Squalenoylation Favorably Modifies the in Vivo Pharmacokinetics and Biodistribution of Gemcitabine in Mice

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ABSTRACT:

Gemcitabine (2’,2’-difluorodeoxyribosylcyclotinose; dFdC) is an anticancer nucleoside analog active against wide variety of solid tumors. However, this compound is rapidly inactivated by enzymatic deamination and can also induce drug resistance. To overcome the above drawbacks, we recently designed a new squalenoyl nanomedicine of dFdC [4-(N)-trisnorsqualenoyl-gemcitabine (SQdFdC)] by covalently coupling gemcitabine with the 1,1’,2-trisnorsqualenic acid; the resultant nanomedicine displayed considerably greater anticancer activity compared with the parent drug in an experimental murine model. In the present study, we report that SQdFdC nanoassemblies triggered controlled and protracted release of dFdC and displayed considerably greater t1/2 (−3.9-fold), mean residence time (−7.5-fold) compared with the dFdC administered as a free drug in mice. It was also observed that the linkage of gemcitabine to the 1,1’,2-trisnorsqualenic acid noticeably delayed the metabolism of dFdC into its inactive difluoro-deoxyuridine (dFdU) metabolite, compared with dFdC. Additionally, the elimination of SQdFdC nanoassemblies was considerably lower compared with free dFdC, as indicated by lower radioactivity found in urine and kidneys, in accordance with the plasmatic concentrations of dFdU. SQdFdC nanoassemblies also underwent considerably higher distribution to the organs of the reticuloendothelial system, such as spleen and liver (p < 0.05), both after single- or multiple-dose administration schedule. Herein, this paper brings comprehensive pharmacokinetic and biodistribution insights that may explain the previously observed greater efficacy of SQdFdC nanoassemblies against experimental leukemia.

Gemcitabine (2’,2’-difluorodeoxyribosylcyclotinose; dFdC) is a nucleoside analog active against a wide variety of cancers including nonsmall cell lung and pancreatic cancers. Marketed as Gemzar, it acts as an antimetabolite, inhibiting ribonucleotide reductase and DNA synthesis and inducing apoptosis (Cappella et al., 2001). However, this compound has a narrow therapeutic index due to rapid enzymatic deamination by deoxycytidine deaminase into its corresponding inactive uracil derivative [difluorodeoxyuridine (dFdU)] (Abbruzzese et al., 1991; Moog et al., 2002; Reid et al., 2004). Therefore, the administration of high doses of drug is needed to achieve the desired therapeutic response but simultaneously, this leads to various adverse effects mainly myelosuppression (Abbruzzese et al., 1991). Another limitation associated with the use of gemcitabine is the induction of anticancer drug resistance due to the down-regulation of deoxycytidine kinase enzyme responsible for the primary phosphorylation of gemcitabine (Ruiz van Haperen et al., 1994; Bergman et al., 2002). To address the problem of deamination, which is the key of the above issues, various attempts have been made to...

ABBREVIATIONS: dFdC, gemcitabine; 2’,2’-difluorodeoxyribosylcyclotinose; dFdU, difluorodeoxyuridine; SQdFdC, squalenoyl gemcitabine; 4-(N)-trisnorsqualenoyl-gemcitabine; LC, liquid chromatography; MS, mass spectrometry; HPLC, high-performance liquid chromatography; THU, tetrahydrouridine; AUC, area(s) under the plasma concentration-time curve; CI, total body clearance; Vss, volume of distribution; dFdCNA, dFdC released from SQdFdC nanoassemblies; MRT, mean residence time.
synthesize lipidic prodrugs (Myhren et al., 2000; Castelli et al., 2006). One of the synthetic options used is to link the 4-amino site of dFdC to a fatty acid derivative, thereby protecting it from deamination by cytidine deaminase (Castelli et al., 2006). This strategy of linking a long chain of fatty acids also aims to improve the lipophilicity of dFdC and, hence, increase its interactions with the lipidic membranes and its cellular uptake (Castelli et al., 2006). However, such compounds were poorly water soluble, which rendered their i.v. administration challenging.

In this context, we recently discovered that by coupling nucleoside analogs to squalene (a natural lipid, precursor of the cholesterol biosynthesis), the resulting compounds self-organized in water as nanoassemblies with an average diameter of 100 to 200 nm, whatever the nature of the nucleoside analog used and the location of the linkage. “Squalenoylation” is a new promising concept in drug delivery (Couvreur et al., 2006).

In vitro, SQdFdC nanoassemblies exhibited a greater cytotoxicity on sensitive and resistant cancer murine and human cell lines than gemcitabine (Couvreur et al., 2006; Reddy et al., 2007). In vivo, SQdFdC nanoassemblies exhibited impressively greater anticancer activity against experimental murine leukemia, producing also higher rates of apoptosis in cancer cells as compared with free dFdC (Reddy et al., 2007). However, the mechanism behind the observed anticancer efficacy of SQdFdC nanoassemblies is not yet understood. Altered pharmacoki-netics and biodistribution in vivo are key issues that may represent a possible explanation.

