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Induction of Intracellular Reductive Stress with a Photoactivatable Phosphine Probe

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Abstract: Reductive stress is a condition present in cells that have an increased concentration of reducing species, and it has been associated with a number of pathologies, such as neurodegenerative diseases and cancer. The tools available to study reductive stress lack both in selectivity and specific targeting and some of these shortcomings can be addressed by using photoactivatable compounds. We developed a photoactivatable phosphonium probe, which upon irradiation releases a fluorescent molecule and a trialkyphosphine. The probes can permeate through the plasma membrane and the photoreleased phosphine can induce intracellular reductive stress as proven by the detection of protein aggregates.

Keywords: Photoactivation · Protein aggregation · Reductive stress · Tributylphosphine

1. Introduction

1.1 Reductive Stress

Redox homeostasis is essential for regulating fundamental processes such as cellular signaling pathways, transcriptional and post-transcriptional activities, and protein folding.[1] Redox homeostasis can be defined as an intracellular equilibrium between oxidative and reducing species. A balanced redox environment is maintained by the reduced to oxidized ratios of redox molecular duos, such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H/NAD(P)+) and glutathione (GSH/GSSG).[2,3] The reduced (GSH) and oxidized (GSSG) glutathione couple is the major redox buffer inside the cell and it is also responsible for maintaining individual redox environments needed in some organelles.[2]

For example, the cytosol needs, in general, a more reducing environment for the reversible oxidation and reduction of the cytosolic protein thiols and it has a range of GSH/GSSG of 100:1.[2] Mitochondria also need a more reductive potential and the reported range of GSH/GSSG is from 20:1 to 40:1. In contrast, the endoplasmic reticulum (ER) has a more oxidative environment necessary for the folding of secretory proteins through the formation of disulfide bridges.[2] Therefore, in the ER, the ratio of GSH/GSSG varies from 1:1 to 3:1 with millimolar concentrations of both components present.

When there is a shift in this balance, oxidative or reductive stress can occur, and these processes are linked to many pathologies, such as diabetes, inflammation, and neurodegenerative diseases.[4,5] Whereas oxidative stress has been studied intensively,[6] there is still a need for biological tools to investigate reductive stress. Pharmacological agents, such as N-acetyl-l-cysteine[2] or dithiothreitol (DTT)[7] can be used to effectively induce reductive stress, but these compounds lack selectivity and spatial resolution.

1.2 Photoactivatable Compounds

Photoactivatable molecules (often called photoremovable or ‘photocaged’) are an important class of compounds as they provide spatial and temporal control for the release of a variety of chemicals.[8,9] A photoactivatable probe is composed of a photocleavable protecting group (PPG) and an inactive molecule of interest, which can permeate through the plasma membrane and the photoreleased phosphine can induce intracellular reductive stress as proven by the detection of protein aggregates.

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A) The photoreaction should be clean and occur with a high quantum yield of release, $\Phi_{\text{rel}}$. B) the chro-
mophore should have strong absorption at wavelengths higher than 300 nm, where there is weaker absorption of light by the biological environment and less phototoxicity; C) the PPGs should be stable and soluble in the target media (most often aqueous, in case of biological applications); D) the photochemical byproducts should not be toxic and should not absorb at the wavelength of irradiation; and E) to facilitate monitoring the photoreaction, one of the photoproducts should be fluorescent.

Using different PPGs, a multitude of functional groups can be photoactivated, such as carboxylates, phosphates, sulfonates, alcohols, and thiols.[8,19] Photoactivatable phosphines were first described by Weiss and co-workers in the 1970s. They reported the release of a free triarylphosphine following a photolysis reaction, at 300 nm, of either a phosphoramidite or a triphenylphosphonium compound. Peranovich et al. then reported that irradiation of 9-anthracenenylmethyltriphenylphosphonium chloride, at 300 nm in degassed isopropanol, produced triphenylphosphine in 25% yield.[20]

To the best of our knowledge, the first application of a photoactivatable triarylphosphine was reported in 2016 by Deo et al., when they released triphenylphosphine from a ruthenium-arene complex (Scheme 1).[23] The complex contained a photoswitchable o-tosylamide azobenzene ligand and, following irradiation with 406 nm light, changed its conformation from the Z to the E isomer. This isomerization released quantitative amounts of triphenylphosphine, which was used as catalyst for the initiation of an aza-Morita–Baylis–Hillman reaction.

