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ORIGINAL ARTICLE

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Change in pharmacokinetic and pharmacodynamic behavior of gemcitabine in human tumor xenografts upon entrapment in vesicular phospholipid gels

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Abstract *Purpose:* The in vivo pharmacokinetics (PK), biodistribution and antitumor activity of a new liposomal formulation of gemcitabine (GemLip) were compared to the conventional (clinical) formulation of gemcitabine (GemConv). *Methods:* Gemcitabine was entrapped in a vesicular phospholipid gel (VPG) consisting of densely packed liposomes. Redispersed VPG containing GemLip consisted of 33% liposomally entrapped and 67% free gemcitabine. The in vivo efficacies of GemLip and GemConv were compared using the subcutaneously growing human soft tissue sarcoma SXF 1301 and the orthotopically growing human bladder cancer BXF 1299T. PK and biodistribution were evaluated using radiolabeled drug and lipid in SXF 1301 tumor-bearing nude mice. *Results:* GemLip was highly active in SXF 1301 at a gemcitabine dose of 6–9 mg/kg (days 1, 8 and 15; dose near the MTD). In the 6-mg/kg groups, complete tumor remissions were observed in seven of eight mice. Equimolar doses of GemConv resulted in only moderate tumor growth inhibition. Even at equitoxic doses (360 mg/kg given on days 1, 8 and 15, or 120 mg/kg on days 1, 5 and 8) GemConv was less active than GemLip. Furthermore, GemLip was active in the orthotopically growing BXF 1299T bladder cancer model at 6 mg/kg and prevented distant organ metastasis. In the PK study, GemLip achieved a 35-fold higher plasma AUC (1680 mg·h/ml) than GemConv (47.6 mg·h/ml). The serum half-lives were 0.15 h for free gemcitabine and 13.3 h for liposomal gemcitabine (6 mg/kg each i.v.). Moreover, gemcitabine levels in tumors were fourfold higher following injection of GemLip than following injection of GemConv. *Conclusions:* GemLip is a highly effective gemcitabine delivery system which results in superior gemcitabine pharmacodynamics and PK than GemConv. The enhanced in vivo efficacy might be explained by sustained release and passive tumor targeting.

Keywords Gemcitabine · Liposome · Human tumor xenograft · Pharmacokinetics · Metastases · Vesicular phospholipid gel · VPG

Abbreviations *dFdC:* gemcitabine hydrochloride (2′,2′-difluoro-2′-deoxycytidine hydrochloride) · *dFdCDP:* difluorodeoxycytidine diphosphate · *dFdCTP:* difluorodeoxycytidine triphosphate · *dFdU:* difluorodeoxyuridine · *EPC-3:* hydrogenated egg PC · *EPR:* enhanced permeability and retention · *GemConv:* conventional (clinical) gemcitabine solution · *GemLip:* redispersed dFdC containing VPG · *MTD:* maximal tolerable dose · *PC:* phosphatidylcholine · *PD:* pharmacodynamics · *PK:* pharmacokinetics · *RES:* reticuloendothelial system · *T/C:* test/control · *THU:* tetrahydrouridine · *VPG:* vesicular phospholipid gel

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Introduction

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC) is a deoxycytidine antimetabolite showing activity against a wide range of solid tumors, e.g. pancreas, non-small cell lung, breast and bladder cancers [1, 11, 13, 23, 26, 32]. In order to exert cytotoxicity, dFdC has to be phosphorylated to dFdCDP and dFdCTP via dFdCMP by deoxycytidine kinase. dFdCTP is then incorporated into DNA, leading to inhibition of chain elongation and

DNA strand breaks. In contrast, dFdCDP inhibits ribonucleotide reductase and consequently de novo synthesis of deoxynucleotides.

The dynamics of dFdCTP and dFdCDP formation in vivo are complex and only a proportion of dFdC is converted into the active di- or triphosphate forms. The majority of dFdC is rapidly metabolized to inactive dFdU by deoxycytidine deaminase in the blood, liver and kidneys. In addition, the formation of dFdCDP and dFdCTP from dFdCMP is reduced through deamination of dFdCMP by a dCMP deaminase to a considerable extent. This process, however, can be downregulated by the active metabolite dFdCTP after dFdCTP has been formed. Thus, dFdC has the very short plasma half-life of 8–17 min. In order to reach therapeutic drug levels in patients, dFdC is currently administered at a dose of 1000 mg/m^2 as 30-min intravenous (i.v.) infusion. Due to its complex pharmacodynamics (PD), in mice and humans the antitumor effects of dFdC are highly dependent on schedule rather than dose [3, 5, 28] and for some tumors higher response rates are obtained by daily administration or prolonged infusion. Moreover, in vitro studies have shown that the duration of drug exposure is relevant for its antiproliferative activity [29].

