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Ebselen and congeners inhibit NADPH-oxidase 2 (Nox2)-dependent superoxide generation by interrupting the binding of regulatory subunits

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Summary

NADPH-oxidases are a primary source of reactive oxygen species (ROS), which function in normal physiology and, when overproduced, in pathophysiology. Recent studies using mice deficient in Nox2 identify this isoform as a novel target against Nox2-implicated inflammatory diseases. Nox2 activation depends on the binding of the proline rich domain of its heterodimeric partner p22phox to p47phox. A high-throughput screen that monitored this interaction via fluorescence polarization identified ebselen and several of its analogs as inhibitors. Medicinal chemistry was performed to explore structure-activity relationships and to optimize potency. Ebselen and analogs potently inhibited Nox1 and Nox2 activity but were less effective against other isoforms. Ebselen also blocked translocation of p47phox to neutrophil membranes. Thus, ebselen and its analogs represent a class of compounds that inhibit ROS generation by interrupting the assembly of Nox2-activating regulatory subunits.

Introduction

Reactive oxygen species play important roles in physiological functions including host defense and signal transduction, and can also cause “oxidative stress” which plays a central role in inflammation, tissue damage, abnormal cell growth, and fibrosis associated with a variety of diseases. Recent reviews catalog the many examples of the roles of oxidative stress in acute and chronic diseases including for example cardiovascular, nervous, endocrine, respiratory, and excretory systems (Kashihara et al., 2010; Essick et al., 2010; Park et al., 2009; Fatokun et al., 2008; Elahi et al., 2009). While mitochondria and various metabolic enzymes were originally considered to be the sources of ROS in disease, NADPH oxidases (Nox and Duox enzymes) have more recently been recognized as the major source of ROS in many cells and in a multitude of disease states [reviewed in (Lambeth et al., 2008;]
Lambeth, 2007). Rather than generating ROS as minor byproducts of metabolism, Nox enzymes catalyze NADPH-dependent generation of superoxide or hydrogen peroxide as their sole function and with high catalytic efficiencies, e.g., up to 5,000 mol O$_2^-$/mol heme/ per minute for Nox2 (Paclet et al., 2007). Secondary metabolites such as hydroxyl radical and, in the presence of myeloperoxidase, hypochlorous acid can also be formed, particularly in inflamed tissues where they contribute to molecular and cellular damage (Vignais, 2002).

Nox enzymes are a family of 7 transmembrane catalytic moieties (Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2) (Suh et al., 1999) (Lambeth, 2004; Lambeth et al., 2000; Geiszt et al., 2000; Cheng et al., 2001; Shiose et al., 2001; De Deken et al., 2000; Banfi et al., 2004) that show distinct tissue expression and mechanisms of regulation. The most thoroughly studied Nox isoform is Nox2, also known as the ‘phagocyte NADPH-oxidase’ or ‘respiratory burst oxidase’. Nox2 generates superoxide, as do Nox1, 3, and 5, while Nox4, Duox1 and Duox2 produce mainly hydrogen peroxide (Takac et al., 2011). The phagocyte NADPH-oxidase is inactive in unstimulated neutrophils, but becomes activated upon exposure to microbes, microbial products and inflammatory mediators. Nox2 forms a heterodimer with the small membrane subunit p22phox, the C-terminal proline-rich domain (PRD) of which provides a docking site for p47phox directly, and indirectly for other regulatory subunits of the complex (p67phox, p40phox, and Rac1). This interaction is an early and key step in an intricate series of protein-protein interactions that result in enzyme activation. Triggered by phosphorylation of p47phox, which unmask a bis-SH3 domain present in p47phox, the regulatory subunits translocate from the cytosol to the membrane, where the bis-SH3 moiety binds to the PRD of p22phox (Sumimoto et al., 1994; Leusen et al., 1994; Leto et al., 1994) (Vignais, 2002; Lambeth et al., 2007) (Babior et al., 2002). This interaction is essential for the activity of the Nox2 system, since mutations that disrupt this binding prevent superoxide generation (Kawahara et al., 2005) (Sumimoto et al., 1996) (de Mendez et al., 1997). An analogous binding interaction between the same PRD of p22phox and the highly homologous bis-SH3 domain of the regulatory subunit NOXO1 activates Nox1 and Nox3 (Banfi et al., 2003; Banfi et al., 2004). Nox4 also requires p22phox for activity, but the PRD of p22phox is not needed since Nox4 is constitutively active and independent of activating subunits (Kawahara et al., 2005). Nox5 and Duox1-2 do not require p22phox for activity (Kawahara et al., 2005)(Kawahara et al., 2011) (Luxen et al., 2009; Grasberger et al., 2006).

Animal models of various diseases, including studies using Nox isoform-deleted strains of mice, demonstrate a protective role of inhibiting or deleting these enzymes, implicating Nox isoenzyme forms as novel targets for the development of drugs. Conditions in which Nox enzymes have been implicated include hypertension (Nox1)(Matsuno et al., 2005; Lassigue et al., 2001; Gavazzi et al., 2006), diabetic nephropathy (Nox4)(Gorin et al., 2005)(Sedeek et al., 2010), lung fibrosis (Nox4)(Hecker et al., 2009) (Carnesecchi et al., 2011), acute lung inflammation (Nox2) (Snelgrove et al., 2006; Imai et al., 2008), Alzheimer’s disease (Park et al., 2008), traumatic brain injury (Nox2) (Dohi et al., 2010) and others (e.g., see (Lambeth, 2007; Jaquet et al., 2009)). Because Nox2 is particularly important in many diseases that have an inflammatory component (Lambeth, 2007), it represents a promising target for drug development for these conditions.

The status of development of drugs/inhibitors targeting Nox enzymes has been reviewed recently (Lambeth et al., 2008) (Jaquet et al., 2009). Most existing inhibitors are non-selective and/or have properties that preclude their development as drugs. A Nox1 and Nox4-selective inhibitor was recently reported (Laleu et al., 2010) and another report identified a Nox1-selective inhibitor (Gianni et al., 2010). The present studies were undertaken to identify inhibitors that are selective for Nox2. A fluorescence polarization (FP) assay was developed to identify inhibitors that block the interaction of the bis-SH3 domain of p47phox with the
PRD of p22phox. The assay was used to carry out a high-throughput screen (HTS) to identify potential inhibitors of Nox2. Surprisingly, among the hits were the selenium-containing analog ebselen and several of its close analogs including those in which sulfur replaces selenium. Ebselen was previously characterized as a glutathione peroxidase mimetic (Sies, 1993; Parnham et al., 1991; Parnham, 1990; Sies, 1995; Schewe, 1995; Parnham et al., 2000). According to this mechanism, ebselen and its selenium- (but not sulfur-) containing analogs are able to consume hydrogen peroxide in a catalytic cycle that uses thiol-containing compounds such as glutathione as a substrate. The present studies demonstrate a new mode of action for this class of molecules as Nox2 inhibitors that directly target assembly of the Nox2 regulatory subunits.

