Spermine-NBD as fluorescent probe for studies of polyamine transport system in Leishmania donovani

Elodie Jagu, Sébastien Pomel, Stéphanie Perhe, Jean-Christophe Cintrat, Philippe M. Loiseau, Raphaël Labruère

a- Institut de Chimie Moléculaire et des Matériaux d’Orsay (ICMMO), CNRS, Univ Paris Sud, Université Paris-Saclay, 15 rue Georges Clemenceau, 91405 Orsay Cedex, France

b- Chimiothérapie antiparasitaire, UMR 8076 BioCis, CNRS, Univ Paris Sud, Université Paris-Saclay, 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France

c- Service de Chimie Bio-organique et Marquage (SCBM), CEA, Université Paris-Saclay, 91191 Gif/Yvette, France

Keywords: Leishmania donovani, Polyamine transport, Radioisotopic method, fluorescence

* Corresponding author

E-mail address: raphael.labruere@u-psud.fr (R. Labruère).

Abstract

This study describes the synthesis of fluorescent probes as potential substrates for the polyamine transport system (PTS) of Leishmania donovani. A competitive radioassay was used to determine the most efficient probe. We observed that the conjugate spermine-nitrobenzofurazan (Spm-NBD) was able to compete with [3H]-spermidine in L. donovani at a relevant IC50 of 60 µM.
Introduction

During their life cycle, Kinetoplastids are exposed to different biological environments in the insect or in the mammalian host. These different environments have dissimilar composition of nutrients, and parasites need a strong capacity to acclimate for good metabolic activity. Kinetoplastids require these nutrients for survival and growth. The replacement of biosynthetic pathways by transport systems is favorable since the need of energy is lower during a nutrient import than during its biosynthesis. Polyamines are essential compounds for parasite (figure 1) and membrane transporters can uptake polyamines from the extracellular environment. These uptake systems are well described in bacteria and yeast but are poorly characterized at the molecular level for mammalian and protozoan parasites. However, biochemical functionality of the transporter has been studied in some protozoa.

Figure 1: Trypanothione and polyamine biosynthetic pathway. ARG: arginase, ODC: ornithine decarboxylase, SAMDC: S-adenosinomethionine decarboxylase, SpdS spermidine synthetase, SpnS: spermine synthetase, TryS: trypanothione synthetase.

In the frame of Kinetoplastid parasites, Trypanosoma cruzi lacks arginine and ornithine decarboxylase, the two first enzymes in polyamine biosynthesis (figure 1), and is therefore incapable of biosynthesis de novo. T. cruzi is consequently dependent on the uptake of putrescine from the host. Identification and functional characterization of two polyamine transporters in T. cruzi have been described. These transporters were high-affinity transporters that recognized both putrescine and cadaverine but not spermidine and
spermine. The parasite can obtain putrescine from the human host but not cadaverine. A source of cadaverine for *T. cruzi* could be the actinomycetes residing in the gut of the vector. The diamine transporters possess the ability to cover the entire cell surface and thus increase the putrescine uptake when putrescine becomes scarce. This suggests the existence of one or more cellular signaling mechanisms that sense putrescine availability. Due to its transporter and signaling system, *T. cruzi* overcomes its inability to synthesize polyamine *de novo*. Furthermore, *T. cruzi* also has the capacity to transport exogenous spermidine by transporter identified and called TcPAT12, and there is indirect evidence that the parasite could be capable of uptaking extracellular spermine. Recently, the polyamine transporter TcPAT12 was overexpressed in *T. cruzi* epimastigotes. It was shown that the regulation of the polyamine transport induces significant parasite growth when different concentrations of polyamines is used. *Trypanosoma brucei*, on the opposite, displays very low putrescine uptake and no evidence of any polyamine transporter has been found in its genome. *Leishmania infantum* was the first *Leishmania* species for which polyamine transports system has been described. This transport system for putrescine has been identified and characterized by measuring the uptake of radioactively labelled putrescine into the promastigote form of the parasite. These results have been confirmed in *Leishmania donovani* promastigotes in which a second polyamine transporter has been described for spermidine. The influx of spermidine was seven times more effective than putrescine but affinity of both spermidine and putrescine transporters were the same for the respective polyamines. Therefore, transporters showed a marked difference when a competition is made between this diamine and this polyamine meaning that uptake of putrescine and spermidine is supported by two distinct transport systems. The transport of putrescine and spermidine has also been described in *Leishmania mexicana* promastigote and amastigote forms. In this study, the transport was found to be temperature- and pH-dependent. Results from kinetic analyses confirm that putrescine and spermidine use different transporters. Hence, total understanding and molecular characterization of polyamine transporters in Kinetoplastids is far from being achieved.

