

Spiro Diorthoester (SpiDo), a Human Plasma Stable Acid-Sensitive Cleavable Linker for Lysosomal Release

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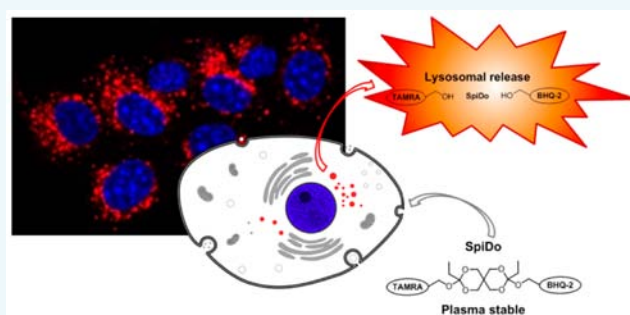
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S Supporting Information

ABSTRACT: pH-sensitive linkers designed to undergo selective hydrolysis at acidic pH compared to physiological pH can be used for selective release of therapeutics selectively at targets and orthoesters have been demonstrated to be good candidates for such linkers. Following an HPLC screening, a Spiro Diorthoester (SpiDo) derivative was identified as a potent acid-labile group for the development of pH-sensitive targeted systems. After incorporation of this linker into activatable FRET-based probe and side-by-side comparison to a well-known alkylhydrazone linker, this SpiDo linker has shown a fast and pH sensitive hydrolysis for mild acidic conditions, a pH sensitive lysosomal hydrolysis, and high stability in human plasma.



INTRODUCTION

In cancer therapy, many successful strategies to increase the therapeutic index of highly cytotoxic drug involve prodrugs that release the active drug once it has reached its target.¹ Such an approach relies on the ability of a linker to be cleaved in response to physiological, pathological, or pathochemical conditions (e.g., enzyme overexpression, redox microenvironment, and pH gradient).^{2,3} Acidic pH-gradients have been extensively used as biological stimuli to trigger the release of delivery systems and, as a result of many studies, several acid-labile structures have been reported over time in the design of drug delivery systems, via protonation mechanisms such as pH dependent microcapsules⁴ or bond breaking mechanisms in polymers⁵ and molecular entities (e.g., acylhydrazone,^{6–9} acetal,¹⁰ silyl ether,^{11,12} *cis*-aconityl,¹³ β -thiopropionate,^{14,15} thiomaleamic,¹⁶ and imidazole^{17,18} derivatives). These chemical groups have been used for both intra- and extracellular drug release. Upon cellular internalization, a delivery system undergoes passage through acidic endosomal/lysosomal compartments, at pH 6–6.8 and 4.5–5.5, respectively, cleaving the labile linker and releasing the drug payload.¹⁹ Alternatively, the extracellular hypoxic tumor microenvironment is reported to be 0.5–1 pH unit more acidic than healthy tissue and blood.

Most of the industrial approaches for antibody–drug conjugates (ADC) encompassed the use of hydrazone linkers

