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Fluorophor-labeled spermidine derivatives as fluorescent markers in optical tumor imaging

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Abstract—Up-regulation of polyamine transporters on the surface of tumor cells and the internalization of biogenic polyamines by active transport processes may be exploited for the accumulation of spermidine derivatives as reporter molecules. We have synthesized and tested fluorophor-labeled spermidine derivatives for the development of a new class of intraoperative tumor imaging agents. In vitro uptake experiments and initial in vivo imaging studies illustrated that fluorophor tagged spermidine derivatives show tumor accumulation.

The discrimination between normal and malignant tissues is a major challenge in tumor surgery and endoscopic tumor diagnosis. Targeted fluorescent dyes resulting in tissue fluorescence differences or the application of photosensitizers—used in photodynamic diagnosis (PDD)—are of substantial value for the endoscopic or intraoperative delineation of primary tumors and metastatic lesions, in particular for oncological applications.[1–3] Most fluorescence markers for intraoperative tumor imaging are based on receptor-targeted dye-peptide or protein-dye conjugates.[4–7] Fluorophor conjugated specific antibodies are used as tumor surface markers.[8]

A number of mammalian tumor cell lines have been shown to contain a high level of an active polyamine uptake system.[9] In addition to biosynthesis cancer cells internalize polyamines by receptor-mediated active transport processes which can result in the accumulation of micromolar polyamine quantities and intra-to-extracellular ratios of the order of 1000.[10,11] The polyamine transport system has a broad structural substrate tolerance.[9] In particular, spermidine conjugates have been found to be good substrates of polyamine transporters.[12,13] The affinity of polyamines and their analogs to polyamine transporters increases with the number of positive charges.[12] Polyamines could therefore be successfully used as carriers for the targeting of anticancer drugs to tumors.[14,15]

We propose of coupling polyamines—such as spermidine—to fluorescent dyes might provide a new class of fluorescent markers in optical tumor imaging which utilize the polyamine transport system in order to accumulate in tumors. We synthesized and characterized spermidine-dye conjugates and evaluated their in vitro and in vivo tumor uptake characteristics.

The chemical structures of compounds **1**–**4**, spermidine, and 2-(methylamino)benzoic acid (MANT) are outlined in Scheme 1. Altogether, a series of three spermidine conjugates has been synthesized that contain the following fluorophores: 5(6)carboxyfluorescein (**1**), acridine yellow G (**3**) or MANT (**4**), along with two spermidine-free control dyes, compound **2** and MANT. MANT allowed direct coupling of spermidine to yield **4**, whereas the other compounds additionally consist of a peptidyl (**1**) or an amino acid as linker moiety (**3**).

Keywords: Tumor imaging; Fluorescence; Spermidine; Intraoperative imaging.
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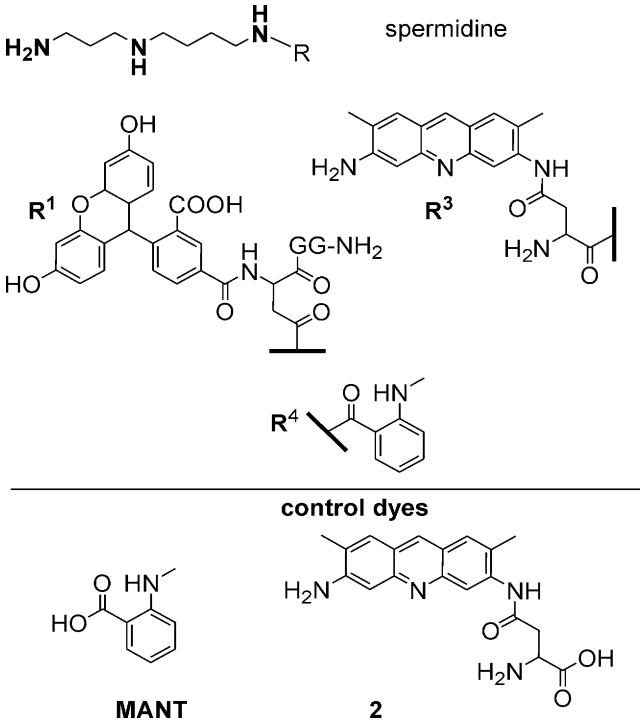
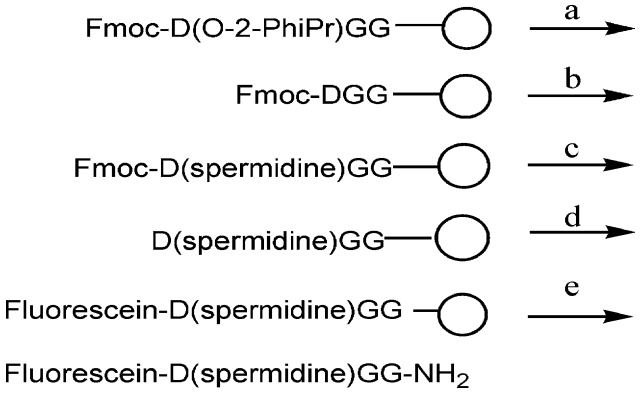


Scheme 1. Chemical structures of the dye conjugates **1**, **3**, and **4** as well as the control dyes.

