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# Cancer cell targeting driven by selective polyamine reactivity with glycine propargyl esters

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**Rapidly growing cancer cells have increased levels of intracellular polyamines compared to normal, healthy tissues. Based on the selective reactivity of glycine propargyl esters, probes were synthesized that show evidence for selective polyamine reactivity, which was then applied for selective cancer cell imaging studies.**

Polyamines are polycationic alkylamines that exist in virtually all forms of life, where their major forms (putrescine, spermidine, and spermine) are typically found within cells at submillimolar or millimolar concentrations. Despite their seemingly simplistic nature, polyamines play a profound role in numerous biological processes;[1] perhaps the most significant being its role in cell growth and proliferation. This is best exemplified by studies that showed aged cells have decreased intracellular polyamine levels and biosynthesis compared to equivalent cells that are younger and still growing.[2]

Due to the rapid and uncontrolled nature of cancer cell growth, several studies have confirmed its correlation with elevated intracellular levels of polyamines.[3] In addition, overexpression of enzymes/proteins related to the polyamine biosynthetic and transport pathways, such as ornithine decarboxylase (ODC)[4] and polyamine transport system (PTS),[5] have been observed.

To exploit the relationship between polyamines and cancer, numerous strategies have been developed. By far the most well-known example is the ODC-inhibitor difluoromethylornithine (DMFO).[6] When coupled with polyamine transport inhibitors,

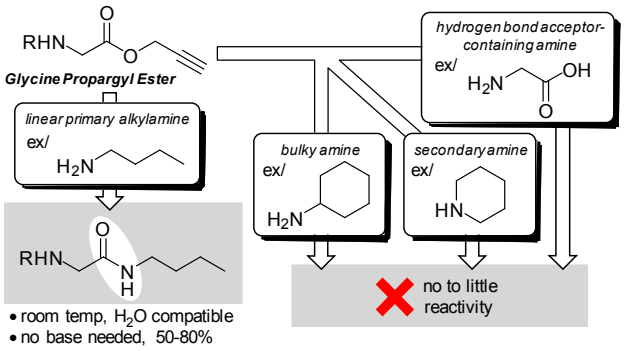


**Fig. 1** Summary of glycine propargyl ester reactivity with linear alkylamines.

an extensive list of studies showed cancerous cell growth can be hindered or reduced.[7] In addition, studies have made use of overactive PTS for selective cancer targeting by employing drug-polyamine conjugates as antitumor agents,[8] and fluorophore-polyamine conjugates as imaging probes[9]

Previously,[10] our lab discovered that glycine propargyl ester-based derivatives could selectively react with linear primary alkylamines (non-secondary amines with long, non-bulky linear substituents), as shown in Fig. 1. Selectivity can be mainly attributed to this reaction relying on hydrogen bonding/intramolecular interactions to stabilize the transition state, causing the facile amidation of an otherwise moderately stable leaving group (propargyl alcohol pKa ~ 13.6).

Within living systems, there are three major groups of

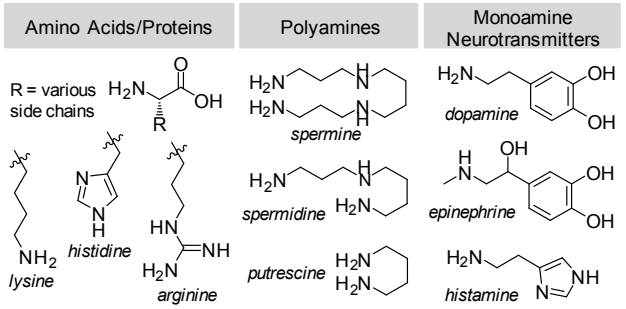


**Fig. 2** Major classes of biological amines.

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biological amines: polyamines, amino acids/proteins and monoamine neurotransmitters (Fig. 2). Amino acids act as important building blocks for not only proteins, but also as intermediates for a wide range of metabolites. Monoamine neurotransmitters act as neuromodulators in the central nervous system and as hormones in the blood circulation. Other minor groups of biological amines also exist, such as lipids and sugars, but their free forms are often present in relatively low abundance.