It has been shown that liposomes provide protection against rapid metabolic inactivation of drugs [34]. Furthermore, liposomes can carry large amounts of drug and are generally very well tolerated [16, 18]. However, it is impossible using currently available technology to stably entrap dFdC into conventional liposomes. dFdC is an uncharged (at physiologic pH), low molecular weight molecule which diffuses rapidly through liposome bilayers. Thus, shortly after liposome preparation and separation of non-entrapped dFdC, rapid diffusion of dFdC out of the liposomes occurs. This limits the shelf-life and therefore clinical use of conventional dFdC liposomes as well as of liposomes loaded with other low molecular weight molecules (e.g. nucleoside analogues such as ara-C and floxuridine) [19, 31].

A new strategy to increase the shelf-life of these formulations is the encapsulation of small, hydrophilic molecules into vesicular phospholipid gels (VPG) [8, 9, 10]. VPGs are composed of very densely packed liposomes, mainly small unilamellar vesicles, and can be prepared by high-pressure homogenization [6, 7]. The volumes of the aqueous phases inside and outside the vesicles have the same magnitude [9]. Due to their high lipid concentrations, these formulations have a semisolid or gel-like consistency.

In this study, we entrapped dFdC into a VPG consisting of hydrated egg PC (EPC-3) and cholesterol (molar ratio 1:1, total lipid concentration 660 m*M*). As reported previously, the diameters of the densely packed liposomes were 60–80 nm [24]. In contrast to conventional liposomal formulations in which the drugs are predominantly entrapped in the aqueous core of the liposomes, in the new dFdC-VPG formulation dFdC is also entrapped in the aqueous phase between the liposomes. The same concentration of dFdC inside and outside the liposomes results in a superior shelf-life of dFdC-VPG. Although dFdC still diffuses through the liposomal bilayers, the dFdC concentration is always in equilibrium between the inner and outer aqueous phases of the liposomes. Thus, the ratio of dFdC between the vesicle core and the aqueous space remains constant during storage. Furthermore, due to the similar volumes of the aqueous phases inside and outside the vesicles in dFdC-VPG, the proportion of dFdC entrapped in the aqueous core of the liposomes is very high (high entrapping efficiency). This is important because, as a consequence of the gel-like consistency of dFdC-VPG, it is not possible to separate non-entrapped dFdC from the dFdC-containing liposomes.

In the study reported here, we aimed to elucidate the antitumor efficacy of dFdC-VPG compared to that of free dFdC (GemConv) and to monitor changes in PD and PK. Gel-like formulations are very viscous. The semisolid dFdC-VPG therefore needs to be diluted prior to i.v. injection and is then designated GemLip. GemLip is a mixture of dFdC-containing liposomes and free dFdC in a defined ratio (dual drug formulation). It contains 33% liposomally entrapped dFdC and 67% in free form. The free dFdC can be considered as a bolus injection.

The non-diluted dFdC-VPG formulation used in this study was stable for more than 8 months at 4°C in terms of PC content, dFdC content and particle size [24]. After dilution, the dFdC concentration outside the liposomes is greatly reduced, and the shelf-life and entrapping efficiency of the formulation become unstable, and therefore the formulation has to be administered immediately.

Two human tumor xenograft models, the soft tissue sarcoma SXF 1301 (growing subcutaneously) and the human bladder tumor xenograft BXF 1299T (growing orthotopically), were used to investigate the tolerability, antitumor activity, and PD and PK behavior of GemLip.

Materials and methods

Materials and drugs

EPC-3 was a kind gift from Lipoid (Ludwigshafen, Germany). [1α,2α(n)-^{3}H]Cholesteryloleylether (^{3}H-Chol) was purchased from Amersham Pharmacia Biotech Europe (Freiburg, Germany). dFdC, ^{14}C-labeled dFdC, 2′,2′-difluoro-2′-deoxyuridine (dFdU) and vindesine (Eldesine) were kindly provided by Eli Lilly (Indianapolis, Ind.). Taxotere was purchased from Rhône-Poulenc (France). 2′-Deoxycytidine, H_2O_2 (30%) were from Sigma (Steinheim, Germany) and tetrahydrouridine (THU) was from Calbiochem-Novabiochem (La Jolla, Calif.). Cholesterol and HPLC solvents were obtained from Merck (Darmstadt, Germany). Minisart filters SRP 4 were obtained from Sartorius (Göttingen, Germany) and scintillation cocktail Hionic-Fluor and tissue solubilizer Soluene-350 from Canberra-Packard (Dreieich, Germany). Thymus aplastic nude mice (nu/nu) of Balb-C genetic background (NCR-nu) were supplied by Charles River (Frederick, Md.).

