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Triple Quad LC-MS for detection of luciferin-6'-p-nitrobenzyl ether – a footprint of peroxynitrite

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Peroxynitrite

Peroxynitrite ($ONOO^-$), a highly reactive oxidizing and nitrating agent is generated via a spontaneous, diffusion-controlled reaction between nitric oxide ($^{\bullet}NO$) and superoxide radical anion ($O_2^{\bullet-}$). Peroxynitrite has been implicated as a key pathophysiological intermediate in various diseases, including acute and chronic inflammatory processes, diabetes, sepsis, ischemia-reperfusion, atherosclerosis and neurodegenerative disorders as well as chemotherapy-induced nephrotoxicity. Low steady state concentrations and unfavorable spectroscopic properties make direct detection of peroxynitrite in cells practically impossible. One of the methods to detect $ONOO^-$ is to use a chemical probe which is selective and/or forms a footprint product after reaction with this oxidant.

Oxidation of arylboronic acid

Over the last decade [1], it has been demonstrated that several oxidants such as hydrogen peroxide, hypochlorous acid, protein hydroperoxides and peroxynitrite convert arylboronic acids ($ArB(OH)_2$) to phenolic products ArOH (figure 1). However, the reactivity of boronates with peroxynitrite ($ONOO^-$) is of special interest. Not only the rate constant of the reaction of boronate probes with $ONOO^-$ is the highest of all tested biological oxidants, but the reaction also typically leads to the formation of minor but $ONOO^-$-specific products ($ArNO_2$, ArH) in addition to the major phenolic product (ArOH). Since the minor pathway is specific only for the reaction of boronates with peroxynitrite, formation of these products ($ArNO_2$, ArH) may serve as a unique footprint providing a diagnostic marker for peroxynitrite formation in cells-free and cellular systems.

Peroxy-caged luciferin (PCL-1) as probe for peroxynitrite detection

Bioluminescent imaging (BLI) is commonly used for sensitive monitoring of various biomolecular processes in cells and living animals. Generally, BLI employs firefly luciferase and its highly specific substrate luciferin (Luc-OH) to produce light in the presence of ATP, O_2 and Mg^{2+}. One of the first luciferin-based bioluminogenic probes designed for imaging of hydrogen peroxide in living systems was peroxy-caged luciferin, PCL-1 (see figure 2A) [2]. The hydroxyl group of Luc-OH is alkylated by the boronobenzyl moiety in PCL-1 which prevents recognition of the probe by luciferase enzyme. Upon reaction of PCL-1 with H_2O_2, the boronate moiety is replaced by the hydroxyl group, leading to the elimination of quinone methide and formation of the luciferase substrate Luc-OH.

However, peroxynitrite oxidizes arylboronic acid nearly one million times faster than does H_2O_2. The high rate constant of the reaction of PCL-1 probe with $ONOO^-$ ($k \sim 10^6 M^{-1}s^{-1}$ [3]) made it possible to detect peroxynitrite in the presence of its physiological scavengers (e.g., CO_2, glutathione, albumin) in cell-free and cellular systems. Recently, we characterized in detail the reaction intermediates and stable products formed in the reaction between PCL-1 probe and selected inflammatory oxidants (H_2O_2, HOCl, $ONOO^-$) [4].

In that study, a protocol was proposed based on profiling all products formed during peroxynitrite-induced oxidation of PCL-1. The scientists detected: (i) major primary phenolic intermediate

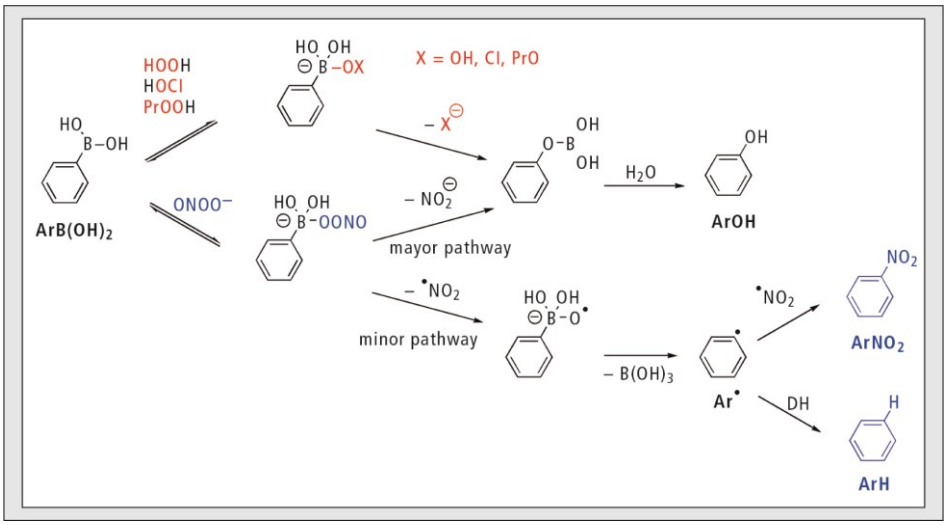

Fig 1: Oxidative conversion of arylboronic acid ($ArB(OH)_2$) induced by different oxidants (hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), protein hydroperoxides (PrOOH), peroxynitrite ($ONOO^-$)). Reaction between peroxynitrite and arylboronic acid leads to the major phenolic product ArOH and the minor peroxynitrite-specific products ($ArNO_2$, ArH).

