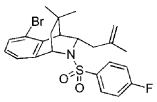
Entry	Compound	Structure	Nox2[a] (μM)	Nox2[b] (μM)	Nox1[b] (μM)	Nox4[a] (μM)	Nox5[b] (μM)
1	**7**, CID3323417		40 ± 9.7	46 ± 11	31 ± 0.7	>100	20 ± 3.1
2	**9**, CID4005560		25 ± 10	40 ± 2.8	60 ± 36	NI	36
3	**8**, CID3160422		>100	>100	>100	NI	>100
4	**13**		78 ± 19	31 ± 3.8	32 ± 18	>100	18 ± 1
5	**11a**		30 ± 7.5	68 ± 33	>100	>100	50 ± 9.0
6	**11b**		>100	>100	>100	NI	>100
7	**11c**		>100	>100	>100	NI	>100
8	**11d**		40	88 ± 22	>100	>100	>100
9	**11e**		12 ± 0.5	48 ± 23	>100	>100	31 ± 11
10	**11f**		51	68 ± 22	>100	>100	79 ± 30
11	**11g**		20 ± 1.9	20 ± 4.9	>100	>100	>100
12	**11h**		32 ± 1.9	38 ± 11	>100	NI	>100
13	**12a**		64 ± 35	9.0 ± 0.9	>100	>100	14 ± 0.6
14	**12d**		NI	NI	ND	ND	ND
15	**12b**		>100	>100	>100	NI	>100
16	**12c**		79	>100	>100	>100	>100

Table 1 (*Contd.*)

Entry	Compound	Structure	Nox2[a] (μM)	Nox2[b] (μM)	Nox1[b] (μM)	Nox4[a] (μM)	Nox5[b] (μM)
17	**14a**		27 ± 4.7	>100	NI	>100	>100
18	**14b**		>100	>100	>100	NI	>100

[a] Amplex® Red assay. [b] L-012 assay; ND = not determined; NI = not inhibitory (no inhibition seen in the slope of the curves); >100: indicates a notable slope decrease that allowed for extrapolation of an IC_{50} > 100 μM. Nox2 activity was measured in whole COS-Nox2 cells stimulated with 5 μM PMA. Constitutive Nox1 activity was measured in transiently transfected Cos-Nox1 cells. Nox5 activity was measured from HEK-Nox5 cells stimulated with 1 μM PMA and 0.5 μM ionomycin. Constitutive Nox4 activity was measured using transiently transfected COS-Nox4 cells. Data are expressed as % of vehicle control and represent the mean ± SEM of 3–7 independent experiments.

was used to synthesize the hit compound **7** in 68% yield from 5-bromoisoquinoline (Scheme 1). The bridged cyclic structure of **8** was confirmed by comparison of the ^{1}H and ^{13}C NMR spectra to those reported for **8**.[59] Compounds **7** and **8** were also used as building blocks for the generation of a series of bridged analogs. Accordingly, tertiary amines **11a–h** were formed *via* reductive amination of **7** and **8** with a variety of aldehydes (Scheme 1).

The original screen of this library had revealed that substitution at the 5-position of the isoquinoline was necessary for activity (**7** *versus* **8**); accordingly, an additional series of biaryl analogs was synthesized to better explore the functional group tolerance at that position. These compounds were prepared by Suzuki–Miyaura cross coupling with aryl boronic acids (Scheme 2). Both the secondary amines (prepared in Scheme 1) and the tertiary amines **11d** and **11g** were coupled to give the C5-aryl-substituted tetrahydroisoquinolines **12a–d**.

To explore whether the alkene moiety was required for the activity of the hit **7**, the disubstituted alkene was reduced in the presence of the aryl bromide using 5% rhodium on carbon to form **13** in 91% yield (Scheme 3). Additionally, to discern the importance of the secondary or tertiary amino functional group, an alkyl and an aryl sulfonamide **14a** and **b** were prepared by reaction with the corresponding sulfonyl chlorides.

As mentioned, an acceptable selective Nox2 probe would not only be inactive against other Nox isoforms but should also lack non-specific effects such as inhibition of another major source of $O_2^{\cdot-}$ in mammalian cells or ability to scavenge $O_2^{\cdot-}$. To test that the observed Nox inhibition of the tetrahydroisoquinolines was not due to non-specific activities, ROS production by XO was measured in the presence of various concentrations of the analogs. None of the compounds tested showed any significant effect on ROS levels as detected by Amplex® Red, and the activity was compared to complete inhibition by DPI (see ESI†). These data corroborate that these compounds neither inhibit XO, scavenge ROS, nor interfere with the assay signals.

Another undesirable side effect would be the reduction of ROS production in the assays due to cell death rather than an inhibition of Nox2. To verify that this was not the case for these tetrahydroisoquinolines, the effect of various concentrations of the compounds on cell viability was determined using a commercially available CytoTox-Glo assay (Promega). This assay measures the concentration of proteases released from cells that have lost membrane integrity (*i.e.*, dead cells). Only compounds **7**, **9**, and **13**, and only at the highest concentration tested, had any effect on cell viability (see ESI†).

