Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 3193–3196

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

Markus Wolf,[a,*] Ulrike Bauder-Wüst,[a] Rüdiger Pipkorn,[b] Helmut Eskerski[c] and Michael Eisenhut[a]

[a]*Department of Radiopharmaceutical Chemistry, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany*
[b]*Peptide Synthesis Facility, German Cancer Research Center (DKFZ), TP3 Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany*
[c]*Clinical Cooperation Unit Nuclear Medicine, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany*

Received 6 February 2006; revised 15 March 2006; accepted 16 March 2006
Available online 18 April 2006

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]

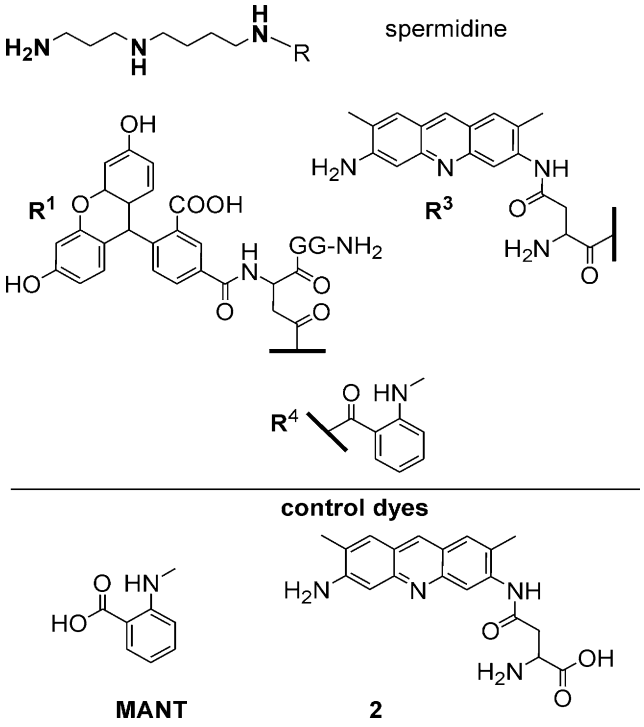
A number of mammalian tumor cell lines have been shown to contain a high level of an active polyamine uptake system.[9] In addition to biosynthesis cancer cells internalize polyamines by receptor-mediated active transport processes which can result in the accumulation of micromolar polyamine quantities 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.
* Corresponding author. Tel.: +496221422422; fax: +496221422430; e-mail: Markus.Wolf@dkfz.de

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.03.046

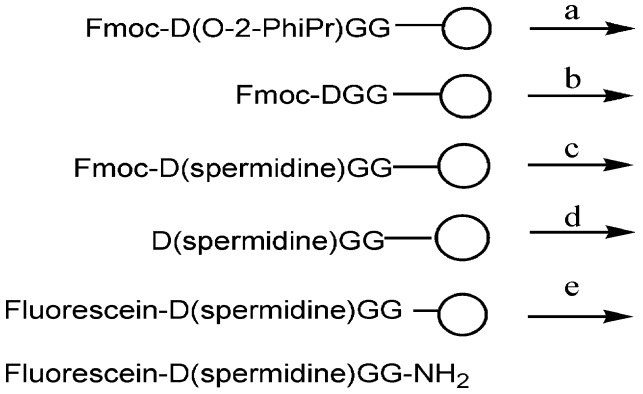


Scheme 1. Chemical structures of the dye conjugates **1**, **3**, and **4** as well as the control dyes.

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

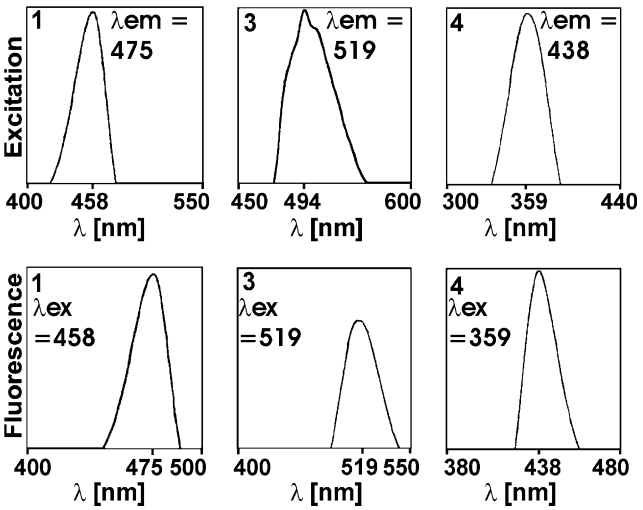


Figure 1. Excitation and fluorescence spectra of compounds **1**, **3**, and **4**.

