Published in final edited form as:

Bioorg Med Chem Lett. 2015 January 1; 25(1): 16–19. doi:10.1016/j.bmcl.2014.11.041.

A selective phosphine-based fluorescent probe for nitroxyl in living cells

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Abstract

A novel fluorescein-based fluorescent probe for nitroxyl (HNO) based on the reductive Staudinger ligation of HNO with an aromatic phosphine was prepared. This probe reacts with HNO derived from Angeli's salt and 4-bromo Piloty's acid under physiological conditions without interference by other biological redox species. Confocal microscopy demonstrates this probe detects HNO by fluorescence in HeLa cells and mass spectrometric analysis of cell lysates confirms this probe detects HNO following the proposed mechanism.

Keywords

Nitroxyl; Nitric Oxide; Staudinger ligation; Phosphine; Fluorescence microscopy

The discovery and establishment of nitric oxide (NO) as an important signaling agent involved in many physiological processes including blood pressure control, neurotransmission, and the immune response initiated the further study of the biological roles of many redox-related nitrogen-containing compounds.^{1–5} Nitroxyl (HNO), the one-electron reduced and protonated derivative of NO, possesses distinguishable physiological and pharmacological properties from NO.^{6–8} HNO-releasing pro-drugs increase cardiac inotropy and lusitropy and elicit arterial and venous dilation without building tolerance, properties that make these compounds intriguing candidates for the treatment of congestive heart failure.^{9–16} These cardiac outcomes occur via selective and covalent thiol modification

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Supplementary Material

Experimental details including synthetic procedures, NMR spectra, mass spectrometry and kinetic data.

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that increases myocardial calcium cycling and enhances the calcium sensitivity of the myofilament.^{17–19} Such biological properties drive the search for new chemical HNO donors as well as the definition of an endogenous biochemical pathway of HNO formation.^{20–28}

Development of new HNO donors and understanding endogenous HNO production requires robust HNO detection methods. Early methods of HNO detection (e.g., identification of N₂O, trapping with thiols or ferric heme proteins) either lack the sensitivity or the selectivity to unambiguously demonstrate endogenous HNO formation. These limitations have led to the development of new HNO detection strategies that include a group of Cu^{II}-based fluorescent complexes and HNO-specific electrodes.^{29–33}

Organophosphines are suitable for HNO detection based on their documented ligation reaction with HNO, their rapid rate of HNO trapping and lack of cross-reactivity with other physiologically relevant nitrogen oxides, such as NO, nitrite, nitrate, and peroxynitrite.^{34–36} In light of this phosphine-mediated ligation chemistry described for HNO, we and others envisioned the development of phosphine probes with fluorophore leaving groups.^{37–39} The reaction of HNO with two equivalents of phosphine nucleophiles produces phosphine oxides (**2**) and the corresponding phosphine azaylides (**3**), forming the chemical basis of these newly reported HNO detection strategies (Scheme 1). In the presence of an internal electrophilic ester, these azaylides undergo Staudinger ligation to yield the thermodynamically stable amide (**4**, Scheme 1) and the corresponding ester-derived alcohol. Based on this mechanism, we designed and synthesized **1**, which produces HNO-dependent fluorescence by generating the known fluorophore, fluorescein monomethyl ether (**5**, Scheme 1).

Compound **1** was prepared by the straightforward coupling of 2-(diphenylphosphino)benzoic acid with fluorescein methyl ether, a previously reported fluorescein derivative,⁴⁰ in 61% yield (Scheme 2).

We first investigated the feasibility of **1** to detect HNO in buffered solution. In the initial ligation experiment, solutions of **1** (40 μM) were incubated with increasing concentrations of Angeli's salt (AS, 0–5 eq.) in 1:3 MeCN:PBS (containing 0.1 mM EDTA, pH 7.4) at ambient temperature and excitation at 465 nm led to a concentration-dependent increase in emission intensity at 520 nm with a 73.8-fold maximum response observed at 5 eq. AS (Figure 1, Panel A). The solution changed from clear to yellow over 20 minutes with a maximum fluorescence achieved at 40 minutes and fluorescence intensity remained unchanged after 2 h (Figure 1, Panel B and SI Figure S4). Such a time course may suggest that ligation is the rate determining step in fluorescence generation. Use of higher amounts of AS results in more intense fluorescence (Figure 1). Control experiments with increasing amounts of nitrite, the by-product of AS decomposition, and **1** (40 μM) do not generate a fluorescence response, illustrating that the response depends on the HNO-mediated release of the fluorophore.

The commercial availability, water solubility and rapid HNO release rate make AS the donor of choice for most chemical and biological studies, including recent studies regarding

HNO detection with both phosphine and Cu^{II}-based fluorophores.^{30–33,37–39} Treatment of **1** with *p*-bromo Piloty's acid, a structurally distinct HNO donor, also results in fluorescence enhancement proportional to *p*-bromo Piloty's acid concentration (SI Figure S5). The slower continual release of HNO from this compound⁴¹ may better mimic its endogenous production and may result in the slightly decreased observed fluorescence response. These results demonstrate HNO detection by **1** from a source besides AS.

