

Development and characterization of small-molecule inhibitors of NADPH-oxidase 4 for the treatment of oxidative stress-mediated diseases

Ph.D. thesis

Gábor Borbély

Semmelweis University
Doctoral School of Pharmaceutical and Pharmacological Sciences



Supervisor:

Dr. György Kéri professor, Doctor of HAS

Official reviewers:

Dr. Laszlo Tretter, professor, Doctor of HAS

Dr. István Venekei, associate professor, Ph.D.

Theoretical exam committee:

President:

Dr. Tamás Török, professor, Doctor of HAS

Members:

Dr. Valéria Kecskeméti, M.D., Ph.D.

Dr. Rita Farkas, Ph.D.

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1. Introduction

It was believed for a long time, that under physiologic conditions the reactive oxygen species (ROS) are byproducts of the normal aerobic metabolism and generated by the biological reactions of mitochondria, microsomal electron transport chains, and phagocyte cells and -enzyme systems. Based on their reactivity they were marked as harmful molecules which cause irreversible oxidative injury of macromolecules of the body and are responsible for processes such as aging. Based on their reactivity, they were considered as harmful molecules causing the irreversible oxidative damage of the macromolecules of an organism, and responsible for the process of aging.

Nicotinamide adenine dinucleotide phosphate-oxidases (NADPH-oxidase, NOX) were the first identified examples of a system of which ROS generation is the primary function. During their activity, they transport electrons across biological membranes. The electron acceptor is O₂ and the end product of the electron transfer is superoxide. At present the NOX family counts seven members: NOX1-5 and DUOX1 and DUOX2. Although all seven NOX isoforms catalyze the reduction of molecular oxygen, they differ in their tissue distribution, subunit requirements, domain structure, and the mechanism by which they are activated.

The NOX4 is highly expressed in a variety of cell types including endothelial cells, vascular smooth muscle cells, hematopoietic stem cells, fibroblasts, and kidney cells.

Although the activity of the majority of NOX enzymes depends on the presence of p22^{phox} subunit and activator or organizer subunits (p22^{phox}, p40^{phox} and p47^{phox}, p67^{phox} or their homologues NOXO1 and NOXA1), NOX4 is also active in cells that do not express the latter cytoplasmic factors. According to our present knowledge, the NOX4 requires only one subunit (p22^{phox}) for its activation. Forming a complex with the p22^{phox}, it is constitutively active without any further activating mechanism.

Since the discovery of NOX enzymes, the scientific results of the past decades shed light on the commanding function of ROS in basic cellular functions like the regulation of cell growth, signaling, differentiation and apoptosis.

By virtue of their activity, NOX isoforms may represent a “double-edged sword” for the organism's homeostasis. On one hand ROS participate in the regulation of several important physiological processes such as the maintenance of endothelial function and the control of vascular tone, the host defense by killing invading microbes, hormone biosynthesis, fertilization, and cellular signaling. On the other hand the relative excess of ROS (oxidative stress) may play an important role in the development of numerous renal and cardiovascular related diseases including endothelial dysfunction, hypertension, atherosclerosis, angiogenesis, fibrosis, extracellular matrix deposition, inflammation, hypertrophy, cardiovascular and renal remodeling and heart failure.

The different NOX isoforms show complex expression in different cells and regions of the vasculature. Although the NOX1, NOX2 and

NOX5 also expressed in the vascular endothelial cells, among these NOX isoforms, the NOX4 expression correlates most strongly with the total NADPH-oxidase activity of the cardiovascular system.

There is enormous interest in the importance of NOX4 in cardiovascular damage. The action of antioxidants is easily overpowered by high concentrations of the ROS, hence they are least efficient at the site of pathologic overactivity of NOX4. Based on the scientific literature, in particular on studies with NOX-deficient mice, it is increasingly clear that inhibition of NOX4 is a promising pharmacologic concept for the treatment of oxidative stress-related diseases. During the past decade, interest has greatly increased in identifying novel and specific NOX inhibitors both as potential therapeutic agents and powerful tools to gain information about enzyme function.

