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Brief Article

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,[†,‡,∥] and György Kéri[†,‡,⊥]

[†]*Vichem Chemie Research Ltd., Budapest, Hungary,* [‡]*Rational Drug-Design Laboratory CRC,* [§]*Department of Medical Chemistry, and* [∥]*Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary,* [⊥]*Pathobiochemistry Research Group of Hungarian Academy of Sciences, Department of Medical Chemistry, Semmelweis University, Budapest Hungary,* [#]*Department of Physiology, Faculty of Medicine, Semmelweis University, Budapest, Hungary, and* [∞]*Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary*

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NOX enzymes are the major contributors in many oxidative damage related diseases. Unfortunately, at present no specific NOX inhibitor is available. Here, we describe the discovery and development of novel NOX4 inhibitors. Compound libraries were tested in a cell-based assay as a primary screen, monitoring H_2O_2 production. Twenty-four compounds inhibited Nox4 activity with low-micromolar IC_{50} values of which three were selected for further drug development.

Introduction

It has been demonstrated that ROS[a] are involved in diverse biological processes including host defense, signal transduction, oxygen sensing, proliferation, apoptosis, and response to mechanical strain. ROS generation of the vascular system has been observed for a long time and is also known to play an important role in the pathogenesis of vascular atherosclerosis, inflammation, and fibrotic disorders.[1] In recent years, a number of ROS-producing NOX isoenzymes have been identified. At present the NOX enzyme family counts seven members: NOX1−5, Duox 1 and 2.

There is no specific vascular NOX isoform but rather a complex expression of different NOX isoforms in different cells. In endothelial and vascular smooth muscle cells, NOX4 appears to be the most common and abundant isoform.[2,3] NOX4 expression is strongly correlated with total NOX activity and endothelial function in human coronary arteries.[4] Oxidative excess in hypertensive patients leads to diminished NO[5] and correlates with the degree of impairment of endothelium-dependent vasodilatation and with cardiovascular events.[6] ROS also appears to be involved in the mediation of endothelial injury leading to programmed cell death or apoptosis.[7] An important precise strategy would be to achieve the specific inhibition of NOX4, which is the major source of ROS in the cardiovascular system, thus preventing CVDs.

It is known that numerous compounds from both natural and synthetic sources have NOX inhibitory effect such as thiol-modifying compounds, endogenous compounds (neopterin), natural compounds (norathyriol, gomisin C, abruquinone, magnolol, honokiol, prodigiosin, apocynin, gliotoxin) (Figure 1a), synthesized compounds (perhexiline), and aryliodinium compounds (DPI).[8] The iodinium-derived DPI is the most commonly used NOX inhibitor. It is a nonspecific inhibitor of many flavoprotein dehydrogenases and a few heme proteins acting by abstracting electron and forming a radical, which then inhibits the electron transporter by a covalent binding step.[9]

1 (S17834),[10] **2**,[11] **3**,[12] **4**,[13] and **5** (VAS2870)[14,8] are inhibitors developed by different pharmaceutical companies (Figure 1b). **5** inhibits oxLDL -induced superoxide release from human endothelial cells.[15] Enhanced vascular formation of ROS in response to oxLDL has been described in several studies *in vitro* and *in vivo*.

Another group of synthetic NOX inhibitors are statins, widely used drugs to treat CVDs, which have an indirect NOX inhibitory action by inhibiting the small G-protein Rac isoprenylation.[16]

Although it seems to be a good and validated target so far, there is no potent and specific NOX4 inhibitor available. In this study we present hit selection, development, and biological characterization of novel NOX4 inhibitors.

Results and Discussion

Transfected free-style 293 F HEK cell line is the basis of the cellular assay which overexpresses the constitutively H_2O_2-producing NOX4 enzyme. The Western blot analysis using rabbit polyclonal NOX4 antibody showed increased NOX4 expression in the transfected HEK 293 FS cell line (Figure 2a). The H_2O_2 production of the transfected HEK 293 FS cell line was confirmed by using Amplex Red assay (Figure 2b). The detection is based on the indirect measurement of the quantity of H_2O_2 produced. The LPO enzyme produces *o*,*o*′-dityrosine in the presence of H_2O_2 and L-tyrosine (Figure 2c). The quantity of the *o*,*o*′-dityrosine can be measured by a spectrofluorometric plate reader.

