Response to Pick

JAQUET, Vincent, RUTTER, A Richard

Abstract

In his letter, Dr. Pick criticizes our use of relative values when representing the NOX2 inhibitory action of a novel small molecule (GSK2795039) in a semi-recombinant NOX2 membrane assay. To address this concern, we performed additional experiments using the superoxide inhibitable assays cytochrome C and water soluble tetrazolium salt (WST-1) reduction. In this letter, we document turnover values between 80 and 100 mol O2(•-) /s/mol cytochrome b558 in our semi-recombinant assay and confirmed that GSK2795039 inhibits the NOX2 isoform in the submicromolar range. Antioxid. Redox Signal. 23, 1251-1253.

Reference


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to proceed for 90 min, with BHK membranes, and for 30–60 min (at 37°C), with PLB-985 membranes, is suggestive of very low reaction rates. In conventional Nox2 cell-free assays, kinetics are recorded for 5 min (or even shorter intervals) at 24°C to assure the linearity of the slope.

References


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Abbreviations Used

BHK = baby hamster kidney

$O_2^* = superoxide$

TO = turnover

Response to Pick

Vincent Jaquet,1 and A. Richard Rutter2

Abstract

In his letter, Dr. Pick criticizes our use of relative values when representing the NOX2 inhibitory action of a novel small molecule (GSK2795039) in a semi-recombinant NOX2 membrane assay. To address this concern, we performed additional experiments using the superoxide inhibitable assays cytochrome C and water soluble tetrazolium salt (WST-1) reduction. In this letter, we document turnover values between 80 and 100 mol $O_2^*$/s/mol cytochrome b$_{558}$ in our semi-recombinant assay and confirmed that GSK2795039 inhibits the NOX2 isoform in the submicromolar range. *Antioxid. Redox Signal*. 23, 1251–1253.

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To the Editor:

We read with great interest the letter by Dr. Pick regarding our publication describing the discovery of GSK2795039, a novel NOX2-specific small-molecule inhibitor (2).

The letter acknowledges the validity of GSK2795039 as a bona fide NOX2 inhibitor; however, it raises concerns about the proper use of the units used when documenting the inhibitory activity and mode of action of GSK2795039 in NOX2 semi-recombinant assays. It addresses the fact that relative values (% control) or absorbance values for the water-soluble tetrazolium salt (WST-1) assay do not inform on whether the levels of the enzyme activity in the assay are consistent with enzyme turnover (TO) rates reported for semi-recombinant NOX2 assays using primary neutrophil cell membranes or related cell lines, such as PLB-985 cells, and are therefore inappropriate values to demonstrate pharmacological inhibition. In his letter, Dr. Pick indicates that both the amount of membranous NOX2 heme and superoxide anion radicals (O2∗·) generated must be measured to address the NOX2 activity in absolute values (TO values in mol O2∗·/s/mol cytochrome b558 heme). Another point raised by Dr. Pick was the 30–60 min measurements used in the WST-1 assay, as it may suggest very low reaction rates.

Thus, to address these important points, we performed additional experiments using membranes prepared from the cell line PLB-985 differentiated into neutrophil-like cells following the detailed methodology published by Pick (4). We calculated the amount of cytochrome b558 in PLB-985 membranes to be 56 pmol/ml by subtracting the redox differential absorbance spectra (measured with an Agilent Technologies Carry 60 UV-Vis spectrometer, 0.5 nm resolution, path length = 1 cm) from differentiated PLB-985 membranes prepared from wild type and NOX2-deficient cells. We calculated the molar amount of NOX2-generated O2∗· by measuring the slope of the superoxide dismutase (SOD)-inhibitable cytochrome C and WST-1 detection probes during the linear part of the slope (first 5 min) using FlexStation® 3 Reader. Figure 1 shows GSK2795039 concentration plotted against TO. Noninhibited values correspond to 102.6 ± 1.6 (cytochrome C) and 83.5 ± 6.1 (WST-1) mole O2∗·/s/mol cytochrome b558 heme, closely matching expected values using these cells (1). The strong inhibitory activity of GSK2795039 was confirmed with pIC50 = 6.26 ± 0.15 when measured using cytochrome C and pIC50 = 6.27 ± 0.05 with WST-1.

Identification of NOX inhibitors is a challenging task because reactive oxygen species (ROS) detection systems are prone to artifacts (3, 5). For this reason, we characterized the NOX2 inhibitory activity of GSK2795039 using multiple systems, including four different ROS detection probes and two assays measuring the decrease of substrates (oxygen and NADPH) of the reaction catalyzed by NOX2 in both cellular and semi-recombinant membrane assays (2). The late time points that we used to measure the NOX2 activity in the WST-1 assay correspond to the values of the plateau reached when the reaction is over. We agree that using the linear part of the reaction is superior as it allows calculating the TO values necessary for comparison between different studies. However, the specificity of our values were robustly proven as the signal was inhibited by SOD, indicating that WST-1 measures O2∗·; it was absent when NADPH was omitted and when membranes isolated from NOX2 knockout PLB-985 membranes were used (2). The new set of experiments presented here provides the absolute values requested by Dr. Pick and adds further evidence that GSK2795039 is a potent NOX2 inhibitor.

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References


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**Abbreviations Used**

- **ROS** = reactive oxygen species
- **SOD** = superoxide dismutase
- **TO** = turnover
- **WST-1** = water soluble tetrazolium salt