Probing the Use of Triphenyl Phosphonium Cation for Mitochondrial Nucleoside Delivery

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Mitochondria targeting, Lipophilic cation, Nucleoside prodrug, Cellular localization, Fluorescein.

ABSTRACT: Herein, we report the design, the synthesis and the study of novel triphenyl phosphonium-based nucleoside conjugates. 2'-Deoxycytidine was chosen as nucleosidic cargo as it allows the introduction of fluorescein on the exocyclic amine of the nucleobase and grafting of the vector was envisaged through the formation of a biolabile ester bond with the hydroxyl function at the 5'-position. Compound **3** was identified as potential nucleoside prodrug, showing ability to internalized efficiently into cells and to colocalized with mitochondria.

Mitochondria, the well-known cellular powerhouse, are subcellular organelles that are responsible for most of the cellular energy production through the synthesis of adenosine 5'-triphosphate (ATP). They are also essential for numerous cellular processes,[1-3] including calcium signaling, cell growth and differentiation, cell cycle control and cell death. Therefore, mitochondrial defects or dysfunctions are involved in a wide number of diseases.[2, 4-6] Consequently, there is considerable interest in delivering bioactive molecules selectively to mitochondria in order to interfere with mitochondrial processes, but mitochondria is known for its highly hydrophobic inner membrane and negative membrane potential, which results in limited diffusion. In this respect, the bioactive molecules (i.e., drugs and probes) must be designed to localize themselves into mitochondria or to be conjugated to a known mitochondrial vector through a permanent or biolabile linker.[7, 8] Thus, triphenyl phosphonium (Figure 1, TPP+)[9, 10] and mitochondria-penetrating peptides (MPPs)[11-13] are two of the well-developed carrier molecules that have both been shown to be able to deliver small molecules specifically to mitochondria. More precisely, TPP+ has been used for decades to study mitochondrial processes and has also been successfully conjugated to many drugs, few of them being used in the clinic or are currently in clinical trials (for examples MitoQ,[14-16], MitoVES,[17, 18,] or MitoTAM[19, 20], Figure 1). The common feature of these mitochondrial targeted drugs is that the TPP+ moiety has been attached to the active entity through a permanent linker and its presence does not seem to perturb the intrinsic activity of the drug.

To our knowledge, this approach has never been applied to nucleoside analogues, a well-known class of antiviral and anticancer drugs.[21,22,23] Indeed, in the literature the terms antiviral

nucleoside analogues and mitochondria are usually associated with toxicity phenomenon.[24,25] Considering the classical mode of action of nucleoside analogues (i.e. phosphorylation steps mediated by cellular or viral kinases, and then interference of the nucleoside 5'-triphosphate with the nucleic acid polymerization process), the development of a mitochondria targeting carrier for nucleoside delivery must include a biolabile linker to address the release of the nucleoside analogue once inside the mitochondria. Therefore, we design first model compounds **1-3** (Scheme 1) with an ester bond-based linker, and three different chain lengths were envisaged in order to study their impact on mitochondrial targeting.

