# Squalenoyl nanomedicine of gemcitabine is more potent after oral administration in leukemia-bearing rats: study of mechanisms

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**In an earlier report, we demonstrated the superior anticancer efficacy of orally administered squalenoyl gemcitabine (SQdFdC) nanomedicine over its parent drug gemcitabine on rats bearing RNK-16 large granular lymphocytic (LGL) leukemia. In the present communication, we investigated the mechanisms behind this observation both at the cell and tissue level. The mechanisms were investigated by performing cytotoxicity, cell uptake, and biodistribution experiments. In the presence of cytidine deaminase, SQdFdC nanoassemblies resisted deamination and exerted significant anticancer activity *in vitro* against RNK-16 LGL leukemia cells, whereas the cytotoxicity of free gemcitabine decreased by ~83-fold, indicating its degradation due to deamination. Additionally, the SQdFdC showed considerably higher intracellular accumulation and retention compared with gemcitabine ($P<0.05$). Unlike gemcitabine, the cellular access to SQdFdC was not influenced by nucleoside transporters. When administered orally to rats, unlike $^{3}$H-gemcitabine, the $^{3}$H-SQdFdC absorbed slowly, but exhibited an improved pharmacokinetics and tissue distribution profile, particularly in the lymphoid organs (the major organs of metastasis). The resistance to deamination, followed by the improved pharmacokinetic and tissue distribution, and greater accumulation and retention at the level of cancer cells, are the key factors for the superiority of SQdFdC nanoassemblies over free gemcitabine against RNK-16 LGL leukemia in rats. *Anti-Cancer Drugs* 19:999–1006 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Gemcitabine is a nucleoside analogue with significant anticancer activity against a wide variety of cancers such as non-small cell lung cancer, breast, pancreas, bladder, ovarian, and head and neck cancers [1–6]. Especially in the cases of non-small lung cancer, pancreatic cancer, and breast cancer, gemcitabine has been approved as a first-line agent. Owing to its hydrophilic character, this compound can be transported intracellularly only through an active mechanism driven by membrane nucleoside transporters including hENT1, hENT2, hCNT1, and hCNT3 proteins [7]. It is now well documented that the deficiency of membrane nucleoside transporter activity induces resistance to gemcitabine [8], which is an important cause of treatment failure.

Gemcitabine (Gemzar) is available on the market solely for intravenous administration, but its activity is also limited by a short half-life because of a rapid metabolism involving cytidine deaminase, which converts gemcitabine into difluorodeoxyuridine (dFdU) [9,10]. In this context, we have recently developed a new strategy known as 'squalenoylation', involving the conjugation of nucleoside analogues to squalene, a natural precursor in cholesterol biosynthesis [11]. The resultant squalenoyl prodrugs possessed amphiphilic character and spontaneously self-organized into nanoassemblies (NA) of 100–300 nm in water. Likewise, coupling of gemcitabine to 4-(*N*)-trisnorsqualenic acid resulted in the formation of squalenoyl gemcitabine (SQdFdC), which when dispersed in water formed NA of 130-nm mean diameter [12]. After intravenous administration, this SQdFdC nanomedicine displayed impressively higher anticancer activity over free gemcitabine against experimental leukemia, developed either by intravenous injection of the leukemia cells leading to metastasis [13] or by subcutaneous grafting inducing solid tumour [14]. At equitoxic doses, the SQdFdC nanomedicine injected intravenously led to 75% long-term survivors, which was not observed after treatment with free gemcitabine [15]. Additionally, the biodisposition studies performed following single-dose intravenous injection in mice revealed

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that the SQdFdC administered in nanoassembly form lowered the rapid metabolism of gemcitabine because of deamination in blood, and markedly increased the half-life and area under the curve (AUC) of gemcitabine [16].

Nevertheless, the development of an oral medication of gemcitabine is desirable for improved patient compliance and comfort and it would also represent significant progress because it does not require expensive patient hospitalization. In this context, and because squalene is a lipid that is absorbed orally [17], we have previously investigated the anticancer efficacy of SQdFdC NA after oral administration to rats bearing large granular lymphocytic (LGL) leukemia [12]. If gemcitabine exhibited significant improvement in the survival of leukemia-bearing rats, the SQdFdC NA were still more efficient than gemcitabine in increasing the life span of the animals and the number of long-term survivors [12]. However, the mechanism behind the observed superior anticancer efficiency after oral feeding of SQdFdC NA over free gemcitabine remained unknown.

Thus, the present communication aims to clarify the reasons for the observed anticancer activity of SQdFdC NA both at the cellular level *in vitro*, and by in-vivo biodisposition studies after oral administration.

