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Squalenoyl nucleoside monophosphate nanoassemblies: New prodrug strategy for the delivery of nucleotide analogues

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ABSTRACT

4-(*N*)-1,1′,2-Trisnor-squalenoyldideoxycytidine monophosphate (SQddC-MP) and 4-(*N*)-1,1′,2-trisnor-squalenoylgemcitabine monophosphate (SQdFdC-MP) were synthesized using phosphoramidite chemistry. These amphiphilic molecules self-assembled to about hundred nanometers size nanoassemblies in aqueous medium. Nanoassemblies of SQddC-MP displayed significant anti-HIV activity whereas SQdFdC-MP nanoassemblies displayed promising anticancer activity on leukemia cells. These results suggested that squalene conjugate of negatively charged nucleotide analogues efficiently penetrated within cells. Thus, we propose a new prodrug strategy for improved delivery of nucleoside analogues to ameliorate their biological efficacy.

Nucleoside analogues (NAs) have demonstrated widespread utility as antiviral and anti-cancer therapeutics. These drugs mimic physiological nucleosides in terms of uptake and metabolism and are incorporated into newly synthesized DNA resulting in synthesis inhibition causing premature chain termination. Some of these compounds also inhibit key enzymes involved in the generation of the purine and pyrimidine nucleotides and RNA synthesis.[1,2]

Following entry into the cells, the NAs are converted into monophosphate derivatives by the action of cellular kinases and are further phosphorylated to the diphosphate and finally into the active triphosphate form. Thus, the monophosphorylation by kinases becomes a rate-limiting step for the activation of these drugs. In many cases, NAs are poor substrates for the cellular kinases needed for their activations and down-regulation of kinases represents an important mechanism of cellular resistance.[3]

For all the above reasons, a direct delivery of the nucleoside monophosphate (NMP) into the cells has been considered as a potential strategy for overcoming the rate-limiting primary phosphorylation step. Unfortunately, NMPs were found to be inefficient because they are not transported by nucleoside transporters and thus poorly penetrate freely through the cellular membranes owing to their high polarity.[4] Therefore, in order to mask the phosphate negative charge and enable the modified nucleotide to enter the cells, many pronucleotides modified on the phosphate moiety by masking groups were designed.[5–9]

Although many imaginative and clever approaches have been tried, no single platform method has been proved to be successfully applicable to all the NAs. In particular, the delivery of 2′,3′-substituted nucleotides remains troublesome.[10] In this context, we have recently disclosed that the linkage of NAs to squalene (**3**) led to amphiphilic molecules that spontaneously self-organized in water as nanoassemblies of 100–300 nm, irrespective of the nucleoside analogue used.[11] This has been assigned to the highly coiled and compact conformation of the squalene moiety in water.[12] For example, nanoassemblies of 4-(*N*)-trisnor-squalenoylgemcitabine (SQdFdC, **5**) derived from Gemcitabine (dFdC, **1**) a difluorinated analogue of deoxycytidine currently marketed as Gemzar® exhibited superior anticancer activity in vivo in mice against experimental leukemia both after intravenous and oral administration.[13–16] The squalenoylation of other antiretroviral nucleosides such as ddC (**2**) and didanosine (ddI) also led to potent prodrugs when tested in primary cultures of HIV-infected lymphocytes.[11,17]

Due to the expanding interest of lipophilic pronucleotides as potential pharmaceuticals, we have investigated the possibility of delivering monophosphate nucleosides into the cells by taking advantage of the new squalenoylation methodology. Thus, in this article, we have summarized our efforts regarding the synthesis of squalenoyldideoxycytidine monophosphate (SQddC-MP **6**) and

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squalenoylgemcitabine monophosphate (SQdFdC-MP, **7**), the elaboration of nanoassemblies from these prodrugs and the primary biological evaluations of their nanoassemblies (Fig. 1).

