Small-molecule optical probes for cell imaging of protein sulfenylation and its 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 H₂O₂-mediated oxidative burst (i.e. exogenously administered H₂O₂). We present preliminary cellular imaging studies with these probes to analyse the oxidation process in cells treated with the anticancer drug cisplatin.

Keywords: optical probe, dimedone, sulfenic acid, cellular microscopy, fluorescence co-localization, 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 , $\text{HO}^\bullet$ and $\text{O}_2^{\bullet-}$ 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 sulfenyl-amide 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 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 (Figure 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 H₂O₂ (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 Figure 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.

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

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 minutes 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 H₂O₂. For the detection of sulfenylation in live cells under exposure to H₂O₂, cells were pre-treated for 15 minutes in DPBS or DMEM⁻ containing 1 mM of **1** or **2**. A 100 mM solution of H₂O₂ 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 H₂O₂ 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 minutes 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 minutes).

2.1.4 Assessing protein sulfenylation in response to cis-platin treatment. MCF7 and MDA-MB-231 were treated for 1 hour 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 minutes.

2.1.5 Cell fixation and removal of unbound dimedone-based probes. After each experiment, samples were prepared for fluorescent microscopy analysis as follows: to remove the excess of **1** or **2**, the solution in the wells was removed and cells were rinsed three times with DPBS. Cells were fixed by treatment with a 70% ice-cold ethanol solution in DPBS v/v for 40 minutes on ice. Then, the 70% ethanol solution was removed and cells rinsed five times with DPBS at room temperature. Finally, the wells were filled with a 50 % w/v glycerol (Sigma-Aldrich, Bio-Reagent purity grade) in DPBS to perform microscopy analysis.

2.2 Light Fluorescence Microscopy. These studies were carried out with a Nikon Eclipse-Ti inverted microscope equipped with motorized stage and perfect focus system (PFS) to address focus drift in axial fluctuation during tile scan acquisition. The NIS-Elements Nikon software was used throughout the microscopy experiments to control the stage movement, exposure time in individual channels and camera recording set up. The Intensilight Hg lamp illumination system (Nikon) was used throughout the experiments for excitation of fluorophores in the samples in combination with the appropriate dichromatic beam splitter filter cubes for the wavelengths of interest. For **1**, a UV-2B filter cube was used ($\lambda_{\text{ex}} = 330\text{-}380\text{ nm}$; DM= 400 nm LP; $\lambda_{\text{em}} = 435\text{ nm LP}$). For **2**, a HYQ-FITC cube was used ($\lambda_{\text{ex}} = 460\text{-}500\text{ nm}$; DM= 505 nm LP; $\lambda_{\text{em}} = 510\text{-}560\text{ nm}$). Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI) was visualized with a DAPI filter ($\lambda_{\text{ex}} = 325\text{-}375\text{ nm}$; DM= 400 nm LP; $\lambda_{\text{em}} = 435\text{-}485\text{ nm}$). Mitochondria signal from MytoTracker™ Deep Red staining was obtained with a CY5 HYQ filter ($\lambda_{\text{ex}} = 590\text{-}650\text{ nm}$; DM= 660 nm LP; $\lambda_{\text{em}} = 663\text{-}738\text{ nm}$). For each experiment, a tile scan of 10x10 fields of view ($5120\text{ px} \times 5120\text{ px}$; $0.27 \frac{\mu\text{m}}{\text{px}}$; $1382\text{ }\mu\text{m} \times 1382\text{ }\mu\text{m} \rightarrow 1.94\text{ mm}^2$) in each individual channel was acquired with a Nikon 60x, NA 1.49, oil immersion objective and the epifluorescence signal was collected with an Andor iXon DU-897 EMCCD camera set at 16-bit, with an overclocked readout at 1 MHz and conversion gain at 2.4x, with the electron multiplier set at 80.

3. Results and Discussion

3.1 Synthesis of the dimedone-based optical probes

The coumarin-based optical probe **1** was synthesized *via* six steps as shown in Scheme 1.

Succinimidyl-activated 7-methoxycoumarin-3-carboxylic acid **4** was obtained as previously reported [35] and reacted with commercially available *N*-Boc-1,4-butanediamine **5** to yield compound **6**. After standard TFA-mediated protecting group removal, intermediate **7** was coupled with the previously described dimedone analogue **9** [27], using EDC, HOBt and Et₃N. The removal of the methyl group from intermediate **10** was carried out using LiOH to yield probe **1**.

Scheme 1. Synthetic route for coumarine-based optical probe **1** and EDANS-based optical probe **2**.
Reagents and conditions: **a)** NHS (1 equiv), DCC (1.1 equiv), DMF, 5.5 h, 0°C → rt; **b)** DMAP (0.1 equiv), DIPEA (2 equiv), **5** (0.95 equiv), CH₂Cl₂, N₂, 5 h, rt; **c)** TFA/CH₂Cl₂ 1:3, 1.5 h, rt; **d)** TsOH (0.05 equiv), CH₃OH, 1 h, rt; **e)** HOBt (1.0 equiv), Et₃N (1.4 equiv), EDC (1.4 equiv), **9** (1.1-1.4 equiv), DMF, N₂, 24-48 h, rt; **f)** LiOH (4 equiv), THF/MeOH/H₂O 4:1.6:1.6, 16 h, rt; **g)** CAN (0.1 equiv), CH₃CN/H₂O 1:1, 6 h, 100 °C.