## Materials and methods

Gemcitabine was purchased from Sequoia (Sequoia, UK) with 98% minimum purity, and [5′-$^3$H]-gemcitabine hydrochloride was obtained from Moravek Biochemicals (USA). Squalene and dextrose were purchased from Sigma-Aldrich Chemical Co., France. The scintillation liquids Ultima Gold and Hionic Fluor were from Perkin Elmer (USA). RPMI 1640 GlutaMAX I and fetal bovine serum were purchased from Dulbecco (Invitrogen, France). Penicillin and streptomycin solution were purchased from Lonza (Verviers, Belgium). Cytidine deaminase (CD) was purchased from Tebu-bio, France. Tissue culture inserts (having 0.02-μm pore size) were purchased from Techno Plastic Products (TPP) AG, Switzerland.

### Synthesis of *4-(N)-trisnorsqualenoylgemcitabine* (squalenoyl gemcitabine)

To a stirred solution of 1,1′,*2-tris*-norsqualenoic acid (0.5 g, 1.2 mmol) in anhydrous THF (3 ml), triethylamine (0.150 g, 1.5 mmol) was added dropwise. The mixture was cooled to –15°C and a solution of ethyl chloroformate (0.135 g, 1.2 mmol) in anhydrous THF (3 ml) was added dropwise. The mixture was stirred at 0°C for 15 min and a solution of gemcitabine hydrochloride (dFdC HCl) (0.37 g, 1.2 mmol) with triethylamine (0.24 g, 2.4 mmol) in anhydrous DMF (5 ml) was added dropwise to the reaction at the same temperature. The reaction was stirred for 72 h at room temperature, and the reaction mixture was then concentrated *in vacuo*. Aqueous sodium hydrogen carbonate was added and the mixture was extracted with ethyl acetate (3 × 50 ml). The combined extracts were washed with water, dried on $MgSO_4$, and evaporated. The crude product was purified by chromatography on silica gel and eluted with $CH_2Cl_2$/MeOH/$Et_3N$: 100 : 2 : 1 then with $CH_2Cl_2$/MeOH/$Et_3N$: 100 : 5 : 1 to give pure 4-*N*-squalenoylgemcitabine (0.46 g, 57%) as an amorphous white solid. $(\alpha)_D = 3.1$ ($c = 0.95$, $CH_2Cl_2$); IR (neat/cm) *v*: 3500–3150, 2950, 2921, 2856, 1709, 1656, 1635, 1557, 1490, 1435, 1384, 1319, 1275, 1197, 1130, 1071, 814; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$: 9.15 (s large, 1H, NHCO), 8.16 (d, 1H, $J = 7.5$ Hz, H6), 7.47 (d, 1H, $J = 7.5$ Hz, H5), 6.18 (t, 1H, $J = 7.0$ Hz, H1′), 5.22–5.15 (m, 5H, H*vinyl*), 4.49 (m, 1H, H3′), 4.86–4.09 (m, 3H, H4′, 2H5′), 2.55 (m, 2H, $COCH_2CH_2$), 2.38–2.28 (m, 2H, $COCH_2CH_2$), 2.13–1.91 (m, 16H, $CH_2$), 1.69–1.55 [m, 18H, $C=C(CH_3)$]; $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$: 173.7 (CONH), 163.0 (CO), 155.8 (C), 145.4 (CH), 135.1 (C), 134.9 (2 C), 132.7 (C), 131.1 (C), 125.7 (CH), 124.4 (CH), 124.3 (CH), 124.2 (2 CH), 122.3 (t, $J_{CF} = 260$ Hz, $CF_2$), 97.7 (CH), 85.8 (m, CH), 81.6 (CH), 69.2 (t, $J = 21$ Hz, CH), 59.7 ($CH_2$), 39.7 (2 $CH_2$), 39.5 ($CH_2$), 36.5 ($CH_2$), 34.3 ($CH_2$), 29.6 ($CH_2$), 28.3 ($CH_2$), 26.8 ($CH_2$), 26.7 ($CH_2$), 26.6 ($CH_2$), 25.6 ($CH_3$), 17.6 ($CH_3$), 16.0 (2 $CH_3$), 15.9 ($CH_3$), 15.8 ($CH_3$); MS (–ESI) $m/z = 644$ [$(M\text{-}H)^-$, 100%]; Anal. Calcd for $C_{36}H_{53}F_2N_3O_5$: C, 66.95, H, 8.27, N, 6.51. Found: C, 66.76, H, 8.40, N, 6.39.

