Trimer hydroxylated quinone derived from apocynin targets cysteine residues of p47\textsuperscript{phox} preventing the activation of human vascular NADPH oxidase

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Enzymatically derived oligophenols from apocynin can be effective inhibitors of human vascular NADPH oxidase (Nox). An isolated trimer hydroxylated quinone (IIIHyQ) has been shown to inhibit endothelial NADPH oxidase with an IC\textsubscript{50} ~ 30 nM. In vitro studies demonstrated that IIIHyQ is capable of disrupting the interaction between p47\textsuperscript{phox} and p22\textsuperscript{phox}, thereby blocking the activation of the Nox2 isoform. Herein, we report the role of key cysteine residues in p47\textsuperscript{phox} as targets for the IIIHyQ. Incubation of p47\textsuperscript{phox} with IIIHyQ results in a decrease of ~ 80% of the protein free cysteine residues; similar results were observed using 1,2- and 1,4-naphthoquinones, whereas apocynin was ineffective. Mutants of p47\textsuperscript{phox}, in which each Cys was individually replaced by Ala (at residues 111, 196, and 378) or Gly (at residue 98), were generated to evaluate their individual importance in IIIHyQ-mediated inhibition of p47\textsuperscript{phox} interaction with p22\textsuperscript{phox}. Specific Michael addition on Cys196, within the N-SH3 domain, by the IIIHyQ is critical for disrupting the p47\textsuperscript{phox}–p22\textsuperscript{phox} interaction. When a C196A mutation was tested, the IIIHyQ was unable to disrupt the p47\textsuperscript{phox}–p22\textsuperscript{phox} interaction. However, the IIIHyQ was effective at disrupting this interaction with the other mutants, displaying IC\textsubscript{50} values (4.9, 21.0, and 2.3 μM for the C111A, C378A, and C98G mutants, respectively) comparable to that of wild-type p47\textsuperscript{phox}.

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observed effect on diabetic rats was due to the conversion of apocynin into active metabolites. Indeed, Johnson et al. [20] and Kanegae et al. [24] found a dimer derived from apocynin to be an effective inhibitor, but its precise mode of action has not been determined, although oxidized oligophenolic compounds have been shown to bind to thiol groups and contribute to NADPH oxidase inhibition [25].

In our previous work, we reported on a broad complex mixture of derived oligophenols (up to heptamers) obtained from the peroxidase-catalyzed oxidation of apocynin and consisting of demethylated, hydroxylated, and quinone forms [26]. We isolated and fully characterized a trimer hydroxylated quinone (IIIHyQ), which showed strong inhibitory activity (IC$_{50}$ 30 nM) against endothelial cell-based NADPH oxidase. The IIIHyQ was able to disrupt the in vitro interaction between a His-tagged p47$_{phox}$ (His-p47$_{phox}$) and a PRR peptide biotin-p22$_{phox}$ (IC$_{50}$ 1.60 μM) [26]. A linear correlation existed between the inhibitory activity against EC NADPH oxidase and the ability to disrupt the interaction between biotin-p22$_{phox}$ and His-p47$_{phox}$, suggesting that apocynin-derived oligophenols are capable of preventing p47$_{phox}$-p22$_{phox}$ interaction in vivo. We now hypothesize that IIIHyQ may bind to critical cysteine residues of p47$_{phox}$; and in particular Cys196 located in the N$\cdot$SH3 domain (Fig. 1C), via Michael adducts. Indeed, similar adducts are well known to occur between benzoquinone, naphthaquinone, anthraquinone, or dopamine quinone with Cys residues in proteins [27,28].

In this work, we have focused on gaining a more complete understanding of the mechanism of NADPH oxidase inhibition by apocynin-derived IIIHyQ. We evaluated the effect of IIIHyQ on the interaction of biotin-p22$_{phox}$ and His-p47$_{phox}$. Four p47$_{phox}$ mutants were expressed and purified, each containing a simple Cys replacement, thus sequentially eliminating potential targets for quinone binding. This approach resulted in the identification of Cys196 as a critical target for IIIHyQ leading to the potential prevention of p47$_{phox}$-p22$_{phox}$ interaction. We also evaluated the toxicity of the IIIHyQ compared with apocynin using PC-12 cells. This set of results establishes apocynin-derived oligophenols, and particularly metabolism-generated quinones, as candidates for potential therapeutic applications.