Preparation of dFdC-VPG

"*Empty*" *VPG*

VPG consisted of EPC-3 and cholesterol (1:1 molar ratio) in a total lipid concentration of 40% (wt/wt) (660 m*M* lipid) and were prepared as described previously [10]. In brief, EPC-3 and cholesterol were dissolved in chloroform/methanol (2:1 v/v). Solvents were removed under reduced pressure at 40°C in a water bath using a rotatory evaporator resulting in a thin lipid film. Solvent traces were removed under vacuum for 24 h. The dry lipid film was hydrated using an appropriate volume of phosphate buffer (150 m*M*, pH 7.3) and the resulting semisolid lipid dispersion was treated in a high-pressure homogenizer (Micron Lab 40, 70 MPa, 10 cycles; APV Gaulin, Lübeck, Germany). The resulting "empty VPG" was autoclaved and stored at 4–8°C [33].

dFdC-VPG

For the entrapment of dFdC into the empty VPG, a newly developed passive loading technique was employed [20]. Empty VPG (350 mg) was transferred into sterile 2-ml Eppendorf tubes under sterile conditions. Appropriate volumes of dFdC stock solution (15 mg/ml in 150 m*M* phosphate buffer, pH 7.3) were added and the components were thoroughly mixed using sterile plastic spatulas. To facilitate the diffusion of dFdC into the liposomes, the mixtures were incubated at 60°C for 4 h.

Dilution of dFdC-VPG (preparation of GemLip)

dFdC-VPG was diluted as described previously [10]. Six sterile glass beads (diameter 1 mm) were added to each dFdC-VPG tube to facilitate the subsequent dilution process by functioning as a ball mill. Phosphate buffer was added in two portions to a final total lipid concentration of 217 m*M*. After each addition of buffer, the mixture was shaken for 45 s at maximum speed, the beads acting as a ball mill (Retsch MM2, Haan, Germany). The resulting GemLip was immediately separated from the glass beads by drawing the dispersion into a syringe (Omnifix-F, 1 ml, 0.45×12 mm 26 G needle) and injected into the animals.

Lipid and dFdC stability and liposomal dFdC content were determined using HPTLC (lipid analysis) [25] and HPLC (dFdC analysis). The HPLC method for dFdC determination in plasma was used as described below. In contrast to the plasma measurements, calibration standards were prepared from phosphate buffer. For determination of liposomal entrapped dFdC, non-entrapped dFdC was removed from GemLip by filtration over cationic exchange resin AG 50 W X-8 (Bio-Rad, Munich, Germany) activated with concentrated NaCl solution. Maximum exchange capacity was 1.2 mg dFdC/g resin.

Radiolabeled dFdC-VPG

Double-labeled dFdC-VPG was prepared as described above. The lipid composition contained in addition [1α,2α(n)-^{3}H]cholesteryl-oleylether (11.2 kBq/mmol lipid). The stock solution of dFdC contained in addition ^{14}C-dFdC (37.8 MBq/mmol dFdC).

Animal experiments

All animal experiments were performed in accordance with German Animal License Regulations (Tierschutzgesetz) identical to UKCCCR Guidelines for the welfare of animals in experimental neoplasia [38]. Female thymus aplastic nude mice at 8–10 weeks of age with a body weight of 21–23 g were used for the experiments. The animals were kept under a natural day/night cycle and received water and rodent diet (Lage, Germany).

MTD

The MTDs of GemLip and GemConv were determined in non-tumor-bearing animals. Both types of preparations with increasing dFdC concentrations were administered into the tail vein on days 1, 8 and 15. The total lipid dose of GemLip was always 2.17 mmol/kg. Drug-related toxic effects in terms of body weight changes and abnormalities in behavior were monitored over 21 days. Deaths were considered as drug-related if they occurred within 7 days of the last injection.

Assessment of anticancer efficacy

Fragments (3–5 mm) of soft tissue sarcoma SXF 1301 were transplanted subcutaneously between the hind- and foreflanks of the nude mice. Fragments (2 mm) of the human bladder cancer BXF 1299T were engrafted orthotopically by sewing them onto the outer bladder wall of the apex vesicae.

Subcutaneously growing SXF 1301

Treatment was initiated when mean tumor volumes reached 150–400 mm^3. GemLip was given at doses of 6 and 9 mg dFdC/kg and a total lipid dose of 2.17 mmol/kg and GemConv was given at doses of 6 and 360 mg dFdC/kg. The preparations were administered i.v. on days 1, 8 and 15. Control groups received drug-free redispersed VPG (2.17 mmol/kg). The anticancer efficacy of GemLip administered once-weekly at 9 mg/kg was also evaluated in comparison with that of GemConv administered daily at equitoxic doses of 3 mg/kg given for five consecutive days. The anticancer efficacy of GemLip at 6 mg dFdC/kg was further compared with that GemConv at a dose of 120 mg dFdC/kg given on days 1, 5 and 9. Tumor growth was followed by serial caliper measurements (tumor volume = (length×width^2)/2 [15].