The synthesis of squalene conjugates **4** and **5** has been previously described.[11] Briefly stated, 1,1′,2-trisnor-squalenic acid (**8**) available in few steps from squalene (**3**),[18,19] was first activated with ethyl chloroformate before coupling to ddC (**1**) or gemcitabine (**2**). Alternatively, SQddC (**4**) was obtained in 50% yield using the usual HBTU/HOBt peptide coupling reagent.[11]

Many very efficient procedures are available for the phosphorylation of nucleosides,[20] however we discovered at the outset of the work that the presence of the isoprenylated side chain bound to the cytosine nucleus was much more troublesome than we originally thought. For example, attempts at phosphorylating the 5′-hydroxyl group of SQddC (**4)** using various phosphorus(V) reagents were unsuccessful. The use of phosphoramidite reagents was therefore investigated for the introduction of the desired phosphate group.[21] After some unsuccessful attempts using bis(2-cyanoethyl) diisopropylamidophosphite (**9a**), due to the harsh conditions required for complete deprotection of both cyanoethyl groups of phosphotriester **10a**,[22] we turned to the use of bis(9*H*-fluoren-9-ylmethyl) diisopropylamidophosphite (**9b**). The 9-fluorenylmethyl group was demonstrated by Watanabe et al. to be an efficient base-labile protecting group allowing for complete deprotection of phosphotriesters by means of an excess of a tertiary amine under anhydrous conditions.[23] The phosphoramidite **9b** was prepared in a one-pot process from PCl_3 according to the Waldmann procedure.[24] Condensation of SQddC (**4**) with **9b** in the presence of 1*H*-tetrazole followed by hydrogen peroxide oxidative work-up gave the expected phosphotriester **10b** in 80% yield.

The final deprotection of the phosphate group was effected upon treatment with a large excess of triethylamine in acetonitrile to give the triethylammonium phosphate salt **11a** in 95% yield as an amorphous white solid. Similarly, treatment of **10b** with an excess of dimethylethanolamine afforded the more pharmacologically acceptable dimethylethanol-ammonium phosphate salt **11b** in 98% yield (Scheme 1).

With a viable synthetic route in hand, we turned our attention to the more challenging case of gemcitabine. Initial efforts to directly phosphorylate the primary alcohol of **5** were not successful due to competitive reaction at the 3′-OH group. Therefore, our synthetic approach of **7** started by protecting group manipulation to provide the C-3′ protected derivative **14** as depicted in Scheme 2. Thus, treatment of **5** with 4,4′-dimethoxytrityl chloride in pyridine[25] followed by protection of the 3′-OH group as *tert*-butyldimethylsilyl ether provided the fully protected derivative **13** in 75% overall yield.

Removal of the DMTr group at C-5′ using acetic acid was unsatisfactory due to the partial lost of the silyl protecting group. By contrast, upon treatment with formic acid in methanol at 0 °C, the expected alcohol **14** was obtained in 77% yield.[26] Condensation of **14** with **9b** as described for **10b** provided the corresponding phosphotriester. At this stage the C3′–OH was unmasked using HF.pyridine[27] to afford **15** in 69% yield. The deprotection of the phosphate group was effected as described above, upon treatment with triethylamine or dimethylethanolamine in acetonitrile to give