### Experimental procedure for the synthesis of tritiated *4-(N)-trisnorsqualenoylgemcitabine* [5′-$^3$H]squalenoyl gemcitabine

$^3$H-SQdFdC (2.89 mCi/mmol) was synthesized in a similar manner, but using $^3$H-gemcitabine (1.6 mCi/mmol). 3.5 mCi of tritiated gemcitabine ([5′-$^3$H]gemcitabine) was mixed with cold gemcitabine hydrochloride (10 mg) to obtain 10 mg of tritiated gemcitabine hydrochloride (3.5 mCi, 0.033 mmol). The synthesis of tritiated 4-(*N*)-trisnorsqualenoyl-[5′-$^3$H]gemcitabine was then achieved according to the experimental procedure described above to provide pure tritiated 4-(*N*)-trisnorsqualenoylgemcitabine ([5′-$^3$H]SQdFdC) (0.017 g, 84%, 84.2 mCi/mmol). The specific activity was adjusted to 2.89 mCi/mmol by mixing this material together with cold SQ-dFdC (343 mg) to finally obtain 360 mg of [5′-$^3$H]SQdFdC. The [5′-$^3$H]SQdFdC was stored at –20°C in a solution (60 ml) of acetone/ethanol (1 : 1).

### Preparation of squalenoyl gemcitabine nanoassemblies

SQdFdC NA were prepared by nanoprecipitation as follows: SQdFdC was dissolved in ethanol (4 mg/ml) and added dropwise to an equal volume of a 5% aqueous dextrose solution, which induced the spontaneous formation of NA. Evaporation of ethanol at 37°C using rotavapor resulted in the formation of aqueous suspension of pure SQdFdC NA. The mean particle size was confirmed using a nanosizer (Coulter; N4MD). For in-vitro and in-vivo experiments, freshly prepared SQdFdC NA were used.

### Cell culture

RNK-16 LGL leukemia cells (obtained from Dr T. Sawyers, NCI, Frederick, Maryland, USA) were cultured at 37°C and 5% $CO_2$ in RPMI 1640 supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin, 2 mmol/l L-glutamine, 100 mmol/l sodium pyruvate, and 100 mmol/l nonessential amino acids (cell culture medium).

### Cytotoxicity studies

The cytotoxicity of gemcitabine and of SQdFdC NA towards RNK-16 LGL leukemia cells was determined using the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl tetrazolium bromide (MTT) test, measuring mitochondrial dehydrogenase activity. The cells were suspended in the medium, placed in 96-well plates and incubated for 24 h at 37°C in a humidified atmosphere of 5% $CO_2$ in air. At 24 h post incubation, various dilutions of gemcitabine and of SQdFdC NA were made in the cell culture medium and added to the wells containing the cell suspension. Each dilution was tested in triplicate. After 72 h of incubation at 37°C, 20 µl of a MTT solution in the cell culture medium (5 mg/ml) were added to each well. After incubation for 2.5 h at 37°C, the culture medium was removed and formazan crystals were dissolved in 200 µl formazan crystal dissolving medium [dimethyl sulfoxide: sodium lauryl sulfate (1 : 1)]. The absorbance of converted dye, which is proportional to the number of viable cells, was measured at 570 nm, with background subtraction at 650 nm using a microplate reader (Metertech Σ 960; Fisher Bioblock, Illkirch, France). The percentage of surviving cells was calculated as the absorbance ratio of treated to untreated cells.

The cytotoxicity assays were performed with or without incubation with CD (0.5 µg/ml). For the assay involving cytidine deaminase, the cells were incubated with CD for 15 min, followed by the addition of various concentrations of either gemcitabine, or SQdFdC NA.

Additional cytotoxicity tests were also performed after inhibition of the cell membrane nucleoside transporters by incubating the cells with nucleoside transporter inhibitors such as nitrobenzyl thioinosine (NBTI) and dipyridamole. Practically, following 24 h preincubation at 37°C in a humidified atmosphere of 5% $CO_2$ in air, the cells were incubated with 1 µmol/l NBTI or 10 µmol/l dipyridamole for 30 min. The inhibitors were then removed from the wells by centrifugation, and the cell culture medium was added followed by incubation with various concentrations of gemcitabine or SQdFdC NA. At 72 h post incubation, the cells were processed for the MTT assay as described above.

A cytotoxicity study using tissue culture inserts has been performed using 6-well plates. RNK-16 LGL leukemia cell suspension was placed in the wells, followed by the hanging of an insert into the well. The SQdFdC nanoassembly dispersion was then placed on the insert membrane. Thus, the insert membrane (0.02-µm pore size) forms a barrier between the cells and the nanoassembly dispersion (130-nm size). The well plates were then incubated for 24, 48, and 72 h at 37°C, 5% $CO_2$ in a humidified chamber. At corresponding time intervals, the insert membrane was removed and the cells were collected by centrifugation, and the cytotoxicity study was performed by the MTT assay as described above.