mouse fibroblasts 3T3YMT-MP expressing Phi-Yellow (a yellow fluorescent protein) and B16 or MH3924A cells in the presence of compound **4**. The cells were imaged on the coverslips on which they were grown following 1, 4, and 24 h incubations with 50 μM of one of the compounds. Conjugate **1** was bound on the cell surface and not internalized by both cell lines (Fig. 2). Unlabeled tumor cells and tumor cells incubated with the control dyes **2** (control for **3**) or MANT (control for **4**) showed no fluorescence. B16 and MH3924A cells internalized conjugates **3** and **4** (Fig. 2) into granular cytoplasmatic structures, probably endosomes and lysosomes. Tumor cells incubated with **3** or **4** in the presence of benzyl viologen, a potent polyamine uptake inhibitor, showed no fluorescence. The non-tumor cell line (3T3YMT-MP) showed yellow fluorescence due to yellow fluorescent protein expression in their mitochondria and slight blue fluorescence, whereas the tumor cells showed intensive blue fluorescence (Fig. 2). Tumor cell associated blue fluorescence was more intensive compared to non-tumor cell associated blue fluorescence suggesting that dye **4** is preferably internalized into tumor cells. Quantitative uptake studies showed a 19% increase in cell associated fluorescence for B16 melanoma and 16% for MH3924A cells after a 24 h incubation with **4** versus unlabeled tumor cells.

The affinity of polyamines and their analogs to polyamine transporters increases with the number of positive charges.[12] As compound **1** shows three negatively and two positively charged groups under physiological conditions, it is bound to polyamine receptors without a subsequent internalization. To achieve intracellular uptake, the net charge of the conjugates must be positive under physiological conditions, which can be realized by the conjugation of neutral (MANT) or basic fluorophores (acridine yellow G) to spermidine. This hypothesis was supported by the internalization of **3** (five positive charges) and **4** (two positive net charges).

Cellular toxicities of the dyes **1**, **3**, and **4** were tested in a colony forming assay with MH3924A and B16 cells.

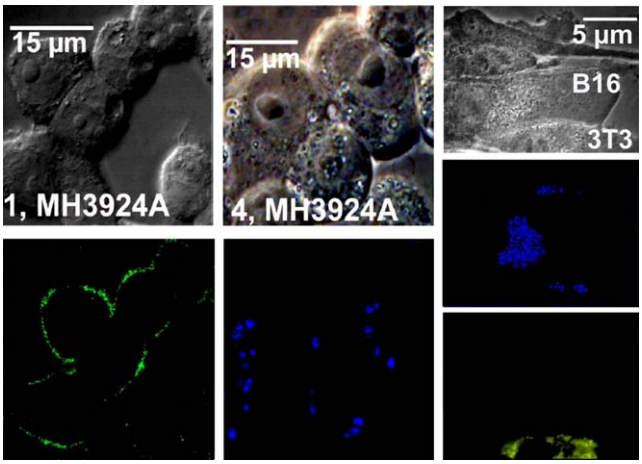


Figure 2. Confocal laser scanning micrographs of cultured MH3924A cells after 24-h incubation with **1** or **4**, and B16 melanoma cells (blue fluorescence) co-cultured with 3T3YMT-MP cells (yellow fluorescence and slight blue fluorescence) after a 4-h incubation with 50 μM of **4**.

Eight days after 4 h incubations with **1** or **4** at concentrations of 1–250 μM, colony forming unit ranged between 100% and 98% (percentage relative to untreated controls). Colony formation was completely inhibited following 4 h incubations with 25 μM of conjugate **3**.