Due to its linear and non-bulky nature, it was hypothesized that polyamines may have preferential reactivity with glycine propargyl esters. Furthermore, the imbalance of intracellular polyamine concentrations between cancer and normal cells is expected to allow glycine propargyl ester-based imaging probes to selectively target rapidly growing cancer cells, which was investigated in this study.

Various glycine propargyl ester probes (Fig. 3) were synthesized and detailed in the supplementary information. Cbz-containing glycine propargyl ester **1** was used for product identification studies, while kinetic and cell-based qualitative assays used the fluorophore probes **9** (linked to 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS)), and **13** (linked to 5-carboxytetramethylrhodamine (TAMRA)), respectively. In the case of probe **9**, a quencher 4-((4-(dimethylamino)phenyl)azo)benzoic acid (DABCYL) is also utilized to make use of Förster resonance energy transfer (FRET) interactions.

Given that polyamines can be structurally defined as linear primary alkylamines, it was speculated that glycine propargyl ester reactivity could be selective for polyamines over other biological amines, such as amino acids and monoamine neurotransmitters. To prove this, Table 1 (HPLC traces in Fig. S1-S3) compares the reactivity of polyamines to representative molecules of the other biological amine classes (i.e. lysine and norepinephrine). Results show that when incubated with the glycine propargyl ester-based probe **1**, the highest yields were obtained with spermine, spermidine and putrescine (59-67% single amidation product, 11-23% double amidation product, entries 1-3) compared to norepinephrine (0%, entry 4) and lysine (2%, entry 5). Observations also show the near complete recovery of probe **1** with norepinephrine and lysine, which is in stark contrast to when polyamines are present. In an additional experimental scenario, representative molecules of each class (i.e. spermine, lysine, and norepinephrine) were mixed together

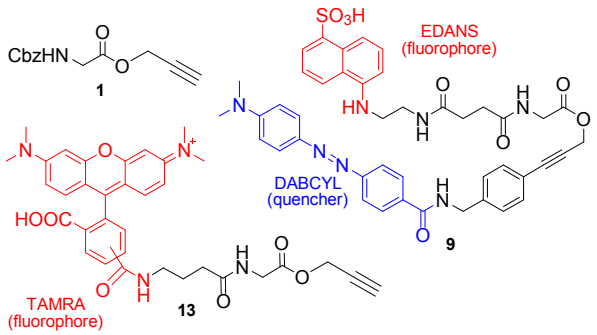
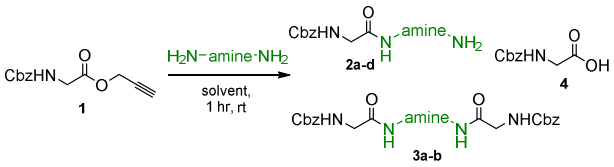
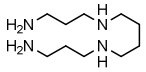
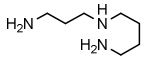
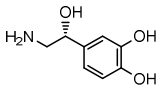
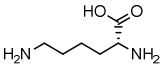



**Fig. 3** Glycine propargyl ester-based probes used in this study.

**Table 1** Exploring biological amine selectivity and reactivity




| entry | amine | single linkage yield[1] | double linkage yield[1] | hydrolysis product **4**[1] | recovered **1**[2] |
|---|---|---|---|---|---|
| 1 |  | 59%[3], 56%[4], 47%[5] (**2a**) | 23%[3], 18%[4], 14%[5] (**3a**) | 17%[3], 29%[4], 14%[5] | 1%[3], N/A[4], N/A[5] |
| 2 |  | 67%[3] (**2b**) | 11%[3] (**3b**) | 21%[3] | 1%[3] |
| 3 |  | 66%[3] (**2c**) | N/A[3] | 22%[3] | 7%[3] |
| 4 |  | N/A[3] | N/A[3] | 7%[3] | 92%[3] |
| 5 |  | 2%[3] (**2d**) | N/A[3] | 6%[3] | 85%[3] |

[1]isolated yields via HPLC purification, [2]isolation of remaining ester starting material, [3]1:1 dioxane/PBS Buffer 7.4, [4]1:1 dioxane/DMEM media, [5]1:1 dioxane/rat serum; N/A = speculated product not found. Reactions were standardized to 0.04 mmol of amine and ester in 0.2 ml of solvent (200 mM).

with probe **1** (Fig. 4). As shown by the HPLC trace and isolated yields, probe **1** reactivity continues to favour polyamines over norepinephrine and lysine.