Orthotopically growing BXF 1299T

In the orthotopically growing human bladder tumor xenograft BXF 1299T treatment was initiated after 12 days when the tumors were palpable and of a size suitable for caliper measurement in situ. Tumor growth was followed by serial caliper measurements (tumor volume = (length×width^2/2 [15]. The median starting tumor volume was 43–45 mm^3. GemLip (6 mg dFdC/kg) was administered on days 1, 8 and 15. The effects of GemLip on primary tumor growth and development of peritoneal metastases were evaluated in comparison with the effects of Vindesine (1.5 mg/kg on days 1, 8 and 15) and Taxotere (20 mg/kg on days 1, 8 and 15), at their MTDs. Metastases were examined after 35 days macroscopically and microscopically. Micrometastases were determined by immunohistochemistry (human pancytokeratin and CEA 18) [30].

PK studies

^{3}H/^{14}C-labeled GemLip or ^{14}C-labeled GemConv were injected i.v. at equimolar doses of 6 mg dFdC/kg. After 5, 15 and 30 min, and 1, 2, 4, 8, 12, 24 and 72 h after injection three mice of the GemLip group were killed. The time-points for the GemConv group were 5, 15 and 30 min, and 1 and 4 h. Blood was collected into vials containing 20 µl 1% THU solution and centrifuged, and the resulting plasma was stored at –80°C. Tumors, livers, spleens, hearts, lungs and kidneys were rapidly excised, rinsed in saline, weighed and stored at –80°C. Elimination of dFdC and dFdU from plasma was quantified by HPLC as previously described [12] using a Waters HPLC system (Eschborn, Germany) equipped with analytical and guard columns Adsorbosphere NH_2 (5 µm particle size, 250×4.6 mm) from Alltech Associates (Deerfield, Ill.). The column temperature was 35°C. The mobile phase consisted of cyclohexane, 1,2-dichloroethane, methanol, purified water, glacial acetic acid

and triethylamine (630/150/220/1/0.5/1 by volume) and the flow rate was 1.5 ml/min. dFdC and dFdU were detected at 278 nm. Plasma calibration standards were prepared from THU-treated plasma containing dFdC and dFdU ranging from 50 to 2400 ng/ml. 2′-Deoxycytidine was used as internal standard. For the determination of ^{14}C and ^{3}H in tissues, the livers were homogenized in 1 ml PBS using an Ultra-Turrax T25 homogenizer (Janke & Kunkel, Staufen, Germany). Liver homogenate (200 µl), and whole tumors, lungs, kidneys, hearts and spleens were incubated in 1 ml Soluene-350 at 55°C for 2 h. The samples were bleached twice with 20 µl 30% H_2O_2 for 20 min. ^{14}C and ^{3}H activities were measured using a Tricarb 1900 CA analyzer and Hionic Fluor as scintillation cocktail (both Canberra-Packard, Dreieich, Germany).

Results

Preparation of GemLip (diluted dFdC-containing VPG)

VPGs without drug were prepared by high-pressure homogenization (660 m*M* lipid) and subsequently autoclaved to ensure sterility. Then various amounts of dFdC were entrapped in the VPGs using a newly developed passive loading technique during which VPG and dFdC were blended and effective diffusion of dFdC into the liposomes was achieved at a slightly increased temperature (60°C). No degradation of dFdC was observed during this process. The proportion of dFdC entrapped in the liposomal vesicles of the VPG (entrapping efficiency) was predetermined by the core to external aqueous phase ratio of the VPG, and therefore remained constant. In these experiments it was $33.2 \pm 4.2\%$.

Immediately prior to i.v. administration, dFdC-VPGs were redispersed (diluted) with buffer by vigorous shaking to obtain a fluid liposomal dispersion (GemLip; final lipid concentration 217 m*M*). GemLip is a dual drug formulation consisting of free and liposomally entrapped dFdC. The redispersion process using a ball mill results in a dispersion of small liposomes. Their mean diameters as determined by quasielastic light scattering ranged from 60 to 80 nm as previously described, and no particles larger than 1 µm could be detected [24]. A typical liposome size distribution of redispersed dFdC-VPG (GemLip) is shown in Fig. 1. Non-dispersed dFdC-VPGs can be stored at 4°C for at least 8 months without change in liposome size or dFdC entrapping efficiency.

In the case of GemLip, one-third of the total administered dose of dFdC was injected liposomally entrapped and two-thirds as free drug. The total injected dose of liposomes was identical throughout all experiments and treatment groups corresponding to a lipid concentration of 2.17 mmol/kg independent of dFdC dose.