We tested ∼1000 compounds from our ML at 10 μM in the H_2O_2/Tyr/LPO cellular assay for NOX4 inhibition. A total of 73 compounds proved to be effective, resulting in

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

[a]Abbreviations: NOX, NADPH oxidase; ROS, reactive oxygen species; Duox, dual oxidase; NO, nitric oxide; CVDs, cardiovascular diseases; DPI, diphenyleneiodinium; oxLDL, oxidized low-density lipoprotein; HEK, human embrional kidney; LPO, lactoperoxidase; EVL, extended validation library; FI, fluorescence intensity; ML, master library; RFU, relative fluorescence unit.

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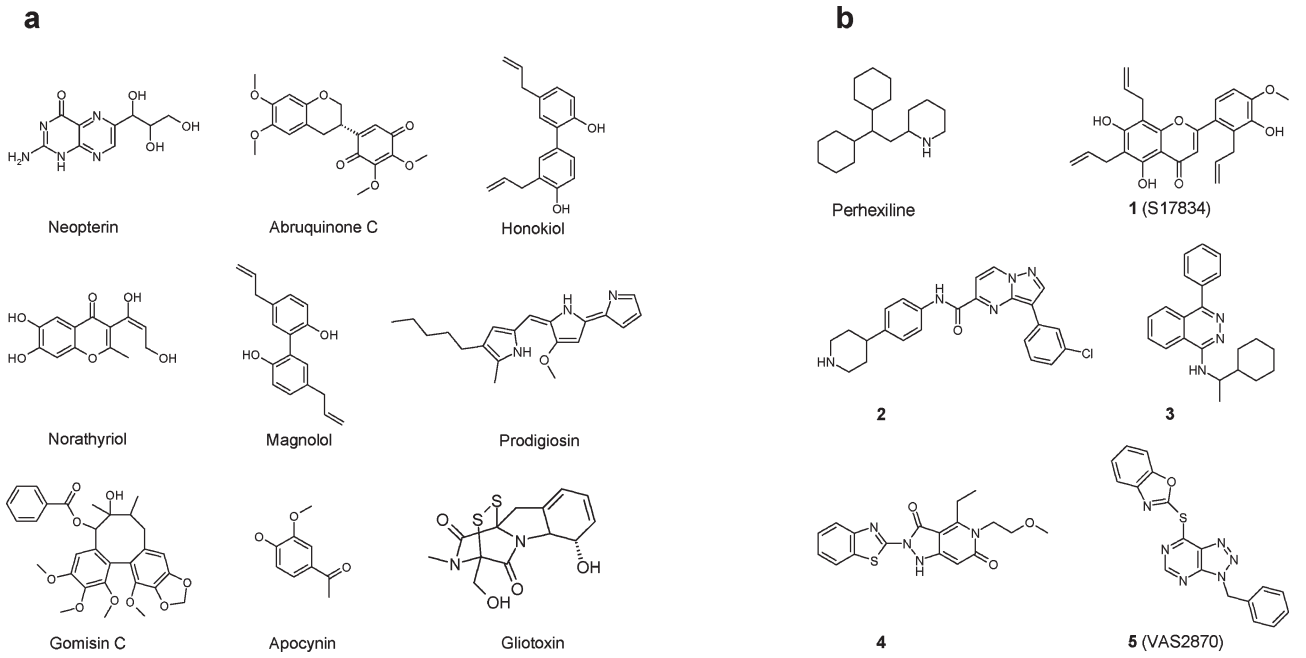

Figure 1. Structures of natural (a) and synthetic (b) compounds with proven NOX inhibitor activity.