Luc-Bz-OH; (ii) stable phenolic product, Luc-OH, formed after elimination of quinomethide QM, and (iii) minor products: luciferin-6'-p-nitrobenzyl ether Luc-Bz-NO_2 and luciferin-6'-benzyl ether Luc-Bz-H (figure 2A).

Formation of Luc-Bz-NO_2 in the reaction of PCL-1 with $ONOO^-$ includes the incorporation of part of the oxidant into the product and provides an opportunity to specifically detect and identify peroxynitrite in biological systems.

LC-MS protocol

LC-MS analyses of PCL-1 and its oxidation products were performed using Shimadzu Triple-Quad-LC-MS coupled to Shimadzu Nexera X2 UHPLC system. Analysis was done on Cortecs C18 column (Waters, 50 mm x 2 mm, 1.6 µm) equilibrated with 10% of acetonitrile in water containing 0.1% of formic acid. The compound was eluted by increasing the acetonitrile concentration in the mobile phase from 10 to 80% over 4 minutes. The flow rate was set at 0.5 mL/min and the flow was diverted to waste during the first minute and after 4 minutes, counting from the time of injection.

PCL-1, Luc-OH, Luc-Bz-NO_2 and Luc-Bz-H were detected as positive ions using multiple reaction monitoring (MRM) mode, with primary/fragment ion pairs of 415 > 135, 281 > 235, 416 > 234 and 371 > 91, respectively. Luc-Bz-OH was detected in positive mode using single ion monitoring (SIM) mode, set at the m/z value of 387 [4].

Observations

Oxidation of PCL-1 by excess H_2O_2 leads to the formation of the primary phenol Luc-Bz-OH, detectable when sample was analyzed immediately (< 2 min) after mixing (figure 2B, C). After one hour incubation, no Luc-Bz-OH was observed and the probe had been completely converted into luciferin (Luc-OH). Similar behavior was noticed when peroxynitrite was used instead of H_2O_2, but in addition to the phenolic products, Luc-Bz-NO_2 formation was observed. Furthermore, when the reaction was performed in the presence of 2-propanol (2-PrOH), applied as a hydrogen atom donor to the phenyl radical, additional product Luc-Bz-H formed in the reaction of Luc-Bz• with 2-PrOH was detected (figure 2C). Application of the probe to the