Materials and methods

Materials

Apocynin, soybean peroxidase (SBP), solvents, phosphate-buffered saline (PBS) tablets, H$_2$O$_2$, thiazolyl blue tetrazolium bromide, Tween 20, 3.3',5.5'-tetramethylbenzidine (TMB), sodium caseinate, fetal bovine serum, heparin, and endothelial growth supplement were purchased from Sigma–Aldrich (St. Louis, MO, USA). Endothelial cells, PC-12 cells, and F12K medium were purchased from the ATCC (Manassas, VA, USA). Escherichia coli BL21(DE3), E. coli Top 10 competent cells, isopropyl-β-D-1-thiogalactopyranoside (IPTG), lucifer yellow iodoacetamide, and N- and C-terminus affinity columns (ProBond System) were purchased from Invitrogen (Carlsbad, CA, USA). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Antibodies were purchased from Upstate Biotechnology (Waltham, MA, USA). High-affinity streptavidin-coated 96-well plates were purchased from Pierce.

LC–MS analyses were performed on a Shimadzu LCMS-2010A. Samples for LC–MS were separated in an Agilent Zorbax 300SB-C$_{18}$ column (5 μm, 2.1 × 150 mm). Silica gel 230–400 mesh was purchased from Natland International Corp. (Morrisville, NC, USA). Thin-layer chromatography (TLC) plates were purchased from Merck (Whitehouse Station, NJ, USA). Microplate reader analyses were performed in a PerkinElmer HTS 7000 bioassay reader.

Enzymatic production of IIIHyQ from apocynin

IIIHyQ was synthesized via SBP-catalyzed oxidation of apocynin as described previously [26,29]. Briefly, apocynin (6 mmol) was dissolved in 5 ml of dimethylformamide and transferred to 490 ml phosphate buffer (50 mM, pH 7). SBP (5 ml of a 1 mg/ml solution) was added and the reaction was initiated by using a syringe pump to introduce H$_2$O$_2$ (30% w/v) at 0.1 ml/min for 12 min to afford 12 mmol H$_2$O$_2$. Finally, the reaction was stopped after 2 h. Soluble and precipitated phases were separated by centrifugation, and ethyl acetate was added to the supernatant to extract organic compounds. The extracted supernatant fraction was dried and stored at −20 °C under argon. Dried powder (290 mg) was dissolved in chloroform and loaded onto a silica gel column (15 g) and eluted with a gradient of petroleum ether:ethyl acetate (2:1 to 0:1). Unreacted apocynin was recovered in the early fractions (210 mg, R$_f$ 0.62 with petroleum ether:ethyl acetate, 1:1) and further elution with pure ethyl acetate furnished the IIIHyQ as a white powder (14 mg, R$_f$ 0.34 with petroleum ether:ethyl acetate, 1:1). TLC, NMR, and high-resolution mass spectrometry analyses were performed as previously reported [26].

Site-directed mutagenesis

Four mutants of His-p47$_{phox}$ were obtained by site-directed mutagenesis using the original plasmid (pET-28a (+), 5369 bp) used for production of recombinant His-p47$_{phox}$ wild type, CB8C, C111A, C196A, and C378A. Primer design was performed following the guidelines of the QuickChange Lightning site-directed mutagenesis kit from Stratagene (Santa Clara, CA, USA); primers (reverse, R, and forward, F) for each mutant were CB8C, GCCACATTACCGAG-TACGCTCGACGTCAGAGCTGGC; CB8R, GCCAGCTACATGACGCTG-GAGCCCTACTCGGTAACTGTCGC; C111AF, CACCAAGATCTCCCGAGCTCCCAACCTCTCGACCT; C111AR, AAGTTCGAGGCGGGGAGCTCGGGA-GATCTTTGGT; C196AF, GCCGCTTGGTGTTGCTGCTGCTGAAAGTGGATACGAAACCAGC; C196AR, GCCTTGCTCTCATCTGAGGGACACCCCGCCCT; C378AF, CCTCACTCTGAACCCCGCTTCGGAGACCAACAG; C378AR, GCCCTGGCTCTCAGCGCTACGGCGGAGCTCGGAGATG. The calculated melting temperature for each primer was ≥78 °C and the cycling parameters used for PCR are described in the instruction manual for the mutagenesis kit from Stratagene. After PCR, DNA was inserted into E. coli Top 10 cells and plasmid purified samples were sent for sequencing (MC Lab, San Francisco, CA, USA) to confirm the correct mutations (see the supplementary material for primer design).