The cell endocytosis inhibition experiments were performed by preincubation of the cells for 1 h either at 4°C or in the presence of phenylarsine oxide (PAO). Then, the PAO was removed from the wells and this was followed by the addition of various concentrations of gemcitabine and SQdFdC NA as described above.

### Absorption and biodistribution studies after oral administration

The animal experiments were carried out according to the principles of laboratory animal care and legislation in force in France. Tritiated versions of gemcitabine or of SQdFdC were used to study drug absorption and biodistribution following oral administration. Practically, 3.5 µCi of either $^3$H-SQdFdC or $^3$H-gemcitabine were mixed with nonradiolabeled SQdFdC or gemcitabine, respectively, (to obtain dose equivalent of 5 mg/kg gemcitabine) and administered orally to F344 Fischer rats (4–5 weeks old, weighing about 75–90 g). $^3$H-gemcitabine was administered as a solution in 0.9% saline, and $^3$H-SQdFdC as NA in 5% aqueous dextrose. At various time intervals, blood samples were collected from rats and the plasma was immediately isolated by centrifugation at 3000 rpm and mixed with 10 ml scintillation cocktail (Ultima Gold). At 24 h post administration, the rats were humanely sacrificed and the organs were isolated, blotted with tissue paper, dissolved in soluene at 60°C, and mixed with 10 ml of scintillation cocktail (Hionic Fluor). The radioactivity of plasma and tissues was measured using a β-counter (Beckman, LS 6000TA; Beckman Coulter, USA).

### Data and statistical analysis

The pharmacokinetic data were treated using the Wagner–Nelson method and the AUC values were determined using GraphPad software. Statistical analysis was performed using Student's *t*-test and one-way analysis of variance (ANOVA) and wherever relevant using GraphPad software (GraphPad Inc., San Diego, California, USA).

## Results

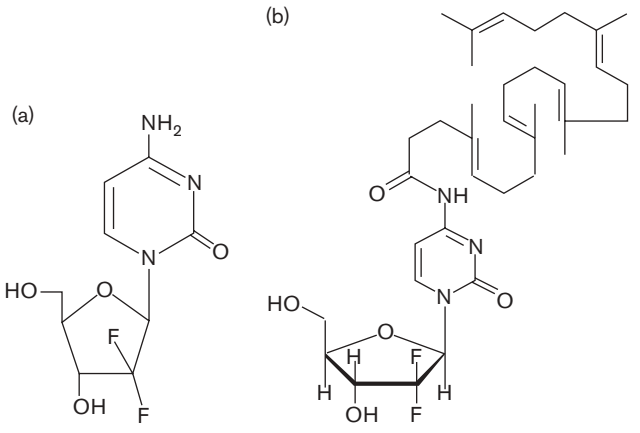
SQdFdC NA displayed 135-nm mean diameter. The stability of SQdFdC suspension has been checked by $^1$H NMR. In the spectrum, the C-5 and C-6 vinylic

protons on the base display very different chemical shifts in the case of gemcitabine (8.06 and 6.15 ppm) and SQdFdC (8.05 and 7.48 ppm). After storing the SQdFdC NA for 2 days at room temperature, the $^{1}H$ NMR spectroscopy performed in dimethylsulfoxide showed no trace of decomposition of SQdFdC. This clearly establishes the stability of SQdFdC NA during the experimentation time.

Gemcitabine (Fig. 1a) exhibited a 2.8-fold greater cytotoxicity than SQdFdC (Fig. 1b) NA on RNK-16 LGL cells (Fig. 2). However, to imitate the in-vivo situation where SQdFdC will come into contact with extracellular deaminases before reaching the cancer cells, we have added CD to the cell culture medium before further 72 h-incubation with either free gemcitabine or SQdFdC NA. In these conditions, a significant decrease in the cytotoxicity of gemcitabine occured, as indicated by an increase in $IC_{50}$ value by ~83-fold compared with that in the absence of CD (Fig. 2). On the contrary, the $IC_{50}$ value of SQdFdC NA in the presence of CD remained almost unchanged. Thus, the anticancer activity of gemcitabine was highly sensitive to the presence of cytidine deaminase, while this was not so with SQdFdC NA.

