Bioorganic & Medicinal Chemistry Letters 19 (2009) 2474–2477

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of conjugated spermine derivatives with 7-nitrobenzoxadiazole (NBD), rhodamine and bodipy as new fluorescent probes for the polyamine transport system

Yves Guminski [a], Martial Grousseaud [a], Sandrine Cugnasse [a], Viviane Brel [b], Jean-Philippe Annereau [b], Stéphane Vispé [b], Nicolas Guilbaud [b], Jean-Marc Barret [b], Christian Bailly [b], Thierry Imbert [a,*]

[a] Division de Chimie Médicinale, Centre de Recherche Pierre Fabre, 17 rue Jean Moulin, 81106 Castres cedex, France
[b] Centre de Recherche en Oncologie Expérimentale, Institut de Recherche Pierre Fabre, 3 rue des Satellites BP 94244, 31432 Toulouse cedex 4, France

ARTICLE INFO

Article history:
Received 11 February 2009
Revised 12 March 2009
Accepted 12 March 2009
Available online 18 March 2009

Keywords:
Polyamine transport system
Spermine
Fluorescent diagnostic probe
Nitrobenzoxadiazole

ABSTRACT

The synthesis of a series of conjugated spermine derivatives with benzoxadiazole, phenylxanthene or bodipy fluorophores is described. These fluorescent probes were used to identify the activity of the polyamine transport system (PTS). N_1-Methylspermine NBD conjugate **5** proved to have the optimal fluorescence characteristics and was used to show a selectivity for PTS-proficient CHO versus PTS-deficient CHO-MG cells. It can therefore be used as a tool for the selection of cells sensitive to cytotoxic compounds vectored through the PTS.

Natural polyamines such as spermine, spermidine and putrescine are aliphatic polycations at physiological pH that are involved in cell growth and differentiation, and are essential for the metabolism and proliferation of cancer cells. Internalisation of polyamines from extracellular sources can compensate for the low availability of such substances within the cell. This cellular uptake process involves the highly regulated polyamine transport system (PTS),[1] which is over-activated in most cancer cells.[2] Chemical selectivity for recognition of the PTS tolerates large modifications in polyamine structure.[3] We therefore took advantage of this property to preferentially vectorize cytotoxic compounds towards cancer cells. An epipodophyllotoxin-spermine conjugate, designated here as F14512, was recently designed as a cytotoxic agent targeted towards tumor cells via the PTS.[4]

In order to investigate the activity of the PTS and identify cancer cells that exhibit a high uptake of polyamines, we designed a series of conjugated molecules featuring a fluorescent probe tethered to a polyamine moiety (i.e. spermine), as shown in Figure 1.

Using these probes, fluorescence detected within the cell would be expected to be dependent upon the efficiency of PTS activity. Consequently, the positively marked cancer cells would be expected to be more sensitive to polyamine conjugated antitumor compounds, such as F14512. Excitation of the appropriate fluorescent probe would also be expected to be attainable using laser sources that exist in most currently available clinical flow cytometers. The selection of fluorescent chemicals was therefore based on optimal wavelength of excitation/emission, quantum yield, selectivity for PTS-positive cells and minimum bleaching upon light irradiation. Small modifications of the spacer could confer a high variability of these parameters. Phenylxanthenes such as fluorescein, rhodamine, lissamine, benzofurazanes such as 4-nitrobenzo[1,2,5]oxadiazole derivatives (NBD) and bodipy were selected as potential fluorophores for this purpose. Initially, bodipy, whose optical properties and chemistry have been recently reviewed,[5] represented an interesting candidate, considering its generally high fluorescence quantum yield. We report here the synthesis of these novel conjugated compounds and the comparison of their fluorescence properties in biological systems for identifying PTS-positive cancer cells. The relationships between fluorescence characteristics and chemical structures of substituted benzoxadiazoles were first investigated.[6] Synthesis of conjugated spermine-NBD derivatives are depicted in Scheme 1.

