Characterisation of Nox4 Inhibitors from Edible Plants

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Key words
- NADPH oxidase 4
- Nox4
- ROS
- inhibitors
- dehydrogenase domain
- broken cell assay

Abstract
NADPH oxidases transport electrons from cytosolic NADPH through biological membranes to generate reactive oxygen species. NADPH oxidase 4, broadly expressed in humans, is an interesting pharmacological target, since its activity is deregulated in several diseases, including pulmonary fibrosis, diabetic nephropathy, and cardiac hypertrophy. Whereas several candidate NADPH oxidase 4 inhibitors were recently described, most of these compounds are either unselective or toxic. Here we set out to identify new NADPH oxidase 4 inhibitors from edible plants, in an attempt to decrease the number of hits with toxic side effects. We screened a compound library prepared from edible plants for new bioactives with the ability to inhibit the activity of NADPH oxidase 4. Using both cell-based and cell-free assays, we identified several compounds with significant inhibitory activity towards NADPH oxidase 4. For selected compounds, the activity profile towards NADPH oxidase 2 and NADPH oxidase 5 was established, and controls were carried out to exclude general reactive oxygen species scavengers. A number of promising NADPH oxidase 4 inhibitors from edible plants was identified and characterised. Several new chemical entities are disclosed which act as NADPH oxidase 4 inhibitors, and the efficacies of our best hits, in particular several diarylheptanoids and lignans, are comparable to the best available pharmacological NADPH oxidase 4 inhibitors. These findings will provide valuable tools to study mechanisms of NADPH oxidase inhibition.

Abbreviations
- AR: Amplex Red
- AUC: area under the curve
- CL: chemiluminescence
- DHE: dihydroethidium
- DPI: diphenyleneiodonium chloride
- EC50: effective concentration at 50% activity
- ETC: electron transport chain
- HEK 293: human embryonic kidney 293 cells
- MBP: maltose binding protein
- NBT: nitrotetrazolium blue chloride
- Nox: NADPH oxidase
- Nox4DH: Nox4 dehydrogenase domain
- PBMC: peripheral blood mononuclear cells
- PMA: phorbol 12-myristate 13-acetate
- ROCS: rapid overlay of chemical structures
- ROS: reactive oxygen species
- SF9: Spodoptera frugiperda insect cells

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction
ROS induce damage to biological systems and were shown to contribute to ageing in cells. Aberrant ROS levels are linked to a variety of cancers [1] and many other diseases, e.g., vascular disease [2–6]. Whereas ROS are obligatory byproducts of mitochondrial oxidative metabolism, Nox2, the founding member of the Nox family [7,8], was found responsible for the oxidative burst of phagocytes, where ROS are deliberately produced by Nox2 to enforce pathogen elimination [7,8]. In humans, the Nox family consists of seven enzymes, i.e., five NADPH oxidases (Nox1 through Nox5; for review, see [7,9]) and two Nox homologues (Duox1 and Duox2) [10], along with several subunits and regulatory proteins. All Nox proteins are transmembrane proteins with six (Nox1–Nox5) or seven (Duox1,2) transmembrane domains. In each case, four conserved histidines...
bind two heme groups that are essential for the electron transport from cytosolic NADPH through membranes to generate ROS. An integral part is also covered by the FAD and NADPH binding domains that are similarly conserved [2,7]. Both Nox2, a multisubunit NADPH oxidase best known for its role in the host defense against pathogens, and Nox5 require activation by upstream stimuli, such as PMA (Nox2) or calcium agonists (Nox5) for full activity; in contrast, Nox4, in complex with its obligatory subunit p22\textsuperscript{phox}, is constitutively active [7]. Nox4 was identified in kidney [11] and was later found to be expressed in a variety of different cell types [12–19], and regulation occurs primarily at the mRNA level [20]. Whereas altered Nox4 activity has been found in a large variety of diseases, including pulmonary fibrosis, diabetic nephropathy (for review, see [4]), and benign prostatic hyperplasia [21], experiments with Nox4\textsuperscript{-/-} mice revealed a protective role of Nox4 in the vascular system [22], and Nox4 deletion was shown to predispose to diet-induced obesity [23].