Anticancer efficacy of GemLip versus GemConv

Determination of MTD

Dose-finding studies with GemLip were performed in non-tumor-bearing nude mice and a dose between 6 and 9 mg dFdC/kg administered once weekly i.v. was identified as the MTD, which resulted in a body weight loss of up to 15% and fewer than 20% drug-related deaths (data not shown). The MTD of GemConv with the weekly schedule was found to be between 360 and 480 mg dFdC/kg, confirming the literature values [3].

Activity in subcutaneous growing SXF 1301

In a first set of experiments, the in vivo activities of GemConv and GemLip were compared using equimolar doses of 6 mg dFdC/kg (Table 1, experiments 1 and 6). Complete tumor remissions were observed in four of five mice 4 weeks after initiation of treatment with GemLip. In marked contrast to liposomal dFdC therapy (T/C 0%), treatment of SXF 1301 with GemConv resulted in only a moderate tumor growth inhibition with an optimum T/C of 39%. The difference in median tumor volume between GemLip-treated and control animals was statistically significant ($P < 0.025$), but the difference

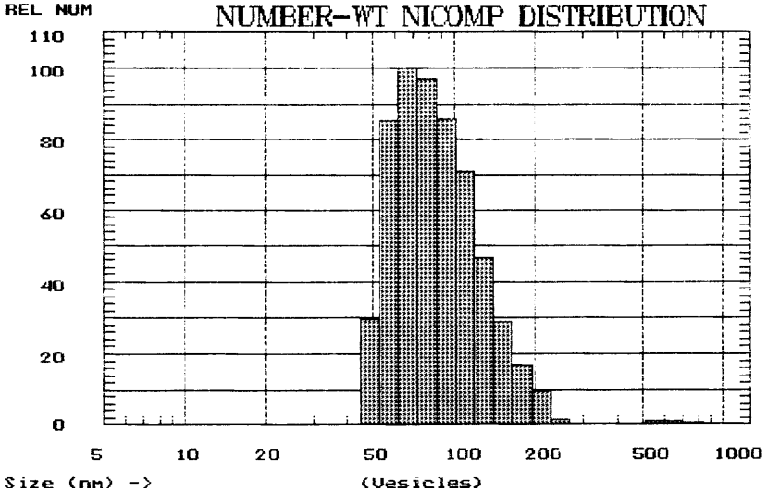

Fig. 1. Typical liposome size distribution of redispersed dFdC-VPG (GemLip) as measured by quasielastic light scattering. The mean diameter of the liposomes was 72.1 nm (98.8%). A minor peak can be observed at 627.1 nm (1.2%); the fit error was 1.92

Table 1. In vivo activity of GemLip and GemConv in the subcutaneously growing soft tissue sarcoma xenograft SXF 1301 (T/C test/control, *n.s.* not significant)

Experiment no.	Treatment	dFdC dose (mg/kg)	Schedule (days)	Optimal T/C (%)	Growth delay (days)	Doubling time (days)[a]	Activity[c]	P value[b]	Toxic deaths	Body weight loss (%)[d]
1	GemLip	6	1, 8, 15	0	–[e]	–[e]	+ + + +	< 0.025	1/5	13.5
2	GemLip	6	1, 8, 15	0	–[f]	–[f]	+ + + +	< 0.001	0/3	–
3	GemLip	9	1, 8, 15	4.5	22	26	+ + +	< 0.001	1/6	–
4	GemConv	360	1, 8, 15	12.6	11	15	+	< 0.001	0/6	–
5	GemConv	120	1, 5, 9	3.0	34	36	+ + +	< 0.001	1/3	8
6	GemConv	6	1, 8, 15	39	17	25	+	n.s.	1/5	–
7	GemConv	3	1–5	19.8	7.4	11	+	< 0.001	0/6	–

[a]From 100% (day 0) to 200%
[b]Mann-Whitney nonparametric U-test vs control
[c]+ + + + T/C < 25% and T_x/T_0 < 10%, complete remission; + + + T/C < 25% and T_x/T_0 10–75%, partial regression; + + T/C < 25% and T_x/T_0 75–125%, tumor stasis; + T/C 25–50%, tumor inhibition; – T/C > 50%, inactive
[d]Maximal weight loss as a percentage of weight at start of experiment
[e]Complete response in four of five animals, GD ∞
[f]Complete response in three of three animals, GD ∞

between GemConv-treated and control animals was not significant.

Since a dFdC dose of 6 mg/kg was not the MTD for GemConv, a second experiment was performed using equitoxic dose levels (Table 1, experiments 3 and 4). Injection of GemConv at a dose close to the MTD (360 mg/kg) on days 1, 8 and 15 did not result in complete tumor remissions, but resulted in progression or partial regression with an optimal T/C of 12.6%. In the latter experiment using equitoxic doses of GemLip (9 mg/kg) and GemConv, GemLip was more active than GemConv. Despite significantly better antitumor activity with a T/C of 4.5% ($P < 0.05$), in this experiment GemLip did not induce any complete remissions.

