Supporting information

Small - molecule inhibitors of NADPH oxidase 4

Gábor Borbély[*,a,b], István Szabadkai[a], Zoltán Horváth[a], Péter Markó[a], Zoltán Varga[a], Nóra Breza[a], Ferenc Baska[a,b], Tibor Vántus[c], Mónika Huszár[c], Miklós Geiszt[d], László Hunyady[d], László Buday[e], László Őrfi[a,b,f], György Kéri[a,b,c]

[a]*Vichem Chemie Research Ltd., Budapest, Hungary,* [b]*Rational Drug-Design Laboratory CRC, Semmelweis University, Budapest, Hungary,* [c]*Pathobiochemistry Research Group of the Hungarian Academy of Sciences,* [d]*Department of Physiology, Semmelweis University, Faculty of Medicine, Budapest, Hungary,* [e]*Department of Medical Chemistry, Semmelweis University, Budapest, Hungary,* [f]*Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary*

Contents

*To whom correspondence should be addressed. For G. B.: Phone, +36-1-4872087; Fax, +36-1-4872081; E-mail, gabor.borbely@kkk.org.hu

Abbreviations: NCL, Nested chemical library; EVL, Extended validation library; HPLC- MS, High performance liquid chromatography- mass spectrometry; NMR, Nuclear magnetic resonance; AcN, Acetonitrile; EDTA, Ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; DMEM, Dulbecco's modified eagle medium; FCS, foetal calf serum; LPO, lactoperoxidase; HRP, horseradish peroxidise

Experimental Section

Compounds. Vichem's NCL ™ technology is a novel hit finding method for rational drug design of inhibitors of various cellular targets. Test compounds were selected from NCL of kinase inhibitors, which is a chemically diverse library containing more than 1600 kinase inhibitory leads (EVL) and more than 14 000 molecules based on more than 110 core structures and several hundred scaffolds [S1,S2]. The novel targets shall be tested first against the EVL. The resulting hits, which are typically also biologically and chemically validated, will provide sufficient input to computer assisted design of new analogues. The hit compounds presented in the paper were obtained via the already published synthetic procedures. (**Table S1**)

The structure and purity of all compounds were verified by HPLC- MS and NMR analysis. All of the compounds have at least 95% purity by HPLC. The NCL includes most of the preclinically and clinically relevant, published kinase inhibitors and a large series of patented compounds that we have developed previously. Small focused sublibraries were built around the two known NOX4 inhibitors (**1**, **5**) and the newly identified hit molecules: oxalyl-hydrazides, thienopyrimidines, pyrrolo-pyrimidines, pyrazolo-pyrimidines, quinolines.

Purity. Quality control was performed on a Waters HPLC with Waters Alliance 2795 separation system. Compounds were detected both on a Waters 996 DAD and a Waters SQD MS detector. The analyses were carried out on an XBridge C18 column (5 cm x 4.6 mm x 3.5 μm) where the flow rate was 2 ml/min and the injected amount was 5 μg. Two different mobile phase compositions were applied. Basic composition (pH = 8): mobile phase "A" was 5 mM NH_4HCO_3. Acidic composition (pH = 3.5): mobile phase "A" was water + 0.1% HCOOH. In both cases mobile phase "B" was AcN. Mass spectra were recorded using positive and negative electrospray ionization modes where source block temperature was 110°C, desolvation temperature 250°C, desolvation gas 500 L/h, the cone gas 80 L/h. Capillary voltage and the cone voltage were 3000 V and 30 V, respectively. All of the compounds showed at least HPLC purity of 95%. The experimental conditions and results are summarised in **table S2**.

Cell culture. All the cell culture assays were carried on HEK 293 FS cells (Invitrogen, Carlsbad, CA, USA) which were transfected with an expression vector (pcDNA3.1) containing NOX4 cDNA. NOX4 expression of stable clones was examined by Northern blot analysis and PCR (data not shown).

Western blot analysis. Cells were lysated in 500 μl of 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 2 mM EDTA, 50 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 200 μM PMSF, "complete" protease inhibitor cocktail (Sigma-

Aldrich). Protein concentration was determined using BCA™ Protein Assay Kit according to the manufacturer's protocol (Thermo Scientific). Aliquots were denaturated for 6 min at 95°C in Laemmli buffer, separated on SDS-PAGE and then blotted onto nitrocellulose membrane. Following blocking in BSA (5%), proteins were detected using rabbit polyclonal NOX4 antibody (Novus Biologicals) at dilution of 1:2500. Visualization was carried out using horseradish peroxidise coupled secondary antibody (Santa Cruz), enhanced chemiluminescence kit (Amersham Biosciences) and X-ray-type photo films (Kodak).

H_2O_2/Tyr/LPO cellular assay. The H_2O_2 producing activity of the NOX4 enzyme was determined as described [S3]. The HEK 293 FS cell line was held in DMEM (Gibco) containing 5% FCS (Gibco) and 1% Penicillin-Streptomycin antibiotics (Gibco) in 5% CO_2 at 37°C. HEK 293 FS cells were grown to 100% confluency, removed from the culture flask (T-75) (Corning) and suspended in extracellular medium (called H-medium) containing: 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.8 mM $CaCl_2$, 10 mM Na- HEPES, 5 mM glucose, pH 7.4. The assay was performed in 384 microtiter plates (Greiner 781076, Sigma-Aldrich). ROS generation was measured at 10 μM compound concentrations in triplicates, in total volume of 50 μl. The final cell number was 20 000 cells/ well/ 40 μl H-medium. The incubation time was half an hour at 37°C and it was stopped by the addition of 50 μl H-medium supplemented with 2 mM of L-tyrosine (Sigma-

Aldrich) and 160 mU/ml of LPO (Sigma-Aldrich). Fluorescence was measured with photometric microplate spectrofluorometer using excitation and emission wavelengths of 330 ± 40 nm and 405 ± 10 nm. To identify the false positive inhibitors a H_2O_2 assay was performed from the H_2O_2/Tyr/LPO cellular assay where the H_2O_2 producing HEK 293 FS cells were substituted with 3 μM of H_2O_2 (Sigma-Aldrich).