In parallel, EDANS-based optical probe **2** was synthesised in three steps (Scheme 1). Commercially available EDANS fluorophore **11** was reacted with the protected dimedone derivative **9** [27] to

generate intermediate **12** through the same coupling conditions used to synthesise compound **10** (Scheme 1). The methyl removal on **12** was performed with cerium(IV) ammonium nitrate [36] to afford the EDANS optical probe **2**.

The identity and purity of **1** and **2** were confirmed by a combination of analytical and spectroscopic techniques (i.e. HPLC, ^1H and ^{13}C NMR spectroscopy, HR-MS and IR spectroscopy – see Supporting Information). The emission spectra for **1** and **2** were recorded and the excitation/emission wavelengths ($\lambda_{\text{ex}}/\lambda_{\text{em}}$) are: 348/403 nm and 335/500 nm respectively.

3.2 Sulfenic cysteine labelling and detection via microscopy approaches in live MCF7 cells

We first established that the probes were cell permeable (see Figure 3) and non-toxic at the concentrations required for imaging (see below for discussion). Subsequently, we determined their optimal concentration for the best signal-to-noise ratio in cellular imaging experiments employing the MCF7 breast cancer cell line. In addition, as a control, we recorded the fluorescence of the probes before and after addition of H_2O_2 to verify that the oxidant did not modify their photophysical properties; no major changes on the emission of the probes were observed (see Figure S1, Supporting Information).

Initially, different concentrations of **1** and **2** were tested in the absence/presence of 1 mM H_2O_2 (Figure 2). H_2O_2 is known to modulate signal transduction through oxidation of cysteine residues in proteins and is the oxidant of choice to study redox behaviours *in cellulo* [22,25]. Given the kinetically labile nature of the SOH moiety and its dependency on the redox state of the cell [1,11,14], it is expected that the presence of the reactive dimedone probe in the cell prior the treatment with H_2O_2 would allow a faster and more quantitative trapping of sulfenic groups while they form during an oxidative burst. Thus, live MCF7 cells were pre-treated for 15 minutes with either **1** or **2** in order to allow diffusion of the probe within the cell compartments prior to the

beginning of each experiment. After incubation with the probes, H₂O₂ was added directly into the wells to a final concentration of 1 mM for the treated sample, whereas the controls were not exposed to H₂O₂, and cells were further incubated for 30 minutes under normal conditions. Immediately after, cells were fixed with a 70% ice-cold ethanol solution and extensively washed to remove the unbound probe before analysis by fluorescent microscopy (see Methods for details).

The mean single cell fluorescence intensities of both probes **1** and probe **2** were calculated by scanning the image of a 1.9 mm² area in each well representing a single experiment and then identifying single cells in the scanned area. In order to extract quantitative information from the signals in the fluorescent channel of the probes, we developed an in-house method (macro run code available in the Supporting Information) in Fiji software for the quantification of the single cell fluorescent intensity as depicted in Figure S2. Briefly, the entire image acquired in each channel of interest was flattened to correct the illumination profile in the field of view and background subtracted. Single cell boundaries were identified to calculate the mean signal intensity from each individual cell, expressed as the ratio between the total pixel intensity and the integrated area within the cell boundaries. The identification of single cell boundaries was achieved via thresholding of pixel intensity between the background and the cell signal and used to create a mask for the algorithmic identification of each single cell in the whole area of the image with defined size and circularity after appropriate segmentation of touching cells (Figure S2). The results obtained using either probe **1** and **2** are shown in Figure 2. For increasing concentration of the probes (i.e. 0.05, 0.2 and 1 mM) in the absence of H₂O₂ (blue bars), the mean intensity of single cell fluorescence appears constant for probe **1** and with a proportional increase for probe **2**. These differences might be attributed to the distinct reactivity of each probe as well as their solubility and consequently cellular distribution. The sulfonic acid moiety of the EDANS fluorophore confers higher water solubility to **2** and may allow a faster and more homogeneous diffusion of this probe within the cytosol, resulting in a more efficient trapping of SOH groups within the cell compartments.

Furthermore, the auto-fluorescence signal in the blue-green fluorescent channels arising from intrinsic cell compounds like NAD(P)H, collagen and riboflavin was found to be below the intensity of the lowest concentration of probes tested (0.05 mM) in both the UV and FITC channels used to visualize **1** and **2**, respectively (Figure S3, Supporting Information). These observations highlight the ability of our probes to monitor the presence of SOH groups at the basal level of protein oxidation in MCF7 cells with **2** displaying a clear dose-dependent behaviour. Furthermore, when the cells were treated with 1 mM H₂O₂ (red bars in Figure 2) a marked increase in the mean fluorescent signal intensity for single cells was recorded for both probes. The increase in signal was found proportional to the increase in concentration of the probe used in each experiment, showing the selective response of the probes to hydrogen peroxide treatment (associated to SOH formation).

Figure 2. Mean intensity signal from MCF7 cells treated in absence and with increasing concentrations (i.e. 0, 0.05, 0.2 and 1 mM) of probes **1** (DAPI channel) and **2** (FITC channel). Blue bars represent the basal levels of SOH measured, whereas red bars show the SOH levels after treatment with 1 mM H₂O₂ in DPBS for 30 minutes at 37°. Error bars are the standard errors of the mean.

