Phosphine-based detection strategies for both nitroxyl (HNO) and S-nitrosothiols (RSNO) were investigated and compared. Phosphorus NMR studies show that azaylides derived from HNO or organic RSNO efficiently participate in subsequent reductive ligation required for fluorescence generation in properly substituted substrates. S-Azaylides derived from biological RSNO containing free amine and carboxylic acid groups primarily yield phosphine oxides suggesting these groups facilitate nonligation pathways such as hydrolysis. The fluorescence response of a phosphine-based fluorophore toward the same RSNO confirms these differences and indicates that these probes selectively react with HNO. Flow cytometry experiments in HeLa cells reinforce the reactivity difference and offer a potential fast screening approach for endogenous HNO sources.

Nitroxyl (HNO), the one-electron reduced/protonated form of nitric oxide (NO), shows distinct physiology and pharmacology from NO. Specifically, HNO inhibits the activity of various thiol-containing enzymes and regulates cardiovascular signaling, making it an intriguing candidate for many physiological disorders such as alcoholism and congestive heart failure (CHF). The lack of fast and reliable HNO detection methods applicable to living cells limits the biological understanding of HNO and identification of endogenous sources. New detection approaches have been developed for robust HNO identification including copper-based fluorescent complexes, a cobalt-porphyrin electrochemical method, a membrane inlet mass spectrometry (MIMS) approach, and a series of phosphine-based fluorescent probes. Organophosphines have been intensively studied due to their fast and selective reaction with HNO compared with other nitrogen oxides. Despite various structures, all of the reported phosphine probes react with HNO in a similar fashion (Scheme 1). Two equivalents of phosphine (1) react with HNO to produce equal amounts of phosphine oxide (2) and azaylide (3), which in the presence of an electrophilic ester, undergoes Staudinger ligation to yield amide (4) and the corresponding fluorescent alcohol (5, Scheme 1).

S-Nitrosothiols (RSNO) represent an important type of post-translational modification that preserves and amplifies NO signaling and regulates protein activity. Variation and dysregulation of RSNO levels are associated with the etiology of diverse diseases. Faster and specific detection and quantification of RSNO will better elucidate their behavior in vivo and define a better understanding of their therapeutic potential. Current RSNO detection depends on indirect assays that limit the overall specificity of the measurements. A recent report on reductive ligation of phosphines with some model RSNO provides insight into new RSNO detection approaches. Like HNO, phosphines react with RSNO to give phosphate oxide (2) and an S-azaylde (6), which undergoes ligation to form a sulfamide (7) and an alcohol (5, Scheme 1). The reactivity of phosphines with RSNO has been exploited to develop fluorescent and mass spectrometry-based probes for RSNO detection that possess similar structures to those for HNO.

While increasing efforts have been made in designing new phosphine-based fluorescent probes for HNO and RSNO detection, little attention has been paid to the cross reactivity of HNO and RSNO with the same phosphine-based detection systems. Prior experiments consistently demonstrate that HNO induces a greater fluorescence response than S-nitrosoglutathione (GSNO) or S-nitrosocysteine (CysNO) upon reaction with the same phosphine. Such differences suggest a relative specificity for HNO over RSNO in their reaction with organic phosphines. The overall similarity of these two described reaction pathways complicates phosphine-based detection strategies of both species and opens the possibility of false positive results in vivo. Concerns regarding the reliability of these probes exist, and in vivo screening of HNO with these probes remains risky without a rationale for the diminished fluorescence response from GSNO. Here, we directly investigated the reactions of two phosphines (1a–b) with HNO and RSNO and reveal important differences vital for the fluorescence response. Unlike the HNO-derived azaylde or the S-azaylde described in earlier reports, the S-azaylde formed from the reaction of phosphines with GSNO or CysNO does not efficiently participate in the reductive ligation needed for fluorophore generation. This difference was further confirmed by using 1b to detect HNO (vs. RSNO) in HeLa cells using flow cytometry. Fluorescence generation described in Scheme 1 relies on productive ylide formation and reductive ligation with release of a competent fluorophore. For the reaction of 1 with HNO, a productive ligation sequence should yield an equivalent of.
phosphine oxide amide (4), phosphine oxide ester (2), and the alcohol (or fluorophore, 5). Given that 2 and 5 could arise from either oxidative or hydrolytic pathways, 4 represents a distinct indicator of a productive ligation process. For the RSNO reaction, the sulfenamide (7) represents an analogous ligation product, which can be reduced to 4 in the presence of excess phosphine (Scheme 1).12