Thus, in this communication, the pharmacokinetics of the SQdFdC nanoassemblies were assessed after i.v. administration in healthy mice, compared with dFdC, by simultaneous quantification of the drug concentrations in plasma using liquid chromatography (LC)-tandem mass spectrometry. The in vivo metabolic kinetics of SQdFdC versus dFdC conversion into the inactive metabolite, dFdU, was also investigated, using an additional LC-MS method. Finally, the organ distribution of SQdFdC nanoassemblies was estimated and compared with that of dFdC by using the corresponding tritiated compounds.

Materials and Methods

Drugs and Reagents. dFdC was purchased from Sequoia (Pangbourne, UK) with 98% minimum purity, and [5-3H]Gemcitabine hydrochloride was obtained from Moravek Biochemicals (Brea, CA). Deoxycytidine, used as internal standard in the pharmacokinetic analysis, was obtained from Sigma-Aldrich (St. Louis, MO) with 99% purity. dFdU was synthesized by Jena Bioscience (Jena, Germany) with 95% minimum HPLC purity. Reagents and HPLC-grade solvents were provided by Thermo Electron Corporation (Waltham, MA), formic acid by Merck (Darmstadt, Germany). Ultrapure water was prepared using a Milli-Q system (Millipore Corporation, Billerica, MA). Drug-free heparinized human plasma was obtained from EFS (Rungis, France). Tetrahydrodronium (THU) was provided by Calbiochem (San Diego, CA). Dextrose was purchased from Sigma-Aldrich. Soluene, Ultima Gold, and Hionic-Flour were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Synthesis of SQdFdC and [5-3H]SQdFdC. The synthesis of SQdFdC was performed as previously described (Couvreur et al., 2006). Practically, triethylamine (0.15 g, 1.4 mmol) was added, under nitrogen, to a stirred solution of trisnorsqualenolic acid (0.50 g, 1.2 mmol) in anhydrous THF (4 ml). The mixture was cooled to 0°C and ethyl chlororormate (0.135 g, 1.2 mmol) in anhydrous THF (3 ml) was added drop-wise. The mixture was stirred at 0°C for 15 min, and a solution of gemcitabine hydrochloride (0.37 g, 1.2 mmol) and triethylamine (0.15 g, 1.4 mmol) in anhydrous DMF (5 ml) was added drop-wise to the reaction at the same temperature. The reaction was stirred for 72 h at room temperature, and the reaction mixture was 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 MgSO4, and concentrated. The crude product was purified by chromatography on silica gel and eluting with CH2Cl2/MeOH/Et3N, 100:2:1, then with CH2Cl2/MeOH/Et3N, 100:5:1, to give pure 4-(N-squalenoyl-gemcitabine hydrochloride (0.017 g, 84%, 84.2 mCi/mmol) as a white solid. (αD)3 = 3.1 (c = 0.95, CH2Cl2); IR (neat, cm−1) 3500–3150, 2950, 2921, 2856, 1709, 1656, 1557, 1490, 1435, 1384, 1319, 1275, 1197, 1130, 1071, 1H NMR (300 MHz, CDCl3) δ 9.15 (broad s, 1 H, NCO), 8.16 (d, 1 H, J = 7.5 Hz, H6), 7.47 (d, 1 H, J = 7.5 Hz, H5), 6.18 (t, 1 H, J = 7.0 Hz, H1), 5.22–5.15 (m, 5 H, HCC = C(CH3)), 4.49 (m, 1 H, H3z), 4.86–4.09 (m, 3 H, H4' and H5'), 2.55 (m, 2 H), 2.38–2.28 (m, 2 H), 2.13–1.91 (m, 16 H, CH2), 1.69–1.55 (m, 18 H, C = C(CH3)); 13C NMR (75 MHz, CDCl3) δ 173.7 (CONH), 163.0 (HNC = N), 155.8 (NCON), 145.4 (CH, C6), 135.1 (C), 134.9 (2 C), 132.7 (2 C), 131.2 (C), 125.7 (CH), 124.4 (2 CH), 124.2 (2 CH), 122.4 (2 CF3), 97.7 (CH, C5), 81.7 (2 CH), 69.2 (CH), 59.9 (CH2), 39.7 (2 CH2), 39.5 (CH2), 36.5 (CH2), 34.3 (CH3), 28.3 (2 CH3), 26.8 (CH2), 26.7 (CH2), 26.6 (CH2), 25.6 (CH3), 17.6 (16.0 (3 CH3), 15.8 (CH3); MS (-ESI) m/z = 644 (M+H−, 100%); Anal. Calc. for C50H76N2O3, C, 66.76, H, 8.04, N, 6.39.