t-Butoxycarbonyl (BOC) substituents as protective groups at the nitrogen atoms of spermine derivatives were selected and yielded efficient results. Putrescine, spermidine or other polyamine derivatives could be prepared in the same way. TriBOC spermine **1** hydrochloride was prepared as described.[7] This three step procedure was slightly modified and could be carried out in a single operation.[8] Coupling of the primary amine of triBOC spermine **1**

* Corresponding author. Tel.: +33 5 63 71 42 03; fax: +33 5 63 71 42 99.
E-mail address: thierry.imbert@pierre-fabre.com (T. Imbert).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.03.052

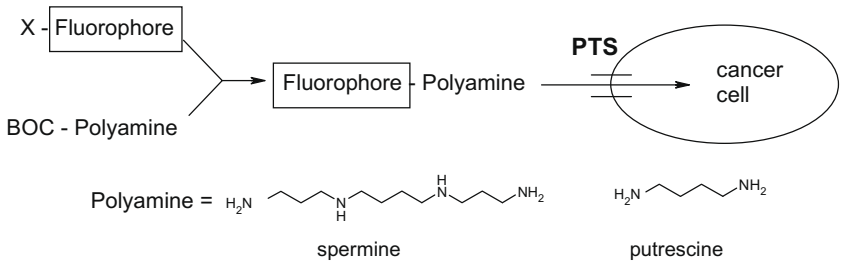
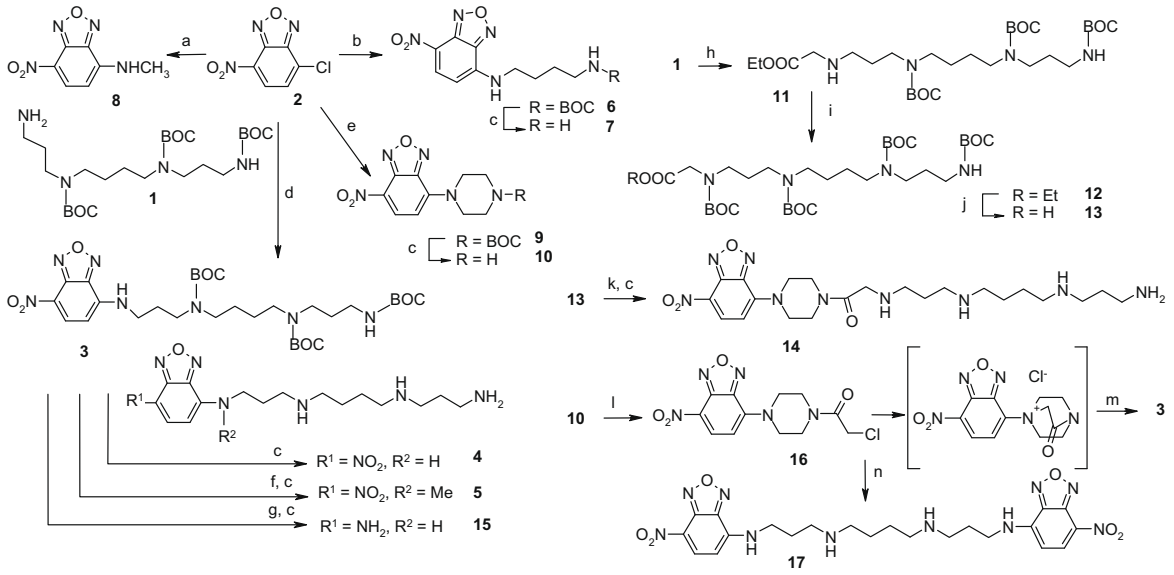


Figure 1.