The inhibitors described so far do not directly block the NOX enzymes but rather interfere with upstream signal-transduction pathways or act as antioxidants or ROS scavengers like superoxide dismutase (SOD) and peroxidase mimetics, or N-acetyl cysteine. Other molecules exert a direct inhibitory effect on the oxidase complex, but also inhibit other enzyme systems sharing common structural binding sites (for example cytochromes P-450, mitochondrial electron-transport chain, and nitric oxide synthase). Compounds that decrease the level of NADPH (electron donor), such as inhibitors of pentose phosphate pathway, can also preclude NOX activation.

The application of non selective NOX inhibitors should be taken with some caution. There is concern for the use of NOX inhibitors in humans since the complete loss of NOX2 function in chronic granulomatous disease (CGD) leads increased life threatening infections because of the malfunction of the immune system. However, CGD carriers do not have obvious symptoms if the 5–10% of their neutrophil cells can generate ROS. This suggests that room exists for safe inhibition of excessive ROS generation by NOX enzymes.

At present no specific NOX4 inhibitor is available for biochemical and/or clinical applications. The diphenylene-iodonium (DPI) which is the most widely used compound in *in vitro* experiments is efficient but nonspecific and toxic. The plant derived apocynin, which was efficient in *in vivo* experiments modeling oxidative stress-mediated diseases like hypertension, atherosclerosis and stroke, is relatively nontoxic, but its inhibitory effect on NOX enzymes has never been rigorously proven. Since the scientific achievements of the past decades just increased the complexity of the picture regarding the NOX4 enzyme function, probably the need for discovery and development of novel, NOX4 specific inhibitors are more exigent.

2. Aims

In collaboration with the chemical research partners we aimed to develop and characterize small-molecule NOX4 inhibitors for oxidative stress-mediated diseases. We set up the following strategy:

- Development of a simple but efficient cellular assay for the identification of NOX4 inhibitors and lead molecule optimization through the measurement of dose dependent inhibition of ROS production.
- Examination of dose dependent inhibition of active compounds in enzyme assay.
- Identification of false hits and validation of NOX4 specificity.
- Evaluation of structure-activity relationship.
- Characterization of early ADME(T) parameters of the promising lead compounds.

3. Methods

- *Compounds*: the compound library contained more than 1000 molecules constructed around 108 core structures. Their purity criteria were higher than 98% which were verified by NMR, LC-MS and analytical HPLC methods.

- *Cell lines*: 293 FS (human kidney epithelial cells), EA.hy926 (vascular endothelial cells).

- *H₂O₂/Tyr/LPO cellular assay*: The NOX4 overexpressing 293 FS cells were suspended in H-media (5×10^5 cells/ml), seeded into 384 microtiter plates and incubated for 30 minutes in the presence of various inhibitors. For H₂O₂ detection, 1 mM tyrosine and 1 μ g/ml lactoperoxidase (LPO) supplemented H-media was added to each well and fluorescence was measured following 30 minutes incubation (excitation: 330 ± 40 nm, emission: 405 ± 10 nm).

- *SDS-PAGE and Western blot*: samples were separated in 10% akrilamide gel (90V) and blotted onto PVDF or nitrocellulose membrane for 1 hour (400 mA). Following blocking in BSA (5%) and 0,1% Tween 20 containing TBS, membranes were incubated with anti-NOX4 or anti-p22^{phox} antibodies overnight at 4°C. Horseradish peroxidase (HRP) coupled anti-rabbit IgG secondary antibodies were bound to the primary antibodies. Visualization was carried out using ECL Advanced Western Blotting Detection kit.

- *Amplex Red assay*: the H₂O₂ concentration was detected using 50 μM Amplex Red reagent and 0,1 U/ml HRP containing KRPG solution or H-media. After 30 minutes incubation time, resofurin fluorescence was measured at 590 nm.

- *Immunohistochemistry*: cells (5x10⁴ cells/well) were let to adhere on cover slips in 24 well plate (overnight), than fixed and permeabilized with PETMF for 10 minutes. It was followed by washing them in PBS (three times), than they were incubated with primary antibodies (in 5% BSA containing PBS) for one night, followed by Cy3 conjugated secondary antibody treatment. For the staining of nuclei, Hoechst 33342 was used.