Furthermore, it has been suggested that the polyamine transport system could be a relevant drug target for the discovery of antikinetoplastid agents. The inhibition of polyamine biosynthetic pathway by Eflornithine, a drug impairing the ornithine decarboxylase (ODC) activity, is often compromised since most of the protozoan can import them from the
extracellular medium. This disadvantage could be exploited by targeting the polyamine uptake mechanism to either block the polyamine uptake or use the transport as a drug targeting system. Such an approach may be particularly effective in the case of *T. cruzi* which is polyamine auxotroph. Furthermore, this could be applied for other protozoa since their polyamine transport system has been demonstrated more active than human polyamine transport system.\(^\text{10}\) Due to the necessity for the parasite to use profusely polyamines, this transporter could be a viable drug target for the development of antikinetoplastid agents. The purpose is to capitalize on the efficient polyamine transport systems to target specifically the Kinetoplastids (leishmania/Trypanosome) and to facilitate the internalization of pharmacologically active compounds covalently linked to the polyamines. The literature is still scarce for this type of strategy against Kinetoplastids.\(^\text{11}\) It was hypothesized that bisbenzyl-polyamines from Marion Merrell Dow Research Institute could bind to the polyamine transporter of *L. donovani* promastigotes and lead to an intracellular depletion of putrescine and spermidine.\(^\text{12}\) Furthermore, Lizzi et al.\(^\text{13}\) envisioned that these polyamine-quinone conjugates could be transported via a similar mechanism in *T. cruzi*.

In addition, polyamine transport system can be highjacked to deliver drugs. However, it is important to prove that polyamine-analogues were incorporated via polyamine transport system. The most widely used method for measuring polyamine transport is the radioisotopic method that measures the radioactivity uptake with radiolabeled polyamines. Despite the fact that this method is very accurate and explicit, it is expensive and needs an appropriate laboratory with authorized workers. We then became interested in identifying a fluorescent probe able to selectively compete with natural polyamines. To our knowledge, such probe has not been reported so far in Kinetoplastids. It would thus allow the accurate determination of the affinity of structurally diverse polyamines for the polyamine transport system. Several fluorescent polyamine probes were previously synthesized in order to study polyamine transporters in mammalian. Cullis et al.\(^\text{14}\) have prepared polyamines conjugated with the fluorescent chromophore MANT (*N*-Methylanthraniloyl), 1 and 2, in order to localize the modified polyamines (Figure 2). Flow cytometry studies have shown that spermidine-MANT derivatives were rapidly uptaken in Chinese hamster ovary cell line (CHO) with a plateau within several hours. With PTS-deficient mutant (CHO-MG), the authors demonstrated that non-specific uptake was very slow. Moreover, observation using conventional fluorescence
microscopy provided clear evidence that the polyamine-fluorophore has been internalized. Furthermore, a fluorescence-based assay was published identifying the activity of the polyamine transport system in tumor cells. The authors synthesized a series of spermine conjugates to different fluorophores: phenylxanthenes, rhodamine, lissamine, benzofuranes such as 4-chloro-5-benzofurazan (NBD) and bodipy. Potential fluorescent probes were chosen as a function of their optimal wavelength of excitation/emission, quantum yield, selectivity for PTS-positive cells and minimum bleaching upon light irradiation. All the synthesized fluorescent probes were evaluated for spectral properties, their cellular incorporation were also tested for competition assays against the drug F14512, a spermine-epipodophyllotoxin conjugate. In addition, probes internalization was compared between CHO cells and its PTS-deficient mutant (CHO-MG) in order to attest specific incorporation of fluorescent probes by polyamine transporter. The best fluorescent polyamine probes identified were \(N^1\)-spermine-nitrobenzoxadiazole 3 and \(N^1\)-methylspermine-nitrobenzoxadiazole 4 (Figure 2) which displayed high fluorescence intensity, strong competition with the drug F14512, and marked differential uptake in CHO and CHO-MG cells.