Given the pleiotropic effects of Nox4 on a variety of physiological and pathophysiological processes, isotype-specific inhibitors of Nox4 would be potentially very useful [2,3,24,25]. Whereas various approaches have been used to inhibit Nox4, experiments with Nox4\textsuperscript{-/-} mice revealed a protective role of Nox4 in the vascular system [22], and Nox4 deletion was shown to predispose to diet-induced obesity [23].

Given the pleiotropic effects of Nox4 on a variety of physiological and pathophysiological processes, isotype-specific inhibitors of Nox4 would be potentially very useful [2,3,24,25]. Whereas extracts of 	extit{Piper sarmentosum} regulate expression of Nox4 [26], inhibition of Nox4 catalytic activity was not described for such extracts. Various efforts to search for new Nox4 inhibitors have been published in recent years, but so far there are no Nox4 inhibitors clinically available [21,27], although preclinical studies seem to support potential benefits in certain settings [28]. Also most known inhibitors are either unspecific or toxic [29,30]. However, there are attempts to chemically synthesize inhibitors from promising lead compounds, but the claimed effects so far remain uncertain [24,25,31–34]. This study aimed to identify formerly unknown Nox4 inhibitors from edible plants, in an attempt to satisfy ADMET criteria and avoid toxicity, which is becoming a high priority in this field [35].

Materials and Methods

Chemicals
All reagents were purchased from Sigma-Aldrich, unless stated otherwise.

Test compounds
The majority of the compounds used for the screening were provided as purified compounds in solid state by AnalytiCon Discovery. For details of sample preparation, see Supplementary Methods as Supporting Information. As control compounds, we used DPI (Sigma), VAS2870 (Vasopharm), as well as honokiol and magnolol (both kindly provided by Hermann Stuppner, Innsbruck University). All control compounds were provided at >95% purity.

Cell culture
Peripheral blood mononuclear cells were isolated fresh every day from volunteers and suspended in RPMI medium. Osteosarcoma cells (U2-OS) were acquired from ATCC, and human embryonic kidney (HEK 293 FT) cells were bought from Invitrogen. Both were grown in DMEM (Sigma) and provided with fresh medium every 2 and 3 days, respectively. DMEM was supplemented with 10% FCS (Biochrome AG, heat inactivated at 56°C for 40 min), 4 mM L-glutamine (Gibco), and 1/100 penicillin/streptomycin (Gibco). SF9 cells, a gift from Alexandra Lusser (Division for Molecular Biology, Medical University Innsbruck), were incubated in SF9 II medium (Gibco) with 10% FCS, 1.4 mM glutamine, and pen/strep at 27°C.

Nox dependent chemiluminescence
Chemiluminescence was performed in white 96-well plates, tissue culture-treated, optical flat bottom (Matrix, Nunc, or Perkin Elmer), essentially as described before [36]. For details, see Supporting Information.

ROS-scavenging assessment by DHE staining
U2-OS cells were treated with rotenone and stained with dihydroethidium to assess ROS levels, as described [37]. For details, see Supporting Information.

Preparation of cell membranes and Nox4 in vitro assay
Membrane fractionation and Nox4 activity measurement was performed similar to a method previously described [24,27,34,38]; for details, see Supporting Information.

Shape-based alignment
In a retrospective analysis of the hits in the Nox-dependent chemiluminescence assay, we performed a shape-based analysis applying ROCS (version 3.1.1. OpenEye Scientific Software. http://www.eyesopen.com), as described [39,40]. For details, see Supporting Information.