Results

Design of a High-Throughput Screening Assay

As described above, activation of Nox2 in vivo depends on the binding of p47phox to p22phox (Vignais, 2002; Lambeth et al., 2007) (Babior et al., 2002), which is mediated by binding of the PRD of p22phox to a deep binding pocket formed at the interface between the two SH3 domains that together comprise the single functional bis-SH3 domain of p47phox (Sumimoto et al., 1996; Groemping et al., 2003). Using the expressed, purified protein in which GST is fused to the bis-SH3 domain of p47phox (residues 156-285, referred to as GST-p47-bis-SH3), a fluorescence polarization assay was developed to measure the binding of a synthetic, rhodamine-labeled peptide corresponding to the PRD of p22phox (rho-PRD). GST-p47-bis-SH3 lacks the autoinhibitory region and thus does not require phosphorylation to bind the PRD. At a constant rho-PRD concentration, increasing GST-p47-bis-SH3 produced a saturable increase in polarization (Fig. 1A), corresponding in several experiments to a $K_d$ of 20-40 nM. GST alone had no effect on FP (Figure 1A). Using rho-PRD peptide bound to the GST-p47-bis-SH3, we tested whether an MBP fusion protein linked to the entire C-terminal region of p22phox (residues 132-195, which includes the PRD, and which we call MBP-p22-C), could displace rho-PRD from its binding site on the bis-SH3 domain, which would result in decreased fluorescence polarization. Fig. 1B demonstrates that MBP-p22-C but not MBP alone decreased fluorescence polarization, corresponding to a calculated $K_i$ of 60 nM. Thus, the binding affinity of MBP-p22-C is similar to that of the PRD alone; the latter therefore represents the major binding determinant. The kinetics of binding were monitored upon addition of multiple concentrations of the GST-p47-bis-SH3 to rho-PRD. Binding was complete by the time the first measurement was made and the fluorescence polarization was stable over the course of more than 8 hours (data not shown). In addition, FP and binding constants were minimally affected by DMSO concentrations up to 1%. Thus, the binding of rho-PRD to GST-p47-bis-SH3 is rapid, tight, specific, and stable. We reasoned that the competition for binding that results in a decrease in FP could be used as the basis for a high-throughput screen (HTS) for inhibitors that target the p47phox-bis-SH3 binding pocket.

High-throughput screening identifies ebselen and analog as hits

We performed the FP assay adapted to a 1536- well HTS format to screen $>200,000$ compounds in the MLSCN library, using a concentration of $20 \mu M$ compound. (Details of the screen and results have been deposited in PubChem AID: 1274 and AID: 1275 (National Center for Biotechnology Information)). The 1200 compounds that produced the greatest apparent decrease in FP were rescreened in HTS format to obtain dose responses and autofluorescence information. Fifty five compounds were chosen on the basis of a) IC$_{50}$ less than 15 $\mu M$; b) low or no interfering autofluorescence; and c) approximately ideal dose response curves the shapes of which corresponded to single site binding. Individual dose response curves were then repeated using the FP binding assay, and dose responses for
inhibition of Nox2-dependent ROS generating activity were carried out in a cell-free system that uses isolated neutrophil membranes as a source for Nox2, plus recombinant, purified phox regulatory subunits (Uhlinger et al., 1992). Compounds with IC$_{50}$ values greater than 10 μM or those that interfered with a control activity assay in which superoxide was supplied via a xanthine/xanthine oxidase system were eliminated from further consideration. Among a small number of bona fide inhibitors was ebselen, a previously published compound that has been characterized to have glutathione peroxidase-like catalytic activity (Sies, 1993; Parnham et al., 1991; Parnham, 1990; Sies, 1995; Schewe, 1995; Parnham et al., 2000). Ebselen showed an IC$_{50}$ of 0.3 μM in the FP binding assay and 0.6 μM in the cell-free Nox2 activity assay (Figure 2A). The effect on the Nox2 system occurred at much lower concentrations than the 10 μM concentration reported for ebselen’s glutathione peroxidase activity (Muller et al., 1984) and also its reported 17 μM IC$_{50}$ for inhibition of horseradish peroxidase (Mishra et al., 2006). Another hit compound is a close analog of ebselen in which sulfur replaces selenium. This compound, which we call Thr101, showed moderate activity (IC$_{50}$ = 4 μM) in both the binding and Nox2 activity assays (Figure 2B). Interestingly, sulfur analogs of ebselen lack glutathione peroxidase activity (Leurs et al., 1989). Neither compound affected the assay system when superoxide was provided by a xanthine/xanthine oxidase system (open circles in lower panels of Fig. 2). Additional commercially available analogs of ebselen were obtained and tested, and several of these also proved to be inhibitors of Nox2 (vide infra).

Ebselen markedly inhibited the binding of purified recombinant MBP-p22-C and GST-p47-bis-SH3 assessed in a pulldown assay using glutathione agarose beads (Figure S1), confirming the results from the FP binding assay.

**Analog synthesis and Structure Activity Relationship (SAR) study**

To optimize the potency of ebselen-like molecules towards the Nox2 target, we synthesized novel analogs of this series including both selenium- and sulfur-containing versions. Replacement of selenium or sulfur with oxygen (TG4-225-2), carbon (Thr140), nitrogen (JM3-71b,c) or sulfonamine (Thr 141) resulted in complete loss of activity (Table S1). Likewise, replacement of the middle five-membered ring system with benzothiazins (TG4-182, TG4-184), benzothiazine-dione (TG4-185) or benzothiazoles (NOX-AS3-1, Nox-AS3-2) also yielded inactive compounds (Table S1). We therefore synthesized a series of compounds that retained the central ring but varied the substituents on the other two rings (Table S1).

Approximately 30 selenium and sulfur analogs were made and tested in both the FP binding assay and the cell-free Nox2 activity assay. Figure 3 shows the correlation between EC$_{50}$ values for the approximately 30 active compounds in the FP binding assay (X-axis) versus IC$_{50}$ values for the same compounds in the Nox2 activity assay (Y-axis) (R = 0.7 (p<0.0001), R$^2$ = 0.5; slope significantly nonzero (p<0.001)). An additional 15 analogs failed significantly to inhibit binding of rho-PRD to GST-p47-bis-SH3 at concentrations up to 25 μM. Data for three representative inactive compounds in which a carbon was substituted for the selenium are shown (g, h, and i) in Figure 3. The correlation points to the interruption of the p47phox p22phox interaction as the mode of action of these both Se- and S-containing analogs in this chemical series.