![Figure 2: MANT and NBD polyamine probes.](image)

Based on the latter study, we hypothesized that reported “polyamine-fluorophore” conjugates could be also efficient in Trypanosomatids. We directly used the most relevant probes from the work published earlier. In this study, we examined the behavior of several
polyamine fluorescent probes with *Leishmania donovani* and determined whether incorporation into parasites is achieved through polyamine transport system.

**Synthesis**

Five probes were envisioned for the study of the kinetoplastid polyamine transport system. Putrescine and spermine derivatives have already been synthesized by Guminski et al.\textsuperscript{15} In addition, we chose to prepare two spermidine probes attached to the two different terminal amines. Each probe is obtained from the nucleophilic aromatic substitution of 7-chloro-4-nitrobenzofurazan (NBD-chloride) (scheme 1). Fluorescent control 6 displaying no polyamine moiety was obtained by reacting methylamine and NBD-chloride in methanol in 61% yield. All the polyamine probes were prepared according to protocols described by Guminski et al.\textsuperscript{15} Mono-protected putrescine 5a was reacted with NBD-chloride to give an intermediate Boc-putrescine-NBD 7a in 50% yield after recrystallization from diisopropyl ether. Boc-protected spermidine/spermine (5b-d) and NBD-chloride have been coupled according to a general procedure by reacting primary amine with NBD-chloride in presence of Cs\textsubscript{2}CO\textsubscript{3} in acetonitrile to give protected intermediates 7b, 7c and 7d in yield ranging from 21 to 80% after purification on flash column chromatography on silica gel. All these intermediates were deprotected in the same conditions, i.e. stirring overnight in a HCl solution 4M in dioxane to afford 8a (put-NBD) as a putrescine derivative, 8d (Spm-NBD) as a spermine derivative and, 8c (N\textsuperscript{1}-Spd-NBD) and 8b (N\textsuperscript{3}-Spd-NBD) as spermidine derivatives linked to different nitrogen atom of this unsymmetrical polyamine. On these two steps, the global yields were good, except for 8d (21%) and 8a (25%). All the compounds have been used directly without any synthesis optimization.
Scheme 1: Synthesis of polyamine-NBD derivatives. Reagents and conditions: (a) NH₂CH₃, MeOH, reflux, 2h; (b) NaHCO₃; (c) Cs₂CO₃, Acetonitrile, reflux, 2h; (d) HCl/Dioxane 4M, rt, Overnight.

Spermidine transport

In this work, we chose to evaluate the polyamine transport system of *L. donovani*, a pathogenic strain commonly used in parasitology laboratories for in vitro assays.

In order to ensure that the fluorescent probes use the polyamine transporter, we used a radiolabeled competitive assay with [³H]-spermidine. The spermidine transport in *Leishmania*
*L. donovani* promastigotes has been studied with [3H]-tetrahydrochloride spermidine and competition have been carried out with three NBD-probes (spermidine and spermine) and the control methyl-NBD 6.