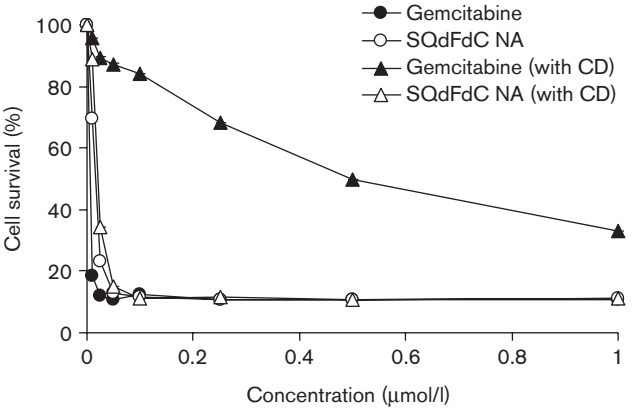
Intracellular accumulation studies performed following incubation of the tritiated versions of gemcitabine or of SQdFdC NA with RNK-16 LGL leukemia cells revealed that the $^{3}H$-SQdFdC NA exhibited significantly greater intracellular concentrations as compared with $^{3}H$-gemcitabine (Fig. 3). The intracellular concentration of $^{3}H$-gemcitabine slightly increased at 3 h post incubation, but decreased later. On the other hand, the $^{3}H$-SQdFdC NA showed an increase of 27% at 3 h post incubation and remained the same up to 6 h post incubation.

**Fig. 1**



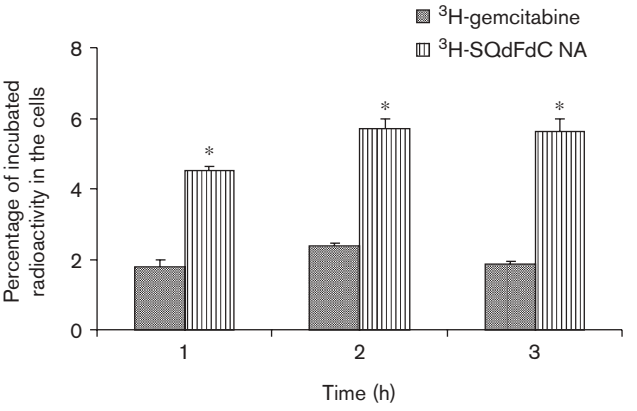

Chemical structures of (a) gemcitabine and (b) 4(*N*)-trisnorsqualenoyl gemcitabine [squalenoyl gemcitabine (SQdFdC)].

**Fig. 2**




Cytotoxicity profiles of gemcitabine and squalenoyl gemcitabine (SQdFdC) nanoassemblies (NA) at 72 h post incubation in the absence and in the presence of cytidine deaminase (CD) in the cell culture medium. CD (0.5 μg/ml) was first added to the cells and incubated for 15 min, followed by the addition of gemcitabine or SQdFdC NA. $n=3$. The values are the mean ± SD.

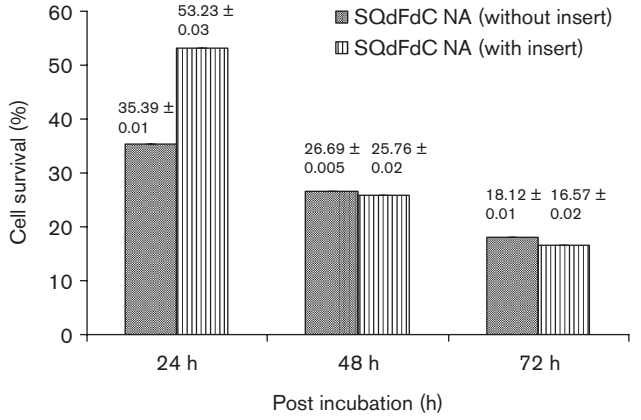
**Fig. 3**




Comparative intracellular accumulation and retention in RNK-16 LGL leukemia cells of $^{3}H$-gemcitabine and $^{3}H$-SQdFdC nanoassemblies (NA) at various time intervals after incubation. $n=3$. The values are the mean ± SD. *$P<0.05$, statistical analysis was performed using analysis of variance, considering 95% confidence interval at significance level $P<0.05$. SQdFdC, squalenoyl gemcitabine.

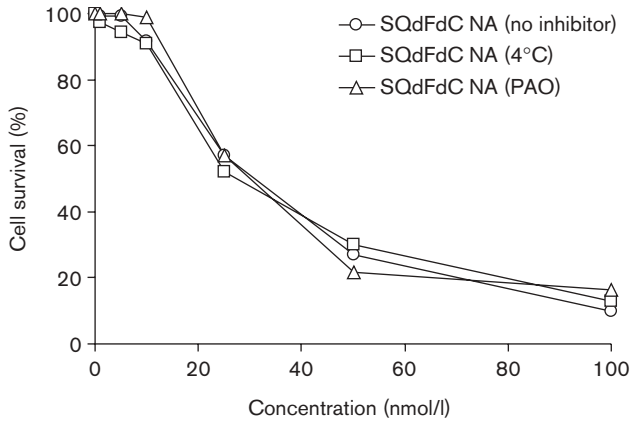
To investigate the mechanism of absorption of SQdFdC NA following oral administration, a tissue culture insert was used to mimic the intestinal membrane. When the SQdFdC NA (130 nm as measured by laser light scattering) were separated from the cells by inserts with a pore size (0.02-μm pore diameter) smaller than the diameter of NA, the SQdFdC NA maintained their cytotoxicity after 48 and 72 h incubation. On the contrary, at 24 h post incubation, the cell viability (%) remained 53% in the presence of the insert, whereas it was only 35% without the insert (Fig. 4).