Several conclusions can be made with regard to structural features that affect activity. For the sulfur analogs, the parent compound of the sulfur-containing series, Thr101, has the...
same potency in both binding and activity assays, about 4 μM (Figure 2B). An isothiazolone analog with a p-COOOMe group (Figure 3/Table S1, l) shows ~10 fold higher activity compared with the parent compound in both the binding and cell-free activity assays: 500 nM and 800 nM, respectively. Replacing p-COOOMe with a p-COOEt (Figure 3/Table S1, m) or a p-COOtBu (Figure 3/Table S1, s) caused a slight decrease in potency. The p-COOtBu group at this position proved to be even less potent than the parent compound, with an IC₅₀ = 10 μM in the FP assay, while longer chains in this position failed to bind at all, suggesting a limitation in the length of the binding cavity at this position. Switching the methyl ester substitution from the para- to the ortho-position resulted in a complete loss of activity. A fluoro-derivative (Figure 3/Table S1, j) showed somewhat increased potency compared to the parent compound with an IC₅₀ of 1.5 μM in the activity assay, but was similar to the parent compound in the binding assay. However, adding a second fluorne substituent at the position para to sulfur in the benzoisothiazolone ring (Table S1, k) improves binding by 3-fold and activity by 5-fold making the compound nearly as effective as ebselen itself.

Replacement of the substituted phenyl ring on the right side of the molecule with a thiozole rings (Figure 3/Table S1, n), thiodiazole rings (Figure 3/Table S1, p, r) or pyridine ring (Figure 3/Table S1, e) were less effective than the p-COOOMe analog (Figure 3/Table S1, l). An isothiazolone analog (Figure 3/Table S1, τ) that lacks the phenyl-fused ring on the left side of the molecule also has significantly decreased activity.

For the selenium-containing compounds, the parent compound is ebselen (b), which has submicromolar potency in both binding and activity assays (Figure 2A). Similar to the sulfur series, substitution with a p-COOOMe on the right-hand ring, (Figure 3/Table S1, α) modestly increases the binding potency (FP assay) compared to the parent compound. The combination of a fluoro-substitution on the left side ring, along with a 1,4-dioxole ring fused to the right-hand ring (Figure 3/Table S1, w) significantly improved the potency, while a 1,3 dioxane ring fused to the right-hand ring (Figure 3/Table S1 δ) had little effect. In contrast either two fluorines (β) or two methoxyl groups (χ) on the left-hand ring decreased the activity about 2-fold.

**Ebselen analogs are selective for Nox1 and Nox2 over Nox4**

Our HTS was designed to identify inhibitors that show selectivity for Nox2, an isoform that requires regulatory subunits for activity, and should not inhibit Nox4, which does not require activation via protein:protein interactions. Isolated Nox enzymes are labile (Tamura et al., 1992), particularly when removed using detergents from membranes. Therefore we tested the isoform selectivity of selenium and sulfur analogs of ebselen in assays using several cell-based systems: 1) human neutrophils, which express high levels of Nox2 but not any other Nox isoform; 2) two HEK cell lines, one that stably expresses human Nox1 (HEK/Nox1rb) and one that stably expresses Nox4 (HEK/Nox4rb; both stable cell lines were the generous gift of Dr. Ralf Brandes); and 3) HEK cells transiently transfected with Nox5 (Kawahara et al., 2011) which activates via binding of its EF hand to its dehydrogenase domain (Tirone et al., 2010). These assays use L-012 luminescence as a reporter for superoxide (e.g., the product generated by Nox1, Nox2 and Nox5) or (in the presence of horseradish peroxidase) for hydrogen peroxide (the ROS product generated by Nox4) (Takac et al., 2011). As seen in Figure 4 and Table 1, both selenium and sulfur analogs of ebselen showed more than an order of magnitude selectivity for Nox1 and Nox2 over Nox4 in intact cells, supporting the proposed mode of action. In addition, four of five compounds tested showed a 5-fold or greater selectivity of Nox2 over Nox5. These compounds did not interfere with assays in which ROS was supplied via xanthine/xanthine oxidase (Figure 3 and Table 1) or directly as H₂O₂ (Figure 4 and Table 1), nor did they affect cell viability of either neutrophils or HEK cells below 30 μM (data not shown). Except for Thr101 which showed an IC₅₀ around 10 μM, these compounds inhibited Nox4 in HEK cells with IC₅₀ values greater than 50 μM.
The apparent effect of Thr101 on Nox4 activity was indistinguishable from its effect on the control assay in which H$_2$O$_2$ was supplied directly in place of cells. Thus, the apparent inhibition of Nox4 at high concentrations of this compound is likely to be an assay artifact, and it can be concluded that this series of inhibitors does not in general inhibit Nox4.

Interestingly, some compounds showed differences in selectivity between Nox1 and Nox2, which probably reflects different interactions with the homologous but not identical bis-SH3 domains of NoxO1 and p47phox. Nox5 activity depends on calcium binding (Banfi et al., 2001), phosphorylation (Jagnandan et al., 2006), and binding of its EF hand to its DH domain (Tirone et al., 2010) all of which are potential targets for these compounds; other unknown mechanisms also cannot be excluded. The overall trend for selectivity of these compounds is Nox2 ≫ Nox1 > Nox5 ≫ Nox4.

Effect of ebselen on membrane translocation of p47phox and p67phox

In intact neutrophils, activation of cells with phorbol esters results in the translocation of p47phox and p67phox to the plasma membrane, a process triggered by the binding of the p47phox bis-SH3 domain to the PRD of p22phox (Sumimoto et al., 1996). The p67phox protein is associated in a complex with p47phox, which results in its association with the membrane in parallel with p47phox (de Mendez et al., 1996). This assembly results in activation of the Nox2 subunit to produce superoxide in an NADPH-dependent manner. The above results predicted that ebselen should block the association of p47phox and p22phox, thereby preventing translocation of p47phox and p67phox protein to the plasma membrane. As shown in Fig. 5, treatment of neutrophils with phorbol 12-myristate, 13-acetate (PMA), followed by isolation of plasma membranes, induced a marked increase in the amount of both proteins associated with the plasma membrane (lane 2). Ebselen had no effect on translocation in the absence of PMA (lane 3), but completely blocked PMA-stimulated protein translocation (lane 4). p22phox, an integral membrane protein, is shown as a gel loading control. Ebselen at the same concentration did not affect protein-kinase-C-mediated phosphorylation of p47phox or indeed other neutrophil proteins (Figure S3), although it did affect the ability of p67phox to coimmunoprecipitate with p47phox (Figure S4). Thus, ebselen prevents the assembly of regulatory subunits induced by phorbol ester, consistent with its targeting of the PRD-binding site within the bis-SH3 domain of p47phox.