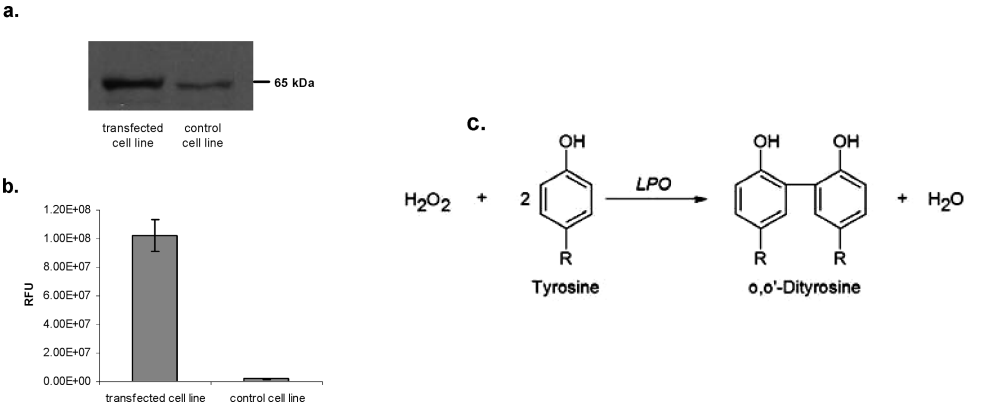

Figure 2. Detection and comparison of NOX4 expression in the transfected and nontransfected HEK 293 FS (control) cell lines by Western blot analysis. (a) Detection of NOX4 activity in the transfected and control HEK 293 FS cell lines, using the Amplex Red hydrogen peroxide/peroxidise assay. (b) Fluorescence was measured with photometric microplate spectrofluorometer using excitation at 551 ± 10 nm and emission at 590 ± 20 nm (b). (c) Indirect measurement of H_2O_2 production in H_2O_2/Tyr/LPO assay. The LPO enzyme produces *o*,*o*′-dityrosine in the presence of H_2O_2 and L-tyrosine. Dityrosine has distinct fluorescent properties. In the presence of H_2O_2 (produced by NOX4 enzyme) the increasing fluorescence intensity can be detected by spectrofluorometric plate reader.

high (between 80% and 100%) NOX4 inhibition compared to the effect of DPI, which is a highly effective but nonspecific NOX inhibitor. Most of the 73 inhibitors belong to the following five core structures: oxalyl hydrazides, flavonoids, oxindols, benzoquinolines, benzothiophenes.

The most potent compounds that showed better than 80% inhibition at a dose of 10 μM were selected to determine their IC_{50} in the same cellular assay. The inhibitory properties of 23 selected compounds (with IC_{50} less than 2 μM) are shown in Table 1a. Most of the selected 23 hit compounds are phenantridinone, flavonoid, aminosalicylic acid, oxindole, oxalylamide, or benzo[4,5]thieno[3,2-*d*]pyrimidine derivatives.

On the basis of the search of core structure similarities between the previously identified 23 hit molecules (Table 1a), we selected 200 analogues from the groups of oxalyl hydrazides, flavonoids, and benzothiophenes with different functional groups and tested them. Table 1b shows the inhibitory properties of the hit compounds from the sublibraries.

Flavonoids (**7a**[17]−**o**) were the most potent inhibitors, which had submicromolar IC_{50}. As Table 1b shows, we have identified numerous effective compounds (**7b**, **7d**, **7e**, **7g**,[18,19] **7h**) among the flavonoid derivatives. On the basis of the above results, the selected most effective NOX4 inhibitors belong to the following core structures (Figure 3).

Phenantridinone Derivatives (6a−h). To be active, phenantridinone derivatives seem to need four hydroxy groups. However in addition to hydroxy groups there needs to be one electron withdrawing group (NO_2, CN) on the benzylic moiety (**6a**, **6b**, **6c**, **6d**[20]). Methylation of hydroxy groups resulted in a loss of activity.

Flavonoids. The most active compound **7a** contains five hydroxy groups. One or more of these hydroxy groups are placed in another position or replaced by a hydrogen or a methoxy in the tested derivatives which affected the activity. In **7a**, **7h**, changing the position of one hydroxy group (meta R^4-R^5 instead of ortho) decreased the activity. In **7a**, **7e** the