**Fig. 4**




Survival of RNK-16 LGL leukemia cells following incubation with squalenoyl gemcitabine (SQdFdC) nanoassemblies (NA), in the presence or absence of inserts. The cytotoxicity test was performed using six-well plates, with or without inserts for separating the cells from the SQdFdC NA (1 μmol/l equivalent of gemcitabine). The pore size of the inserts is 20 nm, whereas the diameter of the NA is 130 nm. $n=3$. The values are the mean ± SD.

**Fig. 5**




Cytotoxicity profiles of squalenoyl gemcitabine (SQdFdC) nanoassemblies (NA) on RNK-16 LGL leukemia cells at 37°C (control), at 4°C, or in the presence of the endocytosis inhibitor phenylarsine oxide (PAO) at 37°C. $n=3$. The values are the mean ± SD.

Incubation at 4°C or with PAO, an inhibitor of endocytosis, did not affect the cytotoxicity of SQdFdC NA on RNK-16 LGL leukemia cells. Clearly, the $IC_{50}$ values of SQdFdC NA were similar whether the cells were incubated at 4°C or with PAO (Fig. 5). A control experiment run to observe the own cytotoxicity, if any, of phenylarsine oxide, revealed no toxicity of RNK-16 LGL leukemia cells even after incubation for 72 h.

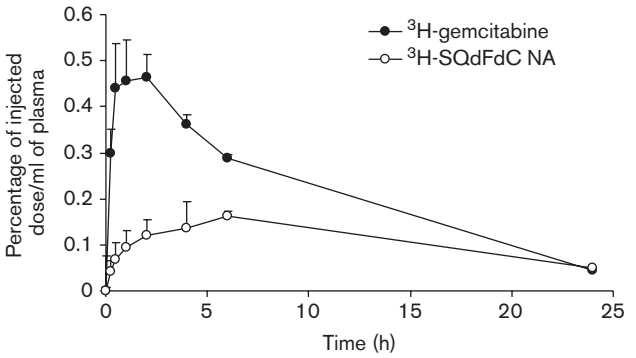
Inhibition of the nucleoside transporters by incubation with nucleoside transporter inhibitors has modified the

**Table 1 Influence of the inhibition of nucleoside transporters on cytotoxicity of gemcitabine and SQdFdC nanoassemblies as represented by the IC50 values, on RNK-16 LGL leukemia cells**

| Treatment | IC50 (nmol/l) |
|---|---|
| Gemcitabine | 7.7 |
| Gemcitabine + NBTI | 9 |
| Gemcitabine + dipyridamole | 18 |
| SQdFdC nanoassemblies | 29 |
| SQdFdC nanoassemblies + NBTI | 25 |
| SQdFdC nanoassemblies + dipyridamole | 32 |

The nucleoside transporter inhibition was carried out using nitrobenzyl thioinosine (NBTI) (1 μmol/l) or dipyridamole (10 μmol/l). $n=3$.
SQdFdC, squalenoyl gemcitabine.

**Fig. 6**




Absorption profiles of $^3$H-gemcitabine (5 mg/kg) and $^3$H-SQdFdC nanoassemblies (NA) (5 mg/kg equivalent) following single-dose oral administration in healthy rats. $n=3$. The values are the mean ± SD.
SQdFdC, squalenoyl gemcitabine.

cytotoxicity profile of gemcitabine (Table 1). Although the nucleoside transporter inhibitor NBTI predominantly inhibits the hENT1 transporter system when incubated with the cells [18], dipyridamole inhibits the combination of hENT1 and hENT2 transporter systems [19]. Following preincubation with the RNK-16 LGL leukemia cells, dipyridamole caused a ~2.3-fold increase in the $IC_{50}$ values of gemcitabine, whereas NBTI only increased $IC_{50}$ by ~1.2-fold, suggesting the transport of gemcitabine into these cells mainly by (but not limited to) the combination of hENT1 and hENT2 transporter systems contrarily to the SQdFdC NA.