Discussion

The assembly of a functional Nox2 complex requires the interaction between p47phox and the PRD of p22phox. In an effort to find inhibitors that are selective for Nox2, we developed a FP assay based on interrupting this interaction and used it to screen compounds in a HTS format. Surprisingly, the HTS identified ebselen and the closely related sulfur-containing analog Thr101. The evidence presented here demonstrates that ebselen and its analogs inhibit Nox2 activity by interrupting the binding of the bis-SH3 domain of p47phox to the PRD of p22phox. While inhibition of NADPH-oxidase activity by ebselen was previously reported (Cotgreave et al., 1989; Wakamura et al., 1990), its mechanism of action remained obscure and it was generally thought that inhibition was due to antioxidant properties of the compound and/or inhibition of upstream signaling. Ebselen analogs inhibit the activity of native neutrophil Nox2 in purified membrane fractions (Figure 2); such fractions produce ROS only when supplemented with p47phox, p67phox, and Rac (Uhlinger et al., 1992). Inhibition of Nox2 activity correlates strikingly with inhibition of rho-PRD binding to p47-bis-SH3 (Figure 3). Ebselen analogs inhibit Nox2 activity in intact neutrophils, which requires p47phox binding to p22phox, at about the same concentration at which they inhibit binding. Because of the homology between the Nox2 cytoplasmic proteins and those of Nox1 and Nox3, ebselen and its analogs are likely to perturb the assembly of these enzyme complexes similarly to Nox2. Consistent with this prediction, in studies using cells co-
transfected with Nox1, NOXO1 and NOXA1, ebselen and several congeners inhibited Nox1 with potencies only somewhat lower than they inhibit Nox2 (Table 1). On the other hand, ebselen analogs inhibit Nox5 with an average of 5-fold or lower potency than they inhibit Nox2, and fail to inhibit the p47phox-independent isoform Nox4 at concentrations below 10 μM (Figure 4 and Table 1). Indeed, apparent inhibition above this concentration appears to be due to interference with the assay itself rather than true inhibition of Nox4. Finally, we showed that ebselen inhibits the translocation of p47phox and p67phox in PMA-activated human neutrophils, consistent with the proposed mode of action.

Ebselen as well as a sulfur-containing analog was previously reported to inhibit superoxide generation from guinea pig alveolar macrophages at 20 μM, and inhibition was proposed to be an effect on protein kinase C (PKC) (Leurs et al., 1989; Wakamura et al., 1990). Ebselen has been shown to inhibit purified PKC enzyme at submicromolar concentrations (Cotgreave et al., 1989) but the effect of ebselen on both protein phosphorylation and superoxide generation in intact neutrophils have reported IC₅₀ values in the range of 20–50 μM (Cotgreave et al., 1989). Inhibition of PKC inhibits phosphorylation of some sites on p47phox, which in turn should inhibit translocation and assembly of the Nox2 complex. The concentration of ebselen used in the translocation experiment shown in Fig. 5 is lower than the IC₅₀ reported by Cotgreave et al. (Cotgreave et al., 1989) for PKC inhibition in intact cells, and as demonstrated in Figure S3 this concentration does not affect PKC-mediated phosphorylation of p47phox or other neutrophil proteins. Taken together, these data indicate that ebselen has a major effect on the interaction between p47phox and p22phox.

Ebselen has been previously reported to decrease oxidative damage in tissues by reducing hydrogen peroxide (H₂O₂) and other hydroperoxides via its glutathione peroxidase catalytic activity (Schewe, 1995). The reaction, which depends on the selenium moiety, oxidizes glutathione or other sulfur-containing substrates (e.g., cysteine, dithioerythritol, dihydrolipoate, etc.) and reduces hydroperoxides to water or an organic alcohol in a series of chemical reactions that regenerate the original organoselenium compound (Schewe, 1995). The optimum concentration of ebselen needed to catalyze H₂O₂ peroxidase activity is about 20 μM (Muller et al., 1984). Based on these studies, it has generally been assumed that ebselen diminishes ROS levels through this mechanism. However, the present studies document that ebselen and its analogs, which include those that contain sulfur rather than selenium, function to inhibit ROS generation in neutrophils at concentrations up to two orders of magnitude lower than those required to observe significant thiol peroxidase activity.

Previous studies have yielded different potencies of ebselen on ROS production in various systems. Superoxide generation in the presence of ebselen was measured in Kupffer cells using cytochrome c reduction, yielding an IC₅₀ of about 10 μM; in the same cells, ebselen inhibited nitric oxide generation measured by conversion of oxyhemoglobin to methemoglobin with an IC₅₀ of about 3 μM (Wang et al., 1992). When luminol luminescence was used as a report of reactive oxygen, ebselen inhibited with an IC₅₀ of about 1 μM, demonstrating that assay sensitivity can affect the results (Wang et al., 1992). In guinea pig neutrophils and membranes isolated from these cells (Wakamura et al., 1990), and in human neutrophils (Cotgreave et al., 1989), superoxide generation measured using several different methods was inhibited by both selenium- and sulfur-containing analogs of ebselen at concentrations similar to those that were effective in the present studies. The authors in the latter two studies suggested that in addition to possibly affecting protein kinase C, these compounds might have a direct effect on the phagocyte NADPH oxidase. However, the site of action was not further characterized. In contrast, the inhibition reported in guinea pig macrophages (Leurs et al., 1989) was at an IC₅₀ of about 20 μM. The difference between our results in human neutrophils and those obtained in guinea pig macrophages may have
resulted from cell type or species differences and/or differences in the assays used. The present studies are in agreement with the Wakamura (Wakamura et al., 1990) and Cotgreave (Cotgreave et al., 1989) studies which used several methods of superoxide detection, increasing the confidence in the results. Our studies also demonstrate that a major site of action of ebselen and its analogs is the interaction between the bis-SH3 domain of p47phox and the PRD of p22phox.

Previous studies that used peptides based on the PRD of p22phox demonstrated binding of the peptides to p47phox (Shi et al., 1996) (Dahan et al., 2002), yet these peptides did not inhibit Nox2 activity in a cell free system, presumably because they did not compete effectively with native p22phox in the membrane for binding. The results reported here used small molecules which would presumably have better access to the binding surface, and also lower likelihood of factors such as conformational restrictions and nonspecific binding to other surfaces which complicate working with peptides.