Considering liposomes as a sustained release formulation, we further investigated whether the efficacy of GemConv could be improved by daily administration. As shown in Table 1 (experiment 7), GemConv at 3 mg/kg (MTD for this schedule) given daily on days 1–5 resulted in tumor growth inhibition with an optimal T/C of 19.8% and a mean growth delay (GD) of 7.4 days. In contrast, the groups treated with GemLip at the MTD (6 and 9 mg/kg) showed GDs of > 22 days or complete remissions (GD ∞) (Table 1; experiments 1, 2 and 3).

Finally, we compared the optimal bolus schedule for GemConv (120 mg dFdC/kg given i.v. on days 1, 5 and 9) [17] with the optimal schedule for GemLip (6 mg dFdC/kg i.v. on days 1, 8, 15) (Table 1, experiments 2 and 5). GemConv in its optimal schedule resulted in a T/C of 3.0 and a GD of 34 days, but GemLip in its optimal schedule showed better PK and PD behavior and led to complete tumor remissions (GD ∞).

Activity in orthotopically growing BXF 1299T

In orthotopically growing bladder carcinoma BXF 1299T, the antineoplastic activity of GemLip was compared with that of Vindesine and Taxotere at their MTDs. Efficacy was assessed in terms of growth inhibition of the primary bladder tumors and their peritoneal metastases. Figure 2 shows the relative median tumor volumes on day 35 after randomization. The T_x/T_0 value of the control group was 1816%, but tumors treated with Taxotere and Vindesine showed a growth delay with T_x/T_0 of 409% and 810%, respectively. The most effective treatment, however, was GemLip which resulted in tumor stasis (T_x/T_0 148%).

Immunohistochemical staining of human cytokeratin which detects human cells of epithelial origin was used to evaluate micrometastases in the lung, liver, peritoneum and regional lymph nodes of the BXF1299T mice. The investigated drugs were effective against macro- and micrometastases (Fig. 3). In the control group, seven of seven animals showed metastases. In the Vindesine-treated group three of six animals, and in the Taxotere-treated group only one of six animals showed metastases. However, none of seven GemLip-treated animals showed detectable metastases.

Plasma and tumor pharmacokinetics

Elimination of dFdC from plasma

The plasma elimination curves of dFdC after injection as GemLip and GemConv at equimolar doses (6 mg/kg) are shown in Fig. 4a, b. Elimination of free dFdC after injection of GemConv followed first-order kinetics with a half-life of 0.14 h (see Table 2). After injection of GemLip, consisting of 33% of liposomally entrapped and 67% free dFdC, a biphasic elimination curve was observed (Fig. 4a). The curve could be described by the biexponential fitting equation:

$$C(t) = U_0 \cdot e^{-k_0 t} + U_1 \cdot e^{-k_1 t} \quad (1)$$

where C(t) is the serum concentration of dFdC at time t upon i.v. administration of the drug, and k_0 and k_1 represent the first-order disposition constants. The corresponding plasma half-lives were calculated according to the equation $t_{1/2} = \ln 2/k$.

After administration of GemLip, the elimination of dFdC was initially dominated by the amount of free,

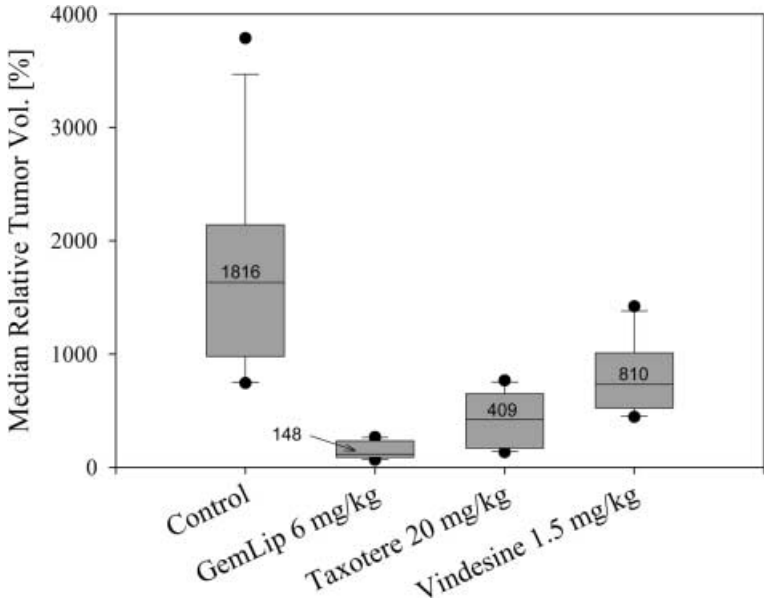

Fig. 2. Effects of GemLip, Vindesine and Taxotere against the primary tumors of orthotopically growing human bladder xenograft BXF 1299T. All formulations were given at their MTDs. Treatment was initiated when the median tumor volume reached 45 mm^3. Data are depicted as median relative tumor volumes at the end of the experiment ($n = 6$ or 7 animals). The reduction in the median relative tumor volume of the GemLip group was significantly different from that in all other groups ($P = 0.0015$ vs Control, $P = 0.0024$ vs Vindesine, $P = 0.039$ vs Taxotere)