Following single-dose oral administration in rats, $^3$H-gemcitabine underwent rapid absorption leading to higher plasma concentration compared to the $^3$H-SQdFdC NA (Fig. 6). The AUC obtained from the percentage (%) injected dose–time profile of $^3$H-gemcitabine [5.25 (h) (%/ml)] was higher than that of $^3$H-SQdFdC NA [2.64 (h) (%/ml)] (Table 2). Two hours after administration, the plasma concentration of $^3$H-gemcitabine diminished rapidly, and at 24 h post administration, it was similar to that of $^3$H-SQdFdC NA. Conversely, the $^3$H-SQdFdC NA exhibited considerably higher plasma half-life ($t_{1/2}$), mean residence time, and volume of distribution ($V_d$) compared

with that of the $^{3}$H-gemcitabine (Table 2). In addition, the clearance (Cl) and elimination ($K_{el}$) of $^{3}$H-SQdFdC NA was noticeably lower than that of $^{3}$H-gemcitabine. The $^{3}$H-SQdFdC NA, though less and more slowly absorbed, displayed an improved tissue distribution profile compared to gemcitabine, especially to the lymphoid organs (spleen and thymus), the major metastatic organs in leukemia [13] (Fig. 7). Additionally, the accumulation of $^{3}$H-SQdFdC NA was also higher than $^{3}$H-gemcitabine in the stomach tissue.

## Discussion

Although earlier in-vivo studies have clearly demonstrated the improved anticancer activity of SQdFdC NA over gemcitabine after oral administration to rats bearing RNK-16 LGL leukemia [12], the in-vitro data obtained here with the same cell line demonstrated, on the contrary, the higher cytotoxicity of gemcitabine versus SQdFdC NA. This could be attributed to the in-vitro experimental conditions, which did not mimic the in-vivo conditions, as the blood contains deaminases whereas the cell culture medium did not. It is worthy to note that the presence of deaminases should have a decisive influence on the activity of gemcitabine, as this nucleoside analogue undergoes rapid deamination because of the action of CD following intravenous administration, resulting in the conversion to the uracil metabolite [20]. Thus, to mimic the in-vivo condition with respect to the presence of cytidine deaminase, the cytotoxicity study has been repeated by including CD in the cell culture medium at a concentration similar to blood. Unlike the case of SQdFdC NA, the presence of CD in the culture medium has considerably decreased the cytotoxicity of gemcitabine. Thus, the SQdFdC NA resisted the deamination process in the presence of cytidine deaminase, which renders the squalenoyl prodrug much more potent than the parent compound in this condition. Additionally, the considerably greater accumulation and retention of the $^{3}$H-SQdFdC NA (as compared with $^{3}$H-gemcitabine) in RNK-16 LGL leukemia cells suggest its prolonged activity when inside the cells causing a high cell kill, which is probably one of the important reasons for the superiority of SQdFdC NA over gemcitabine in the previously reported [12] experimental rat leukemia model.

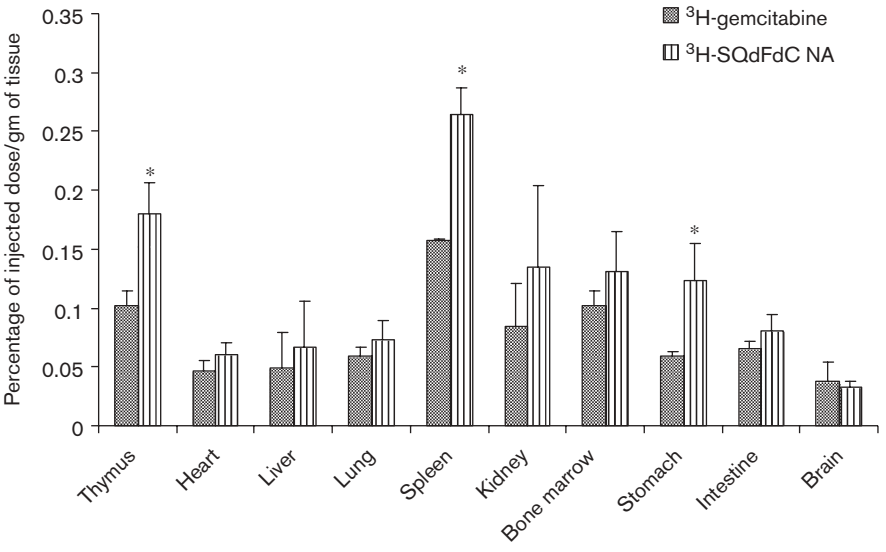
**Table 2 Comparative plasma pharmacokinetic parameters of $^{3}$H-gemcitabine (5 mg/kg) and $^{3}$H-SQdFdC nanoassemblies (5 mg/kg equivalent) following oral administration in healthy rats**

| Pharmacokinetic parameter | $^{3}$H-gemcitabine | $^{3}$H-SQdFdC NA |
|---|---|---|
| $C_{max}$ (%/ml) | 0.46 | 0.16 |
| $T_{max}$ (h) | 2 | 6 |
| AUC (h) (%/ml) | 5.25 | 2.64 |
| $t_{1/2}$ (h) | 7.36 | 29.79 |
| MRT (h) | 6.03 | 10.98 |
| $V_d$ [% (%/ml/ml)] | 0.46 | 0.46 |
| Cl [% h (%/ml)/ml] | 0.20 | 0.05 |
| $K_{el}$ ($h^{-1}$) | 0.09 | 0.02 |

AUC, area under the curve; MRT, mean residence time; NA, nanoassemblies; SQdFdC, squalenoyl gemcitabine.