First of all, an optimization has been done in order to find the concentration of radiolabeled and the needed time of incubation. Experiments were performed at a concentration of 10 µM of [3H]-spermidine and with a reaction time of 10 min. To establish the efficiency of the transporter, [3H]-spermidine transport was measured in the presence of the competitive natural substrate spermidine. In presence of cold spermidine, transport of radiolabeled spermidine was inhibited with an IC\textsubscript{50} values of 34 µM (Figure 3). A second control consisted in evaluating the inhibition of the fluorophore not coupled to polyamine. 6 did not inhibit the [3H]-spermidine transport, and was clearly not actively uptaken by the spermidine transporter. This result confirms that internalization of 6 do not use polyamine.

![Figure 3: Competition assay with (a) cold spermidine and (b) methyl-NBD 6.](image)

Then, the polyamine probes were evaluated by the same method. Each compound was tested in a range of concentration from 10 mM to 1 µM (figure 4). N\textsuperscript{3}-Spd-NBD did compete with the radiolabeled spermidine although a relatively high concentration of the probe was needed. N\textsuperscript{1}-Spd-NBD and Sm-NBD probes were however better at competing with radiolabeled spermidine. In particular spermine conjugate has an IC\textsubscript{50} of 60 µM.
Figure 4: Competition assay with spermidine and spermine probes.

The figure 5 represents a diagram of inhibition capacity of polyamines probes. The ability of the *L. donovani* promastigotes to uptake [*³H*]-spermidine was evaluated in the presence of unlabelled spermidine and in the presence of fluorescent probes, each at concentration of 100, 300 or 1000 µM. Results are plotted as the percentage of spermidine uptake obtained without inhibitor (control).

Figure 5: Competition profile of polyamine probes. Control represents the percentage of [*³H*]-spermidine uptake in absence of inhibitor.

**Conclusion**

In this study, we have highlighted the internalization of fluorescent polyamines by a spermidine transporter in *Leishmania donovani* promastigotes. Using the best fluorescent probes, i.e. the spermine-nitrobenzofurazan (*Spm-NBD*; IC₅₀ = 60µM), a fluorescent-based
competitive binding assay is now possible. This result demonstrates the possibility to avoid radioisotopic method to measure the uptake with radiolabeled polyamines.

Experimental section

Chemistry. All chemical reagents were of analytical grade, obtained from Acros, Alfa Aesar, or Aldrich, and used without further purification. Solvents were obtained from SDS or VWR-Prolabo. Chromatography was performed using silica gel (35-70 μm, Merck). Analytical TLC was performed using Silica Gel 60 F254 pre-coated aluminum plates (Merck). NMR spectra were collected on Bruker DRX 250 (1H at 250 MHz and 13C at 62.5 MHz), DRX 300 (1H at 300 MHz and 13C at 75 MHz) or DRX 360 (1H at 360 MHz and 13C at 90 MHz) spectrometers using MestReNova software. Chemical shifts are reported in ppm (δ) and coupling constants in Hz (J). 1H NMR spectra were performed in CDCl3, MeOD or D2O. High-resolution mass spectrometry (HRMS) analyses were performed by electrospray with positive ionization mode (ESI+). The purity of all compounds used for biological activity test was checked by reverse phase analytical HPLC and confirmed to be ≥ 95%.

N-methyl-4-amino-7-nitrobenzofurazan (6):

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{N} \\
& \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{HCH}_3 & \\
\end{align*}
\]

To 200 mg (1 mmol; 1 equiv.) of 4-chloro-7-nitrobenzofurazane dissolved in 6 mL of methanol was added 101 mg (1.5 mmol; 1.5 equiv.) of methylamine hydrochloride. The reaction mixture was refluxed at 75 °C under N2 for 2h. Then 400 mg (4.8 mmol; 5 equiv.) of sodium hydrogen carbonate dissolved in 6 mL of distilled water, was added dropwise. After stirring overnight and cooling, red crystals of the desired compound precipitated. Red solids were filtered and washed with methanol.

Red crystal (61%). \( R_f \) (Cyclohexane/EtOAc 5:5) = 0.42.