Conjugate **4** was selected for initial in vivo testing based on favorable tumor cell uptake and in vitro cytotoxicity characteristics. In vivo fluorescence imaging experiments were performed on male nude mice Balc C nu/nu (ca. 20 g of bodyweight) bearing subcutaneous MH3924A (rat hepatoma) with a mean diameter of 3–8 mm. All animal experiments were in compliance to the German Animal Guidelines. The polyamine-dye conjugate **4** was injected intravenously as a bolus at a dose of 500 μmol/kg bodyweight via a lateral tail vein. Directly prior to the imaging experiments the mice were anesthetized by intraperitoneal injection of ketamine/xylazine hydrochloride (1:1) at a dose of 1 mL/kg bodyweight. Images were acquired 10 min, 1 and 4 h after the administration of **4** in a constant experimental (exposure time = 60 s, bit range = 0–6) setup using a dual-mode cooled Hamamatsu C4480 12-bit CCD (charge coupled device) camera (Hamamatsu Photonics GmbH) in an open surgery with a large incision in the chest or abdomen. Image acquisition and analysis were performed using Argus 50 software (Hamamatsu Photonics). A monochromatic, ultraviolet 15 Watt handlamp was used as the excitation source. Long-wave pass filters were used to cut off reflected excitation light, and wavelengths between $430 < \lambda < 450$ nm (quantum yield = 0.3) were detected. The working distance was in the range of 5 cm. In control animals that received only PBS buffer injections no contrast enhancement was observed (Fig. 3). Ten minutes and one hour after the administration of contrast agent **4,** fluorescence associated to dye accumulation could be detected mainly in the bladder and in the tumor (MH3924A), to a lesser extent in intestine, liver, and kidneys, but not in the muscle, lungs or other organs and tissues (Fig. 3). Absolute fluorescence signal intensities of tumor and organs were determined by setting regions of interests (= 11.6 mm^2 = 14881 pixels) with Argus software which assures that each region of interest contains the same number of pixels. Signal intensities were expressed as total numbers of counts in the region over 1 min. The bladder showed the highest intensity with 183105 counts, followed by the tumor with 153718 counts, and intestine with 99157 counts. Even 4 h after the application of **4**, contrast enhancement with 115443 photon counts in the whole tumor was observed. The tumor of control animals showed 2103 photon counts. Fluorescence micrographs of excised tumor and selected organs (liver, kidneys, lungs, and intestine) were in accordance with the imaging experiments. Control tissue sections of untreated animals lack auto fluorescence.

The major advantage of polyamine dye conjugates is their potential to accumulate in a wide range of tumor types, as a great number of tumor cell lines have elevated polyamine receptor levels,whereas only a single tumor type can be detected by fluorescent markers based on antibodies and receptor-targeted peptides and proteins.[5,8,9] Additionally after application of **4** no protection from sunlight is necessary, whereas skin retention of photosensitizers

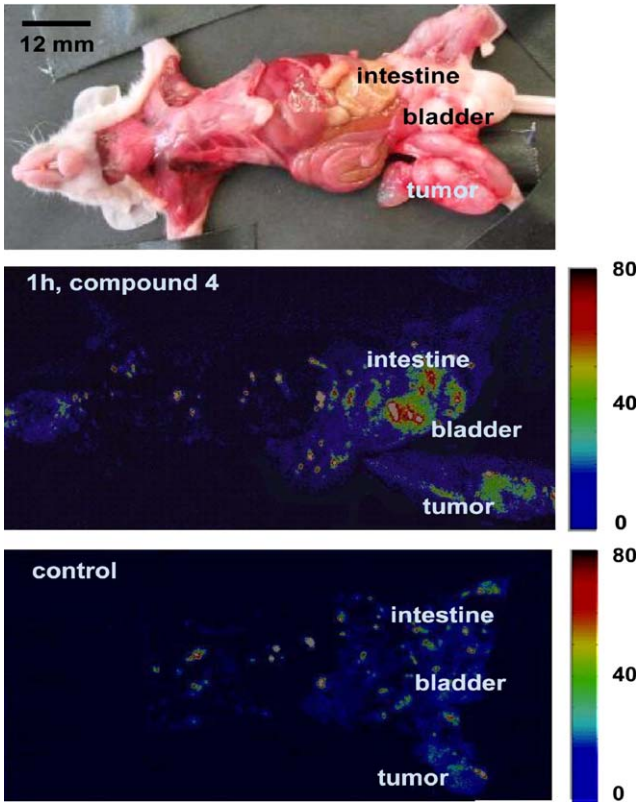


Figure 3. In vivo optical CCD imaging of nude mice carrying MH3924A 1 h after injection of compound **4** or PBS buffer (control). All units are photon counts (Maximal signal per pixel = 80, minimal signal per pixel = 0). Exposure time = 60 s.