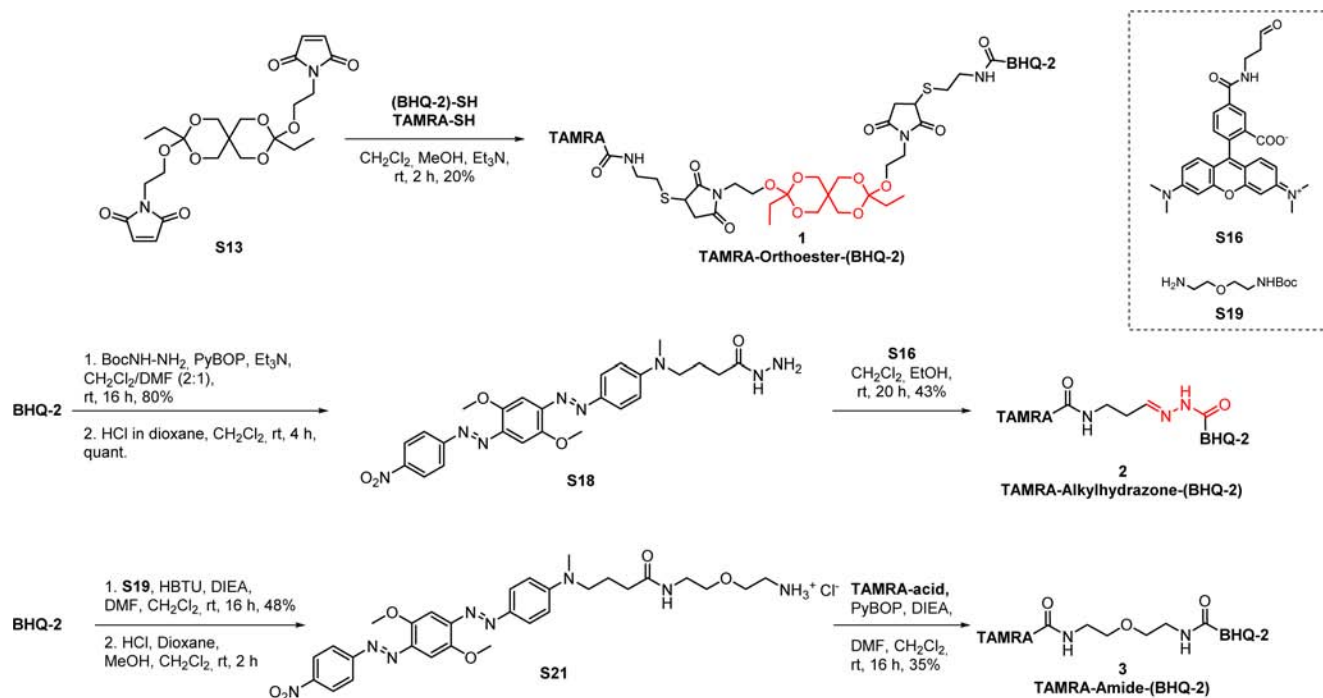
which have been used to facilitate the release of a highly toxic payload after ADC internalization (Mylotarg and Inotuzumab ozogamicin).²⁰ However, both Mylotarg and Inotuzumab ozogamicin have been withdrawn from the market and phase III clinical trial. The withdrawal of Mylotarg was attributed to toxicities related to acylhydrazone linker instability, which resulted in an increased number of treatment-related mortalities.²¹ In the course of bridging the gap between shielding normal tissue from toxic compounds and delivering drugs at therapeutic concentrations to target cells, we have explored the use of a Spiro Diorthoester (SpiDo) for lysosomal release. Heller^{22–28} and Szoka^{29–32} have already prepared a series of polymers based on the diorthoester moiety and exploited their pH-sensitive properties to develop sustained drug release systems. Of particular importance, the half-life of liposomal formulations of Spiro Diorthoester were reported by Szoka and Guo^{29,30} to be approximately 2.5 h at pH 6, and in vivo experiments showed that the lipid derivatives can be used to further modify the delivery properties of the lipidic delivery system in order to enable prolonged circulation time. (e.g., by changing the lipid chain and the polar head). All of these

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Scheme 1. Synthesis of Probes 1–3



literature reports revealed the high potency of orthoesters in the field of bioresponsive polymers and acid-labile liposomes. However, to the best of our knowledge, the precise behavior of the Spiro Diorthoester in response to specific biological stimuli and in the response induced by lysosomal uptake has not been reported on its own. Consequently, its potency to be used as a cleavable linker in targeted systems such as ADCs, which requires both long plasma stability and fast lysosomal cleavage, has not yet been clearly established. The aim of this study is to establish and demonstrate this potency by comparing with the same method, the hydrolysis profile and reactivity of this specific linker to the commonly used acylhydrazone linker. Albeit other pH sensitive groups such as acetals are already known, we decided to solely compare the SpiDo linker to the well-known acylhydrazone linker.