As suggested from the discussion above, ebselen and its analogues can affect multiple targets both by direct effects on the enzymes themselves and by indirect effects such as the consumption of hydrogen peroxide or lipid peroxides that serve as substrates for certain enzymes. Ebselen and its analogs also inhibit endothelial nitric oxide synthase, lipoygenases, c-Jun N-terminal kinase and horseradish peroxidase (Zembowicz et al., 1993) (Shimohashi et al., 2000; Schewe et al., 1994; Mishra et al., 2006). In the case of lipoygenase, inhibition results from depletion of the lipid peroxide substrate by the peroxidase-like activity of ebselen (Tabuchi et al., 1995), while in other cases, the mechanism is less clear. For example, ebselen inhibits horseradish peroxidase with an IC50 of ~17 μM by a mechanism that does not correlate with its glutathione peroxidase activity and in a manner that is independent of a thiol donor (Mishra et al., 2006). In this light, our data indicating that ebselen inhibits co-immunoprecipitation of p67phox with p47phox in PMA-stimulated cells (figure S4) do not discriminate whether this a direct effect of ebselen on the interaction between the C-terminal PRD of p47phox and the C-terminal SH3 domain of p67phox, or whether it is caused by an indirect effect such as an allosteric effect resulting from binding to the bis-SH3 domain of p47phox. Whether ebselen binds to single SH3 domains as opposed to the bis-SH3 domain is a topic for further studies.

The complex chemistry associated with the selenium center in ebselen increases the likelihood that the compound can act by several mechanisms. The mode of action we describe here does not conflict with or rule out other known or proposed modes of action. Ebselen’s glutathione peroxidase activity has been extensively studied, and certainly could contribute to the inhibition of Nox2-dependent H2O2 production when the ebselen concentration is above 10 μM. In some enzymes, ebselen can modify cysteine residues (Ouertatani-Sakouhi et al., 2010). Our binding assay used a recombinant construct consisting of only the bis-SH3 domain of p47phox and a synthetic peptide based on the PRD of p22phox. Inspection of a published structure of p47phox in complex with the PRD peptide of p22phox (Groemping et al., 2003) reveals that the sole cysteine in the p47-bis-SH3 domain points away from the binding groove and does not participate directly in the binding of the PRD. We performed preliminary in silico docking of ebselen into this crystal structure, where it fits with favorable binding energy (data not shown) and all low energy poses are distant from the cysteine. These considerations make it unlikely that cysteine modification contributes to ebselen’s effects on this interaction. Nox1, Nox2, and Nox4 have different patterns of accessible cysteines on their cytoplasmic dehydrogenase domains. In particular, Nox2 has two cysteines (368 and 370) in close proximity, modification of which might be expected to affect activity. Nox1 lacks cysteines at homologous positions, yet selenium and sulfur analogs inhibit Nox1 with micromolar potency. These data argue against chemical modification of Cys368 and Cys370 as a major mode of inhibition of Nox2.
by these compounds. Nonetheless, ebselen and analogs might modify cysteines in other regions of the Nox1 or Nox2 complex, contributing to inhibition. Because sulfur analogs of ebselen are poorly reactive with free cysteines (Leurs et al., 1989), their ability to inhibit Nox1 and Nox2 cannot be ascribed to that mechanism. Our results suggest that many effects, including anti-inflammatory effects, that were previously ascribed to ebselen’s antioxidant activity (Parnham et al., 1987) are likely instead to be the result of ebselen’s direct inhibition of Nox1 and Nox2 activity by preventing assembly and activation of the Nox1 and Nox2 complexes.

**Significance**

NADPH-oxidases (Nox/Duox enzymes) are a primary source of reactive oxygen species (ROS), which function in normal physiology in diverse cell types and, when overproduced, in pathophysiology. The Nox2 isoform is implicated in a variety of inflammatory and neurodegenerative conditions. Recent genetic studies using Nox2-deleted mice identify this enzyme as a novel target for treatment of several diseases. Nox2 enzyme function requires the assembly of a complex consisting of the membrane-bound Nox2-p22phox heterodimer and cytosolic protein factors. The binding of the proline rich domain (PRD) of p22phox to the bis-SH3 domain of p47phox is a crucial step in this assembly. A fluorescence polarization assay that monitors this binding was developed and used in a high-throughput screen identified ebselen, previously characterized as a glutathione peroxidase mimetic, and several of its analogs, as inhibitors of this binding interaction. Ebselen and its analogs also potently inhibited superoxide generation in human neutrophils and in a Nox2 cell-free system, with IC\textsubscript{50} values well below the \( \sim 10 \mu\text{M} \) concentration at which ebselen functions as a glutathione peroxidase mimetic. Medicinal chemistry was performed to explore structure-activity relationships and to increase potency. Nox2 inhibition requires isothiazol-3(2H)-one (sulfur) or selenazol-3(2H)-one (selenium), and moieties such as carboxymethyl or 1,3 dioxane appended to the nitrogen of the phenyl ring in the sulfur series increased potency. Ebselen blocked the phorbol ester-activated translocation of p47phox and p67phox to neutrophil membranes, which requires the same interaction monitored by the fluorescence polarization assay. Neither selenium nor sulfur analogs of ebselen inhibited Nox4, which does not require activating subunits and which produces hydrogen peroxide. These results effectively rule out peroxidase-mimetic activity as the primary mode of Nox2 inhibition by these compounds. Thus ebselen and its analogs thus represent a class of compounds that inhibit ROS generation by interrupting the assembly of Nox2-activating regulatory subunits.

**Experimental Procedures**

**Materials**

Chemicals and enzymes were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Rhodamine-labeled peptide with the sequence Rhodamine-PPTNPPPRPPAE-amide (residues 151–162 of mouse p22phox) was synthesized by the Emory University Microchemical Facility. The peptide was dissolved in water and stored at \( \sim 80 \) C. Ebselen was purchased from Calbiochem EMD Chemicals (Gibbstown, NJ). Thr101 was purchased from Chem Div, Inc. Compounds were dissolved in DMSO at a stock concentration of 10 mM and stored at \( \sim 20 \) C. Antisera against p67phox and p47phox were the generous gifts of Dr. Mark Quinn, University of Montana and of Dr. Jamal El-Benna, INSERM, Paris.

**Compound Synthesis**

Thr101 analogs (isothiazol-3-ones) were synthesized following a reported protocol (Correa et al., 2006) starting from commercially available methyl 2-mercaptobenzoate, and substituted methyl-2-mercaptopbenzoates (see Figure S2). Synthesis of ebselen and its derivatives was
performed as described previously (Chang et al., 2003). Characterization data for representative members is provided in supporting information.