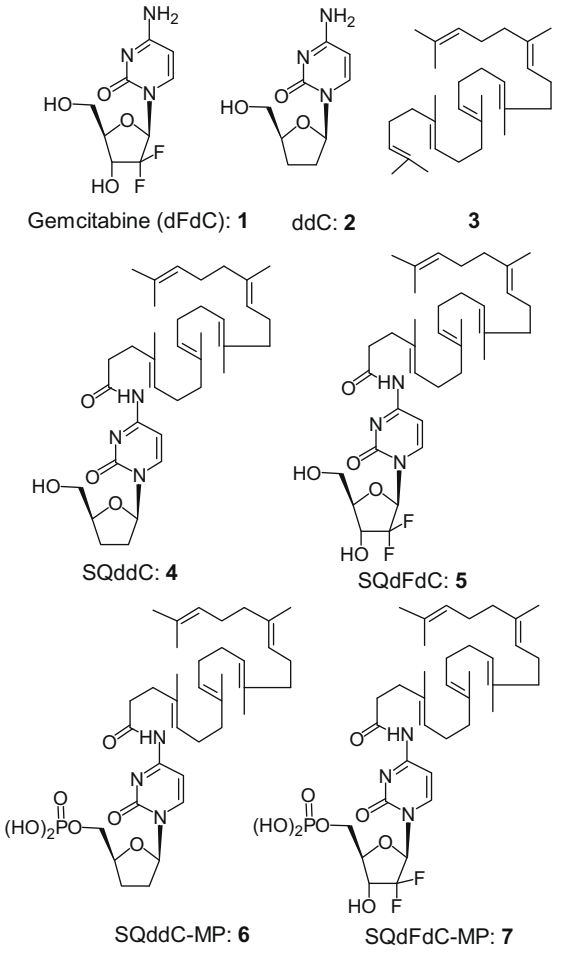
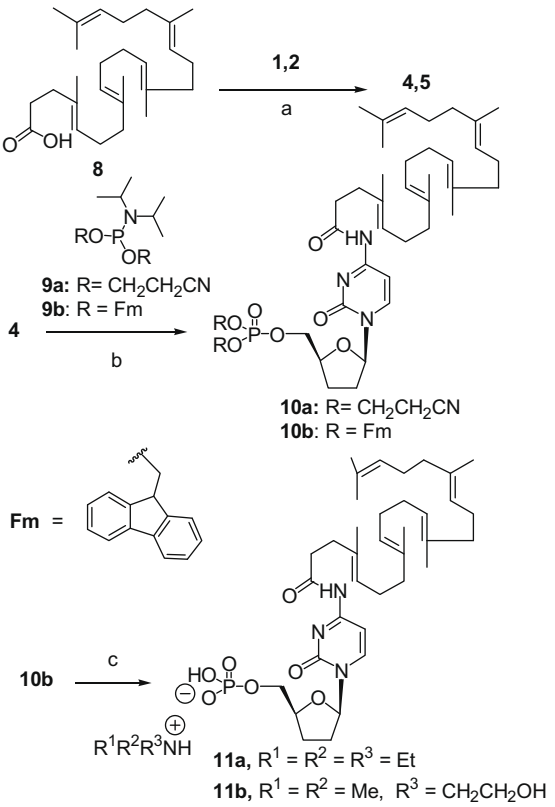
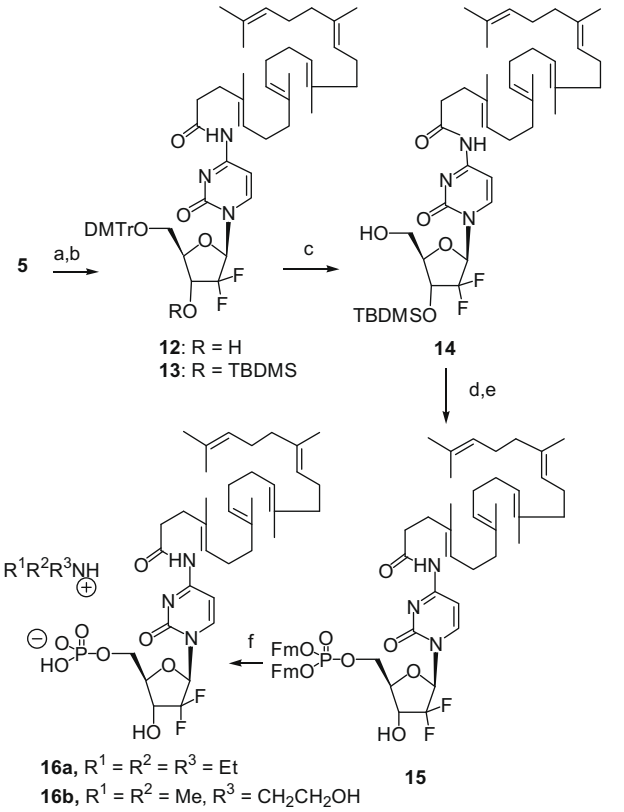


Figure 1. Chemical structure of gemcitabine (dFdC, **1**), dideoxycytidine (ddC, **2**), squalene (**3**), 4-(*N*)-trisnor-squalenoyldideoxycytidine (SQddC, **4**), 4-(*N*)-trisnor-squalenoylgemcitabine (SQdFdC, **5**), 4-(*N*)-trisnor-squalenoyldideoxycytidine monophosphate (SQddC-MP, **6**), 4-(*N*)-trisnor-squalenoylgemcitabine monophosphate (SQdFdC-MP, **7**).