Table 1. Inhibitory Properties of (a) the Most Potent Compounds, Selected for Further Investigation after Primary Screen, and (b) the Derivatives of Newly Identified Hit Compounds in Section a

compd	inh (%) in 10 μM ± 95% CI [a]	IC_{50} (μM) ± 95% CI[b]	inh (%)[c]	inh of cell viability (%)[d]
		(a) Inhibitory Properties of the Most Potent Compounds		
6a	104 ± 3	0.17 ± 0.03	−4.8	90.56
6b	101 ± 3	0.26 ± 0.01	−4.9	112.28
6c	97 ± 3	0.59 ± 0.36	4.8	107.56
6d	93 ± 8	1.58 ± 0.66	−6.11	107.94
6e	95 ± 2	1.58 ± 0.71	−1.05	112.91
7a	94 ± 6	0.68 ± 0.48	−2.64	102.52
7c	86 ± 5	0.79 ± 0.05	6.09	98.85
8a	89 ± 9	0.96 ± 0.13	5.35	101.46
8b	91 ± 8	1.27 ± 0.03	3.83	106.39
8c[e]	82 ± 7	1.34 ± 0.48	72.4	88.71
9a[f]	102 ± 4	0.63 ± 0.61	−0.88	18.69
9b	100 ± 1	1 ± 0.17	−2.94	97.77
9c	102 ± 4	1.13 ± 0.46	0.39	123.23
9d	104 ± 8	1.4 ± 0.42	−5.16	111.6
10c	105 ± 7	1.16 ± 0.71	7.6	99.51
10b	96 ± 10	1.02 ± 0.54	17.42	93.16
11a	98 ± 9	0.24 ± 0.14	17.91	105.56
11b[f]	101 ± 1	1.07 ± 0.48	−2.78	60.51
12	100 ± 8	0.46 ± 0.05	−3.94	99.86
13[f]	108 ± 9	0.52 ± 0.15	−1.15	20.14
14[f]	103 ± 2	0.63 ± 0.41	−0.53	77.52
15[e]	91 ± 11	1.04 ± 0.14	90.71	*g*
16	100 ± 3	1.35 ± 0.21	−5.02	95.71
		(b) Inhibitory Properties of Derivatives of Hit Compounds in Section a		
7b	100 ± 1	0.74 ± 0.14	3.17	120.69
7d	105 ± 1	0.83 ± 0.12	−1.86	104.63
7e	106 ± 1	0.85 ± 0.31	−4.47	110.36
7f[e]	88 ± 12	1.02 ± 0.75	43.41	*g*
7g	105 ± 1	1.13 ± 0.68	−1.2	107.54
7h	105 ± 2	1.2 ± 0.27	2.09	118.71
10a[f]	103 ± 3	0.91 ± 0.09	−4.58	67.17
10d	104 ± 1	1.3 ± 0.19	−3.3	82.36
10e[f]	104 ± 2	1.64 ± 0.78	−3.5	74.9
17	101 ± 2	1.16 ± 1.05	−0.59	85.02
18[f]	102 ± 0	1.27 ± 0.53	−0.64	63.36

[a] All the inhibitory compounds were tested first in H_2O_2/Tyr/LPO cellular assay at a dose of 10 μM. Most of them reached their maximal NOX4 inhibition at this concentration. [b] The IC_{50} values for NOX4 enzyme were estimated by H_2O_2/Tyr/LPO cellular assay, with an eight-point curve using a $^1/_2$ dilution series from 10 to 0.078 μM inhibitor. [c] To identify the redox active small molecules, in the H_2O_2 assay the HEK 293 FS cells of the H_2O_2/Tyr/LPO cellular assay were substituted with 3 μM H_2O_2. The hit compounds were tested in 10 μM final concentration. [d] The inhibition of cell viability was determined by luminescent cell viability assay kit in the presence of 10 μM inhibitory compounds. Cells were incubated with the inhibitors for 72 h. The inhibitory values in the H_2O_2 assay and the cell viability assay are represented as the percentage of live cells. The H_2O_2 production of NOX4-expressing cells was (8.4 nmol/10^6 cells)/h. [e] False positive data. The % inhibition of the compound is higher than 25% in the H_2O_2 assay. [f] False positive data. The % inhibition of the compound is higher than 20% in the luminescent cell viability assay. [g] Not tested.

replacement of one hydroxy group by a hydrogen at position R^3 did not really influence the activity. However, significant decrease can be observed replacing two hydroxy groups by hydrogen in **7a**, **7g**. Similarly methylation of one hydroxy group seems to be tolerable, but compounds containing two or more methoxy goups are significantly less active than the corresponding hydroxy derivatives. This effect is independent of the position of the methoxy goups.