We found that the best ratio between the H₂O₂-treated versus the untreated sample was achieved using 1 mM concentration of the probes. Therefore, this concentration was chosen for all

subsequent experiments. Figure 3 shows representative images acquired for MCF7 cells before (3A, C, E, G) and after (3B, D, F, H) treatment with 1 mM H₂O₂ in the absence (Figure 3, top row) or presence (Figure 3, bottom row) of the probes at 1 mM concentration. These images show significantly higher emission intensity for H₂O₂-treated cells compared to the untreated ones with a 2.4-fold and 4.1-fold increase for **1** and **2** respectively, indicating the ability of both probes to discriminate SOH in MCF7 cells at their basal level and in response to the exogenous oxidative stimuli with H₂O₂.

Figure 3. Representative fluorescent microscopy images for MCF7 cells treated with probe **1** (left panel, cyan colour for the 403 nm emission wavelength in the DAPI channel) and probe **2** (right panel, green colour for the 500 nm emission wavelength in the FITC channel). Images A-B (probe **1**) and images E-F (probe **2**) show the auto fluorescence signal before and after treatment for 30 minutes at 37 °C with 1 mM H₂O₂, respectively. Images C-D (**1**) and G-H (**2**) show MCF7 fluorescence signal in the presence of the probes at 1 mM concentration to reveal the presence of sulfenic acid groups before and after exposure to 1 mM H₂O₂ for 30 minutes at 37 °C. White arrows in each image show the nuclei localization to facilitate cell identification. Calibration bar for pixel intensity: range 0 – 2100 a.u.; scale bar 20 micron.

Considering that the probes were used in the mM range, we evaluated their possible cytotoxicity on MCF7 cells by monitoring the cell growth after incubation for 30 minutes at 37 °C with the probes

at 1 mM concentration. Being allowed to adhere for 24 hours, cells were firstly imaged in bright field for density and morphology evaluation before the treatment. After treatment with the probes, cells were washed to remove the excess of probes, replenish with fresh media and leave to grow under normal conditions. MCF7 cells were monitored for three days and no differences were observed in cell morphology and growth compared to control samples of untreated cells (see Figure S4). This indicates that the probes are not cytotoxic at the concentration (i.e. 1 mM) needed for imaging and quantification of sulfenic acids in our methodology. Considering the higher sensitivity, its cellular distribution and no apparent cytotoxicity, we used probe **2** to carry out further investigation on SOH formation in cells.

3.3 Cellular localization and levels of protein sulfenylation in MCF7 and MDA-MB-231 breast cancer cell lines

We next investigated the sub-cellular localization of SOH residues at the basal level and after oxidative stress induced by H₂O₂ treatment. In addition to MCF7, we also performed experiments on a second breast cancer cell line, namely MDA-MB-231. Despite both being breast cancer cell lines, MCF7 and MDA-MB-231 are well known to differ in many aspects. MCF7 is a luminal breast cancer cell line with an epithelial-like morphology and it is widely used as *in vitro* model for oestrogen and progesterone receptor positive breast cancer studies. On the other hand, MDA-MB-231 is a basal breast cancer cell line with a mesenchymal-like morphology with a complete lack of hormonal receptors which is widely used as *in vitro* model for triple negative breast cancer, a more malignant, invasive and chemo resistant type compared to MCF7 phenotype [37]. We hypothesized that the different molecular features between the two cell lines could results in distinct responses in respect to the production of SOHs when stimulated by exogenous oxidative stress. Figure 4 shows

the microscopy images after incubating the cells with probe **2** (1 mM, 30 minutes) before and after treatment with H₂O₂.

Figure 4. Microscopy images for MDA-MB-231 (top) and MCF7 (bottom) cells treated with 1 mM of probe **2** before (left) and after (right) exposure to 1 mM H₂O₂. Blue channel: DAPI; green channel: FITC (**2**). Images are shown with a contrast suitable to highlight the SOH localization in each cell line. For MDA-MB-231, pixel range intensity shown is 100-3000; for MCF7 the pixel range intensity shown is 100-800.

From these experiments, the staining pattern highlights the presence of SOHs in the perinuclear region of the cells with a punctate nature which becomes more pronounced after incubation with H₂O₂. We recorded major differences between the two cell lines in terms of signal intensity associated to the detection of SOHs. Interestingly, for the basal levels of SOHs we found a 7-fold lower intensity in MCF7 than MDA-MB-231 cells, which suggest different levels of SOH for the two cell lines during their normal proliferation in standard growing conditions. After treatment with

1 mM H₂O₂, the MCF7 cells showed a 4-fold increase in SOH signal whereas for the MDA-MB-231 we recorded a 1.5-fold increase, relative to their starting basal levels. These data support previous findings showing variable basal levels of SOHs in different cells [38-40], which may be related to differences in the redox state of each specific cell line [41-43]. Noteworthy, we observed a distinct response between the two cell lines to the oxidative stress caused by treatment with H₂O₂, with the MCF7 being 2.6 times more susceptible than MDA-MB-231 in increasing the amount of SOHs following the addition of H₂O₂. While we cannot discard the possibility that the differences observed between the cell lines may be partially due to differences in the probe's cellular uptake, this is unlikely to account for a 7-fold increase in intensity in MDA-MB-231 as compared to MCF7. Our data reveal that an intrinsic difference in the basal levels of protein sulfenylation exists for the two different cell lines, which could be due to differences in: i) the overall redox state of the cell and its subcellular compartments (i.e. ROS levels); ii) the diverse signalling pathways responding to increased oxidative stress (which we mimic with a burst of exogenous H₂O₂).