RESULTS AND DISCUSSION

Literature analyses show that various acid-sensitive linkers have been developed to date. However, the difference in experimental condition (buffer, temperature, concentration, etc.) between the different studies makes direct comparison of stability and degradability of different linkers very difficult. To address this problem, we have conducted within our group an HPLC-based hydrolysis assay and recorded the hydrolysis profiles of a broad selection of acid-sensitive groups regularly used in organic chemistry, i.e., acylhydrazone, acetal, dialkylacetal, aryl-1,3-dioxolane, aryl-1,3-dioxane, ethoxybenzylimidazole, oxime, imine, and 3-alkylthiopropionate moieties. From this study, we have identified a Spiro Diorthoester (SpiDo) structure **S3** which displayed a rapid hydrolysis profile selective toward acidic conditions (pH 5.5) relative to physiological pH (see SI Figure S2). For comparison, we prepared acyl and alkyl hydrazones derivatives (**S4**–**S7**) (see SI Figure S3) and compared their hydrolysis profiles (see SI Figure S4). Whereas acylhydrazones **S4** and **S5** showed little hydrolysis at pH 5.5 and 7.4, alkylhydrazones **S6** and **S7** were

more sensitive to acidic conditions, and the most stable alkylhydrazone **S6** was chosen for comparison with **S3**.

With this promising result in hand, we then proceeded to further compare the biosensitivities of SpiDo and alkylhydrazone. To ensure a rapid and efficient method for measuring hydrolysis in vitro, the SpiDo and alkylhydrazone linkers were incorporated into activatable FRET-based probes **1** and **2**, respectively, whose 5-carboxytetramethylrhodamine (TAMRA) fluorescence is quenched by Black Hole Quencher-2 dye (BHQ-2) via intramolecular FRET. Upon acid-catalyzed hydrolysis of the pH-sensitive linker, the TAMRA and BHQ-2 are separated and the fluorescence of TAMRA can be detected. A FRET-based probe **3** without pH-sensitive linker has also been synthesized as a negative control. Of particular interest, we designed the FRET-based TAMRA-Orthoester-BHQ-2 probe **1** on the basis of a previously reported bis-maleimide spiro diorthoester used for protein cross-linking.³³ Starting from already known bis-maleimide Spiro Diorthoester **S13**³³ probe **1** was synthesized in one step by simultaneous treatment of **4** with (BHQ-2)-SH³⁴ and TAMRA-SH³⁴ in 20% yield (Scheme 1). Probe **2** was synthesized in three steps starting from BHQ-2. Coupling of BHQ-2 with mono-Boc protected hydrazine followed by deprotection with HCl gave hydrazide **S18**, which was further condensed with freshly prepared aldehyde **S16**³⁴ to give probe **2** in average yield. Finally, probe **3** was prepared in 3 steps starting from BHQ-2. Coupling of BHQ-2 with synthesized linker **S19**,³⁴ followed by BOC deprotection using HCl, gave **S21** which was further coupled with TAMRA-acid using PyBOP to give probe **3**. The synthesis of probes **1** and **2** required careful attention with the use of mild conditions when handling the highly pH-sensitive group, and at the final stage of the synthesis, the probes were purified by preparative HPLC using NH_3/HCOOH solution (10 mM, pH 8.5)/MeCN as eluent system and no degradation was observed.

Initially, kinetic measurements based on molecular fluorescence were conducted to evaluate the stability of the different probes for 15 h in aqueous buffer solutions at pH 5.5 (to simulate the lysosomal pH) and pH 7.4 (to simulate serum conditions). At pH 5.5, probe 1 showed a rapid activation of fluorescence due to the hydrolysis of the SpiDo linker (Figure 1A). Probe 1 displayed a slight increase of fluorescence at pH

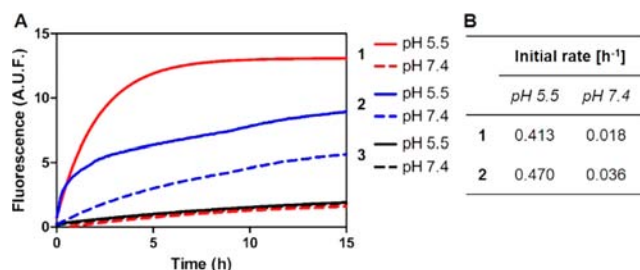


Figure 1. Hydrolysis kinetics of FRET-based probes 1–3. (A) Hydrolysis kinetics of FRET-based probes 1 (red lines), 2 (blue lines), and 3 (black lines) at pH 5.5 (solid lines) and 7.4 (dashed lines). All data were recorded in triplicate at room temperature on a microplate reader and with a probe concentration of 0.5 μ M in phosphate buffers ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ pH 7.4 or $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ pH 5.5, 100 mM). (B) Initial rate kinetics calculated for 10% of conversion of 1 and 2 at pH 5.5 and 7.4 assuming a first-order reaction.