Photoactivatable triarylphtorphines were described in the context of light-activated Staudinger–Bertozzi ligation.[10,24] As seen in Scheme 2, Lam and co-workers reported the cleavage of a triarylphtorphine from 9-anthracenemethyl diphenylphosphorane chloride.[24] The released phosphinothioester was subsequently used as reagent in a Staudinger–Bertozzi ligation, which upon reaction with an azide formed an amide and triarylphtorphine oxide as products.

Shah et al. independently reported the photorelease of a triarylphtorphine and its use as Staudinger–Bertozzi reagent (Scheme 3).[10] The liberated phosphine was used to label an azide-tagged glycoprotein present on the cell surface, either in vitro, in fixed mammalian cells, or in vivo employing zebrafish embryos.

All these reports focused on the photo-activation of triarylphtorphines. The only literature precedence for the release of a triarylphosphine, however, was published in 2012 by Zhuang and co-workers (Scheme 4).[25] The authors described that tris(2-carboxyethyl)phosphine (TCEP), a water-soluble phosphine, quenched the fluorescence of the carbocyanine fluorophore Cy5, following the 1,4-addition of the phosphine to the polymethylene bridge of the dye. The fluorescence of the dye could be recovered after irradiation with UV light. Even though the Cy5–TCEP adduct is not thermally stable, the TCEP-induced fluorescence quenching was successfully applied in super-resolution microscopy and cell internalization assays.

2. Results and Discussion

TCEP and other triarylphtorphines are known to reduce biological disulfide bonds.[26] In an intracellular environment, breaking disulfide bonds can lead to an accumulation of both reducing agents as well as misfolded proteins and the accumulation of unfolded proteins in the ER lumen has been known to induce ER reductive stress.[5,27]

The aim of our work was to develop a photoactivatable compound that upon photostimulation would release a triarylphosphine, for inducing intracellular reductive stress, and a fluorescent reporter, for ease of monitoring, as the only photoproducts.[28]

The probe design was based on the thermally unstable Cy5–TCEP adduct and, using density functional theory (DFT) calculations, various substituents on the cyanine backbone were screened to increase the thermal barrier of the C–P bond cleavage. From this screening, we identified that a N,N-dimethylcoumarin–indolenine hybrid had the best predicted stability and was chosen as model compound for synthesis (Fig. 1A). We tuned the thermal stability of the probes further by varying the substituents on the indolenine core (Fig. 1B).

Probes 1a–d were synthesized by Knoevenagel condensation of the N,N-diethylaminocoumarin aldehyde 2 with Fischer’s bases 3a–d to result in the coumarin–carbocyanine dyes 4a–d (Scheme 5). These dyes were subsequently treated with tributylphosphine to afford probes 1a–d.