For [5-3H]dFdC, 3.5 mCi of 3H-gemcitabine (5-3H)dFdC) was mixed with cold dFdC hydrochloride (10 mg) to obtain 10 mg of tritiated dFdC hydrochloride (3.5 mCi, 0.033 mmol). The synthesis of tritiated 4-(N-trisnor squalenoyl)-[5-3H]gemcitabine was then performed according to the experimental procedure described before to provide pure 4-(N-trisnor squalenoyl gemcitabine (5-3H)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 SQdFdC (343 mg) to obtain finally 360 mg of [5-3H]SQdFdC. [5-3H]SQdFdC was stored at −20°C as a solution of 6 mg/ml in acetonitrile/ethanol (1:1).
Preparation and Characterization of (N)-Trisnorsqualenoylgemcitabine Nanoassemblies. Cold or 3H-SQdFdC nanoassemblies were prepared by the nanoprecipitation technique. Briefly, SQdFdC was dissolved in ethanol (10 mg/ml) and added drop-wise under magnetic stirring (500 rpm) into a 5% (w/v) aqueous dextrose solution. Precipitation of the nanoassemblies occurred spontaneously. Ethanol was completely evaporated using a Rotavapor at 37°C to obtain a aqueous suspension of pure SQdFdC nanoassemblies (5 mg/ml). The mean particle size of the nanoassemblies was determined at 20°C by quasielastic light scattering using a nanosizer (Coulter N4MD, Beckman Coulter, Inc., Fullerton, CA). The selected angle was 90° and the measurement was made after dilution of the suspension of nanoassemblies in water.

Animal Experiments. The animal experiments were carried out according to the principles of laboratory animal care and legislation in force in France. DBA/2 mice (5–6 weeks old) weighing about 15 to 18 g were used for the studies and were purchased from Janvier (Le-Genest-Saint-Isle, France). Mice were provided with standard mouse food and water ad libitum. For pharmacokinetic and biodistribution studies, mice were divided into two main groups for treatment with dFdC or with SQdFdC nanoassemblies, with subgroups of three to five mice for each time point. The control group contained the mice that were not treated with any of the above drugs. The pharmacokinetic and biodistribution studies were performed separately with different animals.

Pharmacokinetic Studies. Drug administration and sampling. SQdFdC nanoassemblies or free dFdC were injected at equimolar doses of 15 mg of dFdC/kg. dFdC was prepared in 0.9% NaCl, and SQdFdC nanoassemblies (36.6 mg/kg SQdFdC) were prepared in 5% dextrose (see Preparation and Characterization of (N)-Trisnorsqualenoylgemcitabine Nanoassemblies). Both compounds were administered by the retro-orbital route. Blood samples were collected into tubes containing 8 µl of THU (10 mg/ml in water) from retro-orbital plexus (from the side other than that administered) after 1, 5, 15, and 30 min and 1, 2, and 4 h of administration (n = 4 or 5). For the long-time pharmacokinetic study, blood samples were collected from the retro-orbital plexus after 2, 4, 8, 24, and 48 h (n = 4). Without delay, plasma was separated by centrifugation at 3000 g for 10 min at 4°C. Extraction and quantification of SQdFdC and dFdC in the plasma samples were performed using a validated high-performance liquid chromatography tandem mass spectrometry (Khoury et al., 2007), and dFdU, the metabolite of dFdC, was assayed by a modified LC-MS method described by Xu et al. (2004).

High-performance liquid chromatography and tandem mass spectrometry. Practically, SQdFdC, dFdC, and dFdU plasmatic concentrations were determined as follows. Briefly, to 100 µl of THU-pretreated mice plasma, 20 µl of 10 µg/ml deoxycytidine (in methanol) was added. The mixture was first vortexed for 10 s, then 1 ml of acetonitrile/methanol [9:1 (v/v)] was added. The samples were vortexed for 10 s and then centrifuged at 15,800 g (Eppendorf centrifuge 5415R, Eppendorf North America, New York, NY) for 20 min at 4°C. The supernatant was transferred to a conical polypropylene tube and evaporated to dryness under a nitrogen flow, at room temperature. The residues were reconstituted in 0.2% formic acid, 95:5 (v/v), followed by 5-min linear gradient up to 100% solvent B [methanol/ammonium formate (5 mM, pH 7.5)/methanol, 98:2 (v/v)] and held for 6 min, then back again to 100% solvent A. The column was then equilibrated for 6 min at the initial conditions before the next injection. Prior to each sample injection, the autosampler was washed with 20 µl of solvent methanol/ammonium formate [5 mM, pH 7.5, 50:50 (v/v)] and then with 30 µl with ammonium formate (5 mM, pH 7.5). The total time of analysis per each sample was 17 min.

Detection was performed with a Quattro-LCZ triple quadrupole mass spectrometer equipped with an orthogonal electrospray source (Waters). dFdU was detected in the positive ion mode using single-ion recording mode of the [M + H]+ ion at m/z 265.0 and 228.0 for dFdU and the internal standard, respectively. The quantification process was performed over the 10 to 10,000 ng/ml concentration range using MassLynx software (Waters).

Pharmacokinetic analysis. Pharmacokinetic calculations were performed by noncompartmental analysis using WinNonlin, version 3.2 (Pharsight, Mountain View, CA). The area under the plasma concentration-time curve (AUC, hours-per-nanogram per milliliter) starting from the first to the last sampling time were calculated using the trapezoidal rule (linear up-log down) with extrapolation to infinity using the ratio Ca/Ke, where Cn was the last measurable concentration. The extrapolated AUC before the first time point and after the last time point did not exceed 15% of the total AUC. The elimination t1/2 was determined using the three last time points of the kinetics. In the SQdFdC nanoassembly-treated mice, total body clearance (C1) and volume of distribution (Vd) of SQdFdC were calculated by using a dose of 36.6 mg/kg (the molar equivalent of 15 mg/kg dFdC). The ratio (r) was defined as the ratio of plasmatic concentrations of dFdC to those of dFdC.