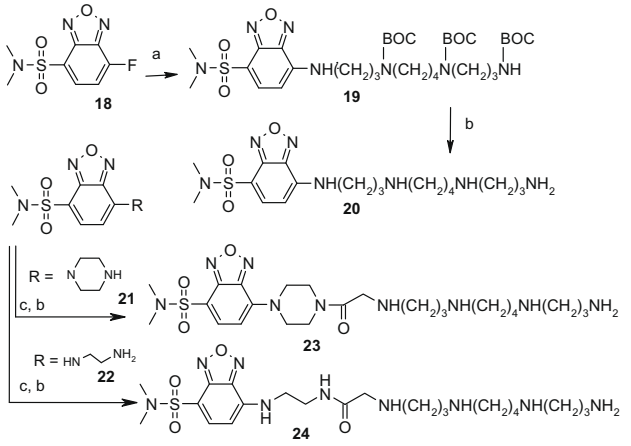


Scheme 1. Reagents and conditions:[16] (a) NH_2CH_3, EtOH, 30 min, rt, 96%; (b) *N*-BOC putrescine, Cs_2CO_3, CH_3CN, 80 °C, 1 h, 80%; (c) TFA, CH_2Cl_2, rt, 1 h or CH_2Cl_2, HCl/dioxane, rt, 1 h; (d) triBOC spermine **1**, Cs_2CO_3, CH_3CN, 80 °C, 1 h, 69%; (e) *N*-BOC piperazine, Cs_2CO_3, CH_3CN, rt, 3 h, quant; (f) CH_3I, Cs_2CO_3, CH_3CN, rt, 4 h, 78%; (g) Pd/C 10%, EtOH, H_2 gas, rt, 1 h, 75%; (h) ethylbromoacetate, NEt_3, CH_3CN, rt, 65%; (i) BOC_2O, NEt_3, THF, rt; (j) 1 N NaOH, EtOH, reflux, quant; (k) **10**, TBTU, Et_3N, CH_3CN, rt, 1 h, 2 steps 40%; (l) chloroacetyl chloride, NEt_3, THF, rt, 54%; (m) triBOC spermine **1**, NEt_3, DMF, rt, 60%; (n) spermine, DMF, rt, 50%.

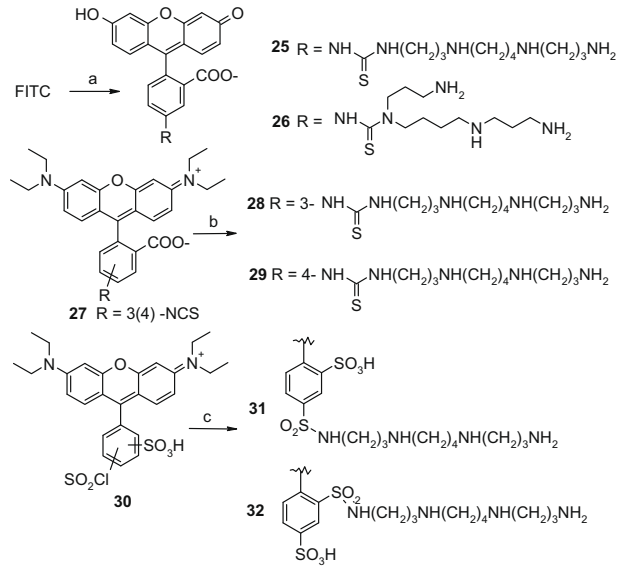
with chloro NBD **2** with Cs_2CO_3 in CH_3CN gave **3**. Removal of the three protective BOC groups with TFA in CH_2Cl_2 led to the crystalline salt of the already known compound **4**.[9] N_1-Methylation at the aniline nitrogen of the BOC-protected **3** led to **5**, following the same acid deprotection. Likewise, the putrescine derivative **7** was prepared in a similar manner, via the BOC protected putrescine derivative **6.** Compound **8**, which was used as a negative control, was obtained from monomethylamine and chloro NBD **2**. A modification of the spacer between the NBD moiety and the spermine chain was achieved, by incorporating an acetamidopiperazine motif. The intermediate NBD piperazine **10** was obtained via its BOC protected derivative **9**. The tetraBOC spermine acetic acid derivative **13** was prepared from the triBOC spermine **1**, by monoalkylation with ethyl bromoacetate to **11**. However, some dialkylated compound was also detected and easily purified. Subsequently, an additional protection with a fourth BOC group at the resulting secondary amine led to **12**, and to the acid **13,** after saponification of the ester group. Coupling of piperazine **10** with the obtained acid **13** was achieved via TBTU method. The water-soluble reagents and intermediates used with this acylation conditions afforded **14**, without a chromatographic step, after HCl cleavage of the protective BOC groups, as described above. The anilino derivative **15** was obtained by catalytic hydrogenation of the protected nitro intermediate **3,** followed by BOC cleavage (TFA). Unexpectedly, the bis NBD-spermine **17** was obtained when chloroacetamide **16** was exposed to non-protected spermine in DMF and triethylamine. It would seem reasonable to suggest that the NBD quinuclidinium salt intermediate obtained in these conditions reacted with the two basic primary amines by displacement of the quinuclidinium moiety as a leaving group. We noticed that compound **3** was also obtained when triBOC spermine **1** was condensed with **16**, via the same displacement of the quinuclidinium motif.