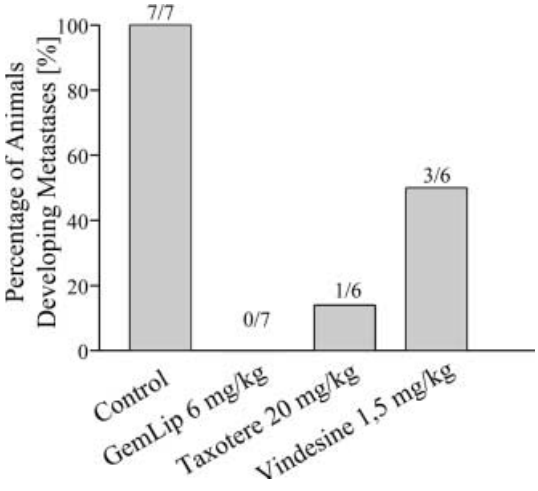

Fig. 3. Development of metastases of orthotopically growing human bladder tumor BXF 1299T on day 35 after initiation of treatment with GemLip, Vindesine and Taxotere at their MTDs. The numbers of animals developing distant organ metastases are shown ($n = 6$ or 7 animals)

non-entrapped drug (U_0, k_0) with a serum half-life ($t_{1/2\alpha}$ 0.15 h) identical to that after injection of GemConv ($t_{1/2\alpha}$ 0.14 h). Thereafter, the liposomally entrapped drug determined the elimination rate of dFdC, which is expressed by the elimination rate constant k_1, and the serum half-life ($t_{1/2\beta}$ 13.2 h). The area under the plasma concentration curve (AUC) of dFdC after administration of GemLip was 1680 μg·h/ml compared to 48 μg·h/ml after injection of GemConv. This represents a 35 times higher availability of dFdC from GemLip.

The elimination curve of dFdC as measured by the ^{14}C label in plasma, paralleled the elimination curve of dFdC measured by HPLC, but showed slightly higher concentrations (data not shown). This can be explained by the fact that the ^{14}C label was still present after metabolic conversion of dFdC to dFdU, so that ^{14}C in plasma represents not only dFdC but also its main metabolite dFdU. Therefore, we investigated the time-course of dFdU in plasma after administration of GemLip and GemConv at equimolar doses (6 mg dFdC/kg; Figs. 4a, b, dotted lines). GemLip led to a rapid increase in dFdU during the first 30 min equivalent to 10% of the total dFdC dose administered, which resulted most probably from the free (external) portion of dFdC. This initial increase was followed by a constant but slow decrease in dFdU over the next 24 h, which could be explained by a slow release of dFdC from the liposomes. At each time-point, the concentration of dFdC was higher than that of dFdU.

In contrast, after administration of GemConv, the concentration of the inactive metabolite dFdU was always higher than that of dFdC. During the first 15 min, dFdU increased rapidly to a dFdC equivalent of 25%, followed by an exponential decrease to about 6% after 4 h (Fig. 4b).

Elimination of the liposomes from plasma

The elimination of the liposomes from plasma was determined by measurement of the ^3H-cholesteryloleate concentration by scintillation counting. Of the injected radioactivity, 70% was rapidly cleared from plasma. However, the elimination rate decreased continuously, and after 24 h 10% of the administered radioactivity could still be detected (Fig. 4c). As described before for the elimination of dFdC, the elimination curve of the ^3H-cholesteryloleate followed a biphasic behavior which could be described by eq. 1 (k_1 0.80 h^{-1}, k_2 0.06 h^{-1}). Corresponding plasma half-lives were $t_{1/2\alpha}$ 0.87 h and $t_{1/2\beta}$ 12.5 h, respectively (Table 2).