Organ Distribution Studies of 3H-SQdFdC Nanoassemblies Compared with 3H-dFdC. Tritiated (3H-) versions of dFdC or SQdFdC nanoassemblies were used to study the organ distribution after i.v. administration in healthy DBA/2 mice. The mice were divided into various groups of three in each and injected i.v. with 3H-dFdC and 3H-SQdFdC nanoassemblies at doses equivalent to 10 mg/kg. The radioactivity at this dose corresponded to 0.9 µCi/mouse for 3H-dFdC and 3H-SQdFdC nanoassemblies, and the final volume of injections remained 100 µl for both drugs. For single-dose biodistribution studies, at 5 min (0.083 h) and 30 min (0.5 h) and 1, 2, 6, and 24 h after i.v. administration, blood was collected from the retro-orbital plexus into heparinized tubes from the respective injected groups, and the mice were then sacrificed. The pharmacokinetic parameters were calculated by WinNonlin, version 3.2 (Pharsight, Mountain View, CA) as described before. For multiple-dose biodistribution studies after the two-dose schedule (injections on days 0 and 4) and the four-dose schedule (injections on days 0, 4, 8, and 13), blood was collected after 4 and 2 days of completion of the schedules, respectively. The blood was centrifuged at 3000 rpm for 5 min at 4°C, and the supernatant plasma was isolated. To 100 µl of plasma, 10 ml of Ultima Gold scintillant was added, and the mixture was vortexed vigorously for 1 min. The vials were kept aside for 2 h to allow the foam to dissipate and then counted in a beta counter. The organs, including the liver, lung, spleen, kidney, muscle, stomach, intestines, and brain, were dissected and blotted using tissue paper to remove adherent blood and any fatty matter. Fecal samples were processed in a similar manner. Approximately 50 to 100 mg of the tissues was weighed and placed in the scintillation counting vials. One milliliter of scintuene was added into the vials containing tissue and kept in an incubator overnight at 60°C to allow complete dissolution. Later, 10 ml of Hionic-Fluor was added to the vials, which were vortexed vigorously for 1 min. The vials were kept aside for 1 h to allow the foam to dissipate. Separately, the urine samples were mixed with 10 ml of Ultima Gold, vortexed for 1 min, and set aside for 1 h for the foam to dissipate. The radioactivity present in the samples and standards was
measured using a beta counter (Beckmann, LS 6000TA; Beckman Coulter). The results obtained for the biodistribution samples were converted and interpreted as percentage of injected dose per gram of tissue or milliliter of plasma or urine. The statistical comparisons were made using Student’s t test using GraphPad software (GraphPad Software Inc., San Diego, CA).

Results

Pharmacokinetic Study. SQdFdC, dFdC, and dFdC released from SQdFdC nanoassemblies in mice plasma. The pharmacokinetic analysis after i.v. administration of SQdFdC nanoassemblies was performed compared with free dFdC. Additionally, the plasmatic appearance of dFdC released from SQdFdC nanoassemblies, i.e., dFdC released from SQdFdC nanoassemblies (dFdCNA), in the blood stream was also quantified. The plasma elimination curves of dFdC and SQdFdC nanoassemblies are depicted in Fig. 2.

The plasma concentration-time curve of dFdC, in mice treated with 15 mg/kg free dFdC, was characterized by non-monoexponential decay, suggesting that dFdC pharmacokinetics followed at least a two-compartment model (Fig. 2, open triangles), as already described in mice and human plasma (Fogli et al., 2002; Immordino et al., 2004; Wang et al., 2005). dFdC rapidly disappeared from plasma with a short terminal t1/2 of 1.6 h and Cl of 2770 ml/h/kg (Table 1).

The plasma drug time course in the group of SQdFdC nanoassembly-treated mice seemed to follow a similar two-exponential decay (Fig. 2, diamonds) with a t1/2 of 8.6 h and Cl of 831 ml/h/kg. dFdCNA presented a higher terminal elimination t1/2 (6.3 h) as compared with that when administered as free drug (1.6 h), but a similar total plasma clearance (2481 ml/h/kg) (Table 1). dFdCNA total plasma clearance was calculated because a complete cleavage of the SQdFdC into dFdCNA was observed. Indeed, the AUC0–∞ of dFdC and dFdCNA were comparable. Additionally, the MRT of dFdCNA was 7.5-fold higher, and its Vss increased 6.8-fold (1074 and 7344 ml/kg for dFdC and dFdCNA, respectively).

dFdU in mice plasma. The plasmatic concentrations of dFdU were measured after i.v. administration of either free dFdC or SQdFdC nanoassemblies. Figure 3 illustrates the plasmatic concentration-time curves of dFdU in the dFdC-treated group (Fig. 3, open triangles) and in the SQdFdC nanoassembly-treated group (Fig. 3, diamonds).

