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Small-molecule optical probes for cell imaging of protein sulfenylation and their application to monitor cisplatin induced protein oxidation

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ABSTRACT

Reactive oxygen species (ROS) are considered versatile second messengers mediating fundamental biological functions. A molecular pathway by which ROS determine functional diversity is the selective oxidation of cysteine residues to form sulfenic acid (SOH) products, known as sulfenylation or *S*-hydroxylation. This crucial post-translational modification is responsible for the alteration of protein stability, function and signalling. Despite considerable advances on the identification of sulfenic residues on individual proteins, improved methods are needed for direct visualization and accurate quantification of the extent of total protein sulfenylation. Herein we present the synthesis of two new cell-permeable fluorescent probes containing dimedone (a cyclic β-diketone with high specificity for sulfenic acids), and apply them to study oxidation processes in individual cells *via* microscopy. The low cytotoxicity, cell permeability and optical features of the probes allowed us to visualize and quantify the oxidation of cysteine residues in live cells during H_2O_2-mediated oxidative burst (*i.e.* exogenously administered H_2O_2). We present preliminary cellular imaging studies with these probes to analyse the oxidation process in cells treated with the anticancer drug cisplatin.

1. Introduction

Reactive oxygen species (ROS) can be generated in response to different stimuli, such as cytokines, G-protein coupled receptor (GPCR) agonists and growth factors, contributing to the so-called 'redox imbalance' due to endogenous oxidative stress in live cells [1]. H_2O_2, $HO^{\bullet}$ and $O_2^{\bullet -}$ are the most common ROS species, being widely involved in bio-macromolecule damage and influencing aging-associated processes, as well as pathological conditions related to cancer and neurodegenerative disorders [2–5]. Cells are commonly exposed to increasing oxidative stress mainly due to mitochondrial disorders, cancer progression and raised metabolic activity [6,7]. It is well-established that the overproduction of ROS may lead to aberrant oxidations of cellular components affecting protein-protein interaction and resulting in different dysfunctions related to homeostatic cell growth, trafficking and apoptosis [6]. For example, elevated ROS can produce aberrant proliferation signals and, consequently, promote malignancy and tumour growth [8]. More recent studies also indicate that ROS can have a role as second messengers in intracellular biochemical mechanisms and pathways [7], often associated to the chemo-selective oxidation of cysteines' thiol in proteins [9].

Sulfenic acids (SOHs) have been often found as first products of oxidation during the reaction of a cysteine thiol/thiolate and ROS. They are crucial players in redox-mediated signalling and protein function, although sulfinic (SO_2H) or sulfonic (SO_3H) acids can also be formed [10–12]. In general, SOHs in proteins are transient and labile, allowing further reversible reduction/oxidation of the intermediates, which in turn could either restore the original thiol or react with local nucleophiles (*e.g.* to form disulfides and sulfenylamide derivatives) [13–15]. Due to the highly reactive nature of SOHs, understanding their physiological roles and quantifying their formation in live cells remains a challenge. On the other hand, considering their wide biological relevance, there is increasing evidence that a deeper knowledge of the connection between ROS

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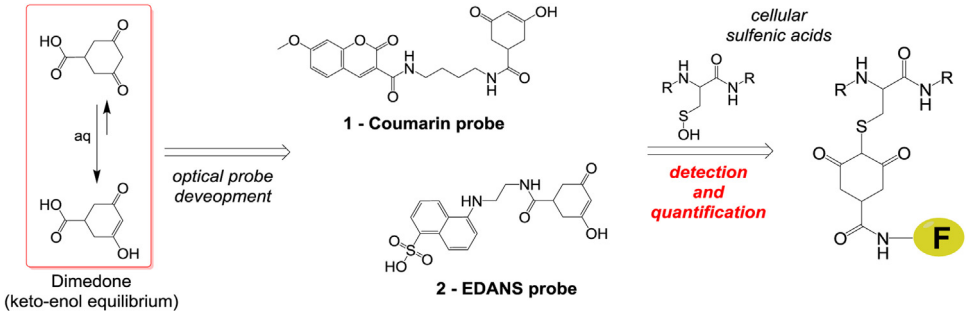

Fig. 1. Optical probes and rational design of the SOH-targeting method (F = fluorogenic residue to allow cellular imaging).

signalling and cysteine sulfenic oxidation could lead to the development of therapeutics that target diseases on the basis of the cellular redox status [16,17].