Production and purification of His-p47$_{phox}$ and biotin-p22

A proline-rich p22$_{phox}$ peptide N-151PSSNPPPRPAEARK165-C, which was biotinylated at the N-terminus and amidated at the C-terminus, was obtained from Genemed Synthesis, Inc. (South San Francisco, CA, USA). A biotin group was attached through a 4-residue spacer consisting of SGSG. The purity of the peptide was 99.99%, EC-derived p47$_{phox}$ (wild type) DNA (6-His tagged) was obtained from the University of Albany and Stratton VA Medical Center and confirmed by DNA sequence analysis (University of Maine). His-p47$_{phox}$ proteins (wild type and mutants) were expressed in BL21(DE3) cells for 9 h using 0.5 mM IPTG at 35 °C. The protein was purified using a Ni-affinity column (ProBond System) and the purity (80%) was calculated with ImageJ software (U.S. National Institutes of Health).

Biotin-p22 and His-p47$_{phox}$ interaction

Interaction of His-p47$_{phox}$ (mutants and wild type) with the biotin-p22$_{phox}$ peptide was assessed using ELISA, as previously described [26,30]. Briefly, experiments were performed in high-affinity streptavidin-coated 96-well plates. To block nonspecific binding sites, each well was reblocked with 300 μl of PBS supplemented with 0.1% (v/v) Tween 20 and 1% sodium caseinate. To each well, 100 μl of biotin-p22$_{phox}$ (2 μM) peptide solution was added and incubated at room temperature for 1 h. After each well was washed four times...
The reaction was stopped with 100 μl by adding 200 μl. Dase activity was performed with a ready-to-use TMB liquid substrate and two additional washes with 300 μl. Unbound IIIHyQ was removed by washing four times with PBS (0.05 M) containing 1% sodium caseinate and incubating at room temperature for 1 h. Each well was washed twice with PBS, and then fresh DMEM (90 μl) was added. ECs were incubated for 30 min at 37 °C with phorbol myristate acetate (PMA; 1 μM) to activate NADPH oxidase. After consecutive washes with PBS and addition of fresh DMEM (90 μl), the ECs were incubated with 1 mM IIIHyQ, apocynin, and PAO (each compound at a concentration of 50 μM) as negative control (each compound at a final concentration of 500 μM). Lucifer yellow iodoacetamide (10 mM) was added and the reaction allowed to proceed at 4 °C overnight in the dark. To separate excess dye and protein conjugate, desalting plates (2 μl desalting plate, 7 kDa MWCO, 0.5 ml) were used three times until complete elimination of background fluorescence was achieved. After removal of the dye, protein concentration was determined by bichinchoninic acid protein assay. Finally, fluorescence spectra were taken at an excitation wavelength of 426 nm, showing a maximum of emission intensity ~530 nm.

**Intracellular production of superoxide via dihydroethidium (DHE) staining**

Qualitative determination of intracellular superoxide was performed via DHE fluorescence. DHE permeates endothelial cells and, in the presence of superoxide and other ROS, is oxidized to the red fluorescent 2-hydroxyethidium and ethidium, respectively, which are trapped with DNA resulting in bright red fluorescence. Although DHE can react with other ROS [31–33], the following method describes the generation of O₂⁻ after the selective activation of vascular NADPH oxidase, which is the progenitor of other ROS (e.g., hydrogen peroxide and hydroxyl radical). Endothelial cells were incubated overnight in black, clear-bottom 96-well cell-binding surface plates in DMEM in the absence of phenol red. DMEM was removed and the cells were washed twice with PBS and then fresh DMEM (90 μl) was added. Cells were incubated with 1 mM IIIHyQ prepared at the desired concentration in a Ni column, wild-type His-p47phox from Invitrogen. After purification in a Ni column, wild-type His-p47phox buffer was exchanged with 10 mM phosphate buffer (pH 7.0) using an Amicon ultracentrifuge filtration device (5 kDa MWCO). Wild-type His-p47phox (50 μM) was then incubated with the IIIHyQ, using 1,2- and 1,4-naphthoquinone as positive controls and apocynin as negative control (each compound at a final concentration of 500 μM). Lucifer yellow iodoacetamide (10 mM) was added and the reaction allowed to proceed at 4 °C overnight in the dark. To separate excess dye and protein conjugate, desalting plates (2 μl desalting plate, 7 kDa MWCO, 0.5 ml) were used three times until complete elimination of background fluorescence was achieved. After removal of the dye, protein concentration was determined by bichinchoninic acid protein assay. Finally, fluorescence spectra were taken at an excitation wavelength of 426 nm, showing a maximum of emission intensity ~530 nm.