With the confirmation that the analogs were not XO inhibitors, ROS scavengers, or cytotoxic, the selectivity of their inhibition for the Nox isoforms was explored. The specificity of **7** and analogs against Nox2 activity was determined by a concentration–response analysis of the effect of the tetrahydroisoquinoline derivatives on Nox2-, Nox1-, Nox4-, and Nox5-dependent ROS generation. These data are summarized in Table 1.

Compounds **7** and **9** inhibited Nox2 with IC_{50} values in the range of 25–40 μM but were also found to be active against both Nox1 and 5 (entries 1 and 2). Compound **8** (entry 3) from the original screen remained inactive against all Nox isoforms. The alkene moiety was found not to be an essential structural element for activity as the saturated derivative **13** retained inhibitory activity of Nox2, 1, and 5 at concentrations comparable to parent compound **7** (entry 4). Based on the assay results with reductive amination products **11a–h**, we concluded that bulky aromatic heterocyclic side chains decreased (entry 8) or completely abolished (entries 6, 7) Nox2 inhibitory activity. In contrast, analogs with relatively smaller *N*-substituents (entries 5, 9–12) were active against Nox2; but many also inhibited Nox5 activity (entries 5, 9, 10). The 5-substituted 3,4-dichloroaryl analog **12a** (entry 13), exhibited comparable Nox2 and Nox5 activity as the hit compound **7** but displayed greater selectivity over Nox1. On the other hand, when this 3,4-dichloro substitution was combined with the tertiary amine groups *N*-pentyl and benzodioxole from the analogs **11d** and **11g**, the hybrid compounds **12b** and **12c** were inactive (entries 15, 16). Further attempts to improve the potency and selectivity of **12a** by forming the C5-substituted pyridyl analog **12d** (entry 14) were unfortunately unsuccessful as this compound was inactive against Nox2 and was therefore not screened against other Nox

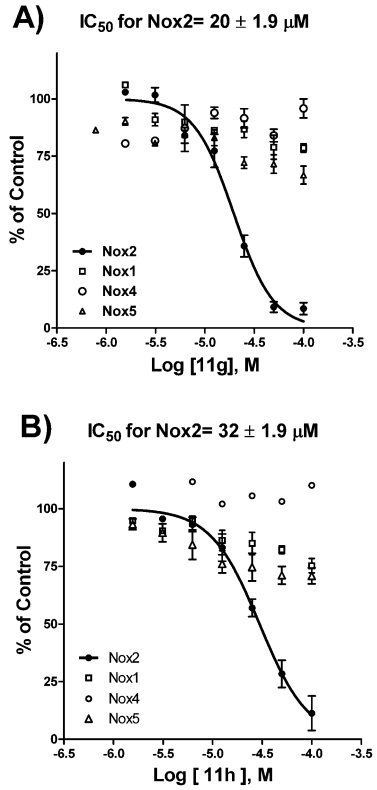


Fig. 5 Concentration–response analysis of compounds **11g** (A) and **11h** (B) for inhibition of Nox1, 2, 4, and 5. IC_{50} values were calculated using the non-linear regression for three parameters analysis which assumes a Hill slope = 1. (See Table 1 caption and ESI† for experimental details.)

isoforms. Additionally, aryl and alkyl sulfonamides in place of the tertiary amine completely abolished activity (entries 17, 18).

Compounds **11g** and **11h** are the most potent and selective among the tetrahydroisoquinoline analogs. Both of these agents showed specificity for Nox2 over Nox1, 4, and 5, as demonstrated by cell-based assays for each independent Nox system (Table 1, Fig. 5). The SAR emerging from lead compound **7** suggests that small substituents such as *n*-pentane (**11g**) and thiophene (**11h**) on the nitrogen atom influence the selectivity of this scaffold toward Nox2 inhibition.

Compounds **11g** and **11h** displayed considerably lower IC_{50} values (~30 nM) when tested in a cell-free system (data not shown). These compounds are likely more potent due to enhanced access to the enzyme in the disrupted cell membrane environment. That notwithstanding, a more complete assessment of IC_{50} values for these compounds and their derivatives will be necessary.

Conclusions

We have identified two selective small molecule inhibitors of Nox2, **11g** and **11h**. These compounds are highly efficacious inhibitors of Nox2 and display virtually no effect on Nox 1, 4, or 5. Furthermore, their inhibitory effects are not due to a nonspecific or undesirable mechanism, such as xanthine oxidase inhibition, ROS scavenging, or cytotoxicity. We anticipate that small molecule inhibitors **11g** and **11h** will prove useful as probes to more fully discern the biological role of Nox2 as compared to other Nox isoforms and could potentially serve as a platform for developing therapeutic agents for the treatment of Nox2-related diseases.

Abbreviations

DPI	Diphenylene idodonium;
LiDS	Lithium dodecyl sulfate;
PMA	Phorbol 12-myristate 13-acetate;
ROS	Reactive oxygen species;
SAR	Structure–activity relationship;
SOD	Superoxide dismutase;
XO	Xanthine oxidase.

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