\(^1\text{H NMR} \) (300 MHz, DMSO): \( \delta = 9.52 \) (bs, 1H), 8.53 (d, \( J = 8.8 \text{Hz} \), 1H), 6.31 (d, \( J = 8.9 \text{Hz} \), 1H), 3.39 (s, 3H) ppm.

HRMS-ESI(+): calcd for C\(_7\)H\(_7\)N\(_4\)O\(_3\): 195.0555, found: 195.0559 [M+H]\(^+\).
**Compound 7a:**

To 941 mg (5 mmol; 1 equiv.) of mono-Boc-protected 1,4-butane diamine dissolved in 25 mL acetonitrile was added 1.2 g (5 mmol; 1 equiv.) of cesium carbonate and 1 g (5 mmol; 1 equiv.) of 4-chloro-7-nitrobenzofurazan. The mixture was refluxed at 80 °C for 30 min. The solvent was removed under reduced pressure and the product was extracted with DCM. The organic layer was washed with brine, dried over MgSO₄ and evaporated. Solids were recrystallized from diisopropyl ether. Orange powder, (50%). Rᵥ(Cyclohexane/EtOAc 3:7) = 0.74.

**¹H NMR** (300 MHz, CDCl₃): δ = 9.54 (bs, 1H), 8.52 (d, J = 8.8Hz, 1H), 6.81 (bs, 1H), 6.42 (d, J = 8.8Hz, 1H), 3.45 (m, 2H), 2.96 (m, 2H), 1.67 (m, 2H), 1.49 (m, 2H), 1.35 (s, 9H) ppm.

**HRMS-ESI(+)**: calcd for C₁₅H₂₂N₅O₅: 352.1610, found: 352.1615 [M+H⁺].

**General procedure for the preparation of compounds spermidine/spermine NBD-BOC 7a-d:**

The Boc-protected compound (1 equiv.) was dissolved in acetonitrile then cesium carbonate (1 equiv.) and 4-chloro-7-nitrobenzofurazan (1 equiv.) were added. The mixture was stirred for 30 min at 80 °C and extracted with DCM. The crude was dried over MgSO₄ and evaporated under reduced pressure. The Boc-NBD intermediate was purified by column chromatography (EtOAc/Cyclohexane 5:5).

**Compound 7b:**

Orange oil, (54%). Rᵥ(Cyclohexane/EtOAc 5:5) = 0.60.

**HRMS-ESI(+)**: calcd for C₂₃H₃₇N₆O₇: 509.2718, found: 509.2703 [M+H⁺].

**Compound 7c:**

Orange oil, (29%). Rᵥ(Cyclohexane/EtOAc 5:5) = 0.60.
HRMS-ESI(+) calcd for C_{23}H_{37}N_{6}O_{7}: 509.2718, found: 509.2726 [M+H]^+.

**Compound 7d:**

Yellow oil, (80%). R_f (Cyclohexane/EtOAc 5:5) = 0.3.

HRMS-ESI(+) calcd for C_{31}H_{52}N_{7}O_{9}: 666.3821, found: 666.3826 [M+H]^+.

**General procedure for the preparation/deprotection of compounds spermidine/spermine NBD 8a-d:**

The protected compounds in solution of 4M HCl in dioxane were stirred for 3h. The solvent was then removed under reduced pressure. DCM was added and the mixture was extracted with water. The aqueous layer was lyophilized to afford compounds 8a-d as hydrochloride salts.

**Compound 8a** (put-NBD):

Orange powder, (quantitative yield).

HRMS-ESI(+) calcd for C_{10}H_{14}N_{5}O_{3}: 252.1091, found: 252.1110 [M+H]^+.

**Compound 8b** (N^3-Spd-NBD):

Orange powder, (quantitative yield).

HRMS-ESI(+) calcd for C_{13}H_{21}N_{6}O_{3}: 309.1670, found: 309.1667 [M+H]^+.

**Compound 8c** (N^1-Spd-NBD):

Orange powder, (quantitative yield).