Scheme 1. Synthesis of tertiary ammonium phosphate salts **11a,b**. Reagents and conditions: (a) (i) $ClCO_2Et$, Et_3N, THF, 0 °C, 30 min, (ii) **1** or **2,** DMF, 20 °C, 3 d; (**4**: 45%, **5:** 57%); (b) (i) **9**, 1*H*-tetrazole, CH_3CN, CH_2Cl_2 (1:1), rt, 3 d, (ii) 30% H_2O_2, 0 °C, 4 h, 80%; (c) $R^1R^2R^3N$, CH_3CN, 24 h, rt, (**11a**: 98%, **11b**: 95%).



Scheme 2. Synthetic approach to the tertiary ammonium phosphate salts **16a,b**. Reagents and conditions: (a) DMTrCl, DMAP cat., Py, rt, 3 d, 83%; (b) TBDMSCl, DBU, CH_2Cl_2, rt, 36 h, 90%; (c) HCO_2H/MeOH/CH_2Cl_2 (2:1:1), 0 °C, 45 nm, 77%; (d) (i) **9b**, 1*H*-tetrazole, CH_3CN, CH_2Cl_2 (1:1), rt, 3 d, (ii) 30% H_2O_2, 0 °C, 4 h, 80%; (e) HF.py, py, rt, 4 h, 69%; (f) $R^1R^2R^3N$, CH_3CN, 24 h, rt, (**16a**: 98%; **16b**, 85%).

the trialkylammonium phosphate salts **16a** and **16b** as amorphous white solids in 98% and 85% yields respectively (Scheme 2).

Nanoassemblies of trialkylammonium salts **11b**, **16a** and **16b** were prepared by straightforward precipitation of an ethanolic solution of these compounds in isotonic 5% aqueous dextrose solution. The mean diameters of the obtained nanoassemblies are depicted in Table 1. These particles had a polydispersity index lower than 0.30 as measured by quasi-elastic light scattering and show the characteristic Tyndall effect of colloids. Interestingly, the increase of drug load up to 5 mg/mL, led to only a slight increase in the particles size that remained below 200 nm. Furthermore, no significant change in the size of the nanoassemblies was detected over a 3-day storage period at 4 °C. Such stability of the nanodispersion can be correlated with the high negative Zeta potential ($z = -43.1$ mV for **16a**) observed.

Transmission electron microscopy (TEM) experiments were performed on nanoassemblies samples **16a** and **16b** which showed spherical particles with mean diameters of 100 nm and 150 nm, respectively (Fig. 2).

Table 1
Measurement of particle size and polydispersity index (PDI) of nanoassemblies

Compd	Concentration (mg mL^{-1})	Size (nm)	PDI
11b	4	109	0.21
16a	1	59	0.27
16a	3	104	0.14
16a	5	144	0.18
16b	2	56	0.29
16b	4	153	0.06

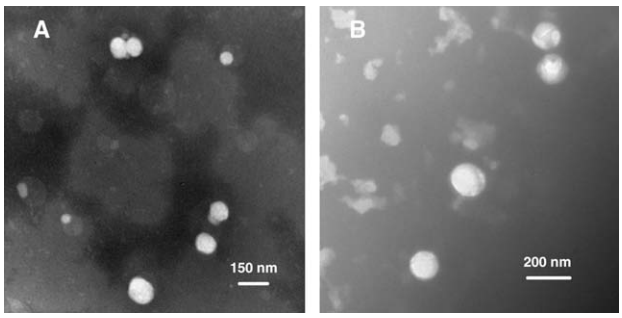


Figure 2. Transmission electron micrographs of nanoassemblies. (A) triethylammonium salt of SQdFdC-MP (**16a**) (3 mg mL^{-1}); (B) dimethylethanolammonium salt of SQdFdC-MP (**16b**) (4 mg mL^{-1}).