based on porphyrin necessitates protection of patients from light for as long as 6–7 weeks.[18] The new generation photosensitizer 5-aminolevulinic acid shows selective uptake in vascular and proliferative tissues.[2] However, its uptake rate is restricted by its hydrophilic nature.[2] Fast, facilitated tumor cell uptake despite the hydrophilic nature of conjugate **4** is an advantage compared to 5-aminolevulinic acid. Due to substantial accumulation of **4** in bladder and intestine, the major disadvantage of compound **4**, the detection of tumors located within these organs would be difficult. In the detection of cancer in bladder and intestine 5-aminolevulinic acid should be more suitable.[2] The data presented in this paper support the further evaluation of conjugate **4** as an optical agent for tumor identification.

Acknowledgments

We thank the following colleagues (all DKFZ, Germany): W. E. Hull for ^{1}H NMR-analyses; H. Spring and M. Müller for assistance with the fluorescence microscope, as well as F. Klimek for assistance in taking pictures with the CCD camera.

Supplementary data

Supplementary data containing experimental procedures and analytical data associated with this article is available. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.03.046.

References and notes

1. Graves, E. E.; Weissleder, R.; Ntziachristos, V. *Curr. Mol. Med.* **2004**, *4*, 419.
2. Lange, N.; Jichlinski, P.; Zellweger, M.; Forrer, M.; Marti, A.; Guillou, L.; Kucera, P.; Wagnieres, G.; van den Bergh, H. *Br. J. Cancer* **1999**, *80*, 185.
3. Wagnières, G. A.; Star, W. M.; Wilson, B. C. *Photochem. Photobiol.* **1998**, *68*, 603.
4. Gurfinkel, M.; Ke, S.; Wen, X.; Li, C.; Sevick-Muraca, E. M. *Dis. Markers* **2003–2004**, *19*, 107.
5. Licha, K.; Hessenius, C.; Becker, A.; Henklein, P.; Bauer, M.; Wisniewski, S.; Wiedenmann, B.; Semmler, W. *Bioconjugate Chem.* **2001**, *12*, 44.
6. Becker, A.; Riefke, B.; Ebert, B.; Sukowski, U.; Rinneberg, H.; Semmler, W.; Licha, K. *Photochem. Photobiol.* **2000**, *72*, 234.
7. Kremer, P.; Wunder, A.; Sinn, H.; Haase, T.; Rheinwald, M.; Zillmann, U.; Albert, F. K.; Kunze, S. *Neurol. Res.* **2000**, *22*, 481.
8. Fibich, G.; Hammer, A.; Gannot, G.; Gandjbakhche, A.; Gannot, I. *Lasers Surg. Med.* **2005**, *21*, 155.
9. Cullis, P. M.; Green, R. E.; Merson-Davis, L.; Travis, N. *Chem. Biol.* **1999**, *6*, 717.
10. Porter, C. W.; Miller, J.; Bergeron, R. *Cancer Res.* **1984**, *44*, 126.
11. Seiler, N. *Curr. Drug. Targets* **2003**, *4*, 565.
12. Seiler, N.; Delcors, J. G.; Moulinoux, J. P. *Int. J. Biochem. Cell Biol.* **1996**, *28*, 843.
13. Holley, J.; Mather, A.; Cullis, P.; Symons, M. C. R.; Wardmann, P.; Watt, R. A.; Cohen, G. E. *Biochem. Pharmacol.* **1992**, *43*, 763.
14. Holley, J.; Mather, A.; Cullis, P.; Symons, M. R.; Wardman, P.; Watt, R. A.; Cohen, G. M. *Biochem. Pharmacol.* **1992**, *18*, 763.
15. Stark, P. A.; Thrall, B. D.; Meadows, G. G.; Abdel-Monem, M. M. *J. Med. Chem.* **1992**, *13*, 4264.
16. Miranda, L. P.; Alewood, P. F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *95*, 1794.
17. Di Carlo, F. J.; Lindwall, H. G. *J. Am. Chem. Soc.* **1945**, *67*, 1999.
18. Pahernik, S. A.; Botzlar; Hillemanns, P.; Dellian, M.; Kirschstein, M.; Abels, C.; Korell, M.; Mueller-Hoecker, J.; Untch, M.; Goetz, A. E. *Int. J. Cancer* **1998**, *78*, 310.