- *Confocal laser scanning microscopy*: visualization was performed with 'Zeiss LSM 510 META' confocal microscope. Cy3 fluorescent dye was excited on 543 nm using He-Ne laser. Emission was detected on 560 nm through a longpass filter. Hoechst 33342 fluorescent dye was excited with the UV emission of a Hg lamp (405 nm). Its emission was detected through a 420-480 nm BP filter. The excitation and emission light was separated by 405/488/543 nm dichroic filters.

- *Preparation of EA.hy926 nuclear fraction*: EA.hy926 cells were centrifuged at 260 g, the pellet was resuspended in hypotonic buffer (NB) and kept on ice for 20 min (swelling). Cells were gently Dounced checking the breaking process with light microscope. Broken cells were layered over a cushion of sucrose solution and centrifuged at 800 g. Pellet was resuspended in NB and the last step was repeated resulting in a cleaner nuclear pellet.

- *Enzyme assay*: 10-10 μM inhibitory compounds were added in the wells of a 384 well plate and mixed with the nuclear fraction of EA.hy926 cells in KRPG buffer ($1,5 \times 10^4$ nucleus/well). KRPG buffer, supplemented with 25 mM NADPH, 20 mM FAD, 100 μM Amplex Red and 0,2 U/ml HRP was added to each well. After the detection reaction (30 min.), fluorescence was measured at 590 nm.

- *Luminescence cell viability- and MTT assay*: cells were treated with the inhibitory compounds in the final concentration of 10 μM for 72 hours. Cell viability was determined with the 'CellTiter-Glo[®] Luminescent Cell Viability Assay' kit according to the manufacturer's protocol. For the MTT assay, 5 mg/ml MTT (dissolved in PBS) was added to each well followed by incubation for 2 hours. Formazan product was dissolved in a solubilization solution and the optical density was measured at 650 nm.

- *Flow cytometry*: cells were treated with the inhibitory compounds in the final concentration of 1 and 10 μM for 72 hours. Trypsinized cells were centrifuged and the pellet was resuspended in 70% ethanol. Right before FACS measurement, the cells were centrifuged, and the pellet was resuspended in 200 mM Na_2HPO_4 (pH 7,8) solution supplemented with 0,5 mg/ml RNase. After 20 minutes incubation, DNA was stained with 50 $\mu\text{g}/\text{ml}$ propidium iodide and samples were counted in flow cytometer. Results were analyzed with WinList 32 software.

- *IMAP assay*: 14-14 μM inhibitory compounds were added in the wells of a 384 well plate. Inhibitors were mixed with appropriate

kinase substrate (100 nM 5FAM-GRTGRRNSI-NH₂ or 400 nM 5FAM-IPTTPITTTYFFFK-NH₂), ATP and 4 nM kinase containing kinase buffer and incubated for 1 hour. For detection 'IMAP™ Evaluation' kit (Molecular Devices) was used according to the manufacturer's protocol. Fluorescence was measured following 30 minutes incubation (excitation: 458 ± 30 nm, emission: 530 ± 25 nm).

- *NOX2 assay*: human neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation, Ficoll-Paque gradient centrifugation and hypotonic lysis of remaining erythrocytes. Cells were kept at room temperature in Ca²⁺-, and Mg²⁺-free medium until use. The surface of the wells of a 384-well plate was precoated with 20 µg/ml human serum albumin (HSA) than blocked with 10% FCS and incubated with HSA antibody for the immunocomplex mediated activation of neutrophils. The treatment was carried out in HBSS solution containing 0,5 mM CaCl₂ and 10 mM MgCl₂.

In the case of phorbol myristate acetate (PMA) activation; first, the surface of the wells was blocked with 10% FCS, than the cells were activated with 100 nM PMA, diluted in 0,5 mM CaCl₂ and 10 mM MgCl₂ containing HBSS. During the experiments, the neutrophils were preincubated with various inhibitors at 1 or 10 µM concentration for 20 minutes. Superoxide release was detected by a cytochrome c reduction test. Absorption was detected at 550 nm and 540 nm (reference) wavelengths.