Absorbance and fluorescence spectra of dyes 4a–d and probes 1a–d were measured in phosphate buffered-saline (PBS). Dyes 4a–d displayed an absorbance maxima ($\lambda_{\text{max}}$) at 570 nm, whereas probes 1a–d exhibited $\lambda_{\text{max}}$ at 415 nm because addition of the phosphine interrupts the conjugation of the molecules. Cuvette experiments were performed to determine the thermal stability of the probes, in the
of the HOMO level of the indolenine, dis-
non photoactivatable.
tron withdrawing, which lowers the energy
The trifluoromethyl group is too elec-
the indolenine core to the coumarin moi-
photoinduced electron transfer (PeT) from
proved that photoactivatable phosphines
photophysical experiments indicated that
of theoretical calculations and additional
GSSG were irradiated with 415 nm light
3). Solutions of probes 1a–d in PBS with
GSSG underwent photoactivation, no reac-
and tributylphosphine. Whereas probes
photoproducts as the corresponding dye
their emission maxima.
the concentration of GSH. Photoirradiation
their stability.
would decrease the thermal stability of the
an electron-donating group, like methoxyl,
thermal stability and good photoactiva-
Because compound 1c had the best
thermal stability and good photoactiva-
tion efficiency, it was chosen for live-cell
experiments. Human cervical cancer cells
(HeLa) were incubated with probe 1c for
10 minutes in imaging medium, washed,
and the fluorescence was recorded in the
red channel ($\lambda_{em} = 561$ nm; $\lambda_{ex} = 605/52$
BP) before and after irradiation with a 405
nm laser (Fig. 3A–C). The increase in fluo-
rescence upon irradiation proved that the
compound is membrane permeable and the
photoactivation is still functional in a cel-
lar environment.
Next, we tested the ability of the pho-
toreleased phosphine to increase the con-
centration of free thiols inside the cell. For
this experiment, cells were pretreated with
monobromobimane (mBB), a thiol sensi-
tive fluorescent probe. Following incu-
bation of 1c and irradiation, we detected a
significant increase in the mBB signal,
which indicated the generation of intra-
cellular reducing species which could be
either the generated thiols or the released
trialkylphosphine (Fig. 3D–E).
As mentioned before, common reduc-
tive stress indicators are the accumula-
tion of unfolded proteins and aggregation
following disulfide bond cleavage.[22] We
employed Thioflavin T (ThT), a common
fluorescent stain for amyloid protein ag-
gregates,[31] to test whether protein aggreg-
ates are formed following irradiation of
1c. HeLa cells that were irradiated with
1c and then treated with ThT exhibited a
significantly higher ThT signal than cells
that were stained with ThT and ir-
radiated, but had not been treated with 1c
(Fig. 3F–I).

dark, in PBS that was supplemented with
GSSG. The role of GSSG was to trap the
released phosphine, thus avoiding re-
attack on the dye. Monitoring the increase
in absorbance at 570 nm, we determined
the half-lives of the probes in absence of
light. The results of this experiment (Table
1, column 2) confirmed our hypothesis that
an electron-donating group, like methoxyl,
would decrease the thermal stability of the
probes, whereas an electron-withdrawing
group, like trifluoromethyl, would increase
their stability.

Photoactivation experiments were car-
ried out in a fluorimeter (Table 1, column
3). Solutions of probes 1a–d in PBS with
GSSG were irradiated with 415 nm light
and the increase in fluorescence was moni-
tored at 630 nm, where the dyes 4a–d have
their emission maxima. $^1$H and $^{31}$P NMR
analyses confirmed the identity of the
photoproducts as the corresponding dye
and tributylphosphine. Whereas probes
1a–c underwent photoactivation, no reac-
tion was observed for 1d. A combination
of theoretical calculations and additional
photophysical experiments indicated that
the phosphine was released following a
photoinduced electron transfer (PeT) from
the indolenine core to the coumarin moi-
ety. The trifluoromethyl group is too elec-
tron withdrawing, which lowers the energy
of the HOMO level of the indolenine, dis-
favoring PeT, and thus probe 1d was not
photoactivatable.

With these probes in hand, we also
proved that photoactivatable phosphines
are capable of reducing GSSG to increase
the concentration of GSH. Photoinfrared

Table 1. Half-lives of phosphine release. (dark
= thermal hydrolysis in absence of light; PA =
hydrolysis during photoporation with 405 nm
light; n.d. = not determined).

| probe | $t_{1/2$ (dark)} (min) | $t_{1/2$ (PA)} (min) |
|-------|-----------------|-----------------|
| 1a    | 9.2(2)          | 4(1)            |
| 1b    | 27.3(3)         | 11(2)           |
| 1c    | 85(2)           | 12(2)           |
| 1d    | >120            | n.d             |

Fig. 1. New probe design based on computational model-
ing. A) Evolution of structures from the Cy5 core struc-
ture to the stable $N,N$-
dimethylycoumarin–in-
dolenine hybrid. B) Further tuning of the stabi-
ity of the phosphonium adduct by variation of substi-
ents on the indolenine core.

Scheme 5. Synthesis of probes 1a–d.
In conclusion, a series of photoactivatable phosphonium probes were synthesized and their properties characterized. The probes are membrane permeable and can be activated with 405 nm light. Live cell photoactivation experiments resulted in an increase in intracellular reducing species and protein aggregates, which are telltale signs of reductive stress. Current work in our lab is focusing on targeting the probes to specific redox sensitive organelles, such as mitochondria and ER.

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