Replacement of the nitro group of the NBD molecule with dimethylaminosulfonyl was considered on the basis of the possible improvement of quantic yield and the expected bathochromic effect described by Uchiyama et al.[10] As depicted in Scheme 2, displacement of the fluoro atom in the *para* position of the nitro group on the NBD moiety of commercially available 7-fluorobenzo[1,2,5]oxadiazole-4-sulfonic acid dimethylamide **18** with triBOC spermine **1** in the presence of Cs_2CO_3 in CH_3CN led to **19.** Cleavage of the BOC groups using TFA generated the spermine conjugate **20**. Piperazine and ethylenediamine benzoxadiazole **21** and **22** were acylated (TBTU, NEt_3, CH_3CN) with tetraBOC spermine acetic acid **13**, and deprotected with TFA to directly yield the crystalline hydrochlorides **23** and **24**, respectively.

Phenylxanthene fluorophores such as fluorescein, rhodamine and lissamine (sulforhodamine B) and their synthetic conjugates were next addressed, as depicted in Scheme 3. Fluorescein derivatives **25** and **26** were prepared directly by the addition of a 6-fold excess of spermine to fluorescein isothiocyanate (FITC) in CH_3CN at room temperature. The resulting crystalline mixture of isomers was separated by preparative HPLC[11], yielding the linear **25** and branched **26** spermine conjugates. Similarly, the reaction of rhodamine isothiocyanate **27**[12] with triBOC spermine **1** (NEt_3 in CH_3CN) resulted in a mixture of both 3- and 4-isomers in equal proportions, which was separated by preparative HPLC followed by cleav-



Scheme 2. Reagents and conditions: (a) triBOC spermine **1**, Cs_2CO_3, CH_3CN, 80 °C, 1 h, 95%; (b) TFA, CH_2Cl_2, rt, 1 h, 60%; (c) tetraBOC spermine acetic acid **13**, TBTU, NEt_3, CH_3CN, rt, 1 h, 52%.

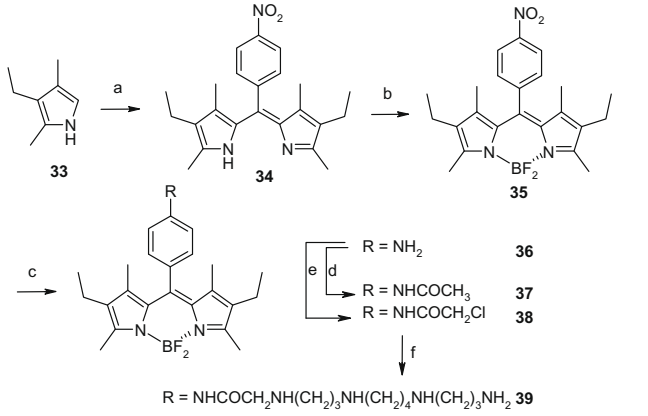


Scheme 3. Reagents and conditions: (a) FITC (1 equiv), spermine (6 equiv)/CH_3CN, rt, 1 h, HPLC→**25** (10%) + **26** (2%); (b) triBOC spermine **1**, NEt_3/CH_3CN, rt, 1 h, 60%, separation of isomers→**28** (7%) + **29** (6%), then HCl/*i*PrOH/CH_2Cl_2; (c) triBOC spermine **1**, NEt_3, CH_2Cl_2, rt, separation of isomers→**31** (46%) + **32** (23%), then TFA/CH_2Cl_2.

age (HCl) of BOC groups to yield **28** and **29**. Lissamine (sulforhodamine B sulfonylchloride) **30** was similarly reacted with triBOC spermine **1** under the same conditions as described above to obtain both 4- and 2-sulfonamides isomers, which were subsequently separated by flash chromatography and deprotected from BOC groups (TFA) to obtain the spermine conjugates **31** and **32**.