To develop an assay that can be used to compare glycine propargyl ester reactivity for a wide range of substrates, the FRET-based probe **9** was synthesized. As shown in Fig. 5 and detailed in Fig. S13, there is a ~13× increase in quantum yield between probe **9** and its spermine-linked amide product **10**. In a further study, shown in the box insert of Fig. 5, investigation of the kinetic activity between spermine and probe **9** gave a calculated apparent second-order rate constant of 19.9 ± 0.1 $\mu M^{-1}min^{-1}$ (calculations detailed in Fig. S14-16).

Using this FRET-based probe, various biological amines of significance from each representative class were tested (Fig. 6

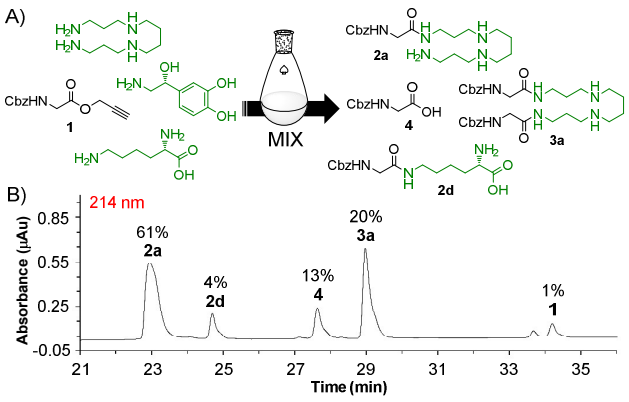



**Fig. 4** HPLC analysis of reaction mixture to observe for polyamine selectivity. Spermine, L-lysine, and norepinephrine (0.04 mmol) were mixed in 1:1 dioxane/PBS buffer pH 7.4 (0.2 ml) for 1 hr. Isolated yields via HPLC purification.

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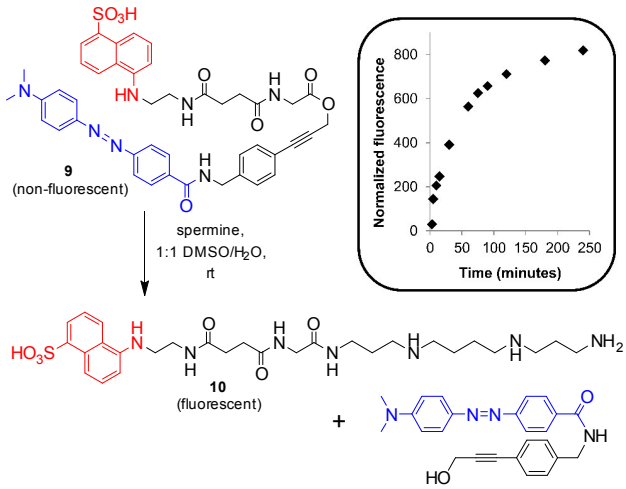


| | ε [M$^{-1}$ cm$^{-1}$]$^1$ | $\lambda_{abs}$ [nm]$^1$ | $\lambda_{em}$ [nm]$^1$ | $\Phi_F$ $^{1,2}$ |
|---|---|---|---|---|
| **9** | 1325 ± 151 | 420 | 420 | 0.0449 ± 0.0002 |
| **10** | 3693 ± 13 | 340 | 490 | 0.5770 ± 0.0293 |

**Fig. 5** Photophysical and kinetic parameters of FRET-based probes. [1]measured in 1:1 DMSO/$H_2O$, [2]relative quantum yields of fluorescence ($\Phi_F$) measured using quinine sulfate as the standard. Box insert shows the kinetic activity of the reaction done in DMSO.

