Characterisation of Nox4 Inhibitors from Edible Plants

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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 obligate 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 redox 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 p22phox, 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-/- 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 functions, screening for potentially useful plant-derived Nox4 inhibitors has been performed in recent years, but so far there are no crystallography data available for Nox4. Despite 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 uncertified [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 Stuppern, 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 seeded on tissue culture-treated, optical flat bottom (Matrix, Nunc, or Perkin Elmer) with growth medium supplemented with 10% FCS, 1.4 mM glutamine, and pen/strep. After 24 h incubation, cells were treated with DHE (5 μM, Invitrogen) and subsequently incubated for 30 min at 37 °C in a 5% CO2 incubator. Then, cells were washed with PBS, pelleted, and resuspended in PBS. Fluorescence was measured using a plate reader (Spectramax M2e, Molecular Devices). DHE fluorescence was normalized to total cell number, which was determined using Trypan Blue (Gibco). Nox4 activity was assessed in intact cells using SF9 cells transfected with the Nox4-mCherry construct (Addgene No: 103840). For DHE staining, cells were treated with 10 µM DHE for 20 min at 37 °C in a 5% CO2 incubator. After washing with PBS, cells were resuspended in PBS and analyzed using a plate reader (Spectramax M2e, Molecular Devices) at an excitation wavelength of 530 nm and an emission wavelength of 580 nm.

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 p22phox 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 Lipinski’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 concentrations 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. A–D Nox4-dependent chemiluminescence measurements of transfected HEK 293 cells using controls (A DMSO, DPI) and substances with strong (B ACD005), medium (C ACD212), and weak (D ACD018) inhibitory effect. E The signal of selected inhibitors at different concentrations 10 min after the start of the experiment. All values were derived from 3 independent experiments.](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.

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<tr>
<th>Substance</th>
<th>Mean [µM]</th>
<th>S</th>
</tr>
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<tbody>
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</tr>
<tr>
<td>ACD 018*</td>
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<td>6.59</td>
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<td>ACD 042*</td>
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<td>ACD 053</td>
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<td>VAS 2870</td>
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<td>1.13</td>
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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 ACD063 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. 3A), 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).

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Table 2  Selected compounds. Given in the table are the compound numbers as used in the paper in the first column. The second column gives the trivial name as known so far, the third column provides the order numbers from AnalytiCon Discovery. Column four shows the 2D chemical structure, and the last line provides the SMILES (Simplified Molecule Input Line Entry) string for each compound.

<table>
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<th>Compound Nr.</th>
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continued
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. 4S). 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’s Rule of Five (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 (Nox4\textsubscript{4304–578}) fragment. Besides ACD005, no Lipinski’s Rule violations are observed.

The two compounds, ACD042 and ACD084, discussed as specific Nox4 inhibitors, showed promising ADME/T characteristics. Nox4 inhibitors have been described by others before. Thus, it was shown that extracts of *Piper sarmentosum* inhibit expression of Nox4 at the transcriptional level [26]. In an approach similar to that employed by the fact that EC50 values for these compounds in cell-based assays are calculated for the best compounds, including GKT137831 at a concentration of 10 µM for each of their best hits. The compounds described here have comparable activity in cell-based assays, but since they are derived from edible plants, we expect better compatibility with ADMET criteria. Based on a screening campaign of substance libraries with Nox4-containing membranes, K values in the two-digit nanomolar range were calculated for the best compounds, including GKT137831 [25,33], which is currently being considered for clinical trials [28]. K values in general describe the kinetic interaction between enzyme and substrate, and their calculation is rather difficult in such an approach, given the fact that the molar concentration of Nox4 in isolated membranes is unknown. The comparison to the inhibitory activity of compounds described here is further complicated by the fact that EC50 values for these compounds in cell-based assays are currently unknown. In a recent study with a cellular model of Nox4-dependent liver fibrosis, it was shown that inhibition of Nox4 activity in hepatocytes required GKT137831 at a concentration of 10 µM [28], suggesting that the most active compounds (ACD005, ACD047, ACD053, ACD66, ACD67; see Table 1) identified here are at least as active as GKT137831, which is considered the best available pharmacological Nox4 inhibitor to date [31].

**References**

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