7.4 but comparable to nonhydrolyzable probe 3. Initial rate kinetics were compared and indicated that the hydrolysis of 1 in acidic conditions was 23-fold faster than at physiological pH (Figure 1B). The hydrolysis half time was found to be 1.5 h (assuming a first-order reaction) and full hydrolysis was completed after 7 h at pH 5.5.

Under the same experimental conditions as with probe 1, alkylhydrazine probe 2 displayed completely different pH-dependent hydrolysis profiles as well as reaction equilibration (Figure 1A). Two different rates were established: (i) a first-order reaction occurring within the first hour associated with a burst release and (ii) a slower rate, which was attributed to an equilibrium state due to the reversible nature of hydrolysis of the alkylhydrazine.³⁵ Running the same experiment with an excess of nucleophilic hydrazine to induce transamination reaction and eliminate the reversibility of alkylhydrazine hydrolysis confirmed the equilibrated reaction (see SI Figure S8). At pH 7.4, significant hydrolysis of probe 2 was observed compared to control probe 3. Kinetic data analysis revealed that hydrolysis of 2 is 13-fold faster in acidic conditions. Interestingly, while initial rates of 1 and 2 appear to be quite similar at pH 5.5, 1 was considerably more stable at pH 7.4 suggesting that SpiDo is more sensitive to pH-variation than the alkylhydrazine linker (Figure 1B).

The biosensitivities of the different probes (1–3) were also studied in tissue cultures and cells were analyzed by confocal microscopy as well as flow cytometry to provide spatial and quantitative information about intracellular probe hydrolysis. First, BNL CL2, a mouse liver cell line, was loaded with the various probes (1 μ M, 90 min) with nuclei stained by Hoechst 33258. Representative images are shown in Figure 2 (images A–C). SpiDo-based probe 1 exhibited the expected fluorescent signal with a vesicular distribution (image A) compared to negative control 3 (image C). In the case of alkylhydrazine-based probe 2, fluorescence activation was also observed, however, with lower intensities (Figure 2B). In order to associate fluorescence activation to intracellular acidic con-

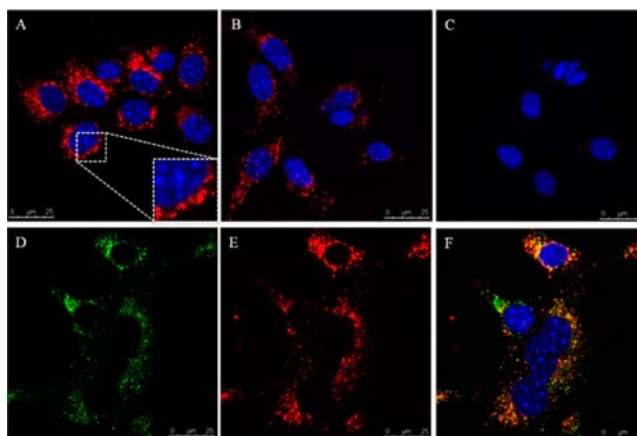


Figure 2. In vitro imaging of BNL CL2 cells loaded with probes 1–3. In vitro imaging of BNL CL2 cells loaded with probes 1 (image A), 2 (image B), and 3 (image C). Distribution and colocalization of 1 with lysosomal probe DND-26 (images D–F). Image D: green channel = lysotracker DND-26 fluorescence, image E: red channel = 1 fluorescence, image F: merge channels. Live cell staining and imaging were carried out by initially staining with the different probes (1 μ M, 90 min) followed by staining with Hoechst 33258 to stain the nuclei (5 μ g/mL, 30 min). For colocalization experiments, cells were stained with LysoTracker Green DND-26 (100 nM, 30 min). Confocal fluorescence microscopy of living cells was performed. Scale bar: 25 μ m.