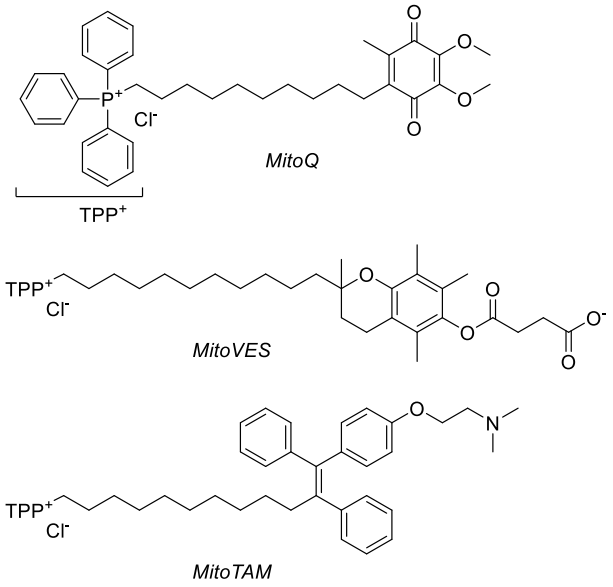
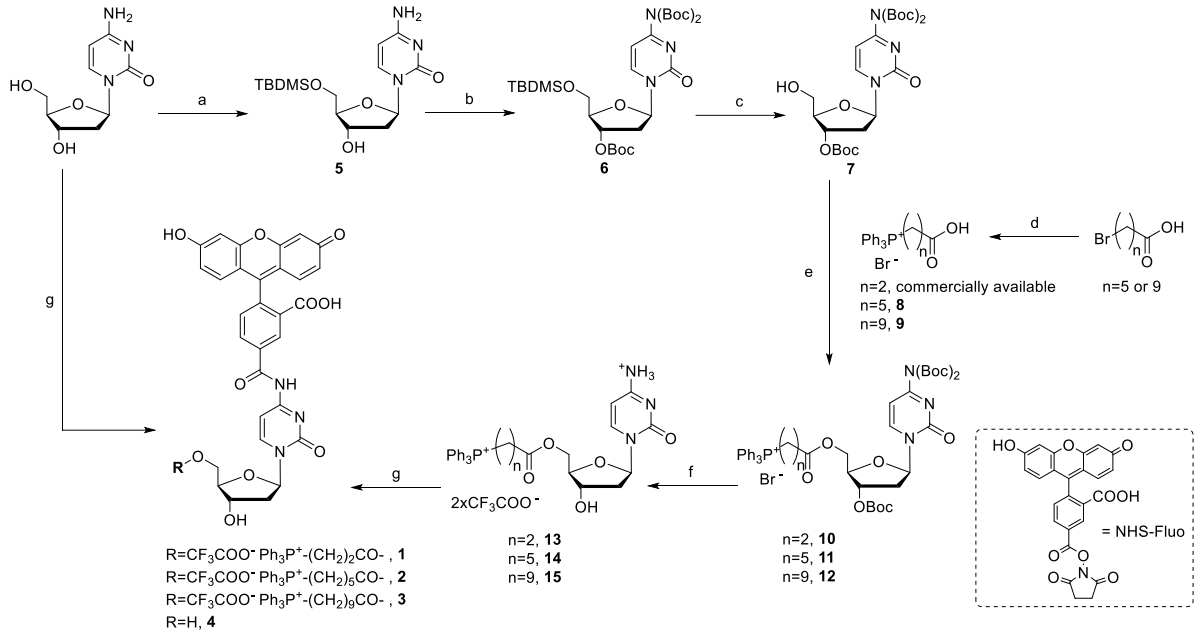


Figure 1. Examples of mitochondrial targeted drugs (Ubiquinone in MitoQ, Vitamin E in MitoVES and Tamoxifen in MitoTAM).

The synthesis of the desired compounds **1** to **3**, as well as reference derivative **4**, is presented in Scheme 1. The initial steps correspond to the selective protection of the exocyclic 4-amino- and the 3'-hydroxyl functions of 2'-deoxycytidine and this was achieved after the introduction of a *tert*-butyldimethylsilyl (TBDMS) group at the 5'-position as temporary protecting group. Thus, commercially available 2'-deoxycytidine was treated by *tert*-butyldimethylsilyl chloride (TBDMSCl) in presence of imidazole, leading to the selective protection of the primary hydroxyl group and derivative **5**. This last is then converted into the fully protected derivative **6** using previously described procedure.[26] Removal of the TBDMS is performed in presence of $NEt_3.3HF$ instead of the common TBAF salts to prevent the migration of the Boc groups.[27] In parallel, preparation of the TPP^+ carboxylic acids **8** and **9** was carried out by nucleophilic substitution of the bromine of 6-bromohexanoic acid or 10-bromodecanoic acid using triphenylphosphine in dry acetonitrile as previously described in the literature.[28]