3.4 Oxidation in mitochondria contributes to protein sulfenylation at the basal level in MCF7 but not in MDA-MB-231

Being the organelles in the cell responsible for the energy (ATP) production, mitochondria are known to produce endogenous ROS species during the process of cellular respiration. As a consequence, high levels of protein oxidation are found in mitochondria and hence they represent an ideal site for the formation of protein-SOH groups [44-46]. Considering that probe **2** showed a punctate staining pattern in the cell lines under study (see Figures 3 and 4), we hypothesised that SOH formation could be derived, at least partially, by the activity of mitochondria and, consequently, localized in their proximity. With this aim, we investigated the localisation of SOH modifications by using **2** in combination with the mitochondria-specific probe MytoTracker™ Deep

Red (MDR) – a well-established dye that accumulates preferentially in active mitochondria; $\lambda_{em}=665$ nm), but covalently binds to the free sulfhydryl groups (SH) within the active mitochondria.

Figure 5 shows representative images for MCF7 and MDA-MB-231 cells after pre-incubation with MDR for 30 minutes, followed by treatment with **2** (1 mM) for a further 30 minutes to trap sulfenic acid moieties at the basal levels in the two cell lines. As can be seen, we found significant co-localisation of both dyes in MCF7 cells indicating the presence of SOH groups in mitochondria (co-localization range: 34-72%). This is analogous to previous studies with a different dimedone-based fluorescent probe in Chang liver cells, which also showed higher levels of protein oxidation in mitochondria [28].

Interestingly, our studies showed that there is minimal co-localisation of **2** and MDR in mitochondria, in the case of MDA-MB-231 cells (co-localization range: 8-22%), with a narrower distribution of probe **2** in the perinuclear region of the cells. These results suggest a different distribution of SOH groups in the cellular compartments, especially in the light of a 7-fold higher level of protein sulfenylation found at basal level for MDA-MB-231 compared to MCF7 (Figure 4).

Figure 5. MCF7 cells (top) and MDA-MB-231 (bottom) stained for 30 minutes with 100 nM of MDR (Cy5-red channel) and probe **2** for the detection of protein sulfenylation (FITC-green channel). Nuclei are stained in blue (DAPI channel). From the merged image the yellow represent the co-localized signal of mitochondria and sulfenic acid groups. Contrast for DAPI and Cy5

channels are the same for the top and bottom images. Contrast for **2** in the FITC channel differs between top and bottom images to better visualize the cellular localization of SOH groups.

The differences in co-staining for the two cell lines might be due to the availability of free SH groups (which represent potential sites for the formation of SOH groups), which in turn might be affected by the MDR probe. MDR binds covalently to thiol groups in proteins through the thiol-reactive chloromethyl moiety, facilitating its retention during the fixation procedure. Therefore, the presence of MDR could reduce the formation of SOH derivatives by blocking some of the available thiol groups within the mitochondria from oxidation by endogenously produced ROS. To investigate this further, MCF7 and MDA-MB-231 cells were treated for 30 minutes in complete media containing MDR at 10, 20, 50 and 100 nM concentrations prior to the addition of **2** (1 mM). As shown in Figure 6, the single cell frequency distribution of **2** (green plots) is characterised by a shift towards lower intensities, which is in an inversely proportional fashion to the increase of MDR signal (red plots) in the sample for MCF7 cells but not for MDA-MB-231. The behaviour observed for MCF7 suggests the presence of a dynamic formation of protein SOH groups within the mitochondria due to the availability of free protein SH groups and endogenous ROS species within the experimental time frame (30 minutes). By covalently blocking the free protein SH groups we were able to detect a decrease in SOH formation with increasing concentration of MDR. In contrast, the MDA-MB-231 cell line behaved differently, indicating a more pronounced oxidation of thiols (SHs) to SOHs within the mitochondria, as demonstrated by the higher 7-fold intensity found at the basal level (Figure 4).

Overall, the results show the presence of multiple sites of SOH formation which in part occurs within mitochondria, as well as in the perinuclear region of the cells where it has been already observed before in connection with the endoplasmic reticulum (ER) [47,48].

Figure 6. Single cell distribution plots for MCF7 (left panel) and MDA-MB-231 (right panel) treated with increasing concentrations of MDR (red plots) and 1 mM of **2** (green plots) to measure protein sulfenylation localized in the mitochondria. Cells were pre-treated for 30 minutes with increasing amounts of MDR as indicated in the plots, followed by incubation for an additional 30 minutes with **2** (1 mM). Standard error of the mean was $\leq 1\%$ of the mean values for the MDR signals and $\leq 5\%$ of the mean values for the FITC channel for probe 2 signal.

3.5 Protein sulfenylation response to cisplatin-induced oxidative stress

The treatment of cell lines with the anticancer agent cisplatin is known to promote apoptosis through a variety of mechanisms, including the disruption of their redox state [31,49,50]. On the other hand, protein sulfenylation is highly dependent on the variation in the redox state within the cell and is reversible, therefore acting as a dynamic, non-definitive signal modification in the

response mechanism to cellular stress. In *in vitro* experiments, cisplatin reduces the cell viability and the proliferative capacity at the doses commonly reached in tissues (10-20 μM) in clinical treatments [33,34]. Interestingly, MCF7 and MDA-MB-231 exhibit a different susceptibility to cisplatin, with the MCF7 showing the faster decrease in cell proliferation and viability compared to the MDA-MB-231 [51]. In MDA-MB-231, cisplatin toxicity appears closely related to a general increase in ROS production and a fast reduction in oxygen consumption immediately after administration, whereas in MCF7 changes are visible only after 5-6 hours [41,52]. On the other hand, cisplatin accumulates in the mitochondria which have been proposed as primary targets for cisplatin-mediated DNA damage [34,50]. There have also been studies showing that cisplatin derivatives induce the formation of sulfenic acid in cysteine Cys34 from Human Serum Albumin [53].