**Cloning and expression of recombinant proteins**—The cDNA encoding the bis-SH3 domain of human p47phox (residues 156-285) was obtained by PCR and subcloned into pGEX4T3 (GE Life Sciences, Piscataway, NJ) in frame with an N-terminal glutathione S-transferase (GST) protein that was used as a purification tag. The C-terminus of human p22phox (residues 132-195, which includes the PRD) was obtained by PCR and subcloned into pMALc2x (NEB, Ipswich, MA) in frame with an N-terminal maltose-binding protein (MBP) that was also used as a purification tag. GST, GST-tagged p47phox-bis-SH3 domain (GST-p47-bis-SH3), MBP, and MBP-tagged p22phox C-terminus (MBP-p22-C) were expressed in and affinity purified from *E. coli* BL21 cells using glutathione agarose for GST-tagged proteins or amylose resin for MBP-tagged proteins, according to the instructions of each plasmid vendor. Proteins were eluted in 20 mM TrisHCl pH 7.5, 150 mM NaCl, 1 mM EDTA supplemented with glutathione or maltose as appropriate, dialyzed overnight against 20 mM HEPES pH 7.5, 25 mM NaCl, and stored at −80°C until use. Protein concentrations were determined using the BCA method (Pierce Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. Proteins were >90% pure as assessed by SDS-PAGE followed by Coomassie Blue staining (data not shown).

**Cell-free assay for Nox2-dependent superoxide generation and xanthine/xanthine oxidase control assay**—Plasma membranes were prepared from human neutrophils as described previously (Curnutte et al., 1987) and stored at −80°C until use. Superoxide generation was assayed as previously described (Ritsick et al., 2007) with minor modifications. Briefly, membranes containing 0.3 μg of protein were mixed with 0.25 μg p67Np47N fusion protein (human p67phox residues 1-210 fused to human p47phox residues 1-286, Ebisu et al., 2001), 0.2 μg Rac1Q61L, 500 nM FAD, 50 μM L-012 (Wako Chemicals, Richmond, VA), and varying concentrations of ebselen or analogs (dissolved in DMSO) in 100 μL of assay buffer (100 mM PIPES, 30 mM NaCl, 35 mM MgCl₂, 1M KCl, pH 7.4); DMSO concentration was kept constant at 1%. We used reported literature values of Nox2 content of human neutrophils (Parkos et al., 1987) (Quinn et al., 1993) to calculate that this assay includes approximately 0.1 pmol of Nox2 catalytic subunit; the recombinant proteins are in excess (9.1 pmol for Rac1Q61L and 5.2 pmol for p67-p47 chimera). The optimal NADPH concentration (10 μM final) was determined empirically, and avoided a quenching artifact seen at higher substrate concentrations. NADPH was added and luminescence was recorded in a Synergy 2 microplate reader (Biotek Instruments, Winooski, VT). After two minutes, 90 μM sodium dodecyl sulfate (SDS) was added and luminescence was recorded for an additional 20 minutes. Maximum luminescence was obtained in 3–5 minutes, and is reported as the mean from 3–5 determinations. In all cases, luminescence of incubations with the general Nox inhibitor diphenylene iodonium (DPI) was less than 10% of that from incubations containing DMSO vehicle alone, consistent with Nox2 as the source of the ROS signal (data not shown). In assay control experiments, 1 μM xanthine oxidase replaced the Nox2 system as the source of superoxide. Xanthine (50 μM) was added to initiate the reaction and luminescence was recorded for 20 minutes.

**Measurement of ROS generation in whole cells**—Neutrophils were isolated from blood collected from human volunteers as previously described (Curnutte et al., 1987) in accordance with Emory University IRB guidelines. Nox2 activity was assessed using luminescence from L-012. 10⁴ freshly isolated human neutrophils were suspended in 100 μl assay buffer to which was added 50 μM L-012, along with DMSO, 10 μM DPI, or varying concentrations of compound. The final DMSO was maintained constant in all samples at 1%. After 2 minutes, 400 nM (final concentration) of phorbol 11-myristate 12-acetate was added and luminescence was recorded for an additional 20 minutes.
(PMA) was added and luminescence was recorded for 20 minutes using a Synergy 2 microplate reader. Maximum luminescence, which was achieved before 5 minutes, was calculated from the average of 3–5 consecutive points. In all cases, luminescence from DPT-treated cells was less than 10% of that from DMSO-treated cells, consistent with a Nox enzyme as the source of ROS (data not shown).

HEK cells stably transfected with Nox1 (HEK/Nox1rb) or Nox4 (HEK/Nox4rb) were the generous gift of Dr. Ralf Brandes (Goethe University, Frankfurt, Germany). $10^6$ HEK, HEK/Nox1rb, or HEK/Nox4rb cells were plated on 10 cm culture plates, incubated for 24–48 hours in supplemented medium and harvested as previously described (Cheng, Ritsick et al., 2004). 24 hours after plating, HEK cells were transiently transfected with 6 μg of eukaryotic expression plasmid containing Nox5 (Kawahara et al., 2011), or HEK/Nox1rb cells were transiently transfected with 1.5 μg of eukaryotic expression plasmid containing NoxO1 and 1.5 μg plasmid containing NoxA1 (Cheng and Lambeth, 2004), per 10 cm plate, using the Fugene (Roche, Indianapolis, IN) system according to the manufacturer’s instructions; transiently transfected cells were incubated for an additional 24 hours. Cells were washed and resuspended in phosphate buffered saline (PBS) (11.9 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) supplemented with 1% glucose. L-012 luminescence was assayed essentially as described for neutrophils above, except that $10^5$ transfected HEK cells were used in a final reaction volume of 111 μl; for HEK cells transiently transfected with Nox5 the reaction mixture was supplemented with calcium and ionomycin (Kawahara et al., 2011); and for HEK/Nox4rb cells the reaction mixture was supplemented with 0.9 units/ml (final) of horseradish peroxidase. In Nox4 assay control experiments, 25 μM H$_2$O$_2$ replaced stably transfected HEK cells. The xanthine/xanthine oxidase assay described above served as the assay control experiment for HEK cells transfected with Nox1 and Nox5.

Assessment of cell viability—Freshly isolated human neutrophils ($5 \times 10^4$ cells) were suspended in PBS supplemented with 1% glucose, or $4 \times 10^4$ HEK/Nox4rb cells were suspended in DMEM supplemented with 10% FBS, and incubated with varying amounts of compound or DMSO vehicle for 10 minutes at 37°C, then assayed for viability using the CellTiterGlo kit (Promega) according to the manufacturer’s instructions. This kit determines the proportion of viable cells based on luminescent quantification of ATP, an indicator of metabolic activity. Data were normalized to 0% viable cells with 1% Triton-X-100, a potent detergent that interrupts cell integrity, as suggested by the manufacturer.