Esterification of commercially available (2-carboxyethyl)triphenylphonium chloride, or intermediates **8** or **9**, with nucleoside derivative **7** proceeded efficiently in the presence of 1-ethyl-3(3-dimethylpropylamine)carbodiimide (EDC) as coupling agent and 4-dimethylaminopyridine (DMAP) as catalyst, leading to the fully protected esters **10-12** in high yields, which were then treated in acidic conditions (trifluoroacetic acid (TFA) solution in dichloromethane) to yield nucleosidic ester derivatives **13-15**.

Introduction of the fluorophore was carried out using commercially available 5-carboxyfluorescein *N*-succinimidyl ester, affording the fluorescent labelled compounds **1-4**, ready to be used in cellular localization studies.



Scheme 1. Synthesis of fluorescent-TPP$^+$-2'-deoxycytidine conjugates. *Reagents and conditions:* (a) TBDMSCl, imidazole, pyridine, rt, 64 h, 79%; (b) Boc_2O, DMAP, NEt_3, dioxane, rt, 19 h, 90%; (c) $NEt_3.3HF$, THF, rt, 19 h, 95%; (d) PPh_3, CH_3CN, reflux, 24 h, 88-97%; (e) EDC, DMAP, CH_2Cl_2, rt, 66 h, 83-92%, (f) TFA/CH_2Cl_2,(1:1, v:v), CH_2Cl_2, 0°C then 6 h, rt, 92-98%; (g) NHS-FLuo, DMF, 70°C, 24 h, 63-82%.

In order to evaluate the impact of the triphenyl phosphonium moiety on the hydrolysis of the ester bond, that is expect to occur after cell penetration in order to deliver the nucleoside (cargo), the stability of compounds **13-15** was tested towards pig liver esterase (PLE, CE/232.773.7), cell culture media as well as cell extracts obtained from breast cancer MCF-7 cells (both media being used during the *in vitro* biological studies). Decomposition studies were followed by HPLC using a previously described method,[29, 30] allowing the direct injection of biological media without pre-treatment of the samples. Compound **3** was also included in the study to check the influence of the presence of the fluorescein-tag on the stability of the biolabile linker in comparison to derivative **15**. The results are given in Table S1 (cf. Supporting information).

First, enzymatic activation by the model enzyme PLE was found to be efficient for all studied compounds with half-lives ranging from less than 15 min to 6.5 h. The kinetic of hydrolysis is highly dependent on the proximity and the steric hindrance of the TPP$^+$ moiety with the ester bond. In the culture media, all compounds are relatively stable, with half-life values of more than 17.8 h, except for compound **13** which exhibited a rapid hydrolysis. In this case, the closest presence of the TPP$^+$ moiety may be responsible for chemical instability, associated with inductive attractive electronic effect weakening the ester bond. Surprisingly, all compounds appeared stable in cell extracts with half-lives ranging from 8.4 h to more than 24 h. These results may be due to a poor esterase content of the extracts or loss of part of this enzymatic activity during the preparation protocol.

When comparing the behavior of compounds **15** and **3**, this last being the same pronucleoside including fluorescein on the *N*-4 position of the nucleobase, the half-lives increased in the absence of the fluorescent tag for the culture media and the cell extracts, whereas compound **3** was less sensitive towards esterase hydrolysis with PLE. All together the stability of both compounds were in the same range. In addition, the metabolite obtained after hydrolysis of compound **3** was identified, by co-injection with authentic sample, as compound **4**. This observation is of importance as it shown that under the experimental conditions of the *in vitro* assays (see below) the fluorescent tag is not lost.

