Biochemical Pharmacology 143 (2017) 25–38

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Characterization of potent and selective iodonium-class inhibitors of NADPH oxidases

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ARTICLE INFO

Article history:
Received 7 February 2017
Accepted 7 July 2017
Available online 11 July 2017

Chemical compounds studied in this article:
diphenylene iodonium, DPI (PubChem CID:3101)
diphenyliodonium, IDP (PubChem CID: 12877)
di-2-thienyliodonium chloride, DTI (PubChem CID: 162551)
FAD (PubChem CID: 643975)

Keywords:
Dual oxidase
NADPH oxidase
Colon cancer
Diphenylene iodonium
Reactive oxygen

ABSTRACT

The NADPH oxidases (NOXs) play a recognized role in the development and progression of inflammation-associated disorders, as well as cancer. To date, several NOX inhibitors have been developed, through either high throughput screening or targeted disruption of NOX interaction partners, although only a few have reached clinical trials. To improve the efficacy and bioavailability of the iodonium class NOX inhibitor diphenylene iodonium (DPI), we synthesized 36 analogs of DPI, focusing on improved solubility and functionalization. The inhibitory activity of the analogs was interrogated through cell viability and clonogenic studies with a colon cancer cell line (HT-29) that depends on NOX for its proliferative potential. Lack of altered cellular respiration at relevant iodonium analog concentrations was also demonstrated. Additionally, inhibition of ROS generation was evaluated with a luminescence assay for superoxide, or by Amplex Red® assay for H_2O_2 production, in cell models expressing specific NOX isoforms. DPI and four analogs (NSCs 740104, 751140, 734428, 737392) strongly inhibited HT-29 cell growth and ROS production with nanomolar potency in a concentration-dependent manner. NSC 737392 and 734428, which both feature nitro functional groups at the meta position, had >10-fold higher activity against ROS production by cells that overexpress dual oxidase 2 (DUOX2) than the other compounds examined ($IC_{50} \approx 200$–400 nM). Based on these results, we synthesized and tested NSC 780521 with optimized potency against DUOX2. Iodonium analogs with anticancer activity, including the first generation of targeted agents with improved specificity against DUOX2, may provide a novel therapeutic approach to NOX-driven tumors.
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1. Introduction

The members of the NADPH oxidase (NOX) family—NOX1-5 and dual oxidases (DUOX) 1 and 2—are conserved transmembrane enzymes expressed in a variety of human tissues. The NOX isoforms share core structural elements, including a cytosolic NADPH binding domain and FAD binding site, and a heme-containing 6-transmembrane domain. Additionally, NOX5 and DUOX1/2 have cytosolic calmodulin-like Ca^{2+}-binding functionality at the N-terminal, and the DUOX enzymes contain an extracellular peroxidase homology domain. By mediating the transport of electrons across the plasma membrane, NOXs are important for redox homeostasis in various non-malignant tissues [1]. The first isoform to be described, NOX2, was discovered in phagocytes (i.e., neutrophils and macrophages) where it catalyzes a respiratory burst in response to pathogens, growth factors, or cytokine exposure [1]. However, the generation of reactive oxygen species (ROS) can stimulate cellular proliferation, contributing to the

Abbreviations: NOX, NADPH oxidase; DUOX, dual oxidase; ROS, reactive oxygen species; DPI, diphenylene iodonium; DTI, di-2-thienyliodonium chloride; DMSO, dimethyl sulfoxide; FMOC, fluorenylmethyloxycarbonyl; IC_{50}, inhibitory concentration 50%; RLU, relative light units.

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http://dx.doi.org/10.1016/j.bcp.2017.07.007
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pathogenesis of pre-malignant, chronic inflammatory conditions such as pancreatitis and Crohn's disease [2–4]. Increased production of ROS has also been linked to enhanced invasiveness and angiogenesis in a variety of malignant conditions [5–7]. Certain NOX isoforms, in particular NOX1, NOX4, NOX5, and DUOX2, are highly expressed in specific epithelial malignancies and malignant melanoma; furthermore, genetic manipulation of NOX1 and NOX4 expression significantly alters the growth of human tumors in vitro and in vivo [8–12]. Consequently, the NOX isoforms constitute attractive targets for the development of anticancer therapeutics.