A number of previous reports have shown the use of dimedone as a valuable reactive group to generate chemical tools which are selective for sulfenic acid detection in cells and tissues (*i.e.* no cross reaction with other functional groups in proteins, as well as with different cysteine oxoforms) [18–21]. The majority of these probes are based on the use of biotin-dimedone constructs both pre-assembled (*e.g.* DCP-Bio1®) or in cell-coupled (*i.e.* azide-tagged analogues for click strategies) [22–24]; these in turn have been used to visualize SOH-modified proteins *via* 'bulk analysis' in cell lysate (*e.g.* ELISA or gel-electrophoresis) and Western blot (*i.e.* affinity enrichment with streptavidin) [25], or by mass spectrometry analysis of labelled proteins [26,27]. Although biotin-dimedone probes have proven to be useful to study protein sulfenylation, they have limitations mainly due to the size of the streptavidin (not allowing live cell measurements) and difficulties in safely interpreting all streptavidin affinity-purified proteins as sulfenic products [25]. A strategy to address this is to use dimedone directly attached to a fluorophore tag for direct SOH visualization. While some examples of this type of probe have been reported [21,28,29], detailed studies to determine their effective application in cell analysis are still sparse.

Herein we report the synthesis of two new cell-permeable and non-toxic optical probes (Fig. 1) to study SOH formation. We show that these probes can detect cysteine sulfenic acid in live cells (*i.e.* MCF7 and MDA-MB-231) by fluorescence microscopy, which allowed us to quantify and monitor cellular localization of oxidised product formation among different cellular compartments during a burst of H_2O_2 (as archetypal ROS species). Our new probes make use of the well-known reaction between sulfenic groups and a range of 5- and 6-membered cyclic β-diketones (*e.g.* dimedones) to form the corresponding thioether (see Fig. 1) [30].

As a proof of concept, we have additionally applied our new optical probes to detect for the first time the increase of sulfenic acid products in live cells following administration of the anticancer agent cisplatin [31], known to induce mitochondrial damage and impairment of respiration and thus resulting in the production of endogenous ROS [32–34]. These studies allowed the quantification of drug-induced SOH levels, proposing the current approach as a useful means to monitor oxidative imbalance in therapeutically relevant contexts.

2. Material and methods

All the experimental details for the synthesis and characterisation of new compounds reported in this paper, the cell culture protocols and image analysis methods, can be found in the Supplementary information.

2.1. Detection of sulfenic acid in live cells with dimedone-based probes 1 and 2

2.1.1. Sample preparation and cell treatment

The relevant solutions of **1** and **2** for the detection of sulfenylation in live cells were prepared freshly before each experiment. Briefly, a 100 mM solution of **1** or **2** in dimethylsulfoxide was obtained by dissolving the solid powder and further dilute to 10 mM in DPBS. From the latter, the working solutions ranging from 0.05 to 1 mM in final concentration were prepared by dilution either in DPBS or in DMEM without FBS (DMEM$^-$) as indicated in the relevant discussion. Before the addition of the solution containing **1** or **2**, the growing media containing FBS was removed and the adhered cells were washed three times with abundant DPBS to remove any trace of FBS. Finally, 100 μL of **1** or **2** working solution was added into each well and incubated for 15 min at 37 °C before the start of each experiment. For each set of experiments samples were prepared in triplicate.

2.1.2. Protein sulfenylation in live cells in response to exogenous H_2O_2

For the detection of sulfenylation in live cells under exposure to H_2O_2, cells were pre-treated for 15 min in DPBS or DMEM$^-$ containing 1 mM of **1** or **2**. A 100 mM solution of H_2O_2 was obtained from the 30% w/w in water stock solution (Sigma-Aldrich, #H3410) immediately before use in DPBS and then added to the pre-treated sample containing **1** or **2** to the desired final concentration of H_2O_2 in the wells.

2.1.3. Co-staining of protein sulfenylation and mitochondria

For mitochondria staining and co-localization experiments with **1** or **2**, the MytoTracker™ Deep Red (Invitrogen, #M22426) was used following the manufacturer instruction. Briefly, cells were incubated with the desired concentrations of 10, 25, 50 and 100 nM of MytoTracker™ for 30 min in DMEM$^+$. After the incubation step, the media was removed, cells were washed with DPBS and DMEM$^-$ containing 1 mM of **2** was added (incubated for further 30 min).

2.1.4. Assessing protein sulfenylation in response to cisplatin treatment

MCF7 and MDA-MB-231 were treated for 1 h in DMEM$^+$ containing 1, 10 or 25 μM cisplatin obtained by dilution from a 3.3 mM (1 mg/mL) stock solution 150 mM in NaCl and 0.01% w/v in mannitol. After incubation, media was removed and cells were washed three times with DPBS to remove FBS and then incubated with DMEM$^-$ and 1 mM of probe **2** for 30 min.