Results and Discussion

To assess the activity of Nox4 in intact cells, HEK cells expressing high levels of p22\textsuperscript{phox} were transfected with an expression vector for Nox4. Stable transfection of HEK cells led to a significant increase of the Nox4 protein and activity, determined by chemiluminescence, which was sensitive to inhibition by the general Nox inhibitor DPI (Fig. 1S). This system was applied to determine the inhibitory activity of plant-derived compounds, selected from a compound library containing 3557 purified compounds, as outlined in Fig. 25 and briefly described below. For the first screening, a structural diverse subset of compounds derived from edible plants was selected, with a focus on compounds inside Lipinsky’s Rule of Five. Results obtained with the diversity set were used to select additional compounds for screening, based on chemical similarity. The third screening was done with compounds mostly not structurally related to the hit compounds. Selected experimental data are shown in Fig. 1 for three selected plant compounds, representative of the activity spectrum (weak, intermediate, strong) obtained for the 14 best hits. DMSO vehicle had no effect on chemiluminescence activity, which was however significantly reduced by DPI when added at increasing concentrations (Fig. 1A). Under these conditions, compound ACD005 strongly suppressed Nox4 activity when assessed at a concentration as low as 0.5 µM (“strong” inhibitory activity) with a clear dose dependency observed (Fig. 1B). Under the same conditions, ACD018 induced a clear-cut inhibition of Nox4 activity when added at a concentration of 7.5 µM (“weak” inhibitory activity) (Fig. 1C, Table 1), whereas ACD212 displayed significant inhibitory activity when assayed at a concentration of 2.5 µM (“intermediate” inhibitory activity), and residual inhibitory activity was still visible at a concentration of 0.5 µM (Fig. 1D). The results of all measurements are summarised in Fig. 1E for 14 selected plant-derived compounds and the pharmacological Nox4 inhibitor VAS2870 [41,42], and approximative
EC50 values were calculated (Table 1). Apocynin, gomisin C, magnolol, and honokiol, described as potential Nox4 inhibitors in the literature [25, 43, 44], were included in our screening program; however, these compounds were at best moderately active in our assays. Thus, EC50 values were 60 µM for apocynin, 25 µM for gomisin C, 13 µM for honokiol, and 150 µM for magnolol. Because of the relatively low inhibitory activity in comparison to our best hits, these compounds were not considered further.

Appropriate controls were included to identify inhibitors specific for Nox4 activity. Thus, we set up a Nox2 activity screen based on normal PBMC isolated from healthy donors, in which Nox2 activity was stimulated by addition of PMA (Fig. 2A), and compounds were applied at a concentration suitable for Nox4 inhibition (see above, Table 1). Some of the Nox4 inhibitors, such as ACD042, did not inhibit Nox2 to any visible degree; in contrast, compound ACD018 displayed Nox2 inhibitory activity close to that of DPI, whereas VAS2780 displayed intermediate inhibitory activity (Fig. 2B). The relative Nox2 inhibition by selected compounds is displayed in Fig. 2C.

To further address Nox specificity we also established a HEK-derived cell line constitutively expressing Nox5. Nox5 expression

![Fig. 1 Identification of Nox4 inhibitors.](image)

**Table 1** EC50 of selected Nox4 inhibitors. This table summarises the calculated EC50 values for several inhibitors, as obtained in experiments with HEK-Nox4 cells. All values where derived from 3 independent experiments with 3 data points each, except for × 2 independent experiments with 3 data points each and ** 1 experiment with 3 data points.

<table>
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<th>± S</th>
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<td>VAS 2870</td>
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Kofler PA et al. Characterisation of Nox4... Planta Med 2013; 79: 244–252
was verified, and Nox5 activity was detectable in these cells after addition of ionomycin (Fig. 3S), as described before [45]. In this system, Nox5 activity was completely inhibited by DPI. Compound ACD066 displayed intermediate inhibitory activity, and VAS2780 was inactive (Fig. 3S). An important source of potentially confounding effects is the well-known ability of many plant-derived compounds to unspecifically quench ROS, in many cases by direct chemical interaction (for recent review of ROS quenching mechanisms, see [46]). Such compounds, often referred to as ROS scavengers, neutralise a large variety of chemical ROS entities and therefore are expected to negatively interfere with the activity of most if not all experimental systems described in this communication. To assess the ability of plant-derived compounds to quench mitochondrial ROS production [37], mitochondrial ROS production was stimulated in U2-OS osteosarcoma cells by the addition of rotenone, an inhibitor of ETC complex I [47]. Subsequently, cells were stained with DHE, a redox-sensitive fluorescent dye recorded at 590 nm [37]. Cells were co-stained with Hoechst 33342, and fluorescence was recorded at 465 nm. Fluorescence intensity of Hoechst 33342-stained cells is proportional to cell numbers and was used to normalise DHE fluorescence. Rotenone-induced signal was reduced to background by the addition of ascorbate (Fig. 3 A), consistent with previous findings [37]. In this experimental system, the selected compounds had no significant activity as ROS scavengers, with the exception of compound ACD066.
Our finding that ACD066 reduced the rotenone-induced DHE signal by roughly 50% suggests a more general mechanism rather than specific inhibition of NADPH oxidase activity for ACD066, which was therefore considered as a general ROS scavenger. Interestingly, the addition of some compounds, such as ACD176 and ACD055, even increased DHE fluorescence (Fig. 3B); this observation was not investigated further as it is not directly related to Nox inhibition.

