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Clickable polyamine derivatives as chemical probes for the polyamine transport system

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Abstract: Polyamines are essential for cell growth and differentiation, but their trafficking by the polyamine transport system is not fully understood. In this paper, we describe the synthesis of several azido-derivatized polyamines for easy conjugation by click chemistry. Attachment of a BODIPY-dye gave fluorescent polyamine probes, which were tested in cell culture. The linear probe series showed superior cellular uptake compared with the probes where the dye was attached to a branch on one of the central amines. Interestingly, the linear probes accumulated rapidly in cancer cells (MCF-7), but not in non-tumorigenic cells (MCF-10A). The fluorescent polyamine probes are therefore applicable to the study of polyamine trafficking, whereas the azido-polyamines may be further utilized to transport cargo into cancer cells by exploiting the polyamine transport system.

Naturally occurring polyamines, such as spermidine and spermine (Figure 1A), are involved in a variety of critical cellular functions.[1] These include gene expression,[2] cell proliferation and differentiation,[3] as well as cellular stress.[4] Their intracellular concentration, which is in the millimolar range, is tightly regulated by several mechanisms: biosynthesis, catabolism and polyamine transport.[5] Whereas the polyamine biosynthesis and catabolism have been well characterized, the molecular players of polyamine transport remain elusive.[5] As most tumors have elevated activity of the polyamine transport system (PTS),[6] chemical probes that exploit the PTS might be used to target cancer cells for imaging and chemotherapy.[7] In addition, polyamine chemical probes may help in elucidating the transporters constituting the PTS. Interestingly, the structural tolerance of the PTS allows the uptake of substituted polyamines to a large extent. This is illustrated by the report of various fluorescently tagged analogs of spermidine and spermine,[8][9][10][11][12][13][14][15] which include N4-spermidine-MANT,[9] spermidine-C2-BODIPY[9] and spermine-NBD[12] (Figure 1B), all made by different types of linkages to the fluorophore. In order to increase the synthetic flexibility towards polyamine analogs, we initiated the synthesis of clickable polyamine derivatives. In this paper, we describe the synthesis of five azide-functionalized polyamines suitable for easy click chemistry mediated conjugation to alkyne-functionalized cargo facilitating different research needs. As an example, we click the polyamines to an alkyne-functionalized BODIPY to monitor polyamine uptake by cancer cells.

For the design of the azido-polyamine derivatives, we took the following aspects into consideration: (1) Conjugation can be performed on the terminal primary amine or at an internal secondary amine. In the past, spermidine analogs substituted at the secondary amine were shown to exhibit better uptake than analogs with substituents at the primary amine.[16] (2) The polycationic character also seems to be important for uptake into the cell.[17] In view of both of these points, we decided to synthesize two different series of compounds: a branched series, in which the azido group is located on a substituent at a secondary amine (Figure 2A), and a linear series in which the azide is located on a substituent at a primary amine (Figure 2B). The resulting azido polyamines carry different numbers of positive charges under physiological conditions. In total, the charges are 2 (compound 3), 3 (compounds 1 and 4) and 4 (compounds 2 and 5). This will aid in getting insight into the relationship between charge, linearity and uptake.

Figure 1. Structures of polyamines and fluorescent polyamine probes. A) Spermidine and spermine. B) Three different fluorophore-polyamine conjugates.

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Supporting information for this article is given via a link at the end of the document.
For the synthesis of the azido polyamines, commercially available spermidine and spermine were used as starting materials. We employed different protecting group manipulation strategies in combination with selective N-alkylation, as described in detail in the supporting information, yielding Boc-protected derivatives 6-10 (Scheme 1). In order to show the applicability of the azido-polyamine derivatives, BODIPY conjugates were synthesized by first deprotection of the Boc-groups on compounds 6-10 using 50% TFA. Subsequently, the products underwent copper-catalyzed click chemistry with BODIPY alkyne building block 16[18] followed by HPLC purification (Scheme 1).