Fluorescence polarization (FP) assay—Assays were performed in black, flat-bottom 384 well microplates (Corning, Corning, NY) in FP buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; 0.01% Tween-20) using a final volume of 50 μl at room temperature. Parallel and perpendicular fluorescence intensities were measured using excitation filter 545 (bandwidth 40) nm and emission filter 620 (bandwidth 40) nm in a Synergy 2 fluorescence microplate reader, and FP values were calculated by the manufacturer’s Gen5 software. To establish feasibility, we measured the FP of 10 nM of rhodamine-labeled peptide corresponding to the PRD domain of p22phox (rho-PRD) in the presence of a range from 0 – 3 μM protein (either GST alone or GST-p47-bis-SH3). The isolated bis-SH3 domain of p47phox is in the open state (without the need for phosphorylation) because it lacks the autoinhibitory domain of the native protein (Yuzawa et al., 2004). Preliminary experiments (not shown) revealed that the binding was somewhat tighter and more stable with the peptide based on the mouse PRD sequence. We therefore used mouse PRD in the HTS. To demonstrate the validity of the assay, we also measured FP of 10 nM rho-PRD, plus GST-p47-bis-SH3 (20 – 40 nM) in the presence of increasing concentrations of MBP or MBP-p22-C. The stability of the assay was evaluated by recording FP values at intervals ranging from 1 to 24 hours. The assay was also carried out at a range of DMSO concentrations up to 5% to investigate the sensitivity to solvent.
For competition experiments, the FP of 10–20 nM rho-PRD plus GST-p47-bis-SH3 (20 - 40 nM) was recorded before and after addition of either MBP-p22-C (500 nM) or varying concentrations of compound (DMSO concentration was adjusted to 1% final). The FP of the free rho-PRD peptide alone in FP buffer was also recorded. To insure that a stable signal had been achieved, FP was measured at several intervals over a total period of 60 min. Values at the 30 minute point time were used.

**Pull-down assay**—In one ml of FP assay buffer, 1.2 nmoles of GST-p47-bis-SH3 was incubated at room temperature for 1 hour with 1.2 nmoles of MBP-p22-C in the presence and absence of DMSO vehicle, 20 μM ebselen, or 10 μM rho-PRR. Control incubations using combinations of purified GST, purified MBP, MBP-p22-C, and GST-p47-bis-SH3 were carried out in the same conditions. Glutathione agarose beads were added, incubated for another hour at RT, washed, pelleted and boiled 10 minutes in 50 μl of 2x Laemml sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8). Samples containing equal of GST or GST- p47-bis-SH3 were subjected to SDS-PAGE and visualized by staining with Coomassie blue.

**Translocation of p47phox and p67phox**—Neutrophils (10⁸ cells) in 3.25 ml assay buffer containing 0.1% DMSO with or without 10 μM ebselen were incubated at 37°C. After 2 minutes, 500 nM PMA or vehicle control was added; final DMSO was kept constant in all samples at 0.2%. Cells were incubated at 37°C for 10 minutes, and pelleted by centrifuging for 10 min at 300 x g. Plasma membranes were isolated from human neutrophils as previously described (Curnutte et al.,1987). An aliquot of each membrane sample was assayed for protein concentration by the BCA method; total protein was extracted from the remainder of the membranes by boiling for 10 minutes in 2x Laemml sample buffer. Protein from each sample was loaded onto a 4–15% TGX gel and subjected to SDS-PAGE. Western blotting was carried out following the protocol suggested by Licor (Licor Biosciences, Lincoln, NB). Blots were probed with either a mixture of antiserum to p47phox and monoclonal antibody to p67phox or with monoclonal antibody to p22phox (Abcam, Cambridge, MA). Protein bands were detected by addition of appropriate Licor fluorescence-tagged secondary antibodies to mouse or rabbit IgG and visualized using a Licor Odyssey imager. Bands were sized and images were subjected to densitometry using the vendor’s software.

**Detection of PKC-mediated phosphorylation of p47phox and co-immunoprecipitation of p67phox with p47phox**—10⁸ neutrophils in 1 ml Hank’s buffer containing 1% DMSO with or without 10 μM ebselen were incubated at 37°C. After 2 minutes, 500 nM PMA or vehicle control was added; final DMSO was kept constant in all samples at 2%. Cells were incubated at 37°C for 15 minutes, and at which time 10 ml ice-cold PBS was added to stop the stimulation. Cells were treated with 2.7 mM DFP and resuspended in 1 ml of ‘RIPA + 11’ buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Na-deoxycholate, 0.1% NP-40) supplemented with complete protease and phosphatase inhibitors (Roche, Indianapolis, USA), 0.5 mg/ml β-glycerol phosphate, 0.5 mg/ml p-nitrophenyl phosphate, and 0.2 mg/ml levamisol. The lysates were sonicated briefly and centrifuged at 100,000 x g for 20 minutes. Rabbit polyclonal p47phox antibody () was used to immunoprecipitate p47phox using standard immunoprecipitation and Western blotting methods. For assessing PKC mediated serine phosphorylation, blots were probed with rabbit antibody to phosphoserine in a PKC- motif (Cell Signaling Technology, Beverly, MA). To detect p47phox and p67phox in the immunoprecipitates and lysates the blots were probed with rabbit polyclonal antibody to p47phox or p67phox respectively. Secondary antibodies included ImmunoCruz HRP-conjugated antibodies(Santa Cruz Biotechnology, Santa Cruz, CA) to avoid heavy chain interference in the immunoprecipitates, or standard hrb-goat-anti-
rabbit or anti-mouse antibodies (BioRad Hercules, CA). Blots were visualized using SuperSignal chemiluminescence reagent kit (ThermoScientific, Rockford, IL) according to the manufacturer’s instructions. To normalize samples, the blots were stripped and probed with mouse monoclonal antibody to actin (Sigma, St. Louis, MO).

**Curve-fitting and statistical analysis**—Data analysis was performed using Prism 4 software (GraphPad Software, La Jolla, CA). Data for binding was fit to a 4-parameter non-linear least squares sigmoidal dose response model, in which parameters are: maximum and minimum polarization values, EC_{50} values, and slope of the curve. Data for inhibition of binding, or for inhibition of activity, were fit to a one-site competition model that uses a nonlinear least squares fit and which reports IC_{50}, K_d, and K_i values. Densitometry values of p47phox or p67phox bands in Westerns were compared by first determining total intensity for the p47phox, p67phox bands, or p22phox bands across all four experimental treatments. The intensity of each band was normalized as the fractional intensity of the band in each treatment to the total intensity of the protein detected across all treatments. P47phox and p67phox intensities were normalized to the intensity of the p22phox band from the same treatment. Normalized intensities were compared by one-way ANOVA.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We gratefully acknowledge the generous gifts of Nox1 and Nox4 stably transfected cell lines from Dr. Ralf Brandes (Goethe University, Frankfurt), antibody to p47phox from Dr. Jamal El-Benna (INSERM, Paris), and antibodies to p47phox and p67phox from Dr. Mark Quinn (Montana State University, Montana). The authors declare that they have no competing interests in this work. The work was supported by NIH R03 MH083234 to SMES and NIH R01 CA084138 and R01CA084138-08S1 to JDL.