In the dFdC-treated group, the highest plasmatic concentrations of dFdU (Cmax 10,375 ng/ml) was measured at 0.5 h postinjection, indicating the rapid deamination of dFdC (Table 1). On the other hand, in the SQdFdC nanoassembly-treated group, a comparable value of Cmax (10,756 ng/ml) was observed at 2 h postinjection. Herein, the tmax of dFdU resulting from the administration of SQdFdC nanoassemblies (at equivalent dose than dFdC) was markedly delayed (Table 1; Fig. 3). Other relevant pharmacokinetic parameters were listed in Table 1. Surprisingly, the AUC of dFdU in the SQdFdC-treated group was 2-fold higher (112,701 versus 54,243 ng/h/ml).

Organ Distribution. Tissue distribution of radioactivity was determined in mice after single or multiple (two doses on days 0 and 4 and four doses on days 0, 4, 8, and 13) i.v. dosing of either 3H-dFdC or 3H-SQdFdC nanoassemblies. Of all the organs evaluated up to 24 h after single-dose injection of 3H-dFdC, higher distribution occurred in spleen (Fig. 4). At 1 h postinjection, the splenic accumulation of 3H-dFdC radioactivity was the highest (37.4% injected dose/g), followed by a constant decrease with time. The overall distribution of 3H-dFdC radioactivity to intestine was relatively higher than the other organs evaluated except the spleen (Table 2; Fig. 4). The accumulation of 3H-dFdC radioactivity in the liver slightly increased at 30 min postinjection (9.3% injected dose/g) but later showed a constant decrease with time.

The distribution of the radioactivity resulting from 3H-SQdFdC nanoassemblies to organs such as heart, kidney, muscle, stomach, and intestine was lower compared with the radioactivity resulting from 3H-dFdC at all the time points studied (Table 2). The low concentrations in the kidneys suggest slower excretion of the 3H-SQdFdC nanoassemblies compared with the 3H-dFdC. Interestingly, the distribution and retention of 3H-SQdFdC nanoassembly radioactivity in the organs of the reticuloendothelial system were significantly higher than that of 3H-dFdC radioactivity at all the time points studied up to 6 h postinjection (Fig. 4). In liver, accumulation of the 3H-SQdFdC nanoassembly radioactivity increased up to 1 h postinjection, followed by a constant decrease in the later time points. In spleen, highest concentrations of the 3H-SQdFdC nanoassembly radioactivity were found up to 6 h postinjection before the decrease, and 7.35% injected dose/g still remaining at 24 h postinjection (p < 0.05). 3H-SQdFdC nanoassembly radioactivity exhibited significantly higher accumulation in lung as compared with the 3H-dFdC radioactivity, up to 6 h postinjection (p < 0.05) (Table 2). Additionally, calculation of pharmacokinetic parameters in organs revealed a higher Cmax and AUC0–∞ values in the liver, lung, and spleen for the radioactivity of 3H-SQdFdC.
The urinary excretion of $^3$H-dFdC radioactivity (in terms of $^3$H-dFdC or $^3$H-dFdU) was biphasic, with rapid initial phase followed by a slow excretion (Fig. 5). $^3$H-SQdFdC nanoassembly radioactivity (in terms of $^3$H-SQdFdC, $^3$H-dFdC, or $^3$H-dFdU) exhibited considerably lower excretion rate ($p < 0.05$) as compared with $^3$H-dFdC radioactivity. Excretion of the $^3$H-dFdC radioactivity was rapid, with maximum amounts excreted at 1 h postinjection (99,255 Bq/ml urine), reaching a minimum (5935 Bq/ml urine) at 24 h postinjection. The $^3$H-SQdFdC nanoassembly radioactivity showed a similar trend, with maximum excretion occurring at 1 h postinjection (39,717 Bq/ml urine). However, the excreted amounts were considerably lower ($p < 0.05$ up to 6 h postinjection) than those observed with $^3$H-dFdC.

$^3$H-dFdC radioactivity underwent a rapid distribution to the brain, initially with peak concentration appearing at 1 h postinjection (3.27% injected dose/g), followed by a decrease later (0.61% injected dose/g at 24 h postinjection) (Table 2). The overall brain distribution of $^3$H-SQdFdC nanoassemblies was lower than that of $^3$H-dFdC as observed from the AUC values (3H-dFdC, 47.86% radioactivity/h/ml; 3H-SQdFdC nanoassemblies, 31.57% radioactivity/h/ml). Other pharmacokinetic parameters are presented in Table 3.

The organ distribution studies were then performed using similar i.v. administration schedules as described by Reddy et al. (2007) on leukemia-bearing mice (i.e., two doses on days 0 and 4, followed by tissue sampling on day 8, or four doses on days 0, 4, 8, and 13, followed by tissue sampling on day 15). In the two-dose injection schedule, the distribution of $^3$H-dFdC radioactivity and $^3$H-SQdFdC nanoassembly radioactivity into organs such as heart, lung, kidney,
with the 3H-dFdC (Fig. 6A). After the four-dose injection schedule, bly radioactivity was again significantly higher (values are the mean ± S.D. of three mice).