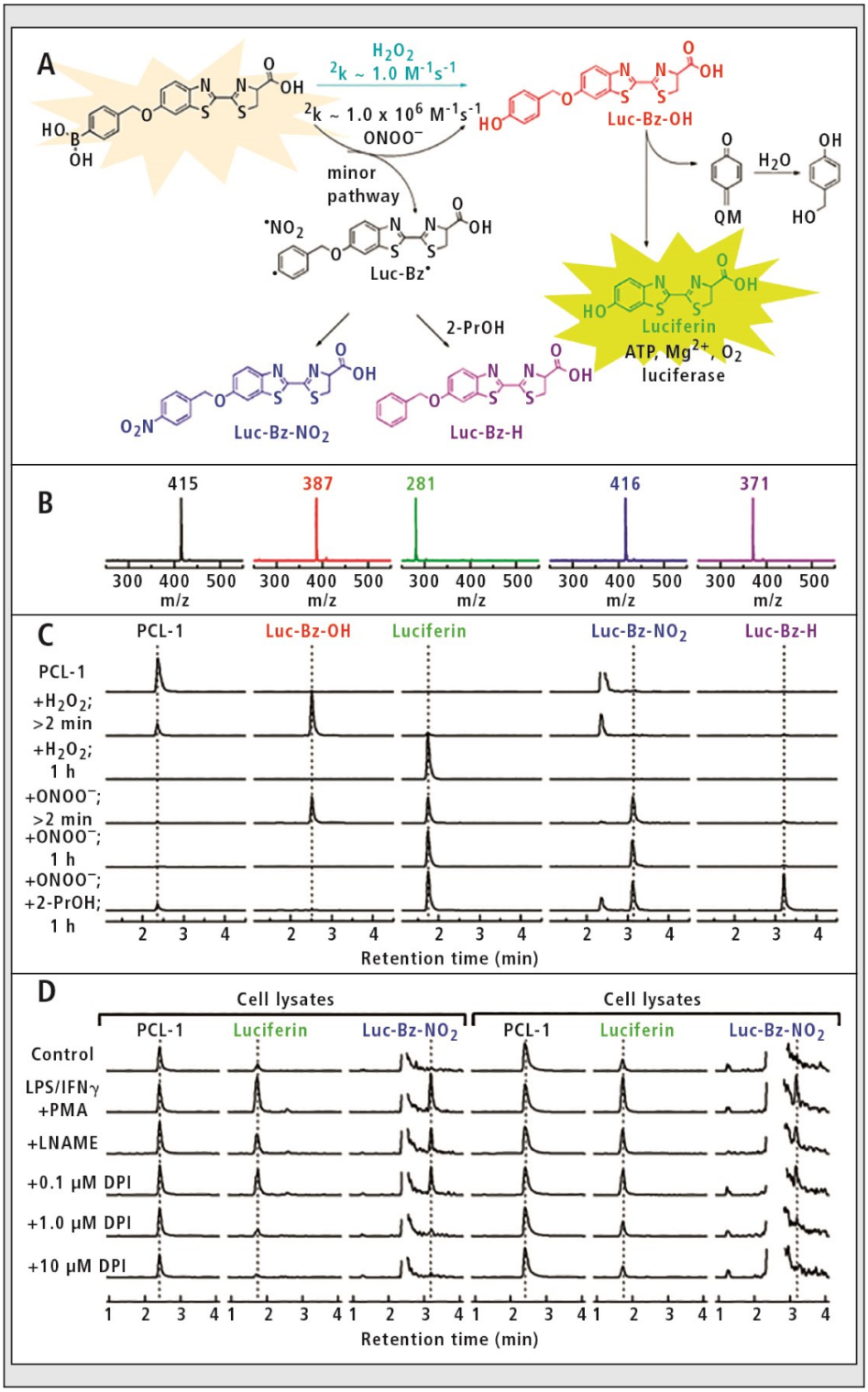

Fig. 2: LC-MS analyses of the products of PCL-1 oxidation.
(A) Scheme of peroxynitrite-induced transformation of PCL-1 probe leading to firefly luciferin and peroxynitritespecific products: luciferin-6'-p-nitrobenzyl ether (Luc-Bz-NO_2), and luciferin-6'-benzyl ether (Luc-Bz-H).
(B) Online mass spectra recorded for each product and
(C) LC-MS traces of the reaction mixtures of PCL-1 (100 µM) alone or after addition of H_2O_2 (10 µM) or $ONOO^-$ (80 µM).
(D) LC-MS/MS traces of PCL-1, luciferin and Luc-Bz-NO_2 detected in RAW 264.7 macrophages activated to produce $ONOO^-$ [modified from ref. 4]

RAW 264.7 macrophages activated to produce both $^{\bullet}NO$ and $O2^{\bullet-}$ led to increased probe oxidation and appearance of the peroxynitrite-specific product Luc-Bz-NO_2 (figure 2D).

Conclusions

The protocol allows LC-MS-based profiling of different oxidation products formed from the PCL-1 probe, including the $ONOO^-$-specific product, luciferin-6'-p-nitrobenzyl ether Luc-Bz-NO_2. Using this method, it was demonstrated that Luc-Bz-NO_2 is formed by activated RAW 264.7 macrophages incubated in the presence of the PCL-1 probe, proving the formation of $ONOO^-$ upon co-production of $^{\bullet}NO$ and $O2^{\bullet-}$ in cellular systems [4]. PCL-1-based bioimaging of $ONOO^-$, in combination with LC-MS-based detection of the Luc-Bz-NO_2 provides an opportunity to detect, localize and identify this inflammatory oxidant in vitro and in vivo.

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The team's current research interests are focused on the application of fluorescent and bioluminescent dyes in the detection of reactive oxygen, nitrogen and sulfur species. The research involves design and synthesis of fluorescent dyes; characterization of novel fluorescent compounds; application of optical probes in cell-free systems as well as in intracellular milieu. The main research goals are achieved by using organic synthesis in combination with chemical and spectroscopic characterization of synthesized probes applying fluorescence and HPLC techniques. In addition, the research works focus on the design and synthesis of novel dyes engineered for functionalization of textile materials and plastics, and development of new photosensitizers of polymerization.

The Institute edcates students in the field of "Dyes and Household Chemicals Technology." The program prepares students for solving of practical tasks which they may encounter in their professional activity.

The Free Radical Research Laboratory led by Dr. Jacek Zielonka, focuses on understanding the role of cellular oxidants and redox signaling pathways in cancer proliferation and anticancer treatments. This includes the development of new, rapid methods for detection of reactive oxygen and nitrogen species, and applicationof high-throughput screening for development of new inhibitors of NADPH oxidases as possible cancer therapeutics. Another research topic being explored is exploitation of the differences in bio-energetic profiles and redox status between normal and cancer cells for development of novel strategies to selectively target cancer cells. Specifically, the scientists develop mitochondria-targeted redox modulating agents to be used alone or in combination with standard-of-care drugs to inhibit the growth of cancer cells in vitro and in vivo.

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