- *Permeability assay*: the 5 mM stock solutions (dissolved in DMSO) of the inhibitors were diluted in PBS buffer until 200 µM. This 200

μM dilution was used for permeability assay. Experiments were carried out in BD Gentest 96 well PAMPA plates following the provider's instructions. The acceptor plate (with 200 μl sample) was placed into the donor plate (with 300 μl sample). Following 5 hours incubation, the absorbance of the samples was detected by UV/VIS spectrophotometry.

4. Results

In our current work we identified and developed novel small-molecule inhibitors of NOX4 enzyme, based on the widely accepted strategy of rational drug design.

- First, we framed a cellular assay into a medium throughput screening (MTS) assay compatible system. Then we characterized the efficacy of a large compound library, synthesized by our chemical partner group. In the LPO catalyzed detection reaction, the H_2O_2 quantity, produced by the NOX4 enzyme, can be determined indirectly through the dityrosine formation. For NOX4 enzyme activity analysis, the properly readied cells were preincubated with the organic small-molecules, developed by our chemical partners. In the course of the one point (10 μM) primary screening, 1100 compounds were tested, 68 of them were further analyzed based on their efficacy. We determined the IC_{50} values of these compounds and got to a group of inhibitors of which IC_{50} values were lower than 2 μM . We were focusing our attention on further characterization of these molecules.

- As the second part of our work, we validated the efficacy of the hit compounds by cross-screening experiments. The analysis of the impact of hit molecules on cell viability is important for ADME(T) characterization. These experiments help to identify the compounds which modify the metabolic activity of the cells. Also important, that it could help to avoid the unnecessary examination of false hits. In line with the cellular experiments the toxicity and antiproliferative properties of the compounds were successfully checked in three different assays (luminescence cell viability- , MTT- and FACS assay). 16 compounds provoked decreased cell viability but they were exhibiting it more than an order of magnitude above the IC₅₀ values measured on NOX4 inhibition. We concluded, that during the primary screening, the experimental parameters (40 minutes incubation time, 0,15 - 10 µM concentration) was not permissive for false-hit selection. In these operating conditions, the antiproliferative or toxic effects of hit compounds might not come into play, at least it was not interfering with the results of NOX4 cellular assay. However, these properties of the compounds should be considered in the later improvements.

- No signaling mechanisms are known to exist which could block the NOX4 enzyme activity via upstream phosphorylation. Some of the molecules of the tested compound library however, have proven kinase inhibitory properties. That is why we wanted to exclude the possibility of any potential off-target activity. We believed that an enzyme assay would have been suitable for this purpose. Thus we were investigating the intracellular localization of NOX4 enzyme in

293 FS cells, which were used in the NOX4 cellular assay and also in EA.hy926 cells which are expressing the NOX4 endogenously. While the NOX4 enzyme showed relatively diffuse expression in the transfected 293 FS cell line, it was mainly concentrated in the nucleus of EA.hy926 cells. Testing the compounds in broken cell assay, 14 of them did not inhibit the NOX4 enzyme even as high concentration as 10 μ M. Another 11 compounds were found to be insufficiently active for further investigations after we determined the IC₅₀ values of them in the broken cell assay.

- In the light of the results gained so far, in cooperation with the chemical partners, we wished to lay down some structural property deduction which was common to most of the hit compounds. Unfortunately the active compounds are structurally very diverse from each other so a reliable structure-activity relationship is difficult to determine. The most striking feature of all chemical structures described so far is the extended double-bond conjugated systems, which are able to mediate electron exchange. This is important in redox reactions and they are also involved in ROS production. As the 3D structure has not yet been solved for NOX enzymes, only hypotheses can be made. Assuming that all the presented NOX inhibitors have a direct effect on NOX enzymes, the large structural diversity of these molecules is indicative of a large binding pocket, allowing a wide range of molecules to interact or discrete binding sites. Such a large binding pocket has been described for the cytochrome P450 enzymes, which also contains a catalytically

essential heme group, in which a large number of molecules are known to interact, even at remote locations.