Bodipy conjugates were prepared according to Scheme 4. Pyrrole **33** was condensed with 4-nitrobenzaldehyde in the presence of TFA, and direct DDQ oxidation led to dipyrrole methene **34** as described.[13] The bodipy moiety was formed by treatment with Et_2O–BF_3/NEt_3, leading to the nitro derivative **35**. Catalytic reduction to aniline **36** and acylation with acetyl chloride or chloroacetylchloride yielded **37** or **38**, respectively. Condensation of **38** with N_1,N_2,N_3-tribenzyloxycarbonyl spermine (triZ spermine)[7,8] generating the conjugated spermine-Bodipy **39** after catalytic deprotection of the Z groups.

Four major criteria were established as prerequisites for selecting optimal fluorescent probes. Firstly, a high fluorescence quantum yield (QY) would be desirable for detection at low concentrations. Secondly, the optimum wavelengths (λ_{ex} and λ_{em}) would preferably be within the 480–530 nm range (green area), and in particular at λ_{ex} 488 nm and λ_{em} 525 nm, to be compatible with currently-available spectrofluometers used for cancer haematology applications in hospital and clinical laboratories. Thirdly, the selected fluorescent probe should have a minimum of self-quenching or photo-bleaching for best observation and recording characteristics (signal stability). Fourthly, and most importantly, the selected probe should display a specific recognition for the PTS in cellular systems.



Scheme 4. Reagents and conditions: (a) *p*-nitrobenzaldehyde, TFA, CH_2Cl_2, rt, then DDQ, rt; (b) Et_2O–BF_3, NEt_3, CH_2Cl_2, 0–5 °C; (c) H_2, Pd/C 5%, THF/EtOH, quant; (d) CH_3COCl, NEt_3/THF, 50%; (e) chloroacetyl chloride, NEt_3, THF,50%; (f) TriZ spermine, NEt_3/DMF, rt, then H_2, Pd/C 10%, MeOH, 25%.

The spectral properties of synthesized fluorescent probes were evaluated using a Spetramax Gemini microplate spectrofluometer (Molecular Devices). The excitation wavelengths tested ranged from 430 to 570 nm and emission fluorescence was recorded at 525, 583 and 680 nm for the green, yellow and red ranges, respectively. The results of the fluorescent measurements are presented in Table 1. Subsequently, the cellular incorporation of fluorescent probes was tested in vitro with the Burkitt's human lymphoma Namalwa cell line, using flow cytotmetry. Briefly, cells were incubated with 1 μM of each probe for 2 hours in serum-free medium supplemented with 2 mM aminoguanidine (to prevent polyamine oxidation in the culture medium). Fluorescence was then measured by a fluorescence-activated cell sorter (FACS) (λ_{ex} 488 nm/λ_{em} 525 nm and λ_{ex} 488 nm/λ_{em} 583 nm). Using this protocol, two parameters were thus studied simultaneously: the cellular uptake of the probe and the stability of its signal. F14512, a recently designed new epipodophyllotoxin derivative tethered with a spermine chain and identified as a novel targeted cytotoxic drug[4] was used for competition assays against the candidate fluorescent probes in order to prove the specific efficiency of uptake through the PTS in Namalwa cells. Finally, selective cell internalization of the fluorescent probe through the PTS was also confirmed in a chinese hamster ovary cell line (CHO) and its PTS-deficient mutant (CHO-MG).[14] Differential fluorescence intensity between the two cell lines provided an index of efficiency for internalisation of compounds through the PTS. The results of cellular uptake and PTS specificity are presented in Table 1.