However, liver and spleen accumulation of 3H-SQdFdC nanoassemblies in muscle, stomach, intestine, and brain was not significantly different. The values are the mean ± S.D. of three mice.

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The organ distribution of radioactivity following single-dose i.v. administration (10 mg/kg) in mice of either 3H-dFdC or 3H-SQdFdC nanoassemblies is shown in Table 2. The urinary excretion profiles of radioactivity after single-dose i.v. administration of 10 mg/kg of either 3H-dFdC or 3H-SQdFdC nanoassemblies is shown in Fig. 5. The organ distribution of radioactivity after the completion of two i.v. doses (10 mg/kg on days 0 and 4) of either 3H-dFdC or 3H-SQdFdC nanoassemblies is shown in Fig. 6A. The accumulation of 3H-SQdFdC nanoassembly radioactivity in spleen (12.7% injected dose/g) was dramatically higher than that of 3H-dFdC radioactivity (2% injected dose/g) (p < 0.05), whereas the distribution in the other organs was similar (Fig. 6B).

**Discussion**

After i.v. administration, dFdC rapidly disappeared from plasma, with a terminal \( t_{1/2} \) of 1.6 h, and was rapidly eliminated with short

**Table 2**

Organ distribution of radioactivity following single-dose i.v. administration (10 mg/kg) in mice of either 3H-dFdC or 3H-SQdFdC nanoassemblies

<table>
<thead>
<tr>
<th>Organ</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>6</th>
<th>24</th>
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<tbody>
<tr>
<td>Heart</td>
<td>7.67 ± 0.79</td>
<td>8.73 ± 5.73</td>
<td>5.32 ± 1.50</td>
<td>6.42 ± 5.54</td>
<td>6.20 ± 2.43</td>
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<tr>
<td>Lung</td>
<td>8.33 ± 0.45</td>
<td>34.59 ± 6.20</td>
<td>7.97 ± 0.41</td>
<td>29.09 ± 6.07</td>
<td>5.60 ± 0.67</td>
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<tr>
<td>Kidney</td>
<td>8.63 ± 0.68</td>
<td>3.56 ± 1.05</td>
<td>6.01 ± 0.76</td>
<td>5.46 ± 1.54</td>
<td>8.05 ± 1.14</td>
</tr>
<tr>
<td>Brain</td>
<td>1.38 ± 0.42</td>
<td>0.41 ± 0.05</td>
<td>3.24 ± 0.75</td>
<td>0.82 ± 0.07</td>
<td>3.27 ± 0.24</td>
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<tr>
<td>Muscle</td>
<td>8.30 ± 0.20</td>
<td>1.66 ± 0.25</td>
<td>5.33 ± 0.30</td>
<td>2.80 ± 0.22</td>
<td>6.20 ± 0.34</td>
</tr>
<tr>
<td>Stomach</td>
<td>5.54 ± 0.69</td>
<td>2.33 ± 0.58</td>
<td>5.57 ± 0.99</td>
<td>4.64 ± 0.21</td>
<td>7.30 ± 0.78</td>
</tr>
<tr>
<td>Intestine</td>
<td>8.45 ± 2.30</td>
<td>2.00 ± 0.25</td>
<td>10.40 ± 1.10</td>
<td>5.88 ± 3.59</td>
<td>11.77 ± 0.13</td>
</tr>
</tbody>
</table>

**Table 3**

Organ pharmacokinetic parameters in mice following single-dose i.v. administration (10 mg/kg) of either 3H-dFdC or 3H-SQdFdC nanoassemblies

<table>
<thead>
<tr>
<th>Organ</th>
<th>Compound</th>
<th>( t_{1/2} )</th>
<th>( T_{max} )</th>
<th>( C_{max} )</th>
<th>AUC ( 0-\infty )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>dFdC</td>
<td>9.62</td>
<td>1</td>
<td>3.27</td>
<td>47.86</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>8.36</td>
<td>6</td>
<td>1.83</td>
<td>31.57</td>
</tr>
<tr>
<td>Heart</td>
<td>dFdC</td>
<td>8.53</td>
<td>0</td>
<td>3</td>
<td>72.56</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>8.40</td>
<td>0</td>
<td>8.73</td>
<td>62.53</td>
</tr>
<tr>
<td>Intestine</td>
<td>dFdC</td>
<td>7.48</td>
<td>1</td>
<td>11.77</td>
<td>108.39</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>7.28</td>
<td>2</td>
<td>7.15</td>
<td>74.44</td>
</tr>
<tr>
<td>Kidney</td>
<td>dFdC</td>
<td>6.43</td>
<td>0</td>
<td>8.63</td>
<td>90.93</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>9.44</td>
<td>1</td>
<td>5.47</td>
<td>69.63</td>
</tr>
<tr>
<td>Liver</td>
<td>dFdC</td>
<td>11.63</td>
<td>1</td>
<td>9.36</td>
<td>67.57</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>10.63</td>
<td>1</td>
<td>29.67</td>
<td>138.88</td>
</tr>
<tr>
<td>Lung</td>
<td>dFdC</td>
<td>10.28</td>
<td>0</td>
<td>8.33</td>
<td>83.87</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>5.17</td>
<td>0</td>
<td>34.59</td>
<td>234.82</td>
</tr>
<tr>
<td>Muscle</td>
<td>dFdC</td>
<td>9.67</td>
<td>0</td>
<td>8.31</td>
<td>77.19</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>7.73</td>
<td>1</td>
<td>3.51</td>
<td>52.16</td>
</tr>
<tr>
<td>Spleen</td>
<td>dFdC</td>
<td>7.21</td>
<td>0</td>
<td>37.45</td>
<td>458.65</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>5.82</td>
<td>6</td>
<td>62.64</td>
<td>845.91</td>
</tr>
<tr>
<td>Stomach</td>
<td>dFdC</td>
<td>12.01</td>
<td>1</td>
<td>7.30</td>
<td>75.02</td>
</tr>
<tr>
<td></td>
<td>SQdFdC</td>
<td>9.65</td>
<td>1</td>
<td>4.65</td>
<td>52.51</td>
</tr>
</tbody>
</table>

