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Cell-free Screening for NOX Inhibitors

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NOX enzymes are major ROS generators in pathological states and represent a promising pharmacological target. In this issue of *Chemistry & Biology*, Smith and colleagues developed an original and elegant cell-free assay to identify specific Nox2 inhibitors in a high throughput manner.

A major breakthrough in redox biology was made at the beginning of 21st century when it was discovered that the NADPH oxidase of the phagocyte (now known as NOX2) was only one member of a whole family of transmembrane proteins catalyzing the production of reactive oxygen species (ROS) in specific cell types. This family comprises seven members of enzymes (NOX1–NOX5 and DUOX1 and 2), which are major ROS generators in both in physiological and pathological conditions.

Excess of ROS is a hallmark of numerous diseases and antioxidant molecules have been extensively used as treatments to neutralize ROS. Unfortunately, this approach has shown little medical benefit, probably because antioxidants can act to decrease ROS levels only once ROS have been produced. Targeting the source of ROS, such as NOX enzymes, has far more potential for a pharmacological strategy as it prevents ROS generation. Developing drugs that act specifically on a particular isoform of NOX would be an even more refined treatment with fewer unwanted effects. Targeting individual isoforms is conceivable as, in spite of high structural similarities, NOX isoforms differ in their mode of activation.

Usual approaches to identify NOX inhibitors are based on detection of NOX-derived ROS using colorimetric, luminescent, or fluorescent probes in whole cells or in so-called semi recombinant systems consisting of purified NOX-containing membranes to which NADPH, FAD, and recombinant cytosolic factors are added. However, although straightforward, this approach shows a number of limitations: (1) ROS are extremely reactive molecules and the probes used to detect them are prone to artifacts; (2) NOX enzymes are not the sole source of ROS in cells and membrane preparations; and (3) more importantly these methods do not discriminate between antioxidant molecules (ROS scavengers) and real inhibitors, i.e., compounds, that are able to block NOX-derived ROS generation by direct interaction with the enzyme (for review, see Jaquet et al., 2009).

In this context, the study by Smith et al. (2012) in this issue of *Chemistry & Biology* is exemplary. In order to screen a chemically diverse library, they developed a completely recombinant system not based on NOX2 activity, but on the blockade of the binding of cytosolic subunits necessary for its activation.

The catalytic core of the NOX2 complex is formed of a transmembrane protein containing two heme molecules and the binding sites for NADPH and FAD (NOX2) as well as the transmembrane protein p22phox. In order to catalyze ROS production, NOX2 requires activation, in a process involving phosphorylation and translocation of cytosolic subunits rac, p67phox, p40phox, and p47phox (Figure 1A). In particular, the interaction between a groove formed by a tandem Src homology 3 (SH3) domain of p47phox and a proline rich region of p22phox is critical. This complex has been crystallized and characterized both in term of structure (Groemping et al., 2003) and function (Sumimoto et al., 1996) (Figure 1B).

This study by Smith et al. (2012) is the first example of rational drug discovery based on available structural information on known binding pocket of the NOX2 complex. The assay consists of measuring changes in fluorescence polarization when a peptide containing the proline rich region of p22phox labeled with a fluorescent molecule (rhodamine) interacts with a recombinant protein encompassing the tandem SH3 domain of p47phox (Figure 1C). It was developed in a high throughput screening format and requires only minute amount of material. Selected hits were validated in functional assays to confirm that they in fact did block NOX2.

Ebselen and a close analog named Thr101 were among the most promising molecules. This finding must have come as a surprise, because ebselen is a renowned antioxidant molecule! Ebselen is a synthetic selenium compound first described as a potent antioxidant with glutathione peroxidase-like activity (Müller et al., 1984). However, the current study convincingly demonstrates that ebselen is a bona fide NOX2 inhibitor, confirming observations made more than twenty years ago (Cotgreave et al., 1989; Wakamura et al., 1990).

Although ebselen is potently inhibiting NOX2, it is known to have numerous other pharmacological effects, and the specific disruption of p22phox-p47phox complex might only be part of its effect on NOX2 as it also potently inhibited NOX5, an isoform completely independent of cytosolic subunits. However, the other analogs tested in this study show a strong correlation between functional and binding assays as well as lower potency on NOX5.