Compounds of the phenylxanthene series displayed potent fluorescent optical properties, but showed no uptake by Namalwa cells and no competition with F14512, as shown in Table 1. Bodipy derivatives were predicted to be potential candidates because of their highly fluorescent optical properties.[6] Accordingly, the spermine-conjugated bodipy **39** displayed a high and stable signal. However, it had no PTS specificity, as demonstrated by a weak dif-

Table 1
Spectral properties, fluorescence intensity in namalwa cells, cellular uptake and polyamine transport system (PTS) specificity of benzoxadiazole, phenyxanthenes and bodipy probes

Compounds	QY[a]	λ_{ex}[b] (nm)	λ_{em}[b] (nm)	Fluorescence intensity[c]	Competition with F14512[d]	Differential uptake in CHO/CHO-MG cells[e]
Benzoxadiazoles						
4	2500	488	540	65	Strong	Yes
5	32,800	500	520	56	Strong	Yes
7	2042	488	550			No
10	1900	480	540	1.1	No	
14	1400	488	550	2.9	No	
15	321	488	530	0	No	
8	10,700	490	510	0.5	No	No
20	229	430	590	0	No	
23	438	440	590	0	No	
17	196	488	540	627	No	No
24	173	440	590			
Phenylxanthenes						
25	130,000	488	520	7.9	No	
26	117,000	490	510	8.1	No	
28	45800	560	580	15	No	
29	ND				No	
31	241,000	570	580	2.9	No	
32	31,700	570	580	1.3	No	
Bodipy						
35	3600	520	550	1387	Weak	
36	362	520	560	3185	Weak	
37	92,300	520	540			
39	50,300	520	540	839	Weak	No

[a] Quantum yield (QY) of fluorescence was determined in DMSO solution as arbitrary units and was corrected from background noise.
[b] Wavelengths (λ indicated are for excitation and emission.
[c] Fluorescence intensity in Namalwa cells: mean fluorescence (measured by FACS) was corrected by the untreated control. Each probe was incubated for 2 h at 1 μM.
[d] 2 μM F14512 and 0.1 μM of each probe were co-incubated for 2 h and cellular fluorescence was detected by FACS and microscopy. 'Strong' competition with F14512 was defined by a decrease of fluorescence by greater than 70% of control without F14512. 'Weak' inhibition was defined by a decrease of fluorescence of 50–70%.
[e] Differential cellular uptake of 1 μM fluorescent probe by CHO versus CHO-MG cells, as visualized/determined by microscopy and FACS after a 2 h incubation period. The uptake of the fluorescent probe was considered different when the fluorescence intensity was at least 10-fold higher in CHO than in CHO-MG cells.

ferential uptake by CHO versus CHO-MG cells. Moreover, the negative control compounds **35**, **36** or **37** (bodipy derivatives lacking the spermine chain) exhibited a higher fluorescence signal than that of **39**, and demonstrated the absence of PTS-specific labelling. In contrast, encouraging results were obtained in the NBD series. Excitation and emission wavelengths for NBD derivatives (**4**, **5**, **7**, **8**, **10**, **14**, **17**) were measurable in the expected range. In Namalwa cells, only **4**, **5**, **17** displayed a significant level of fluorescence. Furthermore, **4** and **5,** in contrast to **17,** showed a competitive uptake with F14512 (Table 1), proving the active cellular internalisation through the target mechanism, i.e. PTS recognition. Compound **4** displayed a less stable signal compared to **5**. Further structure–activity considerations in this series indicated that the bis NBD derivative **17** exhibited the highest fluorescence signal, but displayed no PTS specificity (likely due to allosteric blocking or absence of polyamine recognition). Replacement of the nitro substitution of benzoxadiazole by other groups, such as NH_2 **15** or SO_2NMe_2 **20**, **23**, **24** displayed weak fluorescent potency, with a lower quantum yield that did not satisfy our first selection criterion. The N_1-methyl NBD derivative **8**, which lacked the spermine chain and which was used as a negative reference, displayed high optical fluorescence, but no recognition by the PTS. Evidently, the variation of the linker was responsible for this result, since the piperazine **14** was also not recognized. Finally, compounds **4** and **5** were both tested in CHO and CHO-MG cell lines and, in contrast to the other compounds, showed a differential uptake indicative of a PTS-mediated internalization. Taken together, these results led to the identification of compound **5** that retained all of the required criteria as a fluorescent tool for our research purposes. In conclusion, N_1-methylsperminenitrobenzoxadiazole (N_1-methylspermine-NBD) **5**, was identified and selected as the superior tool for identifying cancer cells with high PTS activity. Very recently, this rationally designed fluorescent probe has been tested in a clinical context, and has proved to be useful in selecting patients with leukaemia for treatment with the new epipodophyllotoxin derivative F14512.[15]