\( *p < 0.05\) compared with that of 3H-dFdC.
The possibility of a third \( t_{1/2} \) and, thus, a longer terminal \( t_{1/2} \), cannot be excluded. However, this latter phase was not observed under our experimental conditions because, at 24 h postinjection, the dFdC plasmatic concentrations were exceedingly below the detection limit of our analytical method. Interestingly, the dFdC\(_{\text{NA}}\) released from SQdFdC nanoassemblies displayed a ~3.9-fold higher \( t_{1/2} \) and ~7.5-fold higher MRT, showing that SQdFdC nanoassemblies considerably modified the pharmacokinetics of dFdC with prolonged blood retention and an increased volume of distribution. According to the pharmacokinetic results, the cleavage of SQdFdC into the pharmacologically active dFdC\(_{\text{NA}}\) seems to occur rapidly, which is in accordance with the previous in vitro experiments performed using cathepsins B and D enzymes (Couvreur et al., 2006). Interestingly, because cathepsin B is overexpressed in cancer cells (Kos et al., 2005), part of the SQdFdC prodrg may be specifically activated in those target cells. However, in the blood circulation, other amidases are likely implicated in the cleavage of SQdFdC into dFdC\(_{\text{NA}}\). On the other hand, the deamination by deoxycytidine deaminase was significantly delayed. This may be explained by the association of SQdFdC with plasma lipoproteins, mainly very low-density lipoprotein, as described previously with squalene (Tilvis et al., 1982; Strandberg et al., 1990), leading to a delayed accessibility of the released dFdC to deamination. This is further supported by calculation of the ratio (\( r \)) of the plasma concentrations of dFdU to those of dFdC in dFdC- and SQdFdC-treated groups (Fig. 7). This ratio (\( r \)) is constantly lower in the SQdFdC nanoassembly-treated group than in the dFdC-treated one, at least up to 8 h postinjection (Fig. 7). The increased \( t_{1/2} \), \( t_{\text{max}} \), AUC, and the altered ratio of dFdU to dFdC (\( r \)) are a consequence of the increase in the effective volume of distribution after SQdFdC administration.

In clinical practice, dFdC is administered at doses as high as 1250 mg/m\(^2\) per day, contributing to the manifestation of adverse effects. The data we report here suggest that squalenoylation of dFdC offers various advantages over free dFdC, such as prolonged \( t_{1/2} \), increased MRT, and volume of distribution. Although an increased AUC of dFdU was calculated, a comparable plasma clearance was observed.

The organ distribution patterns of the radioactivity after single-dose i.v. administration of either \(^{3}\text{H}\)-dFdC or \(^{3}\text{H}\)-SQdFdC nanoassemblies were distinct. Primarily, the lower radioactivity concentrations of \(^{3}\text{H}\)-SQdFdC nanoassemblies in kidney and urine suggest a slow elimination and, hence, longer body retention compared with the \(^{3}\text{H}\)-dFdC radioactivity, which is also supported by the plasma pharmacokinetic data. The significantly higher accumulation of \(^{3}\text{H}\)-SQd-