**Superoxide anion radical-scavenging capacity assay**

The O₂⁻ -scavenging capacity of IIIHyQ was measured by competition with a molecular probe, nitroblue tetrazolium (NBT), after synthesis of O₂⁻ by the hypoxanthine–xanthine oxidase (HPX–XOD) system. Briefly, 200 μl of NBT solution (0.34 mM in PBS), 500 μl of HPX (2 mM in PBS), and 100 μl of IIIHyQ prepared at the desired concentration (or the solvent-only control) were vortex-mixed for 5 s. Then, 200 μl of XOD (0.56 U/ml in PBS) was added and vortex-mixed for 30 s. Samples were taken every 10 min to measure the absorbance at 560 nm. NBT has a yellow color that upon reduction by O₂⁻ forms the blue formazan.

**Cell toxicity assay**

Toxicity of IIIHyQ and apocynin was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using rat adrenal medullar cells (PC-12). The cells were cultured in modified DMEM supplemented with 5% fetal bovine serum, 10% horse serum, and 1% penicillin-streptomycin. The cell suspension (110,000 cells per well) was incubated for 24 h and the medium was then replaced with fresh medium containing IIIHyQ and apocynin (2 μM each). After 24 h, the MTT solution was added and the absorbance was measured at 570 nm.
cells/ml) was separated into aliquots of 90 μl in a 96-well microtiter plate (CellBIND, Corning, Lowell, MA, USA) and allowed to adhere for 24 h. Afterward, 10 μl of IIIHyQ or apocynin solution was added (to cover a range of concentrations from 0 to 1 mM, in 1% dimethyl sulfoxide (DMSO)) to the microtiter plate, and the cells were further incubated for 48 h at 37 °C. The medium was then removed, and the cells were washed with PBS. Next, DMEM (200 μl) and thiazolyl blue tetrazolium bromide (50 μl of 2.5 mg/ml) were added to each well for 3 h at 37 °C. Finally, these solutions were removed, 250 μl of DMSO was added, the plate was shaken (∼75 rpm) for 20 min, and the absorbance was measured at 562 nm. The cytotoxicity values were normalized to the PBS (1% DMSO) control without IIIHyQ and apocynin.

Results and discussion

Interaction of the IIIHyQ with p47\textsubscript{phox}

The mechanism of NADPH oxidase inhibition by apocynin-derived enzymatic oxidation products remains unclear. In phagocytes, myeloperoxidase is expected to convert apocynin into active metabolites [24,35,36]. Along these lines, we generated a library of potential inhibitors of NADPH oxidase, resulting in the identification of a IIIHyQ as a strong inhibitor of the human EC NADPH oxidase via blocking the interaction between p47\textsubscript{phox} and p22\textsubscript{phox} subunits. We hypothesized that the IIIHyQ may bind to critical Cys residues on p47\textsubscript{phox} preventing its translocation to the membrane. p47\textsubscript{phox} contains four Cys residues at positions 98, 111, 196, and 378. Cys196 is located within the N–SH3 domain, which binds the PRR of p22\textsubscript{phox} (Fig. 1B).