Subcellular localization studies were carried out in human breast cancer MCF-7 cells treated with 50 µM of the studied compounds (**1**, **2**, **3** and **4**)

for 24 h and observation was performed with confocal fluorescence microscopy. Results are presented in Figure 2 and showed marked differences in the internalization between tested compounds. Both compounds **1** and **4** are diffused in the cytoplasm, with no obvious co-localization with the mitochondria for reference compound **4** (the TPP^+ vector is absent) and some co-localization for pronucleoside **1**. In addition, this last seems to be more internalized inside the cell than the reference compound **4** with no targeting moiety.

Compound **2** is internalized inside the cells in few localized spots and with very low amount compared to others. Subcellular localization pattern of compound **3** was really different than the other studied compounds, showing significant co-localization with mitochondria.

In order to confirm the distribution behavior of compound **3** in cells using super-resolution microscopy, MCF-7 cells were treated with 100 µM of compound **3** for 24 h and the MitoTracker was used at 100 nM concentration (Figure 3). As shown in Figure 3B, the superimposed images clearly indicate green and red areas overlapping, confirming the colocalization of compound **3** with mitochondria.

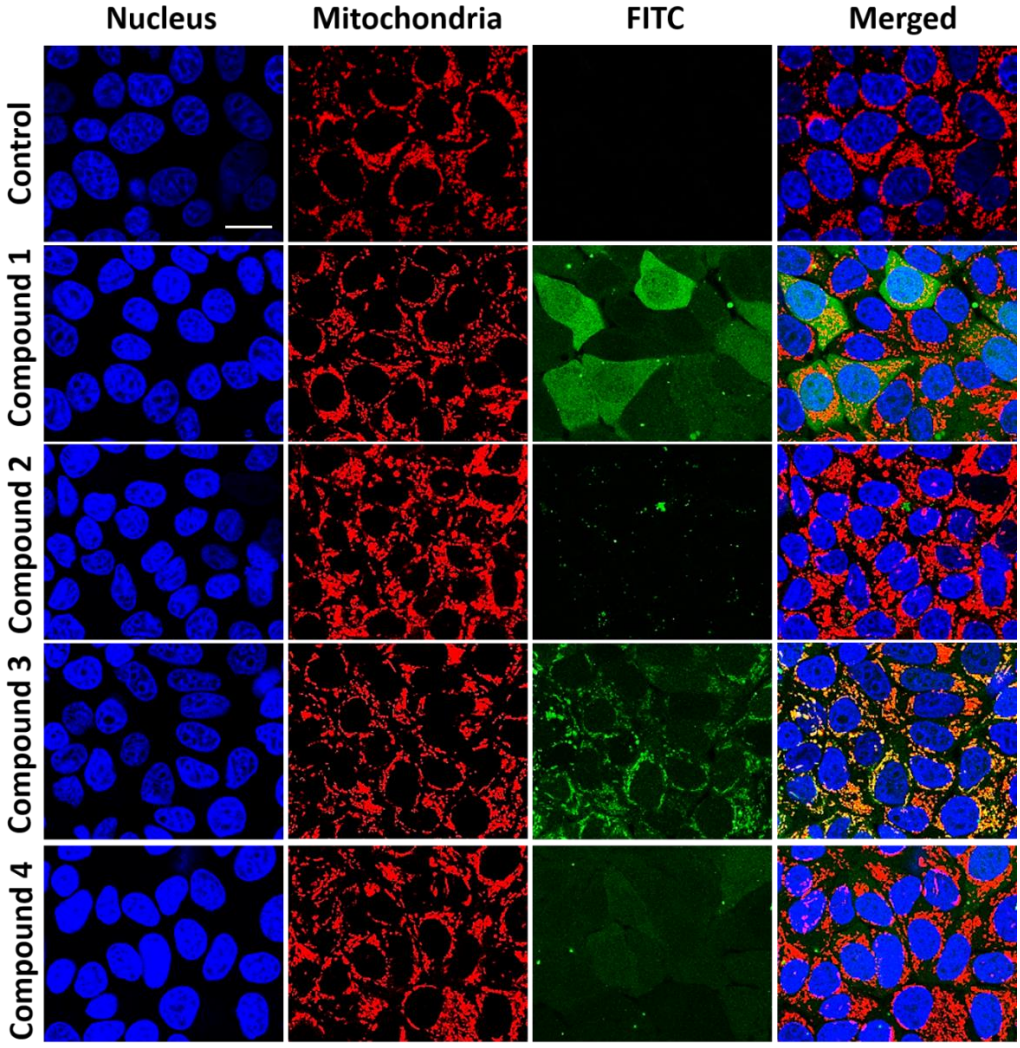


Figure 2. Subcellular localization study in breast cancer MCF-7 cells after 24 h of incubation at 37 °C with 50 µM of FITC-pronucleoside analogues **1**, **2**, **3** and reference FITC-nucleoside **4**. Control refers to untreated cells. To observe mitochondria, cells were treated with 200 nM of MitoTracker™ red CMXRos (Invitrogen, USA). To observe nucleus, cells were treated with Hoechst 33342 (Invitrogen, USA) at a final concentration of 10 µg mL^{-1}. Cells were washed two times with culture medium before observation with confocal fluorescence microscope LSM780 (Carl Zeiss, France) at 488 nm for FITC, 561 nm for MitoTracker and 760 nm for Hoechst, using a high magnification (63x/1.4 OIL Plan-Apo). Scale bar: 20 µm.