ditions, 1 was costained with LysoTracker Green DND-26, which is known to stain acidic compartments in live cells such as lysosomes. To our delight, cells displayed colocalization of the red and green fluorescence as a result of the TAMRA and lysotracker, respectively, resulting in an orange signal in a merged picture (images D–F). This colocalization suggests that the probe is taken up by cells to be specifically hydrolyzed in acidic organelles. Moreover, flow cytometry experiments were conducted for additional quantitative comparison. Cells were incubated with probes 1 and 2 for 4 h, suspended in media and analyzed. Probe 1 exhibited a 2.6-fold higher level of fluorescence (A.U.F.) than probe 2, confirming an increased rate of hydrolysis of SpiDo relative to alkylhydrazine (see SI Figure S9). These cellular results are in good agreement with buffer based experiments (Figure 1).

To further validate that hydrolysis of 1 is triggered by pH and not by any other mechanism, endosomal/lysosomal system acidification was inhibited by chloroquine (CQ) which is a weak base known to quickly raise intracellular pH.³⁶ Cells were pretreated with chloroquine (100 μ M, 2 h) prior to probe incubation (1 μ M, 4 h), and analyzed by flow cytometry. Fluorescence activation in CQ-treated cells considerably decreased by 45% and 35% for 1 and 2, respectively, compared to untreated cells (Figure 3 and SI Figure S10). These results clearly demonstrate that activation of both 1 and 2 occurred intracellularly due to pH variation. The lower effect of CQ-treatment on probe 2 may suggest other mechanisms for its activation, such as transamination reactions with proteins. Finally, we decided to investigate the stabilities of probes 1–3 in human plasma (Figure 4). A strong increase of fluorescence was observed for alkylhydrazine-based probe 2, while the level of fluorescence of SpiDo probe 1 remained at a level comparable to control probe 3.

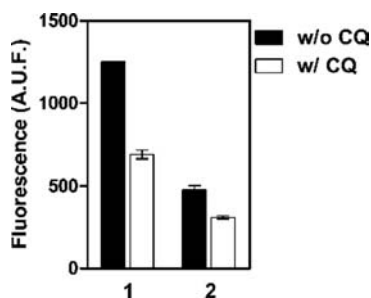


Figure 3. Flow cytometry analysis of probes 1–2. Flow cytometry analysis (2000 cells/assay) of BNL CL2 cells loaded with probes 1 and 2 (1 μ M, 4 h) with (white bars) or without (black bars) CQ-pretreatment (100 μ M, 2 h).

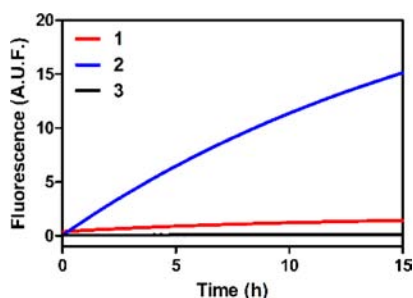


Figure 4. Hydrolysis kinetics of FRET-based probes 1–3 in Human plasma. Hydrolysis kinetics of FRET-based probes 1 (red line), 2 (blue line), and 3 (black line) in Human plasma (50% in PBS). All data were measured in triplicate at 37 $^{\circ}$ C on a microplate reader and with a probe concentration of 0.5 μ M.

CONCLUSION

In conclusion, we have revealed that Spiro Diorthoester (SpiDo) derivative is a potent acid-labile group for the development of pH-sensitive targeted systems. After HPLC-based kinetics of model compounds, the SpiDo motif was inserted into FRET-based probes and the hydrolysis kinetics was recorded and compared to a related acylhydrazone FRET-based probe. This specific SpiDo linker has shown a fast and pH sensitive hydrolysis under mild acidic conditions. The biosensitivities of the different probes were also studied in cellular systems and colocalization experiments showed that the SpiDo FRET-based probe was specifically hydrolyzed in acidic organelles. Finally, stability of both probes was investigated in human plasma and the SpiDo probe proved to be highly stable compared to the acylhydrazone probe. As a result, the profile of SpiDo makes it a very promising acid-labile group for pH-sensitive targeted systems such as internalizing ADCs.

ASSOCIATED CONTENT

Supporting Information

Complete experimental procedures, characterization of new compounds. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00280.

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Notes

The authors declare no competing financial interest.

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