To elucidate the role of specific Cys residues, and particularly Cys196 of p47\textsubscript{phox} in the interaction with p22\textsubscript{phox} in the presence of IIIHyQ, four mutants (C98G-p47\textsubscript{phox}, C111A-p47\textsubscript{phox}, C196A-p47\textsubscript{phox}, and C378A-p47\textsubscript{phox}) of recombinant p47\textsubscript{phox} were generated. Dose-response analysis of IIIHyQ in C98G-p47\textsubscript{phox} and C378A-p47\textsubscript{phox} enzyme-linked immunosorbent assay (Fig. 2A) shows that the apocynin derivative disrupts the interaction with biotin-p22\textsubscript{phox} giving IC\textsubscript{50} values (4.9 and 2.3 μM, respectively; Fig. 2B and E) comparable to the value previously reported using the wild-type p47\textsubscript{phox} (1.60 μM) [26], whereas the IC\textsubscript{50} value using C111A-p47\textsubscript{phox} (21.0 μM) was an order of magnitude higher (Fig. 2C). However, when Cys196 was replaced by Ala, the IIIHyQ was unable to block the interaction over the entire dose range (Fig. 2D). This was interesting, as Cys196 is the only cysteine located within the SH3 domain and particularly within the N-
terminal SH3 domain that binds the PRR of p22\textsuperscript{phox}. Cys196, therefore, seems to be a critical target for preventing protein–protein interactions necessary for NADPH oxidase activation. Several quinones (for example 1,2- and 1,4-naphthoquinones) are known to form Michael adducts with cysteine residues\[^{28}\]. As controls, 1,2-naphthoquinone and 1,4-naphthoquinone were evaluated for their effects on the interaction of His-p47\textsuperscript{phox} with biotin-p22\textsuperscript{phox}. Using wild-type His-p47\textsuperscript{phox}, both quinones were able to prevent this protein–protein association with comparable IC\(_{50}\) values (0.2 and 0.7 \(\mu\)M respectively; Fig. 3A and B). However, when the mutant C196A-p47\textsuperscript{phox} was used, neither naphthoquinone had any effect (Fig. 3C and D).

The reactivity of the IIIHyQ on p47\textsuperscript{phox} Cys residues was determined using the fluorescent dye lucifer yellow iodoacetamide (Fig. 4A). The 1,2- and 1,4-naphthoquinones were used as positive controls, and apocynin was used as a negative control. The fluorescence spectra (Fig. 4B) show a significant decrease (~80%) in total Cys-thiol groups after incubation with the IIIHyQ, as well as the naphthoquinones, whereas incubation with apocynin did not result in appreciable reduction in the fraction of free Cys residues. This result suggests that IIIHyQ is not selective for Cys196, which is consistent with the results of Park and Park using the fluorescent probe \(N,N'\)-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethyleneamine\[^{37}\]. This and the reduction of ~80% of total cysteines indicate that the IIIHyQ is not specific for Cys196, but its modification seems to be critical to prevent the interaction with p22\textsuperscript{phox}.

Effect of the IIIHyQ on NADPH oxidase after enzyme activation

NADPH oxidase inhibition can be the result of at least two mechanisms. One mechanism involves interference of enzyme activation, e.g., by preventing translocation of cytosolic subunits to the cell membrane. A second mechanism involves the inhibition of the already assembled and activated enzyme. Along these lines, it is important to note that few attempts have been made to evaluate inhibition when the NADPH oxidase is already activated and the target sites for the inhibitors may be restricted. For example, PAO is ineffective as an

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**Fig. 3.** ELISA controls with quinone structures. Effects of 1,2- and 1,4-naphthoquinones on the interaction between biotin-p22\textsuperscript{phox} and recombinant His-p47\textsuperscript{phox}. (A) Biotin-p22\textsuperscript{phox}–wild-type His-p47\textsuperscript{phox} interaction with 1,2-naphthoquinone (IC\(_{50}\) 0.2 \(\mu\)M), (B) biotin-p22\textsuperscript{phox}–wild-type His-p47\textsuperscript{phox} interaction with 1,4-naphthoquinone (IC\(_{50}\) 0.7 \(\mu\)M), (C) biotin-p22\textsuperscript{phox}–C196A-p47\textsuperscript{phox} interaction with 1,2-naphthoquinone, and (D) biotin-p22\textsuperscript{phox}–C196A-p47\textsuperscript{phox} interaction with 1,4-naphthoquinone (IC\(_{50}\) values of (C) and (D) were not determined).