The resulting BODIPY-tagged polyamine derivatives display strong absorption and green fluorescence (emission at 504-510 nm; Figure 3). All compounds show Stokes shifts between 10 nm (the alkyne BODIPY 16) and 16 nm (compound 15) (Figure 3 and Table 1). The spectral properties show only very minor differences between the different BODIPY-tagged polyamines, indicating that the polyamine-probe does not influence the electronic properties of the dye (Table 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \lambda_{\text{Abs}} ) (max) (nm)</th>
<th>( \lambda_{\text{Em}} ) (max) (nm)</th>
<th>Stokes shift (nm)</th>
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<tr>
<td>11</td>
<td>495</td>
<td>508</td>
<td>13</td>
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<td>12</td>
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<td>16</td>
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Scheme 1. BODIPY click conjugation towards fluorescent polyamine probes. a) TFA/DCM (1/1). b) DIPEA, CuBr, DCM. c) 2,4-Dimethylpyrrole, DCM, reflux. d) Et3N, BF3OEt2, DCM.
Next, the ability of cells to take up the different fluorescent polyamine conjugates was examined. To this end, the breast cancer cell line MCF-7 was incubated with probes 11-15 followed by confocal fluorescent microscopy (Figure 4A). A substantial difference in the uptake of the various probes was obvious from the observed intensities of the intracellular BODIPY fluorescence. Interestingly, branched probes 11-13 displayed low uptake efficiency in comparison with linear probes 14 and 15. Previous studies reported that spermidine analogues with substituents at the central N\(^4\)-nitrogen were better in competing for spermidine uptake than those with substituents at the primary amines.\(^{[13]}\) However, the substituents only ranged in size from methyl (least bulky) to benzyl (most bulky). Apparently, the biological properties of these substituted polyamines cannot be generalized when attaching larger cargo to the polyamines. In line with our findings, linear fluorophore-conjugated polyamines, such as spermine-NBD (Figure 1) and a cyanine dye connected to the primary amine of various polyamines,\(^{[13]}\) have shown efficient cellular uptake, but no direct comparison with branched counterparts was made. We therefore performed flow cytometry experiments of MCF-7 cells after 4 h treatment with probes 11-15. Results show that uptake of linear probes is more than tenfold higher compared with branched probes (Figure 4B). In order to demonstrate that the PTS is responsible for the uptake, we treated MCF-7 cells with benzylviologen, a known inhibitor of the PTS.\(^{[20]}\)\(^{[14]}\)\(^{[15]}\) prior to incubation with probes 14 and 15. PTS inhibition gave up to 60\% reduction of the mean fluorescence of the cells in comparison with untreated cells (Figure 4C), supporting that the probe uptake is mediated by the PTS.

Uptake of linear probes 14 and 15 was efficient, and intracellular accumulation was observed as early as 1 h after probe addition (Figure 5A). Next, we compared the uptake of both linear probes in two different cell lines: the tumorigenic MCF-7 cell line and the non-tumorigenic breast epithelial cell line MCF-10A. Previous studies have shown that polyamine levels in MCF-7 cells are significantly higher than in MCF-10A cells.\(^{[21]}\) We observed a large difference in fluorescent staining, with almost no uptake by the MCF-10A cells (Figure 5B), in agreement with the higher activity of the PTS in cancer cells.

In summary, we have synthesized novel, clickable azido-polyamine derivatives. By clicking these to a BODIPY dye, fluorescent polyamine probes were easily constructed. Cellular uptake of the linear fluorescent polyamines was much more efficient than of the branched ones. Both linear probes were taken up similarly, suggesting that spermine and spermidine are both effective in delivering cargo and that the total positive charge in these probes does not seem to matter. We also show that linear probes 14 and 15 are taken up by tumorigenic MCF-7 cancer cells, but much less by non-tumorigenic MCF-10A cells, which may be explained by a higher activity of the PTS in cancer cells. Hence, these molecules will be useful tools for future interrogation of the PTS, and experiments along these lines will be undertaken and reported in due course. In addition, the azido polyamines may also be utilized to shuttle alkyne-containing chemical probes into cancer cells, allowing for selective delivery of potent anticancer modalities that may ultimately reduce off-target effects.

**Figure 4.** Uptake of polyamine probes by MCF-7 cells. A) Representative fluorescent microscopy pictures of representative cells during a time course of uptake in MCF-7 cells. Blue color represents nuclear staining by DAPI; green color corresponds to BODIPY emission. B) Flow cytometry of MCF-7 cells after 4 h incubation with 10 \(\mu\)M of the indicated probes and trypan blue. Depicted is the mean fluorescent intensity of a minimum of 10,000 cells. C) Mean fluorescent intensity as measured by flow cytometry of MCF-7 cells after 24 h incubation with the PTS inhibitor benzyl viologen followed by addition of probes 14 or 15 for 4 h.