As the sensitivity to the nucleoside transporters is another key factor of the anticancer activity of gemcitabine,

**Fig. 7**




Tissue distribution profiles of $^{3}$H-gemcitabine (5 mg/kg) and $^{3}$H-SQdFdC nanoassemblies (NA) (5 mg/kg equivalent) at 24 h after single-dose oral administration in healthy rats. *$P<0.05$, statistical analysis was performed using Student's *t*-test, considering 95% confidence interval at significance level $P<0.05$. $n=3$. The values are the mean ± SD. SQdFdC, squalenoyl gemcitabine.

we have investigated the sensitivity of RNK-16LGL leukemia cells toward SQdFdC NA in the presence of nucleoside transporter inhibitors. Unlike gemcitabine, the SQdFdC NA remained poorly influenced by both dipyridamole and NBTI, suggesting that the SQdFdC NA probably do not need nucleoside transporters to have cellular access.

Furthermore, to simulate the intestinal absorption of SQdFdC NA following oral administration, a tissue culture insert of 0.02-μm pore diameter has been used as a model of intestinal membrane to separate the NA (130 nm) from the leukemic cells. In this condition, the SQdFdC NA were able to induce similar cell cytotoxicity as in the absence of the insert, at least for the 48 and 72 h time points. This clearly suggests that the SQdFdC was able to diffuse and exert its anticancer activity as a single molecule without being endocytosed by the cancer cells as NA. To further support this assertion, the SQdFdC NA were tested for endocytosis transport by incubating either at 4°C or with PAO at 37°C. PAO is, indeed, a trivalent monosubstituted organoarsenic compound shown to inhibit endocytosis via clathrin-coated pits [21]. Under these conditions, cytotoxicity of SQdFdC NA remained unchanged. These results support the hypothesis that SQdFdC diffused into the cells as single molecular entities, rather than by endocytosis of NA.

To further investigate the basis for the previously observed [12] superior in-vivo anticancer efficacy of SQdFdC NA over gemcitabine after oral administration, we conducted pharmacokinetics and tissue disposition studies. It was observed that after oral administration, $^{3}$H-gemcitabine underwent rapid absorption followed by a quick decline in plasma concentration. In contrast, $^{3}$H-SQdFdC administered in nanoassembly form was absorbed slowly from the gastrointestinal tract, but displayed improved tissue distribution profile. The higher accumulation of $^{3}$H-SQdFdC in the lymphoid organs such as the spleen and thymus, the major organs of metastasis, may explain the superior anticancer activity of SQdFdC in RNK-16 LGL aggressive leukemia-bearing rats. Indeed, in the case of leukemia, the abnormal cancer cells generally spread to lymphoid organs, especially the spleen [13]. Furthermore, greater accumulation of SQdFdC in the stomach suggests its penetration and retention into epithelial tissue, which accounts for the observed slower absorption profile. This enhanced stomach accumulation of SQdFdC could also be of interest for treating stomach-associated cancers by the oral route.

It is worth noting that it has been shown recently that following multiple oral administrations in mice, a significantly higher hepatic and renal accumulation of gemcitabine triphosphate, dFdU, and dFdU triphosphate occurred [22]. On the other hand, multiple dosing of gemcitabine in human patients led to low systemic exposure of gemcitabine [23], but higher exposure of dFdU with a long terminal half-life ($\sim$89 h). These results raise the important question of whether or not dFdU and its triphosphate metabolite could contribute to activity/toxicity following oral administration of gemcitabine. In the light of these important recent findings, it should be of prime interest to measure dFdU triphosphate levels generated after oral administration of SQdFdC NA.

### Conclusion

This study has highlighted some key factors that could account for the improved anticancer activity of SQdFdC NA when administered orally. These include resistance to deamination, improved pharmacokinetics, and increased accumulation in the lymphoid organs. At the cellular level, greater accumulation and retention in the target lymphocytic leukemia may explain the effective cell killing. Thus, the SQdFdC nanomedicine could be an interesting therapeutic approach in the treatment of leukemia by the oral route.

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