Having established the ability of **2** to image SOH in cells, we were interested in applying this probe to study a process in which cells are subjected to endogenous oxidative stress by the addition of cisplatin. In Figure 7, we show the microscopy results for the treatment of both cell lines (MCF7 and MDA-MB-231) with 1, 10 and 25 μM cisplatin administered for 1 hour followed by incubation with **2**.

Figure 7. Single cell distribution for protein sulfenylation detected with probe **2**. From top to bottom: dose-dependent treatment with cisplatin at (A-E) 0, (B-F) 1, (C-G) 10 and (D-H) 25 μM . Left panel: representative images for MCF7 cells in DAPI (blue channel), FITC (green channel for probe **2**) and merged channels of the single cell distribution plots for the tested concentrations of cisplatin. Right panel: representative images for MDA-MB-231 cells in DAPI (blue channel), FITC (green channel for probe **2**) and merged channels of the single cell distribution plots for the tested concentrations of cisplatin. Red stars in each distribution plot represent the mean value of the population distribution.

The MCF7 cells showed increased protein sulfenylation when they were treated with increasing concentrations of cisplatin, with a general shift of the entire population towards higher signal intensities (Figure 7A-D). Furthermore, a small fraction of the cell population with higher levels of SOHs already appears at the low dose of cisplatin administered (1 μM , Figure 7B) and further increases at higher doses, contributing to a heterogeneous response within the overall population. On the other hand, the MDA-MB-231 cell line (Figure 7E-H) shows an equal and modest increase

in the mean value of the protein sulfenylation across the entire range of cisplatin concentrations compared to the control sample. In MCF7 cells the signal from SOH groups appears initially localized in the perinuclear region at the basal level (Figure 7A). With the increase in cisplatin concentration during treatment, the signal intensity increases in the perinuclear region and it extends into the surrounding cytosolic fraction, as well as localises in the mitochondria with a punctate nature (Figure 7B-D). In contrast, the staining of SOH groups in MDA-MB-231 appears not punctate in nature and narrowly localized in the perinuclear region of the cells in the control as well as in the cisplatin treated samples (Figure 7E-H). We found that the MCF7 cells hold: i) a higher potential than MDA-MB-231 in terms of SOH formation after treatment with 1 mM H₂O₂ (i.e. 4-fold increase in MCF7, 1.5-fold increase in MDA-MB-231) and ii) a significant difference in the presence of SOH groups at the basal level (i.e. 7-fold lower SOH signal for MCF7 than MDA-MB-231). These observations suggest an active and dynamic role of protein sulfenylation in response to cisplatin damage. For cells with a low presence of SOH groups at the basal level (i.e. MCF7), the oxidative stress introduced by cisplatin prompts a rapid increase in SOHs that is proportional to the dose administered, a behaviour not mirrored in the MDA-MB-231 cells which have a higher basal level of SOH and can therefore progress to higher oxidative species like sulfinic and non-reversible sulfonic acids after cisplatin-induced stress.

4. Conclusions

In this work, we have developed two new fluorescent probes (**1** and **2**) for the direct detection and optical visualization of protein sulfenic residues. The two probes are cell permeable and not cytotoxic at the concentrations needed for cellular imaging allowing us to carry out a range of imaging studies in cells. Both these probes are able to detect the increase of SOH cellular levels upon treatment with H₂O₂. Probe **2** – while not as bright as a previously reported dimedone-based

probe with 7-aminocoumarin [28] – has shown to have higher sensitivity and solubility than **1**, as well as a more punctuated localisation in perinuclear regions of the cell. Co-staining microscopy studies with **2** and the well-established dye MDR demonstrate the presence of high levels of SOHs in mitochondria. Our observations also highlight different basal levels of SOH oxidation between two cell lines, with MCF7 cells showing a higher level of SOH formation in mitochondria with respect to MDA-MB-231 cells. We subsequently used probe **2** to monitor changes in protein sulfenylation in cells upon treatment with cisplatin. As expected, addition of the drug resulted in higher levels of SOHs which were detected by our probe (likely due to protein oxidative stress associated with cisplatin treatment).

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References

- [1] C.E. Paulsen, K.S. Carroll, Orchestrating redox signaling networks through regulatory cysteine switches, *ACS Chem. Biol.* 5 (2010) 47-62. <http://dx.doi.org/10.1021/cb900258z>
- [2] B. Halliwell, Oxidative stress and cancer: have we moved forward?, *Biochem. J.* 401 (2007) 1–11. <http://dx.doi.org/10.1042/BJ20061131>
- [3] J.E. Klaunig, L.M. Kamendulis, The role of oxidative stress in carcinogenesis, *Annu. Rev. Pharmacol. Toxicol.* 44 (2004) 239–267. <http://dx.doi.org/10.1146/annurev.pharmtox.44.101802.121851>