Amplex Red assay. The Amplex Red assay contained 50 μM Amplex Red reagent, 0.1 U/ml HRP in H medium and the H_2O_2 producing cells (30 000 cells). In the presence of HRP, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stochiometry to produce the red-fluorescet resorufin [S4]. After 30 min incubation with the suspended HEK 293 FS cells at 37°C, resorufin fluorescence was measured at 590 nm.

Cell viability assay. The HEK 293 FS cells were seeded (1000 cells/ well, day 0) in 96 well cell culture plate in 50 μl DMEM supplemented with 5% FCS and 1% antibiotics (Penicillin-Streptomycin). After 24 h incubation in 5% CO_2 at 37°C, the cells were treated with the inhibitory compounds in the final concentration of 10 μM for 72 h. Cell viability was determined with the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) kit according to the manufacturer's protocol (Promega Corporation, Madison, USA).

Table S1. Published synthetic procedures.

Compounds	References
6 d	[S5]
7 a-h	[S6]
8 a-c	[S7]
9 a	[S8]
9h	[S9]
10 a-i	[S10]
11 c	[S11]

Table S2. HPLC experimental conditions.

Detector	Waters 996 DAD and Waters SQD MS
Column	XBridge C18 5 cm x 4.6 mm x 3.5 µm
Injector	Rheodyne
Mobile phase	A: 5 mM NH_4HCO_3 pH = 8.0 or A: water + 0.1% HCOOH pH = 3.5
	B: AcN
Flow rate	2 ml/min
Injected amount	5 µg

References

[S1] Kéri G, Őrfi L, Erős D, Hegymegi-Barakonyi B, Szántai-Kis C, Horváth Z, Wáczek F, Marosfalvi J, Szabadkai I, Pató J, Greff Z, Hafenbradl D, Daub H, Müller G, Klebl B, Ullrich A. Signal transduction therapy with rationally designed kinase inhibitors, *Cur Sig Trans Therapy.* **2006,** 1: 67-95.

[S2] Kéri G, Székelyhidi Z, Bánhegyi P, Varga Z, Hegymegi-Barakonyi B, Szántai-Kis C, Hafenbradl D, Klebl B, Müller G, Ullrich A, Erös D, Horváth Z, Greff Z, Marosfalvi J, Pató J, Szabadkai I, Szilágyi I, Szegedi Z, Varga I, Wáczek F, Őrfi L. Drug discovery in the kinase inhibitory field using the Nested Chemical Library™ technology. *Assay Drug Dev Technol*. **2005**, 3: 543-551.

[S3] Donkó A, Orient A, Szabó PT, Németh G, Vántus T, Kéri G, Orfi L, Hunyady L, Buday L, Geiszt M. Detection of hydrogen peroxide by lactoperoxidase-mediated dityrosine formation. *Free Radic Res.* **2009**, 43(5):440-445.

[S4] Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP.A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem*. **1997,** 253(2):162-168.

[S5] Dow RL, Chou TT, Bechle BM, Goddard C, Larson ER. Identification of tricyclic analogs related to ellagic acid as potent/selective tyrosine protein kinase inhibitors. *J Med Chem*. **1994,** 37(14):2224-2231.

[S6] Groundwater PW, Solomons KR, Drewe JA, Munawar MA. Protein tyrosine kinase inhibitors. *Prog Med Chem*. **1996,** 33:233-329.

[S7] Chen H, Boiziau J, Parker F, Maroun R, Tocque B, Roques BP,. Garbay-Jaureguiberry C. Synthesis and structure-activity studies of a series of [(hydroxybenzyl)amino] salicylates as inhibitors of EGF receptor-associated tyrosine kinase activity, *J Med Chem.* **1993,** 36: 4094-4098.

[S8] Sun L, Tran N, Tang F, App H, Hirth P, McMahon G, Tang C. Synthesis and biological evaluations of 3-substituted indolin-2-ones: a novel class of tyrosine kinase inhibitors that exhibit selectivity toward particular receptor tyrosine kinases. *J Med Chem*. **1998,** 41(14):2588-2603.

[S9] GB 1,206,401 [**1966**] Denis John Bauer and Peter William Sadler: Isatin thiosemicarbazones, their preparation and pharmaceutical formulations containing them (Application date: Febr. 23. 1961).

[S10] Őrfi L, Wáczek F, Kövesdi I, Mészáros G, Idei M, Horváth A, Hollósy F, Mák M, Szegedi Z, Szende B, Kéri G. New antitumor leads from a peptidomimetic library. *Lett Pept Sci* **1999**, 6(5): 325-333

[S11] Showalter HD, Bridges AJ, Zhou H, Sercel AD, McMichael A, Fry DW. Tyrosine kinase inhibitors. 16. 6,5,6-tricyclic benzothieno[3, 2-d]pyrimidines

and pyrimido[5,4-b-] and -[4,5-b]ĭndoles as potent inhibitors of the epidermal growth factor receptor tyrosine kinase. *J Med Chem*. **1999**, 42(26):5464-5474.

Table of Contents graphic

Small - molecule inhibitors of NADPH oxidase 4

Gábor Borbély*, István Szabadkai, Zoltán Horváth, Péter Markó, Zoltán Varga, Nóra Breza, Ferenc Baska, Tibor Vántus, Mónika Huszár, Miklós Geiszt, László Hunyady, László Buday, László Őrfi, György Kéri