We monitored the reactions of phosphine probes with HNO and GSNO by phosphorus nuclear magnetic resonance (NMR) spectroscopy and HPLC-MS (Figures S3–S8 in the Supporting Information). Treatment of phenyl (diphenylphosphino)benzoate (1a) with Angeli’s salt (AS), a common HNO donor, reveals the formation of 4 (δ = 34.8 ppm), the corresponding phosphine oxide (2a, δ = 33.7 ppm) as expected, and an additional peak (δ = 35.8 ppm), identified by HPLC-MS as 2-(diphenylphosphino)benzoic acid oxide, possibly from the hydrolysis of 2a (Figure 1A). The amide peak (4, δ = 34.8 ppm) indicates productive ligation. In contrast to reported efficient reductive ligation results,12 treatment of this model phosphine with GSNO only yields 2a (δ = 33.7 ppm) with trace amounts of 4 or sulfenamide (7, Figure 1B), revealing inefficient ligation. Incubation of fluorescent probe (1b) with AS gives a similar 31P NMR spectrum showing complete conversion of 1b to 4 (δ = 34.8 ppm), the corresponding phosphine oxide (2b, δ = 33.7 ppm, Figure 1C) and 2-(diphenylphosphino)benzoic acid oxide (δ = 35.8 ppm). The formation of 4 correlates with a strong and rapid fluorescence response.16 Similarly, incubation of 1b with GSNO yields mostly phosphine oxide (2b, δ = 33.7 ppm) and a trace amount of another phosphorus product (Figure 1D). A previously reported coumarin-derived fluorescent HNO probe (P-CM) demonstrates similar results (Figure S1 in the Supporting Information).16 These results clearly show different reactivity of phosphine probes with HNO and GSNO under these conditions. The HNO reaction reliably gives Staudinger ligation products (that yield fluorescence in properly designed compounds) but the GSNO reaction primarily yields phosphine oxide with little evidence of ligation, which corresponds to the low fluorescence response observed in previous reports.

While reductive ligation phosphine-based fluorescent detection strategies for HNO are becoming well established,6 the
The reasons for the

1a – 1a

Figure 3. Fluorescence responses of 1b (40 μM) in CH₃CN/PBS at rt for 60 min after addition of CysNO derivatives (2 equiv). Data are the

results correlate with the amount of ligation product observed by phosphorus NMR (more fluorescence with more ligation product) and support the idea that RSNO structure ultimately controls the stability of the derived S-azaylide.

Additional phosphorus NMR experiments followed by HPLC-MS of the reactions of these S-nitroso cysteine derivatives with triphenylphosphine reinforces these ideas (Figures S2, S13–16 in the Supporting Information). The reaction of N-acetyl CysNO methyl ester with PPh₃ gives an expected 1:1 ratio of phos-

Figure 2. ³¹P NMR for reaction of 1a (0.02 mmol) with CysNO and its deriva-

tives (0.01 mmol): A) N-acetyl-CysNO-methyl ester, B) CysNO-methyl ester,
C) N-acetyl-CysNO, D) CysNO in CD₃CN/THF/Tris-HCl Buffer (100 mM, pH 7.4, 3:1:2) in dark after 30 min.

As described in Scheme 1 for phosphine-based fluorescent probes, successful ligation processes occur accompanied by the generation of fluorescence. Hence, the fluorescence re-

sponse of 1b toward these four CysNO derivatives should remain consistent with the data presented in Figure 2. Indeed, addition of 2 equiv of N-acetyl CysNO methyl ester to 1b induces a significant (10.1-fold) fluorescence increase compared with CysNO methyl ester (2.9-fold), which is much greater than N-acetyl CysNO and CysNO (Figure 3). These fluorescence

To explore this idea, the reaction of various CysNO deriva-
tives with 1a followed by phosphorus NMR and HPLC-MS analysis (Figures S9–S12 in the Supporting Information) provides further information regarding RSNO structure in these reactions. Previous work shows N-acetyl CysNO methyl ester, a fully protected CysNO derivative, reacts with 1a via ligation to give the sulfenamide in 84% yield. In our hands, treatment of 1a with N-acetyl CysNO methyl ester yields three phosphorus-containing products, the expected phosphine oxide (2a, δ = 33.7 ppm), sulfenamide (7a, δ = 34.3 ppm) and the amide (4, δ = 34.8 ppm), a ligation product that forms from the reduc-
tion of 7a by 1a (Figure 2A). Both 7a and 4 result from a re-
ductive ligation process and confirm previous work. However, treatment of CysNO methyl ester, which contains a free amine, with 1a only provides small amounts of 7a and 4 with the phosphine oxide as the major species (Figure 2B). Incuba-
tion of N-acetyl CysNO, which contains a free carboxylic acid, results in primarily phosphine oxide (2a) with a small amount of another downfield phosphorus-containing product (δ = 53.5 ppm, Figure 2C), presumed to a phosphonium salt as judged by HPLC-MS (Figure S11). Reaction of CysNO with 1a yields phosphine oxide (2a) as the only phosphorus-con-
taining product (Figure 2D). These results indicate that the structure of the RSNO influences the final product selectivity possibly by influencing the stability of the S-azaylide. The reactions with RSNO substrates containing free carboxylic acid and amine groups predominantly generate phosphine oxide (Fig-
ure 2B–D) suggesting the presence of these groups facilitate S-azaylide hydrolysis as the most direct mechanism of phosphine oxide formation.