In many instances, it may be an advantage to use molecules with multiple modes of action for complex diseases and the one drug-one target paradigm promoted by the pharmaceutical industry appears to be more of a ''holy grail'' rather than a biological reality. Nevertheless, the protective action of ebselen in pathologies where oxidative stress plays a key

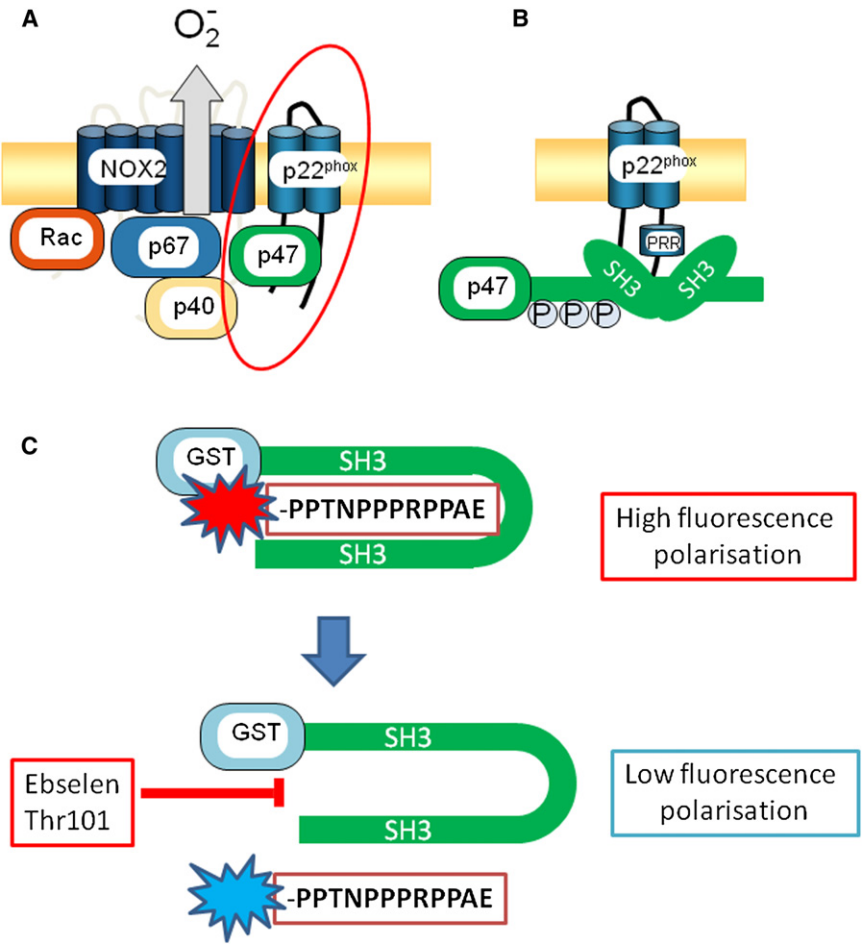

Figure 1. Strategy for the Identification of Specific NOX2 Inhibitors
(A) The NOX2 complex requires the binding of cytosolic subunits for ROS (superoxide anion) generation.
(B) The rationale for identifying NOX2 inhibitors is based on the fact that NOX2 activation is fully dependent on the binding of a tandem SH3 domain of p47phox to a proline rich (PRR) region of p22phox.
(C) In this study, the binding of p22phox-p47phox complex was reproduced in vitro. High fluorescence polarization is measured when a rhodamine labeled peptide encompassing the PRR of p22phox binds to a recombinant protein comprising the tandem SH3 domain of p47phox fused to glutathione-S transferase (GST). Small molecules like ebselen or Thr101 interfering with this interaction induce a change in fluorescence polarization and were identified as hits in the HTS.

role, including stroke or noise-induced hearing loss, should therefore be considered not only in the context of its antioxidant role, but also in light of its potent inhibitory action on NOX enzymes.

In conclusion, the present description of a cell-free high throughput screen represents a milestone for the development of NOX inhibitors jointly with the efforts of the pharmaceutical industry (Gaggini et al., 2011; Laleu et al., 2010). Furthermore, as the crystal structure of the p47phox tandem SH3 domain with the proline rich peptide is known, the way is open for computer-assisted drug design.

Discovering novel small molecules NOX2 inhibitors will be extremely valuable for numerous indications, in particular for central nervous disorders where NOX2 plays a key regulatory role in neuroinflammatory and psychotic diseases (Sorce et al., 2012).

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