- [4] J. Watson, Oxidants, antioxidants and the current incurability of metastatic cancers, *Open Biol.* 3 (2013) 120144. <http://dx.doi.org/10.1098/rsob.120144>
- [5] A. Yarnell, Cysteine oxidation: New chemical tools are poised to help scientists explore the roles oxidized cysteine residues might play in biology, *C&EN News* 87 (2009) 38-40. <http://dx.doi.org/10.1021/cen-v087n040.p038>
- [6] G. Roos, J. Messens, Protein sulfenic acid formation: from cellular damage to redox regulation, *Free Radic. Biol. Med.* 51 (2011) 314-326. <http://dx.doi.org/10.1016/j.freeradbiomed.2011.04.031>
- [7] T. Finkel, Oxidant signals and oxidative stress, *Curr. Opin. Cell Biol.* 15 (2003) 247–254. [http://dx.doi.org/10.1016/S0955-0674\(03\)00002-4](http://dx.doi.org/10.1016/S0955-0674(03)00002-4)
- [8] S. Ragu, G. Faye, I. Iraqui, A. Masurel-Heneman, R.D. Kolodner, M.E. Huang, Oxygen metabolism and reactive oxygen species cause chromosomal rearrangements and cell death, *Proc Natl Acad Sci USA* 104 (2007) 9747–9752. <http://dx.doi.org/10.1073/pnas.0703192104>
- [9] L.B. Poole, K.J. Nelson, Discovering mechanisms of signaling-mediated cysteine oxidation, *Curr. Opin. Chem. Biol.* 12 (2008) 18–24. <http://dx.doi.org/10.1016/j.cbpa.2008.01.021>
- [10] R. Brigelius-Flohé, L. Flohé, Basic principles and emerging concepts in the redox control of transcription factors, *Antioxid. Redox Signal.* 15 (2011) 2335-2381. <http://dx.doi.org/10.1089/ars.2010.3534>
- [11] N.J. Kettenhofen, M.J. Wood, Formation, reactivity, and detection of protein sulfenic acids, *Chem. Res. Toxicol.* 23 (2010) 1633–1646. <http://dx.doi.org/10.1021/tx100237w>
- [12] R. Karisch, B.G. Neel, Methods to monitor classical protein-tyrosine phosphatase oxidation, *FEBS J.* 280 (2013) 459–475. <http://dx.doi.org/10.1111/j.1742-4658.2012.08626.x>

- [13] L. Billiet, P. Geerlings, J. Messens, G. Roos, The thermodynamics of thiol sulfenylation, *Free Radic. Biol. Med.* 52 (2012) 1473-1485. <http://dx.doi.org/10.1016/j.freeradbiomed.2011.12.029>
- [14] A.J. McGrath, G.E. Garrett, L. Valgimigli, D.A. Pratt, The redox chemistry of sulfenic acids, *J. Am. Chem. Soc.* 132 (2010) 16759–16761. <http://dx.doi.org/10.1021/ja1083046>
- [15] J. Pan, K.S. Carroll, Light-mediated sulfenic acid generation from photocaged cysteine sulfoxide, *Org. Lett.* 17 (2015) 6014–6017. <http://dx.doi.org/10.1021/acs.orglett.5b02981>
- [16] D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?, *Nat. Rev. Drug Discov.* 8 (2009) 579-591. <http://dx.doi.org/10.1038/nrd2803>
- [17] G.T. Wondrak, Redox-directed cancer therapeutics: molecular mechanisms and opportunities, *Antioxid. Redox Signal.* 11 (2009) 3013-3069. <http://dx.doi.org/10.1089/ARS.2009.2541>
- [18] P.A. Wages, K.S. Lavrich, Z. Zhang, W.-Y. Cheng, E. Corteselli, A. Gold, P. Bromberg, S.O. Simmons, J.M. Samet, Protein sulfenylation: a novel readout of environmental oxidant stress, *Chem. Res. Toxicol.* 28 (2015) 2411–2418. <http://dx.doi.org/10.1021/acs.chemrestox.5b00424>
- [19] A.T. Saurin, H. Neubert, J.P. Brennan, P. Eaton, Widespread sulfenic acid formation in tissues in response to hydrogen peroxide, *Proc. Natl. Acad. Sci. USA* 101 (2004) 17982–17987. <http://dx.doi.org/10.1073/pnas.0404762101>
- [20] C. Klomsiri, K.J. Nelson, E. Bechtold, L. Soito, L.C. Johnson, W.T. Lowther, S.-E. Ryu, S.B. King, C.M. Furdui, L.B. Poole, Use of dimedone-based chemical probes for sulfenic acid detection evaluation of conditions affecting probe incorporation into redox-sensitive proteins, *Methods Enzymol.* 473 (2010) 77-94. [http://dx.doi.org/10.1016/S0076-6879\(10\)73003-2](http://dx.doi.org/10.1016/S0076-6879(10)73003-2)

[21] L.B. Poole, B.-B. Zeng, S.A. Knaggs, M. Yakubu, S.B. King, Synthesis of chemical probes to map sulfenic acid modifications on proteins, *Bioconjugate Chem.* 16 (2005) 1624-1628.

<http://dx.doi.org/10.1021/bc050257s>

[22] K.E. Crump, D.G. Juneau, L.B. Poole, K.M. Haas, J.M. Grayson, The reversible formation of cysteine sulfenic acid promotes B-cell activation and proliferation, *Eur. J. Immunol.* 42 (2012) 2152–2164.