use of similar compounds for RSNO detection remains to be
fully developed. Early studies show the ability of phosphines to
react with RSNO to form S-azaylides that undergo ligation re-
actions but these discoveries have not been translated to a
robust RSNO detection system. A mass spectrometric

phosphine-based method for GSNO has been reported and
this work also shows that probes with similar structures to 1a–
b do not undergo ligation with GSNO. The reasons for the
observed differences in the ligation reactivity of phosphines
and HNO (reliable) and RSNO (unreliable) remain to be defined.
Kinetic studies on the reaction of phosphines with HNO or
RSNO show that initial phosphine addition occurs rapidly (with
the HNO reaction being slightly faster). Differences in the
reactivity of the azaylidelintermediates (3 or 6) likely play a
role in the observed reactivity as the rate determining step
and rate of classical Staudinger ligations vary significantly with
azaylide stability. Examination of previous work shows that
in general only organic RSNOs or fully protected versions of
biological RSNO derivatives (fully protected CysNO) yield suc-
cessful and reliable reductive ligation. We speculate that the S-azaylides derived from GSNO and CysNO that contain
both free carboxylic acid and amine groups may be less stable
than those from the organic RSNO. Both of these RSNO
exist as a zwitterion at physiological pH, and these functional
groups may react with the S-azaylide facilitating other possible
reaction pathways such as hydrolysis to yield the observed
phosphine oxide.

Additional phosphorus NMR experiments followed by HPLC-
MS of the reactions of these S-nitroso cysteine derivatives with triphenylphosphine reinforces these ideas (Figures S2, S13–16 in the Supporting Information). The reaction of N-acetyl CysNO methyl ester with PPh₃ gives an expected 1:1 ratio of phos-

Figure 3. ³¹P NMR for reaction of 1a (0.02 mmol) with CysNO and its derivati-
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results correlate with the amount of ligation product observed by phosphorus NMR (more fluorescence with more ligation product) and support the idea that RSNO structure ultimately controls the stability of the derived S-azaylide.

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Figure 3. Fluorescence responses of 1b (40 μM) in CH₃CN/PBS at rt for 60 min after addition of CysNO derivatives (2 equiv). Data are the

average ± SD of two independent experiments.
Triarylphosphine probes react rapidly with both HNO and RSNO to generate a 1:1 mixture of the corresponding azaylide and the phosphine oxide (Scheme 1). In the case of HNO, these simple unsubstituted azaylides efficiently undergo Staudinger ligation with properly positioned esters to generate the fluorophore and the amide phosphine oxide (4, Scheme 1). While RSNO species also quickly generate an S-azaylide, these appear to only undergo efficient ligation reactions (required for fluorophore release) with simple organic RSNO or with fully-protected peptide or amino acid RSNO derivatives.

Under predominantly aqueous conditions in the presence of free carboxylic acid and amine groups, the non-fluorescent phosphine oxide represents the major phosphorus-containing product indicating that other nonligation reaction pathways of the S-azaylide dominate. Considering the physiological environment and the presence of amine and carboxylic acid groups in many proteins or in biological constituents, protein RSNO will mostly likely react with phosphine probes to yield phosphine oxides. Such reactivity suggests that phosphine-based fluorescent probes demonstrate selectivity for HNO and will not to be interfered with by RSNO.

To further confirm the reactivity difference, we measured the intracellular fluorescence increase in HeLa cells treated with 1b by adding an HNO donor and RSNO. Intracellular fluorescence was determined by flow cytometry, which generates more statistically reliable data by simultaneous measurement of millions of cells and focuses on normal cells, avoiding faulty data from abnormal and/or dead cells. Figure 4 and Figures S17–18 in the Supporting Information show the mean fluorescence intensity value difference between 1b-treated HeLa cells with different substrates. Direct addition of GSNO to cells does not yield an increase in fluorescence (Figure 4). Possible intracellular formation of protein RSNO by adding diethylenamine (DEA)/NONOate (a well-known NO donor) also does not yield a fluorescence response. Addition of AS to cells containing 1b immediately results in an increase in fluorescence that grows over 30 min. The successful detection of HNO-induced fluorescence in numerous cells and the lack of response from GSNO or protein RSNO-derived from NO treatment confirm the difference in reactivity and reveal the possibility for HNO detection in vivo using these phosphine probes. The combination of such probes with flow cytometry may facilitate the search for endogenous HNO formation from different primary cells.

In summary, we compared the phosphine detection strategies for HNO and RSNO. The reactions of phosphines with HNO and organic or biological RSNO demonstrate clear differences in the ability of the intermediate ylides to undergo ligation leading to fluorophore release. Ylides derived from HNO or organic RSNO tend to participate in the ligation process, while ylides from biological RSNO that contain free carboxylic acid and amino groups do not readily undergo ligation and preferentially react (perhaps through hydrolysis) to form the phosphine oxide. This reactivity difference was confirmed by monitoring the fluorescence response in HeLa cells. Successful detection of HNO in cells using 1b by flow cytometry without biological RSNO interference illustrates the reliability of phosphine probes for HNO and may serve as a fast robust screening approach for endogenous HNO sources.

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