and S4); amino acids/proteins (albumin, L-arginine, L-histidine, L-lysine), polyamines (putrescine, spermidine, spermine), monoamine neurotransmitters (epinephrine, histamine, dopamine, phenethylamine), as well as sphingosine. As expected, the highest levels of fluorescence were observed when polyamines such as spermine and spermidine were tested. Aware that two primary amines are present on polyamines compared to one primary amine of most amino acids and monoamine neurotransmitters, various substrate concentrations were also tested. For example, comparing the fluorescence levels of 2 mM lysine with 1 or 0.5 mM of spermine clearly shows much lower levels of activity. To confirm product formation (Fig. S5), the reaction mixtures containing L-arginine, L-lysine, and spermine were further analyzed by fluorometric HPLC. Observations show that the low levels of fluorescence for mixtures containing L-arginine and L-lysine are mainly attributed to ester hydrolysis, whereas the mixture containing spermine clearly shows desired product, as well as some ester hydrolysis.

Another important observation is that polyamine reactivity rapidly increases from putrescine to spermidine to spermine. This is likely due to the increasing presence of secondary amines, which may play a role in accelerating reactivity through intramolecular basicity. Future studies will likely look to confirm these claims.

To investigate the potential for *in vivo* polyamine targeting within a living biological system, cell-based studies with water soluble probe **13** linked to a cell-penetrable TAMRA moiety was carried out. Initial studies focused on incubation with the MCF-7 breast cancer cell line (protocol detailed in supplementary information). By fluorometric HPLC analysis (Fig. S6 and S7), a key metabolite found in the cellular extract may be the 1,3-diaminopropane-TAMRA linked product, which is formed from spermine oxidase metabolization of spermine-TAMRA. This result led to speculation that fluorescent probe **13** could be preferentially retained within cancer cells following polyamine reactivity due to possible interactions with polyamine biosynthetic and catabolic pathway enzymes. Another valid reason for cell retention would be that the fluorescent probe **13** gains an increase in positive charge following polyamine reaction, thus preventing cell membrane penetration.

Studies have shown that there are disproportionately higher polyamine levels within breast cancer cells compared to their normal cell counterparts.[11] As such, the potential usage of probe **13** to preferentially image cancer cells over normal cell lines was next explored. Imaging results show fluorescence (Fig. 7A and S8-S10) was detected in three different breast cancer cells lines (MCF7, MDA-MB-231, SK-BR-3) when incubated with probe **13**. In contrast, incubation with normal cell lines (MCF10A and lymphocytes) showed no fluorescence (Fig. 7B and S11-S12). This difference is clearly illustrated by comparing the relative fluorescence intensities normalized against the background (Fig. 7C).

In conclusion, we have shown glycine propargyl ester-based compounds have preferential reactivity with polyamines over other biological amines (amino acids/proteins and monoamine neurotransmitters). To prove the viability of employing this reactivity towards biomedical applications, a novel framework for cancer cell targeting/imaging was developed. Through the imbalance of intraceullular polyamines typically found within rapidly growing cells, evidence shows that glycine propargyl ester-based probes can be applied for selective cancer cell