**Figure 5.** Uptake of probes 14 and 15. A) Representative fluorescent microscopy pictures of representative cells during a time course of uptake in MCF-7 cells. Blue color represents nuclear staining by DAPI; green color corresponds to BODIPY emission. B) Comparison of uptake in MCF-7 versus MCF-10A cells after 4 h of incubation with 10 \(\mu\)M of the indicated polyamine probes.

**Experimental Section**

**Synthesis:** the synthesis of all compounds is described in detail in the supporting information.
COMMUNICATION

Click chemistry: Boc-protected azido-polyamine derivatives 6-10 (1.05 eq.) were treated with 50% TFA in DCM for 2 h, after which the solution was concentrated under vacuum, and residual TFA was coevaporated with toluene. To a solution of the crude material and BODIPY 18 (1 eq.), which was synthesized according to previous reports, in DCM were added CuBr (0.5 eq.) and DIPEA (4 eq.). The reaction was stirred for 2-4 days with addition of extra CuBr when the progress of the reaction, as monitored by LC-MS, had stalled. The solvent was removed under vacuum and the products purified by reversed phase HPLC. Fractions containing product were pooled and lyophilized.

Acquisition of absorption and emission spectra: A UV-Visible-NIR Lambda 950 Perkin Elmer spectrometer was used to record the absorption spectra with 1 nm spectral resolution. Attenuators were used to remove the effect of background and noise. The samples placed in a quartz cuvette were sealed and mounted on a Teflon sample holder. Emission spectra were recorded using an Edinburgh Instruments FLS 980 spectrometer on the samples placed in a quartz cuvette (10 mm path length) and sealed by a teflon stopper. The excitation wavelength was set to 470 nm and the emission was collected using a 1-nm step size. The emission was collected in “right-angle mode” through the quartz cuvette and sent to a monochromator and photomultiplier tube (PMT) detector. The wavelength dependence of the detection channel of the fluorimeter was corrected. The optical density of the sample was kept below 0.2.

Cell culture: MCF-7 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich) with 10% fetal bovine serum (Invitrogen). MCF-10A cells were cultured in DMEM/F-12 medium (Fisher Scientific) with 5% horse serum (Aldrich) and 10 μg/mL insulin (Sigma-Aldrich). Cells were cultured at 37 °C under 5% CO2 atmosphere and were split before reaching confluency. Both cell lines were tested for mycoplasma infection and found negative.

Cellular uptake experiments: Cells were seeded in 12 well plates at 25,000 cells/well and cultured overnight in order to adhere. Thereafter, cells were incubated with 10 μM of fluorescent probes 11-15 for the indicated time, washed with PBS, and fixed with 4% paraformaldehyde. DAPI (1:5000, 10 min) was used as a nuclear stain. Images were acquired using a Zeiss LSM 780 (x63 lens and 2x digital magnification) and processed using Image J.

Flow cytometry: Cells were seeded in a 12 well plate at a density of 100,000 cells/well and allowed to attach overnight. Subsequently, cells were treated with 10 μM of probes 11-15 for 4 h and collected by trypsinization. Samples were subjected to flow cytometry on an Attune flow cytometer, where the mean fluorescent intensities were derived from a minimum of 10,000 events per treatment. For the inhibition studies, the experiment was performed as follows: after overnight attachment in 6- or 12-well plates, cells were treated with benzylviologen (50 or 100 μM) or vehicle (PBS) for 24 h. Probes 14 or 15 were added to a final concentration of 10 μM, followed by 4h incubation. Cells were collected by trypsinization and subjected to flow cytometry on an Attune flow cytometer. Each treatment was performed twice in triplicate.

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Keywords: polyamines • fluorescent probes • bioorganic chemistry • click chemistry • imaging agents

Click to enter. Azide-conjugated spermidine and spermine derivatives can easily be clicked onto alkyne-containing cargo. Here, clicking a fluorophore yields spermidine and spermine probes that can be used to follow entry into the cell by the polyamine transport system.