HRMS-ESI(+) calcd for C_{13}H_{21}N_{6}O_{3}: 309.1670, found: 309.1667 [M+H]^+.
Orange powder, (quantitative yield).

**HRMS-ESI(+)**: calcd for C_{13}H_{21}N_{6}O_{3}: 309.1670, found: 309.1661 [M+H]^+.

**Compound 8d (Spm-NBD):**

![Chemical structure of Compound 8d](image)

Orange powder, (quantitative yield).

**HRMS-ESI(+)**: calcd for C_{16}H_{28}N_{7}O_{3}: 366.2254, found: 366.2261 [M+H]^+.

**Biology.**

**Culture of Leishmania donovani.** MHOM/ET/67/HU3, also called LV9, promastigote forms were grown in M-199 medium supplemented with 40 mM HEPES, 100 mM adenosine, 0.5 mg.mL^{-1} haemin, 10% heat-inactivated foetal bovine serum (FBS) and 50 mg.mL^{-1} gentamycin at 26 °C in a dark environment. All the experiments were performed with parasites in their logarithmic phase of growth.

**Radiolabelled experiments.** Promastigotes were counted using a hemocytometer. All transport assays were performed in triplicate. Three microfuge tubes per condition were prepared. Conditions varied in terms of inhibitor concentrations, times of incubation, and whether a putative transport antagonist was added to the transport medium. 500 µL of a 2-butylphthalate/mineral oil (7:1) mixture was added to the bottom of each microfuge tube. Solution of radiolabeled [^{3}H]-spermidine in Assay Buffer* was prepared at a concentration 4x (40 %M) using a mixture between spermidine solution and [^{3}H]-spermidine. Inert oil already present in the microfuge tubes was overlayed with 100 µL of that mixture. The number of scintillation vials required should correspond to the number of microfuge tubes used in the transport experiment. Parasites were centrifuged at 1,500 x g for 10-15 min in order to obtain cell pellet. Supernatant was removed and sedimented cells were suspended in 50 mL of Assay Buffer. This washing step was repeated two more times. Then, after the last washing step, the cell pellet was suspended to afford a concentration of 1x10^6 cells/mL in Assay Buffer. Microfuge tubes containing radiolabeled buffer overlaying the inert oil were placed into the
rotor of a benchtop microfuge without caps. 100 µL of cell suspension were added into the aqueous layer of each tube.

At fixed intervals, as determined by a timer started at the initiation of the experiment, the cells were sedimented through the inert oil layer at 16000 x g for 60 s. This centrifugation step separated the cells from the aqueous layer containing the non-incorporated radiolabeled. This step terminated the transport assay. At the end of each centrifugation steps (60 s), the parasite pellet should be clearly visible at the bottom of the microfuge tube. Without delay, the microfuge tubes were flash frozen with liquid nitrogen. The tip of the frozen microfuge tube containing the cell was cut with a dog nail clipper and dropped into scintillation vial. 200 µL of 2% SDS were added to each scintillation vial to solubilize the cell pellets and this solution was vortexed at maximal speed for 30 min. 3-4 mL of scintillation fluid were added to each vial which were again vortexed at maximum speed overnight. Radiolabel incorporated by parasites was quantified using a liquid scintillation counter. Each sample was counted for 3 min. Additional scintillation vial were prepared in order to assess the number of counts in the radiolabeled transport cocktail (controls).

*Ingredient for 2 liters of Assay Buffer: Glucose 5.1 g, HEPES 16 g, MOPS 10 g, NaHCO₃ 4 g, KCl 695 mg, MgCl.6H₂O 125 mg, NaCl 11.4 g, NaH₂PO₄·2H₂O 1.83 g, CaCl₂·2H₂O 81.4 g, MgSO₄·7H₂O 39.8 g.

**Acknowledgement**

This work was supported by Initiative d’Excellence Paris-Saclay (IdEx). The authors are grateful to the COST Action CM1307 and Laura Darai for technical assistance on organic synthesis.
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