The nanoassemblies of dimethylethanolammonium SQddC-MP (**11b**) were tested for their antiviral activity in vitro on HIV-1-infected PHA-P-activated peripheral blood mononuclear cells (PBMC). Nanoassemblies of **11b** were found to be just as active as ddC but with an improved selectivity index due to a reduced cytotoxicity. In parallel, nanoassemblies of SQddC (**4**) were found to be twice as potent as ddC and nanoassemblies of **11b** in their ability to inhibit virus replication (Table 2).[11] These results showed that there is no significant differences between the three compounds and that nanoassemblies of **11b** displayed an identical pharmacological efficiency as compared to the parent NA. This could be of interest to by-pass resistance phenomenon and to eliminate viral sanctuaries in HIV-infected patients. Nevertheless, the cell mechanisms, for example, uptake, phosphorylation and possible dephosphorylation, involved in this anti-HIV efficiency remains to be defined.

The anticancer activity of trialkylammonium salts of **16a** and **16b** nanoassemblies was tested against L1210 wt (a wild type murine lymphoid leukemia cell line) (Table 3). On this cell line, dFdC exhibited an efficient anticancer activity displaying an inhibitory concentration 50% (IC_{50}) value of 3.2 nM. Owing to the prodrug nature, SQdFdC (**5**) nanoassemblies showed a higher IC_{50} (38 nM) and thus a lower anticancer activity compared with free dFdC, probably because of the lag time associated with cellular penetration and primary phosphorylation, before to result in the active triphosphate formation. On the contrary, nanoassemblies of

Table 2
Anti-HIV activity of ddC (**2**) and nanoassemblies (NA) of **4** and **11b**

Compd	ED_{50} (μM)[a]	TC_{50} (μM)[b]	SI[c]
2	0.12 ± 0.10	4.3 ± 3.4	36
4-NA	0.06 ± 0.02	4.7 ± 3.7	74
11b-NA	0.13 ± 0.09	8.3 ± 2.4	60

[a] Concentration needed to inhibit virus replication by 50% in PHA-P-activated PBMC; results obtained from 3 independent experiments performed in triplicate.
[b] Concentration that reduced cell viability to 50%.
[c] Selectivity index.

Table 3
In vitro anticancer activity of nanoassemblies of **5**, **16a** and **16b**

Compd	IC_{50} on L1210 wt leukemia cell line (nM)	IC_{90} (nM)
1	3.2	10
5-NA	38	73
16a-NA	6.1	31
16b-NA	6.7	28

The inhibitory concentration 50% (IC_{50}) data are an average of three different experiments performed simultaneously using different well plates.

phosphorylated salts **16a** and **16b** displayed a considerably lower IC_{50} values (6.1 nM and 6.7 nM, respectively) and thus a more efficient anticancer activity than SQdFdC (**5**) nanoassemblies, almost similar to that of dFdC (**1**). On the other hand, triethylammonium dihydrogen phosphate incubated as an aqueous solution did not elicit any activity against this cell line, thus ruling out any cytotoxicity of the triethylammonium cation. Thus, the phosphorylated versions of SQdFdC (i.e., **16a** and **16b**), improved the inferior cellular delivery of SQdFdC and at the same time retaining the inherent advantages associated with this non-phosphorylated bioconjugate as compared to that of dFdC.

In conclusion, new lipophilic prodrugs of ddC monophosphate and gemcitabine monophosphate with unmodified phosphate group have been synthesized by covalent coupling of a squalenoyl residue on the N-4 nitrogen of the cytosine ring. The ammonium salts of these bioconjugates self-organized in water as nanoassemblies of 50–150 nm. The nanoassemblies of these derivatives displayed interesting biological activities as anti-HIV and anticancer compounds demonstrating that the conjugation promoted efficient cellular delivery of this negatively charged phosphate esters. The exact mechanism by which these prodrugs enter the cells either by passive diffusion through cell membranes or endocytosis remains to be addressed. In a nut shell, this new prodrug approach open exciting perspectives to develop nucleotide analogues derivatives with improved pharmacological activity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.070.

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