**Fig. 4.** Quantification of cysteines in recombinant His-p47\textsuperscript{phox} incubated with apocynin, IIIHyQ, 1,2-naphthoquinone, and 1,4-naphthoquinone. (A) Chemical representation of the Michael adduct between IIIHyQ and Cys thiol group. (B) Fluorescence spectra of lucifer yellow iodoacetamide complexed with cysteines of wild-type p47\textsuperscript{phox}.
inhibitor once NADPH oxidase is activated [38]. We proceeded to investigate whether the IIIHyQ was an effective inhibitor after NADPH oxidase activation. To that end, we activated the enzyme within ECs with PMA and followed intracellular $O_2^{•−}$ formation using DHE, a cell-permeative dye that reacts with $O_2^{•−}$ to generate 2-hydroxyethidium [31,33]. The production of $O_2^{•−}$ was then examined upon addition of IIIHyQ. As shown in Fig. 5A, the level of $O_2^{•−}$ decreased in the presence of IIIHyQ in relation to apocynin and PAO, although the inhibition was not as striking as addition of IIIHyQ before NADPH activation. Nevertheless, it is interesting that IIIHyQ can achieve some degree of inhibition once enzyme assembly had occurred. One explanation is that IIIHyQ may be able to bind to Cys196 even after the p47$^{\text{phox}}$–p21$^{\text{phox}}$ association takes place. If this occurs, then once IIIHyQ binds to Cys196, the two enzyme subunits dissociate, thereby eliminating enzyme activity. Another possibility is that other protein–inhibitor interactions take place. For example, it has been demonstrated that polyphenols have an affinity for physically interacting with the PPR of proteins and inducing conformational changes [17,18]. Interactions between the N–SH3 domain of p47$^{\text{phox}}$ and PPR of p22$^{\text{phox}}$ occur through Van der Waals and hydrogen bond interactions [9,39]. Similar interactions may occur with polyphenols that can disrupt the association of p47$^{\text{phox}}$–p22$^{\text{phox}}$. Importantly, IIIHyQ does not scavenge free radicals. Similar interactions may occur with polyphenols that can disrupt some of those interactions resulting in a reversible effect on NADPH oxidase. As a control, we determined that the IIIHyQ does not scavenge $O_2^{•−}$ and the reduction of $O_2^{•−}$ levels is due to a loss of enzymatic activity (Fig. 5B).

Fig. 5. (A) Intracellular $O_2^{•−}$ detection by DHE staining in endothelial cells. Inhibitory activity of apocynin, IIIHyQ, and PAO on previously activated NADPH oxidase. (B) $O_2^{•−}$-scavenging capacity of IIIHyQ (0–1 mM): black square, IIIHyQ 0.0 μM; red circle, IIIHyQ 0.01 μM; green triangle, IIIHyQ 100.0 μM; blue triangle, IIIHyQ 1000.0 μM.

Fig. 6. MTT assay. Cytotoxicity of apocynin and IIIHyQ on rat adrenal medullar cells (PC-12).
Toxicity of apocynin and IIIHyQ

Our results suggest that IIIHyQ does not target a specific cysteine and thus may be indiscriminately reactive toward other cysteines residues on other proteins within cells, thereby affecting critical pathways that may damage or kill cells. We therefore evaluated the cytotoxicity of IIIHyQ via the standard MTT assay using PC-12 cells. As shown in Fig. 6, the dose–response plots of apocynin and IIIHyQ were similar and only a 20% loss in viability occurred at a concentration of 1 μM. This value is far higher than the IC50 values obtained during the NADPH oxidase activity assay (30 nM) and for biotin-p22phox-p47phox (1.60 μM) [26], indicating a potentially wide therapeutic window. These cytotoxicity results are similar to those for the stilbene resveratrol and its peroxidase-generated oxidation products [40].

Conclusions

We have studied the mechanism of inhibition of the IIIHyQ derived enzymatically from apocynin, with specific attention given to the interaction of p22phox and p47phox. Cysteine residues of p47phox seem to be the primary target for the quinone, with Cys196 in the N–SH3 domain playing a critical role as a site of inhibition. Specifically, the IIIHyQ is ineffective when Cys196 is replaced by alanine. The IIIHyQ also reacts with other Cys residues in p47phox, however, inhibition is eliminated only when a likely Michael addition to Cys196 occurs. Similar results were obtained with the structurally simpler 1,2- and 1,4-naphthoquinones. Despite the relatively low selectivity of the IIIHyQ on Cys residues, the compound showed minimal cytotoxicity in vitro. Enzymatic oligomerization of apocynin represents an attractive alternative to generate potent inhibitors of NADPH oxidase. Characterization of IIIHyQ and its mechanism of action provides additional insight into the structural features for potent inhibitors of vascular NADPH oxidase, which may prove valuable in the search for effective therapeutic interventions in cardiovascular diseases related to oxidative stress [34].

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