Aminosalicylic Acid Derivatives (8a−c).[21] All of the tested hit compounds **8a**,**b** were found active. The activity is slightly decreased by introduction of additional hydroxy groups, and at the same time, a free carboxylic group seems to be beneficial.

Oxindole Derivatives (9a−i). The most active compound contains a 3,4,5-trisubstituted phenyl moiety, but the substitution of the indolinone ring system (for example, in the fifth position) results in loss of activity (**9a**[22]**−g**). Compounds containing 4-hydroxyphenyl moiety were also active (**9c**,**d**), but in this case, introducing an electron withdrawing group next to the hydroxy function spoiled the activity (**9i**). However in **9c** and **9d** the substitution of indolinone core was tolerated. Substituted semicarbazide derivative of isatin also showed good inhibition (**9b**), but substitution of the isatin core decreased the activity (**9f**, **9h**[23]).

Oxalylamide Derivatives (10a−i).[24] It is not obvious which scaffold can represent the most active compounds because there are inactive and active examples in each of the three compound groups. Dihydroxyphenyl and hydroxymethoxyphenyl substituents are beneficial especially in the symmetrical cases. Replacement of a nitro group by an amino and of a hydroxy group by a methoxy can increase the activity (**10h−d**, **10g−d**).

Benzo[4,5]thieno[3,2-*d*]pyrimidine Derivatives (11a−i). All of the tested compounds have an amino group attached directly to the tricyclic core, e.g., **11c**;[25] however, only those (**11a**, **11b**) that contain an additional amino substituent on the aniline moiety were found active. In this case any other type of substitution resulted in inactive compounds.