MRT as described previously (Immordino et al., 2004). Nevertheless, the possibility of a third \( t_{1/2} \) and, thus, a longer terminal \( t_{1/2} \), cannot be excluded. However, this latter phase was not observed under our experimental conditions because, at 24 h postinjection, the dFdC plasmatic concentrations were exceedingly below the detection limit of our analytical method. Interestingly, the dFdC\(_{\text{NA}}\) released from SQdFdC nanoassemblies displayed a ~3.9-fold higher \( t_{1/2} \) and ~7.5-fold higher MRT, showing that SQdFdC nanoassemblies considerably modified the pharmacokinetics of dFdC with prolonged blood retention and an increased volume of distribution. According to the pharmacokinetic results, the cleavage of SQdFdC into the pharmacologically active dFdC\(_{\text{NA}}\) seems to occur rapidly, which is in accordance with the previous in vitro experiments performed using cathepsins B and D enzymes (Couvreur et al., 2006). Interestingly, because cathepsin B is overexpressed in cancer cells (Kos et al., 2005), part of the SQdFdC prodrg may be specifically activated in those target cells. However, in the blood circulation, other amidases are likely implicated in the cleavage of SQdFdC into dFdC\(_{\text{NA}}\). On the other hand, the deamination by deoxycytidine deaminase was significantly delayed. This may be explained by the association of SQdFdC with plasma lipoproteins, mainly very low-density lipoprotein, as described previously with squalene (Tilvis et al., 1982; Strandberg et al., 1990), leading to a delayed accessibility of the released dFdC to deamination. This is further supported by calculation of the ratio (\( r \)) of the plasma concentrations of dFdU to those of dFdC in dFdC- and SQdFdC-treated groups (Fig. 7). This ratio (\( r \)) is constantly lower in the SQdFdC nanoassembly-treated group than in the dFdC-treated one, at least up to 8 h postinjection (Fig. 7). The increased \( t_{1/2} \), \( t_{\text{max}} \), AUC, and the altered ratio of dFdU to dFdC (\( r \)) are a consequence of the increase in the effective volume of distribution after SQdFdC administration.

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1576 REDDY ET AL.

The organ distribution evaluation performed after the two-dose schedule (days 0 and 4, Fig. 5) and four-dose schedule (days 0, 4, 8, and 13, Fig. 6) revealed again significantly higher accumulation of \(^{3}\text{H}\)-SQdFdC nanoassembly radioactivity in the liver and spleen (\( p < 0.05 \)), compared with \(^{3}\text{H}\)-dFdC radioactivity. As already discussed before, this distribution pattern may give increased activity against cancer metastasis because the lymphoid organs, such as the spleen and liver, are the major organs of metastasis in the case of cancers such as leukemia.

On the other hand, such distribution should have implications on the toxicity of this nanomedicine. However, our recent report (Reddy et al., 2008) revealed no hematologic or hepatic toxicity in mice with either dFdC or SQdFdC nanoassembly treatment, even at maximal tolerated doses as assessed from the blood and biochemical parameter analysis, indicating the safety of these doses. On the contrary, at lethal doses, SQdFdC nanoassembly treatment led before death to lymphopenia and severe thrombocytopenia, whereas dFdC treatment led to lymphopenia and granulocytopenia. Additionally, both of these drugs showed a decrease in spleen weights, whereas the decrease was higher in the SQdFdC nanoassembly-treated group. Interestingly, even at the lethal dose and although the observed liver concentration of SQdFdC, no hepatotoxicity was detected as assessed from the liver enzyme levels (aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase) (Reddy et al., 2008). Regarding the activity, the enhanced lung accumulation of SQdFdC as observed in this study could be of interest for the treatment of lung-associated cancers. The lower tissue distribution to non-reticuloendothelial organs observed here would allow the anticipation that the SQdFdC nanoassemblies would display a lower activity in an s.c. grafted tumor. In fact, on the contrary, SQdFdC nanoassemblies led to a greater tumor control and survival of the mice compared with dFdC in an s.c. grafted P388 leukemia. In summary, the greater efficacy of SQdFdC nanoassemblies observed both in an i.v. leukemia metastatic model and an s.c. solid tumor model coupled to the pharmacokinetic and organ distribution may be explained by: i) a greater distribution to reticuloendothelial organs, which favored improved treatment of metastatic cancer; and ii) triggered controlled and prolonged release of dFdC by SQdFdC nanoassemblies in the vascular compartment, leading to an increased blood \( t_{1/2} \), which, in turn, improve exposure of the tumor site to the drug.

This study has demonstrated that squalenoylation of the anticancer nucleoside analog, gemcitabine, dramatically modified the pharmacokinetics, metabolism, and biodistribution of this compound. Our findings indicate that the squalenoyl gemcitabine nanomedicine as a reservoir of gemcitabine due to its modified distribution compared with free dFdC, then releasing the active molecule, thereby minimizing the rapid exposure of the drug to deamination. Apart from the favorable pharmacokinetics, selective accumulation of the squalenoyl gemcitabine nanoassemblies into the organs of reticuloendothelial system provides the basis to explain the previously observed drug distribution in the reticuloendothelial organs such as the spleen, liver, and lung may possibly be due to the uptake of the gemcitabine nanomedicine by macrophages, probably due to the hydrophobic surface and colloidal nature of this formulation. This biodistribution pattern suggests that a significant amount of SQdFdC was directly captured by these tissues as nanoassemblies, before cleavage. Otherwise, a quite similar organ/tissue distribution as for \(^{3}\text{H}\)-dFdC should have been observed. Although SQdFdC appears to be rapidly cleaved into dFdC\(_{\text{NA}}\) as stated by the pharmacokinetic parameters, the squalenoylation seems to modify the behavior of dFdC through an altered biodistribution pattern of SQdFdC, explaining also the delayed dFdU formation.
superior anticancer activity of gemcitabine nanomedicine in comparison with gemcitabine free. Thus, squalenoylation of gemcitabine offers several pharmacological benefits and, hence, is expected to provide a positive therapeutic advantage in cancer therapy.

References


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