Before attempting HNO detection in cells, **1** was assessed for the selectivity toward HNO compared to other biological redox species. Figure 2 shows the comparative fluorescence response of **1** to excess amounts of NO, NO₂[−], NO₃[−], H₂O₂, H₂S, GSH, *S*-nitroglutathione (GSNO) and *S*-nitrosocysteine (CysNO). Given the ability of phosphines to react with *S*-nitrosothiols,⁴² **1** (40 μM) was treated with HNO, GSNO and CysNO (2 equivalents) to determine the relative fluorescence response under identical conditions. Reaction of **1** with HNO yields a 58.5-fold increase in fluorescence while incubation with GSNO and CysNO only gives a 2.3 and 3.2-fold increase, respectively (SI, Figure S6). These results clearly demonstrate the selective reactivity of **1** with HNO. Nitric oxide (NO) and a series of oxidants (NO₂[−], NO₃[−], H₂O₂) gave no or little fluorescence response. Formation of the phosphine oxide (**2**) by treatment of **1** with H₂O₂ does not yield a fluorescence response indicating that oxidation of these probes does not produce a false fluorescence response. Additionally, **1** does not react with the biological reductants (H₂S and GSH), which may reduce the Cu^{II}-based probes giving potential false positives.^{30–31} These results reinforce the general selectivity of these agents for HNO.

After determining the ability of **1** to detect HNO *in vitro*, we applied this probe for the detection of HNO in cells. Treatment of HeLa cells with **1** (12.5 μM) for 10 min gives little or no background signal as judged by fluorescence microscopy (Figure 3a). Control experiments, show a slow increase in background fluorescence during longer (30 min) incubation, presumably due to esterase-mediated hydrolysis. Addition of AS to the cells immediately initiated intracellular fluorescence (Figure 3b). These findings highlight the ability of **1** to detect HNO within a cellular environment.

Following fluorescence microscopy, the control and AS-treated HeLa cells were lysed and the extracted contents analyzed by mass spectrometry (MS) to confirm the conversion of **1** to the expected products, particularly HNO-derived amide **4** (SI Figures S7–S12). Orbitrap MS analyses of the AS-treated cells show the formation of the phosphine oxide amide (**4**, [M+H]⁺ 322.0991) and mono-methylated fluorescein (**5**) in the cell lysates, confirmed by comparison to authentic standards. Control experiments using cells not treated with AS show the presence of **5** over time, possibly from background hydrolysis, but no evidence of **4**. As **4** results from the HNO-mediated ligation of **1**, this finding confirms the ability of **1** to trap HNO within cells and further validates **4** as a HNO marker. Identical experiments using ¹⁵N-AS, which generates H¹⁵NO, followed by MS reveals that formation of ¹⁵N-**4** and **5** further confirming HNO as the nitrogen source in **4** and the ability of **1** to trap HNO within cells via the proposed mechanism.

In conclusion, the triarylphosphine-based HNO probe **1** was designed and synthesized to produce a fluorescent response based on the fast phosphine/HNO chemistry previously

reported. HNO donors, including AS and 4-bromo Piloty's acid, activate **1** with a concentration-dependent increase in fluorescence intensity. Probes **1** is highly sensitive and selective for HNO detection under physiological conditions. Fluorescence microscopy experiments demonstrate that **1** detects release of HNO from AS in HeLa cells. Subsequent MS analyses of these cell lysates identify the amide phosphine oxide ligation product (**4**), a distinct HNO-derived product, whose detection confirms the suggested mechanism. The successful detection of HNO in HeLa cells with **1** offers an approach for the screening of endogenous HNO sources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (HL62198 to S.B.K.). Flow Cytometry and Tumor Tissue Core services were supported by the Comprehensive Cancer Center of Wake Forest University NCI CCSG P30CA012197 grant. The Thermo Orbitrap LTQ XL mass spectrometer was purchased with support from NSF-CRIF (0947028) from the US NSF. The authors would like to acknowledge Dr. Anita McCauley (Wake Forest University, Biology) for assistance with microscopy.

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Figure 1.

Panel A: Fluorescence responses of **1** (40 μM) to 0.005, 0.05, 0.5, 5 eq. of AS or NaNO_2 in $\text{CH}_3\text{CN/PBS}$ after 2 h incubation at room temperature. Panel B: The ligation induces a distinct color change.

Figure 2. Fluorescence responses of **1** (40 μ M) in CH₃CN/PBS at room temperature for 2 h after addition of 200 eq. of GSH, H₂O₂, NaNO₂, NaNO₃, Na₂S, DEA/NO, GSNO, CysNO, AS.

Figure 3. HNO-induced fluorescence images of HeLa cells treated with **1** (12.5 μM) after 10 min (a), then treated with AS (500 μM) followed by 30 min incubation (b): (top) confocal image and (bottom) bright field. Scale bars represent 20 μm .

Scheme 1.
Proposed mechanism of probe **1** with HNO

Scheme 2.
Synthesis of probe **1**