For further characterisation of the Nox4 inhibitors identified in this study, we assessed their ability to inhibit Nox4 activity in vitro. To this end, membranes were isolated from Nox4-expressing HEK cells (HEK-Nox4) and control HEK cells (HEK-pcDNA), and Nox4 content of the membrane fractions was verified by Western blot (Fig. 4A). NADPH oxidase activity of these membrane fractions was assayed using AR fluorescence measurements, as described [24,27,34]. HEK-Nox4 derived membranes oxidised AR, and this activity was sensitive to DPI inhibition (Fig. 4A), as expected. Addition of compounds ACD005, ACD047, ACD053, and ACD212 reduced Nox4 activity in vitro, and the inhibitory activity of ACD053 was similar to that of DPI, whereas VAS2870 showed rather weak activity in this assay (Fig. 4B). Some of the compounds also inhibited the residual activity obtained with membranes from control (HEK-pcDNA) cells (Fig. 4C), which most likely is not related to Nox4. For normalisation, the values obtained with control-transfected cells were subtracted from the respective values obtained with Nox4-expressing HEK cells. After normalisation, significant Nox4 inhibition was still obtained with compounds ACD053 and ACD047, whereas some compounds failed to inhibit Nox4 activity under these conditions (Fig. 4D). Addition of ACD066 also significantly reduced AR fluorescence in this assay (Fig. 4D); however, due to the proven capability of ACD066 to act as general ROS scavenger (see above, Fig. 3), this compound was excluded from further analysis. DPI fully inhibited Nox4 activity in intact cells (see above, Fig. 1), but after addition of DPI to Nox4-containing membranes, roughly 50% of the chemiluminescence signal persisted, probably reflecting some background signals in the in vitro setting.

Conceivably, the high background signal is due to the fact that much higher (roughly 10 fold) cell numbers are required for one measurement in the broken cell assay, compared to assays performed in intact cells; however, other reasons for the high background cannot be excluded.

Of note, the inhibitory potential of VAS2870 was strongly limited in these experiments, and ACD005 showed only an indistinct trend, relative to experiments in intact cells (Fig. 4D); possible reasons for this are discussed below with the chemoinformatic analysis. The bioavailability and a compound’s ability to penetrate into the cell is a big issue, and not all compounds that can inhibit an enzyme in vitro are expected to reach their protein target in intact cells. This is of particular importance for Nox4, since it is known that, unlike other members of the Nox family, a substantial proportion of Nox4 is found in intracellular membranes [7,48–50].

We also assessed the activity of selected compounds towards a recombinant fragment of Nox4, which retains the dehydrogenase reaction associated with Nox4 enzymatic activity. The protein fragment Nox4(304–578) [51] was cloned in a baculoviral vector, expressed in Sf9 insect cells as an MBP fusion protein and purified by affinity chromatography [51]. Upon incubation with NADPH, FAD, and NBT, significant dehydrogenase activity was detectable, which was abolished by boiling the recombinant protein prior to the assay (data not shown). The reaction could be triggered by addition of NADPH (Fig. 4S) or, alternatively, by the addition of FAD in the presence of NADPH (data not shown), confirming the dependency on both cofactors. Surprisingly, addition of DPI did not lead to sustained inhibition of the diaphorase activity associated with Nox4DH domain; instead, DPI addition in this setting even increased signal intensity (data not shown). The reasons why DPI fails to act as an inhibitor in this setting remain to be clarified. Whereas compound ACD084 significantly inhibited the
<table>
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dehydrogenase reaction catalysed by Nox4. Compounds ACD005 and ACD063 displayed weak but significant inhibitory activity (Fig. 4S); in contrast, compounds ACD069 and VAS2870 were inactive in this assay (Fig. 4S). This may indicate that the mode of inhibition displayed by these inhibitors differs from the other compounds described in this communication. As was pointed out by Jaquet et al. [25], inhibition of Nox enzymes including Nox4 can occur at different stages, including depletion of substrate, interference with transcription or complex assembly, masking of the reaction product, etc. For compounds that inhibit Nox4 in intact cells but not in the Nox4DH assay, inhibition apparently involves Nox4 domains not present in the fragment. Accordingly, the mechanism of inhibition may differ between compounds described here and remains to be identified in all cases.