<http://dx.doi.org/10.1002/eji.201142289>

[23] N. Kaplan, N. Urao, E. Furuta, S.-J. Kim, M. Razvi, Y. Nakamura, R.D. McKinney, L.B. Poole, T. Fukai, M. Ushio-Fukai, Localized cysteine sulfenic acid formation by vascular endothelial growth factor: role in endothelial cell migration and angiogenesis, *Free Radic. Res.* 45 (2011) 1124–1135.

<http://dx.doi.org/10.3109/10715762.2011.602073>

[24] Y.H. Seo, K.S. Carroll, Profiling protein thiol oxidation in tumor cells using sulfenic acid-specific antibodies. *Proc. Natl. Acad. Sci. USA* 106 (2009) 16163–16168.

<http://dx.doi.org/10.1073/pnas.0903015106>

[25] Charles R.L. E. Schröder, G. May, P. Free, P.R.J. Gaffney, R. Wait, S. Begum, R.J. Heads, P. Eaton, Protein sulfenation as a redox sensor, *Mol. Cell Proteomics* 6 (2007) 1473-84.

<http://dx.doi.org/10.1074/mcp.M700065-MCP200>

[26] T.H. Truong, F.J. Garcia, Y.H. Seo, K.S. Carroll, Isotope-coded chemical reporter and acid-cleavable affinity reagents for monitoring protein sulfenic acids, *Bioorg. Med. Chem. Lett.* 21 (2011) 5015–5020.

<http://dx.doi.org/10.1016/j.bmcl.2011.04.115>

[27] K.G. Reddie, Y.H. Seo, W.B. Muse III, S.E. Leonard, K.S. Carroll, A chemical approach for detecting sulfenic acid-modified proteins in living cells, *Mol. Biosyst.* 4 (2008) 521–531.

<http://dx.doi.org/10.1039/b719986d>

- [28] Q. Yin, C. Huang, C. Zhang, W. Zhu, Y. Xu, X. Qiana, Y. Yang, In situ visualization and detection of protein sulfenylation responses in living cells through a dimedone-based fluorescent probe, *Org. Biomol. Chem.* 11 (2013) 7566-7573. <http://dx.doi.org/10.1039/C3OB41434E>
- [29] L.B. Poole, C. Klomsiri, S.A. Knaggs, C.M. Furdai, K.J. Nelson, M.J. Thomas, J.S. Fetrow, L.W. Daniel, S.B. King, Fluorescent and affinity-based tools to detect cysteine sulfenic acid formation in proteins, *Bioconjugate Chem.* 18 (2007) 2004–2017.
<http://dx.doi.org/10.1021/bc700257a>
- [30] V. Gupta, K.S. Carroll, Profiling the reactivity of cyclic C-nucleophiles towards electrophilic sulfur in cysteine sulfenic acid, *Chem. Sci.* 7 (2016) 400-415.
<http://dx.doi.org/10.1039/C5SC02569A>
- [31] A.-M. Florea, D. Büsselberg, Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects, *Cancers* 3 (2011) 1351–1371.
<http://dx.doi.org/10.3390/cancers3011351>
- [32] R. Zabel, G. Weber, Comparative study of the oxidation behavior of sulfur-containing amino acids and glutathione by electrochemistry-mass spectrometry in the presence and absence of cisplatin, *Anal. Bioanal. Chem.* 408 (2016) 1237-1247. <http://dx.doi.org/10.1007/s00216-015-9233-x>
- [33] V.M. Gonzalez, M.A. Fuertes, C. Alonso, J.M. Perez, Is cisplatin-induced cell death always produced by apoptosis?, *Mol. Pharmacol.* 59 (2001) 657-663. <https://doi.org/10.1124/mol.59.4.657>
- [34] R. Marullo, E. Werner, N. Degtyareva, B. Moore, G. Altavilla, S.S. Ramalingam, P.W. Doetsch, Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions, *PLoS One* 8 (2013) e81162.
<http://dx.doi.org/10.1371/journal.pone.0081162>

- [35] G.R. Bardajee, M.A. Winnik, A.J. Lough, Succinimidyl 7-methoxy-2H-chromene-3-carboxylate, *Acta Cryst. E* 63 (2007) o1513–o1514. <https://doi.org/10.1107/S1600536807004175>
- [36] B. Banerjee, S.K. Mandal, S.C. Roy, Cerium(IV) ammonium nitrate-catalyzed synthesis of β -keto enol ethers from cyclic β -diketones and their deprotection, *Chem. Lett.* 35 (2006) 16–17. <http://dx.doi.org/10.1246/cl.2006.16>
- [37] R.M. Neve, K. Chin, J. Fridlyand, J. Yeh, F.L. Baehner, T. Fevr, L. Clark, N. Bayani, J.P. Coppe, F. Tong, T. Speed, P.T. Spellman, S. DeVries, A. Lapuk, N.J. Wang, W.L. Kuo, J.L. Stilwell, D. Pinkel, D.G. Albertson, F.M. Waldman, F. McCormick, R.B. Dickson, M.D. Johnson, M. Lippman, S. Ethier, A. Gazdar, J.W. Gray, A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes, *Cancer Cell* 10 (2006) 515–27. <http://dx.doi.org/10.1016/j.ccr.2006.10.008>
- [38] R. Karisch, M. Fernandez, P. Taylor, C. Virtanen, J.R. St-Germain, L.L. Jin, I.S. Harris, J. Mori, T.W. Mak, Y.A. Senis, A.M. Östman, F. Moran, B.G. Neel, Global proteomic assessment of the classical protein-tyrosine phosphatome and “redoxome”, *Cell* 146 (2011) 826–840. <http://dx.doi.org/10.1016/j.cell.2011.07.020>
- [39] T. Laragione, E. Gianazza, R. Tonelli, P. Bigini, T. Mennini, F. Casoni, T. Massignan, V. Bonetto, P. Ghezzi, Regulation of redox-sensitive exofacial protein thiols in CHO cells, *Biol. Chem.* 387 (2006) 1371–1376. <http://dx.doi.org/10.1515/BC.2006.172>
- [40] V. Gupta, K.S. Carroll, Sulfenic acid chemistry, detection and cellular lifetime, *Biochim. Biophys. Acta* 1840 (2014) 847–875. <http://dx.doi.org/10.1016/j.bbagen.2013.05.040>
- [41] A. Gęgotek, M. Cyuńczyk, W. Łuczaj, A. Bielawska, K. Bielawski, E. Skrzydlewska, The redox status of human breast cancer cell lines (MCF-7 and MDA-MB231) treated with novel