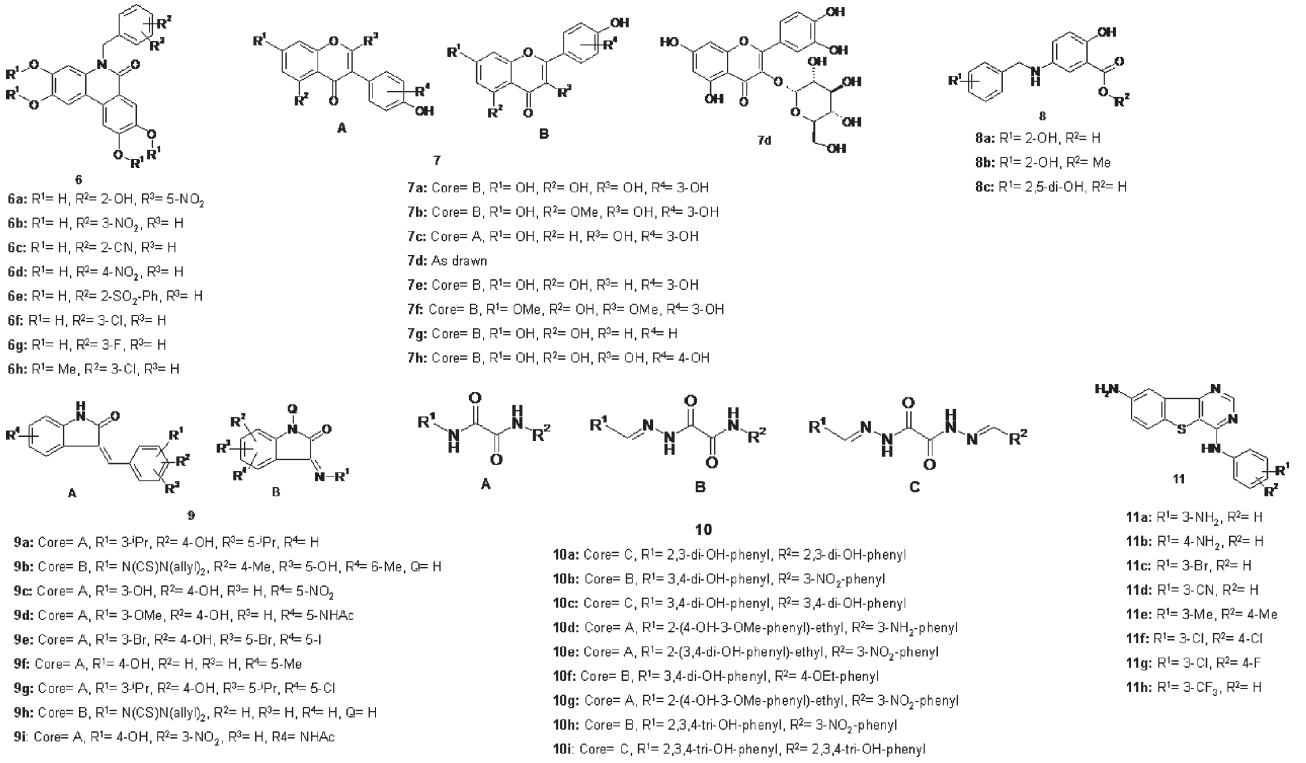

Figure 3. Chemical structures of the selected and examined hit compounds.

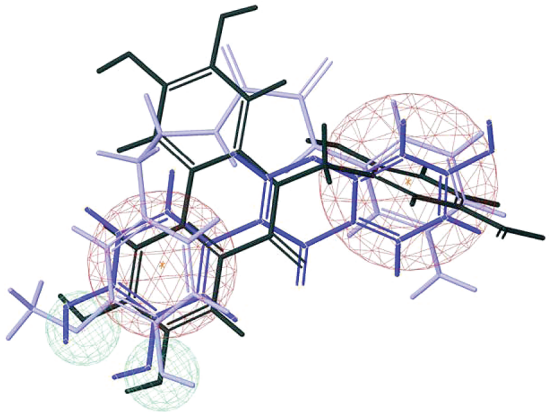

Figure 4. Pharmacophore model: pharmacophore features of hydrogen bond donors, acceptors (green meshed balls) and aromatic rings (red meshed balls). Three different structures are shown (**6a** dark grey, **7c** blue, **10d** light blue).

The H_2O_2/Tyr/LPO cellular assay is based on the indirect measurement of the quantity of the produced H_2O_2. Reduction in the number of viable cells and disintegration of produced H_2O_2 can also cause decreased H_2O_2 concentration. To exclude these false positive results, two additional types of experiments were performed. The luminescent cell viability assay enabled us to identify compounds with cytotoxic effect. Compounds **9a**, **10a**, **10e**, **11b**, **13**, **14**,[19] **18** (marked with footnote f in Table 1) proved to be effective mainly through the reduction of the number of viable cells instead of the NOX4 enzyme inhibition. In our further experiments the HEK 293 FS cells were substituted with 3 μM H_2O_2 because it was comparable to the FI values (~20000 cps) of the positive control of our previous H_2O_2/Tyr/LPO cellular assay experiments. Having tested the hit compounds in this H_2O_2 assay, we could exclude **7f**, **8c**, and **15**, which we considered as false positives (marked with footnote e in Table 1). These molecules showed "inhibitory effect" in the absence of cells (NOX4 enzyme).

Conclusion

Although numerous natural or synthetic NOX inhibitors have been described recently, the currently available small-molecule NOX inhibitors have low selectivity and potency, precluding a pharmacological demonstration of NOX as therapeutic targets in vivo.[14]

The 3D structure of the NOX4 enzyme is unknown, and the structures of the active compounds are very different; therefore, we mapped the common pharmacophore points of the inhibitors. The best hit molecules were used to build the pharmacophore model, and Schrödinger modules[26] were used to create the 3D structure of the compounds. Four pharmacophore points have been found which are demonstrated in Figure 4. The green meshed balls mark the hydrogen-bonding donors and acceptors. These aromatic hydroxyl groups can be found in most of the inhibitors. Other important features are the two aromatic−hydrophobic interaction regions, located 6.5−7.1 Å from each other. These points seem necessary for the NOX4 inhibitory effect because they can be found in most of the tested compounds.

In this study we show for the first time that pathologically relevant selective NOX4 inhibitors can be developed. In addition, we selected a series of novel compounds that efficiently inhibit H_2O_2 formation and may provide a novel strategy to treat ROS derived endothelial dysfunction.

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Supporting Information Available: Experimental procedures for compounds, purity, cell culture, Western blot analysis, H_2O_2/Tyr/LPO cellular assay, Amplex red assay, and cell viability assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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