Favored characteristics for new agents targeting NOX-related ROS formation would include high potency and specificity, increased solubility, and limited toxicity. The preferred mechanism of action of these new compounds would be direct inhibition of NOX enzymatic activity, as opposed to modulation of signaling pathways upstream of NOX, or ROS scavenging. Recent drug screening and rational design efforts aiming to identify isoform-specific NOX inhibitors have uncovered several compounds of interest, although specificity for a single isoform has not yet been convincingly demonstrated. GKT136901 and GKT137831 have specific activity against NOX1, NOX4, and NOX5 [13–15]; ML171 is specific for NOX1 but at low potency [16]; VAS2878 and close derivative VAS3947 have demonstrated activity against NOX1, NOX2, NOX4, and NOX5; recently, peptides with more specific activity against NOX1 have been described [17–20]. However, because of the potential for off-target effects, low potency, and poor solubility [21], most of these agents have, to date, not demonstrated clinical utility as NOX inhibitors.

Historically, NOX function and activity have been interrogated in vitro through modulation with small molecule flavoenzyme inhibitors, such as diphenylene iodonium (DPI), di-2-thienyliodonium (DTI), and apocynin [22]. DPI and DTI inhibit the growth of NOX1-expressing colon cancer cells without altering mitochondrial respiration, if studied at nanomolar concentrations, so that antiproliferative effects may be attributed, at least in part, to a cyclin D_1-dependent G_1 block that is secondary to inhibition of NOX function [23,24]. However, these inhibitors lack specificity for NOX enzymes as they have been shown to disrupt the activity of various flavoproteins [21,25,26], which limits the precision with which they can be utilized in a therapeutic context. DTI is very sparingly soluble in water and all common organic solvents used in biological experiments. Furthermore, apocynin possesses free radical scavenging activity, in addition to NOX inhibiting potential, that limits the interpretation of studies in which it is utilized [27].

In the current study, our goals were to elucidate the mechanisms whereby iodonium-class inhibitors alter ROS production by NOXs, and to optimize the pharmacological properties and NOX isoform selectivity of new iodonium analogs compared to the parent molecule, DPI. Initially, thirty-five compounds derived from DPI were screened for inhibitory effects on cellular proliferation and ROS production in HT-29 colon cancer cells. These iodonium analogs were also evaluated for effects on mitochondrial function, and for NOX isoform selectivity in cell-based assays. We identified four compounds with increased potency compared to DPI in cellular models of NOX activity, and derived a thirty-sixth compound, **521**, with increased selectivity for DUOX2.

2. Materials and methods

2.1. Materials and compounds

Diphenylene iodonium (DPI; NSC 735294, Catalog number: 43088) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The 36 DPI analogs (Figs. 1A and 6A) were synthesized by the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis of the National Cancer Institute (Bethesda, MD, USA) following the general procedure shown in Fig. 1B. DPI and its analogs were dissolved in dimethylsulfoxide (DMSO, Catalog number: D2650; Sigma–Aldrich, St. Louis, MO, USA) at their maximum soluble concentration [23]. All compounds were fully characterized by ^{1}H NMR. Using the techniques described below, all 36 analogs were screened for their ability to inhibit $O_2^{\cdot-}$ production and proliferation in HT-29 cells. The four initial candidate molecules that performed optimally on the basis of their solubility and their ability to inhibit tumor cell growth and ROS production were subsequently evaluated for their effects on mitochondrial function and ROS formation (as shown in the testing funnel; Fig. 1D), and then for their NOX isoform selectivity. A fifth analog (NSC 780521; described below) was prepared after evaluation of the first four to enhance interaction with DUOX2. Compound characterization details are shown below for the 5 lead compounds; data are available upon request for the other analogs.