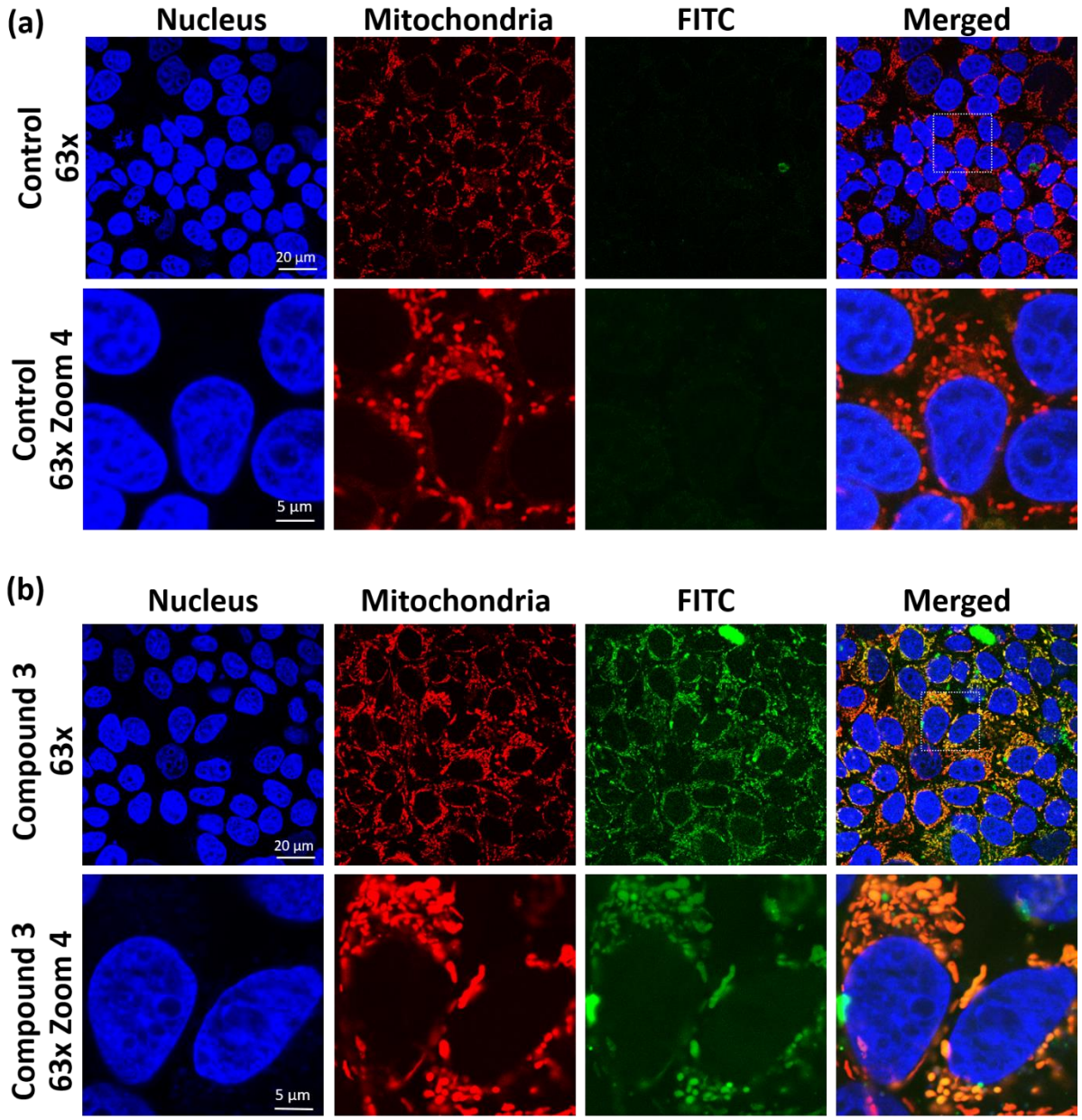


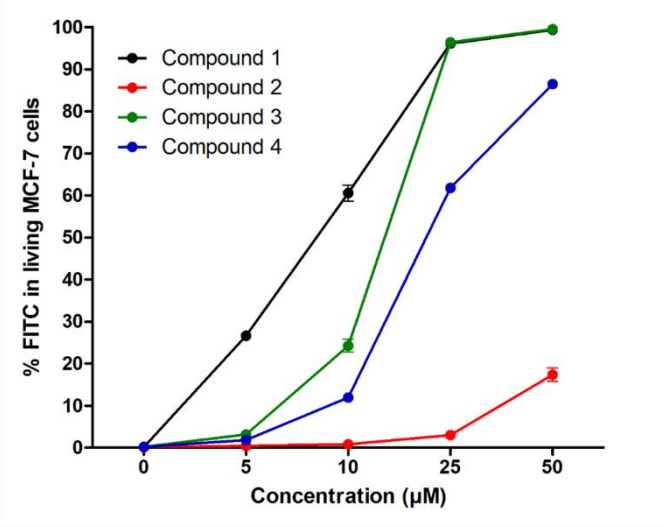
Figure 3. Subcellular localization study using super-resolution microscopy (a) Control of living breast cancer MCF-7 cells (b) Compound **3** in living breast cancer MCF-7 cells after 24 h of incubation at 100 µM concentration. Scale bar: 20 or 5 µm. Control refers to untreated cells. To observe mitochondria, cells were treated with 100 nM of MitoTracker™ red CMXRos (Invitrogen, USA). To observe nucleus, cells were treated with Hoechst 33342 (Invitrogen, USA) at a final concentration of 10 µg mL^{-1}. Cells were washed two times with culture medium before observation with super-resolution confocal fluorescent microscope LSM880 Airyscan (Carl Zeiss, France) at 488 nm for FITC, 561 nm for MitoTracker and 405 nm for Hoechst, using a high magnification (63x).

In parallel to the cellular localization studies, cell viability was also determined as it is crucial to assess cell safety and it will also support the previous results. Thus, cell viability of MCF-7 cells was determined after 72 h of treatment with compounds **1**-**4** at various concentrations (up to 100 µM). The results presented in Figure S1 (cf. Supporting information) showed that compounds **2** induced higher cytotoxic effect in MCF-7 cells than other studied derivatives. Indeed, after 72 h of

treatment, the cell death percentage value reached 61 ± 5% at 10 µM concentration. At the concentration studied for localization study (50 µM) the cell death percentage value is 44 ± 1%, indicating that the cell viability is deeply affected by this compound. In contrast, compounds **1** and **4** did not show any significant cell death up to 100 µM concentration, cell viability percentage values were 72 ± 0.6% and 85 ± 2%, respectively. The compound **3** did not show any significant cell death up to 50 µM concentration, the cell viability percentage value was 84 ± 0.7%. However, increasing the concentration to 100 µM induced a significant cell death compared to control with a value of 60 ± 2%. These results confirmed the low cytotoxic effect of all compounds, except compound **2**, in MCF-7 cells until 50 µM concentration for 72 h of treatment.