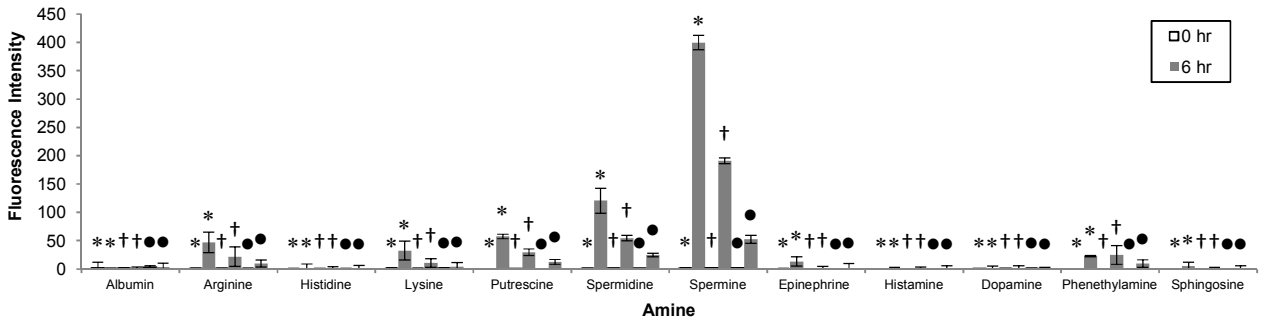


**Fig. 6** Comparison of FRET-based fluorescence generated from reactivity between probe **9** (100 µM) and various biological amines (indicated as ∗2mM, †1mM, ●0.5mM). Reactions were done in 1:1 DMSO/PBS buffer pH 7.4 under various time points.

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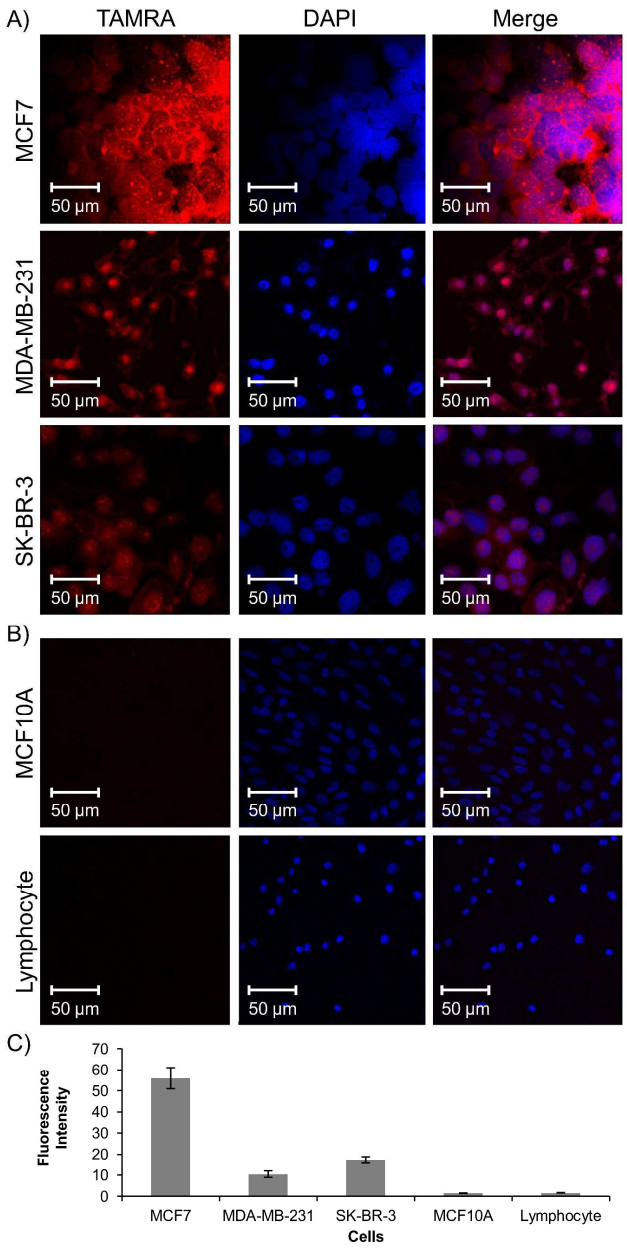


**Fig. 7** A) Cell imaging of breast cancer cell lines MCF7, MDA-MB-231, and SK-BR-3 that were incubated with TAMRA-linked propargyl ester **13** (30 μM). B) Cell imaging of normal cell lines MCF10A and lymphocytes that were incubated with TAMRA-linked propargyl ester **13** (30 μM). C) Comparison of the relative fluorescence intensities normalized against the background.

imaging. Worth noting is that it acts in a different mechanism from thiol-sensing rhodamine propargyl esters.[12] Future studies centred on this work will focus on exploiting this glycine propargyl ester reactivity for other biomedical applications, specifically in anticancer therapies.

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