2.1.1. Dibenziodolium, 3,7-dibromo-, bromide (NSC 740104-T, 104)

Mp 202–205 °C (decomposes). ^{1}H NMR, DMSO-d_6: δ 8.69 (d, 2H); 8.37–8.36 (d, 2H); 8.03–8.01 (dd, 2H). Anal. Calc'd ($C_{12}H_6Br_2I{\cdot}Br$) C,H,Br,I. Yield: 79%.

2.1.2. Dibenz[b,d]iodolium, 3-(methoxycarbonyl)-, salt with 4-methylbenzenesulfonic acid (1:1) (NSC 751140-P, 140)

Mp > 250 °C (decomposes). ^{1}H NMR, DMSO-d_6: δ 8.84 (d, 1H); 8.64–8.62 (d, 1H); 8.61–8.59 (dd, 1H); 8.37–8.35 (dd, 1H); 8.30–8.28 (d, 1H); 7.94–7.90 (m, 1H); 7.82–7.78 (m, 1H); 7.50–7.49 (d, 2H); 7.13–7.11 (d, 2H); 3.96 (s, 3H); 2.30 (s, 3H). Anal. Calc'd ($C_{14}H_{10}IO_2{\cdot}C_7H_7O_3S$) C,H,S,I. Yield: 37%.

2.1.3. Dibenziodolium, 3,7-dinitro-, bromide (NSC 737392-V, 392)

Mp > 270 °C (decomposes). ^{1}H NMR, DMSO-d_6: δ 9.41–9.40 (d, 2H); 8.78–8.76 (d, 2H); 8.65–8.62 (dd, 2H). Anal. Calc'd ($C_{12}H_6IN_2O_4 \cdot Br$) C,H,N,Br,I. Yield: 96%.

2.1.4. Dibenz[b,d]iodolium, 3-nitro-, chloride (NSC 734428-Y, 428)

Mp 282–285 °C (decomposes). ^{1}H NMR, DMSO-d_6: δ 9.40–9.39 (d, 1H); 8.68–8.64 (dd, 2H); 8.59–8.57 (dd, 2H); 7.90–7.87 (t, 1H); 7.81–7.79 (t, 1H). Anal. Calc'd ($C_{12}H_7INO_2{\cdot}Cl$) C,H,N,Cl,I. Yield: 94%.

2.1.5. Dibenziodolium, 1,9-dinitro-, salt with bromide (1:1) (NSC 780521, 521)

MP 207–209 °C (decomposes). ^{1}H NMR, DMSO-d_6: δ 9.02–9.01 (d, 2H); 8.50–8.49 (d, 2H); 8.01–7.98 (t, 2H). Anal. Calc'd (C12H6IN2O4·Br) C,H,N,Br,I. Yield: 93%.

2.2. Cell culture

HT-29, HL-60, UACC-257, and HEK293 cell lines were obtained from ATCC (Manassas, VA, USA). Human HT-29 colon cancer cells were propagated in McCoy's 5 A medium supplemented with 10% FBS (Lonza, Walkersville, MD, USA). HL-60 and UACC-257 cells were grown in RPMI-1640 medium containing 10% FBS. The stable HEK293 cell line expressing both the human DUOX2 and DUOXA2 enzymes was kindly provided by Dr. William M. Nauseef (University of Iowa, Iowa City, IA, USA) and maintained in DMEM:F12 medium supplemented with 10% FBS, 800 μg/ml G418 (Catalog number: 5005; Teknova, Hollister, CA, USA) and 250 μg/ml Zeocin (Catalog number: 46-0509; Invitrogen, Carlsbad, CA, USA) [28]. HEK293 cell lines that stably express the human NOX1 (HEK293 NOX1) and NOX4 (HEK293 NOX4) enzymes were engineered in-

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