- The specificity of the compounds was characterized in the frame of a cellular assay using neutrophil granulocytes. The NOX4 inhibitory compounds were tested in two concentrations (1 and 10 μM) to determine if they inhibit the NOX2 enzyme activity of neutrophil cells. In low dose none of the inhibitors showed activity but in 10 μM we could identify 18 compounds with significant (over 50%) NOX2 inhibition. The specificity analysis was not the only purpose of the NOX2 cellular assay. Decrease in ROS level can be achieved without NOX enzyme inhibition via antioxidant effect. Since both of the NOX4 and NOX2 cellular assays measured the production of ROS if a compound found not to be active in the NOX2 assay it was proof not only for its specificity, but also excluded the possibility that it was acting as ROS scavenger.

- Two important early ADME(T) parameters, the lipophilicity and permeability were characterized for the most promising 11 compounds. The majority of the compounds had optimal lipophilicity, but only 3 compounds showed high permeability. Since the PAMPA assay simulates only the passive, purely physico-chemical penetration of the molecules, we cannot exclude the active transport of the compounds with low permeability properties. It is more likely however, that the diffuse expression of the enzyme was more relevant.

5. Conclusions

- We successfully adapted an earlier published cellular based ROS detecting method for the identification of NOX4 inhibitors. As a result of the screening, more than 6% of the tested compounds proved to be efficient. This high number of the effective NOX4 inhibitors is verifying the reliability of the detection method and also the concept of screening of medium sized but diverse compound libraries.
- The active compounds, identified during the cellular screening were interfering with the NOX4 mediated ROS production. They did not make their effects through the inhibition of any cellular processes (other than NOX4) or molecular targets. This statement is based on the fact that the action of highly efficient, clearly NOX4 inhibitory compounds was reinforced also in the broken cell (enzyme) assay.
- The efficient inhibitors proved to be selective in our experimental setup and they did not exhibit significant NOX2 inhibitory, apoptotic, antiproliferative or antioxidant effects. Thus, these compounds are suitable for the application in experiments which requires selective NOX4 inhibition. They can substitute the insufficiently effective and/or selective NOX4 inhibitory compounds already in use.
- Based on all present information, applying the physico-chemical properties of the effective NOX4 inhibitor compound families, eventually structure-activity relationship (SAR) model was

developed. Based on the SAR model, we could show the possibly beneficial structural properties of a NOX4 inhibitor.

➤ With the characterization of early ADME(T) parameters promising compounds could be selected for drug development. We have a good chance to develop patentable compounds from these structurally diverse and multiple tested hit molecules.

Since different NOX isoforms are expressed even in one identical cell type with different level and intracellular localization, it is clear that they subserve distinct functions. While there has been enormous progress in NOX research, more and more questions are rising. Further investigation is required in order to elucidate the NOX4 (or other NOX enzymes) baseline function, or to find out, which special molecular signaling pathways are targeted by the different NOX isoenzymes. The application of selective NOX4 inhibitors might help to fill in the gaps of knowledge by better understanding of NOX4 enzyme function and the H₂O₂ mediated redox signaling.

6. Publications

List of own publications related to the dissertation:

- **Borbély G**, Szabadkai I, Horváth Z, Markó P, Varga Z, Breza N, Baska F, Vántus T, Huszár M., Geiszt M, Hunyady L, Buday L, Órfi L and Kéri Gy. (2010) Small-Molecule Inhibitors of NADPH Oxidase 4, *J. Med. Chem.*, 53: 6758-6762. **IF: 5,207**

- Breza N, Pató J, Órfi L, Hegymegi-Barakonyi B, Bánhegyi P, Várkonyi E, **Borbély G**, Peták I and Kéri Gy. (2008) Synthesis and characterization of novel quinasoline type inhibitors for mutant and wild type EGFR and RICK kinases, *J. Rec. and Sign. Transd.*, 28: 361-373. **IF: 1,540**