References and notes

1. Gugliucci, A. *Clin. Chim. Acta* **2004**, *344*, 23.
2. (a) Chen, K.; Liu, A. *FEBS Lett.* **1981**, *134*, 71; (b) Scalabrino, G.; Ferioli, M. *Adv. Cancer Res.* **1982**, *36*, 1; (c) Casero, R. A.; Marton, L. J. *Nat. Rev. Drug Disc.* **2007**, *6*, 373.
3. Seiler, N.; Delcros, J.-G.; Moulinoux, J.-P. *Int. J. Biochem. Cell Biol.* **1996**, *28*, 843.
4. Barret, J.-M.; Kruczynski, A.; Vispe, S.; Annereau, J.-P.; Brel, V.; Guminski, Y.; Delcros, J.-G.; Lansiaux, A.; Guilbaud, N.; Imbert, T.; Bailly, C. *Cancer Res.* **2008**, *68*, 9845.
5. Loudet, A.; Burgess, K. *Chem. Rev.* **2007**, *107*, 4891.
6. Uchiyama, S.; Santa, T.; Fukushima, T.; Homma, H.; Imai, K. *J. Chem. Soc. Perkin Trans. 2* **1998**, 569.
7. Blagbrough, I. S.; Geal, A. J. *Tetrahedron Lett.* **1998**, *39*, 439.
8. Spermine (1 equiv) was selectively protected at the primary amine with ethyl trifluoroacetate (1 equiv) in CH_2Cl_2. The reaction mixture was treated with BOC_2O, NEt_3 (4 equiv), and after evaporation, the trifluoroacetyl protecting group was removed with Cs_2CO_3 in MeOH/H_2O (8/2). The solvents were evaporated and the residue treated with iPr_2O and 0.5 N HCl. The oily insoluble organic material in both aqueous and organic solvent was decanted from a 3 layer system, and led to pure triBOC spermine as its hydrochloride salt, without chromatographic separation, with an overall yield of 66%. The analogue triZ spermine was obtained according to the same treatment.
9. Ware, B. R.; Klein, J. W.; Zero, K. *Langmuir* **1988**, *4*, 458.
10. Uchiyama, S.; Santa, T.; Fukushima, T.; Homma, H.; Imai, K. *J. Chem. Soc. Perkin Trans. 2* **1998**, 2165.
11. Purification by preparative HPLC, Sunfire Waters OBD column C18, 5μ, 19 × 250 mm, flow rate 20 mL/min., λ = 220 nm, elution with 5 mM HCl. Pure fractions were lyophilized to obtain hydrochloride derivatives.
12. Commercial rhodamine B isothiocyanate is a mixture of the 3- and 4- isomers.
13. Zeissel, R.; Bonardi, P.; Retailleau, P.; Ulrich, G. *J. Org. Chem.* **2006**, *71*, 3093.
14. Mandel, J.-L.; Flintoff, W. F. *J. Cell Physiol.* **1978**, *97*, 335.
15. Annereau, J.-P.; Brel, V.; Dumontet, C.; Guminski, Y.; Imbert, T.; Broussas, M.; Vispe, S.; Bréand, S.; Guilbaud, N.; Barret, J.-M.; Bailly, C. *Blood*, 2009, submitted for publication.
16. The structures of new compounds were checked by ^1H NMR and mass spectrometry.