Conjugates **1** and **3** as well as compound **2** were synthesized automatically, according to the Merrifield strategy. 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids were coupled in a stepwise manner to the corresponding resin.[16] 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in *N*-methylpyrrolidone (NMP) was used as coupling agent. Fmoc groups were removed using 20% piperidine/NMP. The compounds were cleaved from the resin with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (95:2.5:2.5). Reversed-phase HPLC purification of the crude products yielded the pure conjugates **1**–**4** which were characterized by analytical reversed-phase HPLC, ^1H NMR spectroscopy, and ion-spray mass spectrometry to reveal identity and purity of the novel compounds.

The synthetic approach for conjugate **1** (*N*-5(6)-carboxy-fluorescein-aspartyl(spermidine)-glycylglycyl-amide) is outlined in Scheme 2. Cleavage from the Rink amide resin with subsequent purification yielded **1** ($C_{36}H_{41}N_7O_{10}$, $[M+H]^+ = 731.73$).

Compounds **2** (aspartate(acridine yellow G) and **3** (aspartyl(acridine yellow G)spermidine) were assembled on Fmoc-D (OAll)-Wang resin (OAll = α-allyl ester). The synthesis started with Pd/C (palladium/carbon)-mediated cleavage of the α-allyl ester protecting group. The free side-chain carboxyl group was acylated with a fivefold excess of acridine yellow G. To obtain **2**, the Fmoc group was cleaved with subsequent removal of the compound from the resin. Purification yielded **2** ($C_{19}H_{20}N_4O_3$, $[M+H]^+ = 352.94$). Compound **3** was obtained by cleavage of Fmoc-D-acridine yellow G from the resin. Then Fmoc-D-acridine yellow G was coupled



Scheme 2. Reagents and conditions for the synthesis of **1**: (a) 1% TFA in CH_2Cl_2; (b) spermidine, HBTU/NMP; (c) 20% piperidine/NMP; (d) 5(6)carboxyfluorescein, HBTU/NMP; (e) 2 h, TFA/H_2O/triisopropylsilane (95:2.5:2.5).

with a fourfold excess of spermidine. After cleavage of Fmoc, precipitation with diethyl ether, and lyophilization, the crude product was purified over preparative HPLC, yielding **3** ($C_{26}H_{37}N_7O_2$, $[M+H]^+ = 480.83$) after lyophylization.

2-(Methylamino)benzoic acid (MANT) was prepared as previously described by Di Carlo and Lindwall.[17] Conjugate **4** was obtained by reacting equimolar quantities of spermidine and 2-(methylamino)benzoic acid in the presence of equimolar amounts of the coupling reagent HATU (*O*-(7-azabenzo-triazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate), yielding **4** ($C_{15}H_{26}N_4O$, $[M+H]^+ = 279.08$). Excitation and emission spectra of compounds **1**, **3**, and **4** are shown in Figure 1.

The in vitro uptake characteristics of the fluorescent-labeled spermidine derivatives **1**–**4** into two tumor cell lines (B16 mouse melanoma and MH3924A rat hepatoma) could be directly followed by confocal laser scanning microscopy (a tunable ZeissLSM 510 UV). In a control experiment we co-cultured and co-incubated

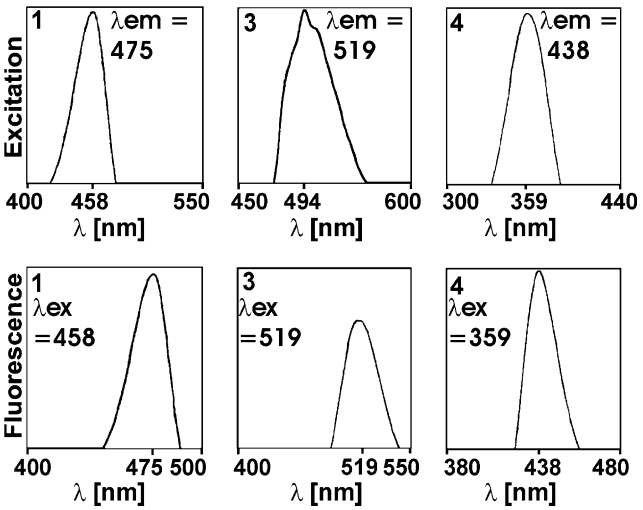


Figure 1. Excitation and fluorescence spectra of compounds **1**, **3**, and **4**.