dinuclear berenil-platinum(II) complexes, *Pharmazie* 69 (2014) 923-928.

<http://dx.doi.org/10.1691/ph.2014.4560>

[42] S.G. Menon, M.C. Coleman, S.A. Walsh, D.R. Spitz, P.C. Goswami, Differential susceptibility of nonmalignant human breast epithelial cells and breast cancer cells to thiol antioxidant-induced G(1)-delay, *Antioxid. Redox Signal* 7 (2005) 711-718. <http://dx.doi.org/10.1089/ars.2005.7.711>

[43] S. Singh, R. Rajendran, K. Kuroda, E. Isogai, M. Krstic-Demonacos, C. Demonacos, Oxidative stress and breast cancer biomarkers: the case of the cytochrome P450 2E1, *J. Cancer Metasta. Treat.* 2 (2016) 268-276. <http://dx.doi.org/10.20517/2394-4722.2016.42>

[44] R.J. Mailloux, X. Jin, W.G. Willmore, Redox regulation of mitochondrial function with emphasis on cysteine oxidation reactions, *Redox Biol.* 2 (2014) 123–139.

<http://dx.doi.org/10.1016/j.redox.2013.12.011>

[45] S.M. Hutson, L.B. Poole, S. Coles, M.E. Conway, Redox regulation and trapping sulfenic acid in the peroxide-sensitive human mitochondrial branched chain aminotransferase, *Methods Mol. Biol.* 476 (2008) 139-152. http://dx.doi.org/10.1007/978-1-59745-129-1_10

[46] Y. Collins, E.T. Chouchani, A.M. James, K.E. Menger, H.M. Cochemé, M.P. Murphy, Mitochondrial redox signalling at a glance, *J. Cell Sci.* 125 (2012) 801-806.

<http://dx.doi.org/10.1242/jcs.098475>

[47] É. Margittai, B. Enyedi, M. Csala, M. Geiszt, G. Bánhegyi, Composition of the redox environment of the endoplasmic reticulum and sources of hydrogen peroxide, *Free Radic. Biol. Med.* 83 (2015) 331-340. <http://dx.doi.org/10.1016/j.freeradbiomed.2015.01.032>

- [48] T. Kakihana, K. Nagata, R. Sitia, Peroxides and peroxidases in the endoplasmic reticulum: integrating redox homeostasis and oxidative folding, *Antioxid. Redox Signal.* 16 (2012) 763-771.
<http://dx.doi.org/10.1089/ars.2011.4238>
- [49] H.-L. Huang, L.-W. Fang, S.-P. Lu, C.-K. Chou, T.-Y. Luh, M.-Z. Lai, DNA-damaging reagents induce apoptosis through reactive oxygen species-dependent Fas aggregation, *Oncogene* 22 (2003) 8168–8177. <http://dx.doi.org/10.1038/sj.onc.1206979>
- [50] N.A. Santos, C.S. Catão, N.M. Martins, C. Curti, M.L. Bianchi, A.C. Santos, Cisplatin-induced nephrotoxicity is associated with oxidative stress, redox state unbalance, impairment of energetic metabolism and apoptosis in rat kidney mitochondria, *Arch. Toxicol.* 81 (2007) 495-504.
<http://dx.doi.org/10.1007/s00204-006-0173-2>
- [51] P. Prabhakaran, F. Hassiotou, P. Blancafort, L. Filgueira, Cisplatin induces differentiation of breast cancer cells, *Front Oncol.* 3 (2013) 134. <http://dx.doi.org/10.3389/fonc.2013.00134>
- [52] H. Alborzinia, S. Can, P. Holenya, C. Scholl, E. Lederer, I. Kitanovic, S. Wölfl, Real-time monitoring of cisplatin-induced cell death, *PLoS One* 6 (2011) e19714.
<http://dx.doi.org/10.1371/journal.pone.0019714>
- [53] N.A. Kratochwil, A.I. Ivanov, M. Patriarca, J.A. Parkinson, A.M. Gouldsworthy, P. del S. Murdoch, P.J. Sadler, Surprising Reactions of Iodo Pt(IV) and Pt(II) Complexes with Human Albumin: Detection of Cys34 Sulfenic Acid, *J. Am. Chem. Soc.* 121 (1999) 8193-8203.
<http://dx.doi.org/10.1021/ja990768n>