Plant-derived compounds described here can be assigned to several activity profiles: several compounds qualify as highly active Nox4 inhibitors, defined here by an EC50 below 1 µM. This includes ACD005, ACD047, and ACD053, all of which also inhibited Nox2 activity. Two additional compounds identified here, ACD042 and ACD084, are of potential interest, because they did not inhibit the activity of Nox2 nor Nox5. They may be considered as specific Nox4 inhibitors, although their EC50 is slightly higher than for the best Nox4 inhibitors described above. In quantitative terms, their activity is similar to the recently described Nox4 inhibitor VAS2870 [52], which in our hands displayed reasonable activity towards Nox4 and Nox2. During screening as well as retrospectively, chemoinformatics tools were applied to guide selection of candidates and to characterize our best hits, respectively. In a shape-based alignment, we identified ACD018 and ACD067 as suitable query molecules to enrich hits within inactive compounds during the evaluation showing the highest area under the receiver operator curve value (Fig. 5S). A retrospective chemical characterisation was done by calculation of selected chemical descriptors allowing an estimation of the drug-likeness for oral uptake according to Lipinski [53] (Table 1S). ACD005 was the only compound showing clear violation of Lipinski’s Rule of Five with more than 10 acceptors, more than 5 donors, and a molecular weight above 500 Da. This candidate Nox4 inhibitor bears a sugar moiety and therefore is very hydrophilic which is reflected in a low logP (o/w). In contrast, high logP (o/w) values are estimated for the compounds ACD018 and ACD212. Although these logP (o/w) values are higher than the criteria of logP ≤ 5, those compounds are classified as drug-like according to Lipinski as one violation is accepted.

The retrospective analysis of the identified hits relies on molecular descriptors calculated in silico. Chemical characteristics within the hits presented here give insight in the chemical requirement for Nox4 inhibitors (Table 2). So, the occurrence of 6 diarylheptanoids (ACD053, ACD055, ACD063, ACD066, ACD067, and ACD084) and the presence of phenol substructures within 12 out of 14 presented compounds indicate that this substructure is beneficial for Nox4 inhibition. For ROCS, the high AUC values for ACD018 and ACD057 are caused by the presence of 4-substituted phenol substructures. This can be seen as a common characteristic within almost all actives except ACD042 and ACD005. In fact, ACD055, which is the only diarylheptanoid without hydroxylation at the aromatic rings, is less active (EC50 = 1.88 µM) than the chemically related compound ACD067 with a phenol. For ACD005, the low AUC value is caused by the sugar moiety unique within the test set. ACD005 was characterised by high activity towards Nox4 in the chemiluminescence assay in the assay with intact cells (Table 1) but a surprisingly low effect on the isolated membranes (Fig. 4D). We assume ACD005 to enter the intact cells although it violates the Lipinski’s Rule of Five due to its sugar moiety. The glycosylation is a typical feature of natural products, and Lipinski’s Rule of Five might not appropriately reflect the consequences on cellular uptake, as this rule of thumb...
was developed from a statistical analysis of non-natural drug molecules [53]. Additionally, the glycosyl bond of ACD005 might be subject of enzymatic hydrolysis depending on the environment within the cells, and the aglycon might be the active form. This hypothesis could explain the reduced activity of ACD005 in the assays performed with isolated membranes and the isolated Nox4 dehydrogenase domain (Nox4304–578) fragment. Besides ACD005, no Lipinski’s Rule violations are observed. The two compounds, ACD042 and ACD084, discussed as specific Nox4 inhibitors, showed promising ADMET characteristics. The two compounds, ACD042 and ACD084, discussed as specific Nox4 inhibitors, showed promising ADMET characteristics. Besides ACD005, no Lipinski’s Rule violations are observed. The two compounds, ACD042 and ACD084, discussed as specific Nox4 inhibitors, showed promising ADMET characteristics.

Conflict of Interest
The authors declare no conflict of interest.

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Koiffer PA et al. Characterisation of Nox4... Planta Med 2013; 79: 244–252