**References**


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Highlights

Development of a HTS based on preventing Nox2 assembly
Congeners of ebselen inhibit Nox2 but not Nox4 activity
Ebselen blocks translocation of cytosolic subunits to neutrophil membranes
Ebselen congeners inhibit Nox2 directly via interruption of assembly
Figure 1. Fluorescence polarization assesses binding of p22-PRD peptide to p47 bis-SH3 domain

A. Fluorescence polarization in mP units of 10 nM of synthetic, rhodamine-labeled peptide corresponding to the PRD of p22phox (rho-PRD) was measured in the presence of increasing concentrations of recombinant GST-p47-bis-SH3 (filled squares) or GST (open squares) as described in Materials and Methods. Points represent means of triplicate measurements, and were fit (R² = 0.99) to a dose response model using non-linear least squares fit as described in Experimental Procedures. The K_d for binding of the peptide to p47-bis-SH3 in this experiment was calculated to be 20 nM. B. Fluorescence polarization of 10 nM rho-PRD in the presence of 20 nM p47-bis-SH3 and increasing concentrations of recombinant MBP-p22-C protein (filled squares) or recombinant MBP alone (open squares) were measured as described in Experimental Procedures. Points represent means of triplicate measurements and were fit (R² = 0.94) to a one-site competition model as detailed in Experimental Procedures, yielding an IC_{50} for MBP-p22-C of 120 nM and a K_i of 60 nM. This experiment is representative of at least three independent experiments. Figure S1 shows the results of a pull-down experiment that supports this figure.
Figure 2. Hits from HTS inhibit both binding of rho-PRD to GST-p47-bis-SH3 and Nox2 activity
Structures of ebselen (A, top) and Thr101 (B, top) are shown. Fluorescence polarization (FP) of 10 nM rho-PRD was measured in the presence of 30 nM GST-p47-bis-SH3 and varying concentration of ebselen (A, middle) or Thr101 (B, middle) as described in Experimental Procedures. The FP value of rho-PRD + GST-p47-bis-SH3 + 500 nM MBP-p22-C was normalized to 0%, while the FP value of free rho-PRD was normalized to 100%. Nox2 activity (in normalized luminescence units) was measured as described in Experimental Procedures, with the activity in 1% DMSO alone normalized to 100%. Inhibition of Nox2 activity in the cell free assay by ebselen (A, bottom) and Thr101 (B, bottom) is shown with filled circles. Control assays in which ROS was supplied using the same detection method by the superoxide-generating xanthine/xanthine oxidase system in place of Nox2 were performed (open circles) in the presence of varying concentrations of ebselen (A, bottom) and Thr101 (B, bottom). Data points represent the means +/- s.d. of triplicate measurements, and were fit to a one-site competition model as described in Experimental Procedures. Experiments shown are representative of two to five independent determinations for each assay.
Figure 3. Ebselen analogs inhibit both binding and enzyme activity
Twenty seven analogs of ebselen that inhibited binding of rho-PRD to GST-p47-bis-SH3 with EC$_{50}$ of 10 μM or less were also tested for effects on Nox2 activity in the cell-free assay described in Experimental Procedures. Sulfur- (black symbols) and selenium-containing (red symbols) analogs inhibit both binding and activity; correlation coefficient R=0.7 (p<0.0001), R$^2$ = 0.5; slope significantly nonzero (p<0.001) as determined in GraphPad Prism. Compounds g, h, and i are representative examples of compounds that did not inhibit in the binding assay and also did not inhibit in the activity assay (EC$_{50}$ or IC$_{50}$ greater than 25 μM). Structures of the compounds in this figure are presented in Table S1, and the synthesis scheme is shown in Figure S2.
Figure 4. Ebselen analogs are selective for Nox2 over Nox4
In panels A and B, ebselen or Thr101 concentrations were varied as indicated, and Nox2 activity was measured in intact neutrophils (filled squares) and Nox4 activity in HEK-Nox4rb cells (filled triangles) as described in Experimental Procedures. In assay control experiments, \( \text{H}_2\text{O}_2 \) was added directly to the assay system (open circles). In additional control experiments, the xanthine/xanthine oxidase superoxide-generating system was used in place of ROS-generating cells; results were similar to those shown in Figure 2, and are omitted here for clarity. Nox activity in the presence of 1% DMSO (vehicle control) was taken as 100% activity. Data points represent means +/- standard deviation of triplicate measurements, and were fit to a one-site competition model as described in Experimental Procedures. The data shown are representative of two to four independent determinations for each assay.
Figure 5. Ebsele inhibits translocation of p47phox and p67phox in PMA-stimulated human neutrophils

Human neutrophils were treated with combinations of DMSO vehicle, 500 nM PMA, and 10 μM ebsele as described in Experimental Procedures. Cells were broken by sonication and separated into cytosol and plasma membrane fractions as described in Experimental Procedures. Proteins were extracted from plasma membrane (Lanes 1–4) or cytosol (lane 5) by boiling in Laemmli sample buffer and separated by SDS-PAGE. After electrophoretic transfer to low-fluorescence polyvinylidene fluoride membrane, membranes were probed with antibodies to p22phox, p47phox, and p67phox as described in Experimental Procedures. Samples were from neutrophils treated as indicated; lane 5 contains protein from untreated neutrophil cytoplasm. The blot shown is representative of three independently isolated batches of neutrophils. Densitometry was performed and analyzed as described in Experimental Procedures; data shown is the mean fluorescence of p47phox or p67phox in neutrophil membranes normalized to p22phox from the same treatment, +/- s.d., obtained from blots from 3 independently isolated batches of neutrophils. Figure S3 shows that 10 μM ebsele does not inhibit PKC phosphorylation, while Figure S4 shows that 10 μM ebsele diminishes the co-immunoprecipitation of p67phox with p47phox.
Table 1
EC\textsubscript{50} values (\(\mu\)M) of ebselen analogs for inhibition of activity, and control assays

Assays testing the effects of compounds on the activity of Nox1, Nox2, Nox4 and Nox5 in whole cells (along with assay controls) were performed as described in Methods.

<table>
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<th>Compound</th>
<th>Structure</th>
<th>EC\textsubscript{50} ((\mu)M) for inhibition of Nox isoform activity in whole cell assays, and EC\textsubscript{50} ((\mu)M) in assay controls</th>
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</table>

*ns = no significant inhibition.