- **Borbély G**, Huszár M., Varga A, Futosi K, Mócsai A, Órfi L, Idei M, Mandl J, Kéri Gy, Vántus T, (2012) Optimization of important early ADME(T) parameters of NADPH oxidase-4 inhibitor molecules. *Med. Chem.*, (in press). **IF: 1,603**

List of own publications not related to the dissertation:

- Hegymegi-Barakonyi B, Székely R, Varga Z, Kiss R, **Borbély G**, Németh G, Bánhegyi P, Pató J, Greff Z, Horváth Z, Mészáros Gy, Marosfalvi J, Erős D, Szántai-Kis C, Garavaglia S, Perozzi S, Rizzi M, Hafenbradl D, Ko M, Av-Gay M, Klebl BM, Órfi L, Kéri G. (2008) Signalling inhibitors against *Mycobacterium tuberculosis*—early days of a new therapeutic concept in tuberculosis. *Curr. Med.*

Chem., 15: 2760-2770. **IF: 4,823**

- Earle M, Seddon K, Gilea M, **Borbely G**, Gilmore B, Gorman S, McLaughlin M. (2010) Antimicrobial system, U.S. Patent Application Serial No. 12/937149.

Summary

Based on the scientific literature, in particular on studies with NOX4 deficient mice models, it is increasingly clear, that the inhibition of NOX4 is a promising pharmacologic concept for the treatment of oxidative stress-related diseases. At present no specific NOX4 inhibitor is available for biochemical and/or clinical applications. In our work we aimed to identify and develop novel small-molecule inhibitors of NOX4 enzyme based on the widely accepted strategy of rational drug design. A cellular assay measuring NOX4 mediated H₂O₂ production was developed and optimized. About 1100 compounds were tested in this assay for NOX4 inhibition in the frame of a medium high throughput screening. Following the examination of the intracellular localization of the NOX4 enzyme in vascular endothelial (EA.hy926) cells, a broken cell assay was developed to check the specificity of the compounds. In this assay we have determined the IC₅₀ values and excluded the compounds with potential off-target activity. For the same reason, the selectivity of the molecules was also tested in a NOX2 based cellular assay. As a result of this assay we could identify not only the NOX2 inhibitors (not selective NOX4 inhibitors), but also the ROS scavengers. The toxicity and antiproliferative effects of the compounds were successfully characterized in three different assays (luminescence cell viability-, MTT- and FACS assay) using two different cell lines (293 FS and EA.hy926). As a part of early ADME(T) characterization, we also tested the lipophilicity and permeability of the compounds. Last but not least, applying the physico-chemical properties of the effective NOX4 inhibitor compound families, eventually structure-activity relationship (SAR) model was developed. Based on the SAR model, we could show the possibly beneficial structural properties of a NOX4 inhibitor. As a result of our scientific work, promising NOX4 inhibitory compounds with low micromolar IC₅₀ values were selected. These compounds might help to puzzle out the function of NOX4 enzyme and treat the oxidative stress-mediated diseases.

List of own publications related to the dissertation:

- **Borbély G**, Szabadkai I, Horváth Z, Markó P, Varga Z, Breza N, Baska F, Vántus T, Huszár M., Geiszt M, Hunyady L, Buday L, Örfi L and Kéri Gy. (2010) Small-Molecule Inhibitors of NADPH Oxidase 4, *J. Med. Chem.*, 53: 6758-6762. **IF: 5,207**
- Breza N, Pató J, Örfi L, Hegyemegi-Barakonyi B, Bánhegyi P, Várkonyi E, **Borbély G**, Peták I and Kéri Gy. (2008) Synthesis and characterization of novel quinasoline type inhibitors for mutant and wild type EGFR and RICK kinases, *J. Rec. and Sign. Transd.*, 28: 361-373. **IF: 1,540**
- **Borbély G**, Huszár M., Varga A, Futosi K, Mócsai A, Örfi L, Idei M, Mandl J, Kéri Gy, Vántus T, (2012) Optimization of important early ADME(T) parameters of NADPH oxidase-4 inhibitor molecules. *Med. Chem.*, (in press), **IF: 1,603**