Lastly, we investigated the cellular uptake of compounds **1**-**4** (Figure 4) in living cells, and quantification of internalization was performed by flow cytometry analysis. Results showed that compounds **1**, **3** and **4** accumulate in a dose-dependent manner, reaching the saturation (>95%) for compounds **1** and **3** when increasing the concentration to 25 µM. Whereas, compound **2** showed lower internalization capability, with only 17 ± 1.6% of internalization percentage at 50 µM concentration. Compound **1** demonstrates a high capability to internalize into the living cells, at relatively low concentrations of 5 µM and 10 µM after 24 h of incubation, with 27 ± 0.9% and 61 ± 1.7%, respectively. This result agrees with the images obtained during the subcellular localization study (Figure 2), where the level of fluorescence was important and observed inside the cells. The cellular uptake ability of compounds **3** and **4** was also significant, reaching the saturation (96 ± 0.1%) at 25 µM concentration for compound **3**, and 62 ± 1% with compound **4** at the same experimental conditions.

Concerning compound **3,** the combined results from quantitative measurement of the cellular uptake and subcellular localization confirmed that this nucleoside conjugate is a good candidate for mitochondrial delivery as it exhibited both high capability to internalize into the living cells (from 25 µM) and a clear differential cell distribution with colocalization with mitochondria.



	Concentration (µM)			
	5	10	25	50
Compound 1	27 ± 0.9	61 ± 1.7	96 ± 0.2	99 ± 0.2
Compound 2	0.4 ± 0	0.8 ± 0	3 ± 0.1	17 ± 1.6
Compound 3	3 ± 0.4	24 ± 1.4	96 ± 0.1	100 ± 0.2
Compound 4	2 ± 0.2	12 ± 0.8	62 ± 1	87 ± 1

Figure 4. Quantitative measurement of the cellular uptake ability of living MCF-7 cells treated with different concentrations of the studied compounds 1-4 for 24 h using flow cytometry. Data are presented as mean ± SEM of three independent experiments.

Due to the crucial role of mitochondria in biological processes, the design and the use of mitochondria-targeted derivatives have led to increase knowledge of mitochondrial biology and to the development of therapeutic approaches. Most of the mitochondria-targeted derivatives incorporated the triphenyl phosphonium moiety as vector, that is covalently and permanently attached to the cargo. Herein, we proposed the association of the triphenyl phosphonium vector to the nucleosidic model *via* a biolabile ester bond. Depending on the length of the acyl-moiety and by balancing the hydrophobic and cationic characters, we identified compound **3** as a mitochondria-targeted nucleoside conjugate showing maximum uptake in MCF-7 cells at 25 µM and a clear differential sub-cellular distribution. In addition, stability studies shown their capacity to undergo esterase triggered release of the nucleoside. These preliminary studies are very encouraging and clearly prompt us to further investigate the potential of this approach.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
1- Experimental procedures for chemical synthesis, *in vitro* biological assays, stability studies (PDF).
2- Copies of NMR and MS spectra, as well as HPLC Traces for final compounds (PDF).

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Author Contributions

M.G. carried out the chemical synthesis and the stability studies, D.E. and S.P. design the study. L.M. and M.G-B designed and L.M., E.G.M., L.L. and M.D. performed all biological experiments. S.P. wrote the first draft and all authors were involved in the preparation of the final manuscripta symbol)

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