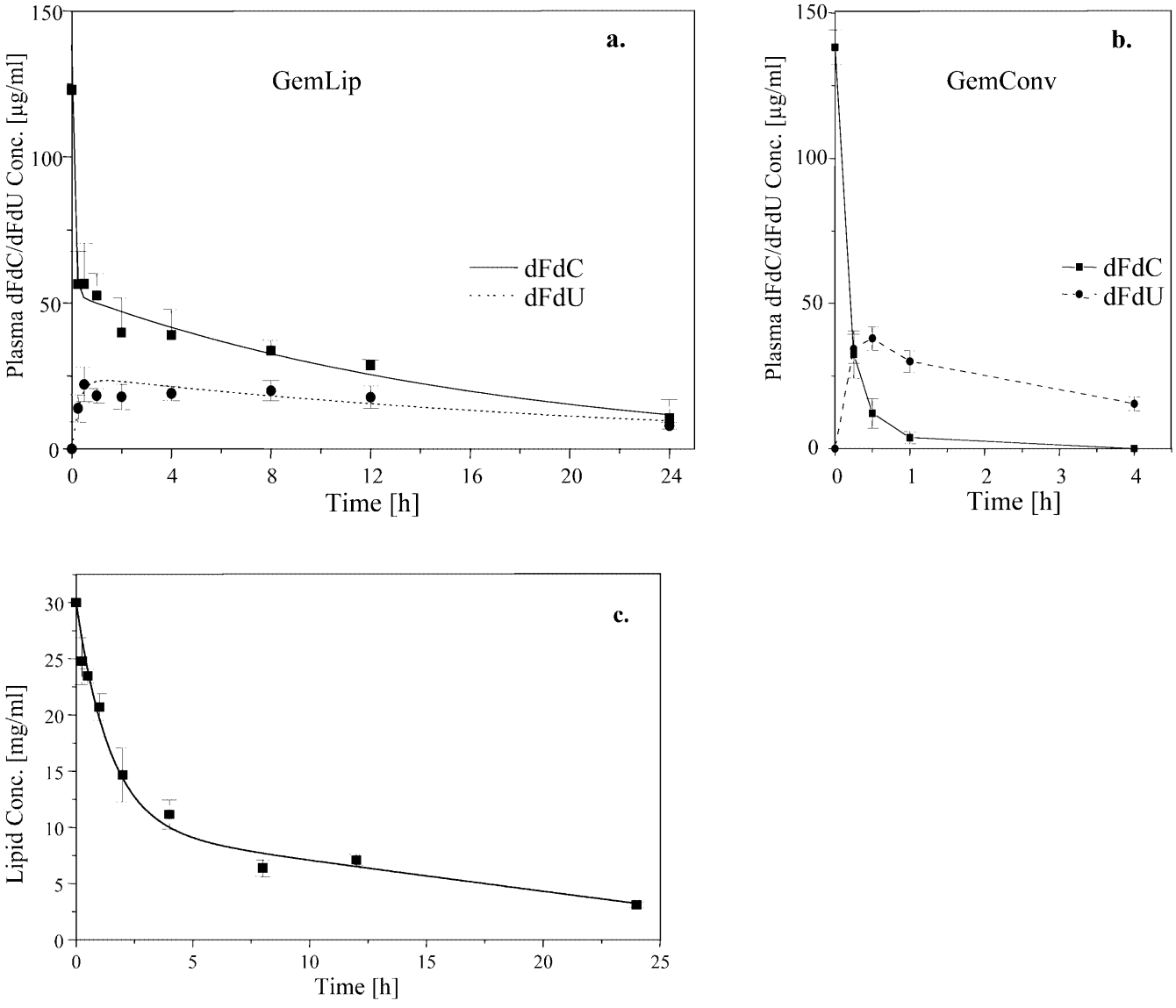

Fig. 4a–c. Elimination of dFdC from plasma and the time-course of dFdU concentration in plasma after injection of $^{14}C/^{3}H$-GemLip (**a**) or ^{14}C-GemConv (**b**) at a dFdC dose of 6 mg/kg ($n=3$) as determined by HPLC. **c** Elimination of ^{3}H-lipid after administration of GemLip (total lipid dose 2.17 mmol/kg) as determined by scintillation counting

Tissue distribution of ^{14}C and ^{3}H

The accumulation of ^{14}C in tumors after administration of GemLip and GemConv, respectively, is shown in Fig. 5. After administration of GemConv, ^{14}C rapidly reached a peak concentration equivalent to 20 µg dFdC/g tumor tissue. Elimination followed first-order kinetics (k_{el} 0.24 h^{-1} and $t_{1/2}$ 2.89 h). Complete clearance was observed after 24 h. After treatment with GemLip, peaks of the same order of magnitude were observed. In contrast, drug elimination was sustained following second-order kinetics (k_1 0.065 h^{-1}, k_2 0.009 h^{-1}, $t_{1/2\alpha}$ 10.66 h and $t_{1/2\beta}$ 77 h). After 72 h a reasonable amount of ^{14}C could still be detected in the tumors. Because of the different elimination rate constants, the tumor AUC of ^{14}C after administration of GemLip was equivalent to 357 µg·h/g dFdC which was about fourfold higher than that for GemConv (89 µg·h/g) (Table 3).

Table 2. Pharmacokinetic parameters for plasma elimination of dFdC and dFdC liposomes (6 mg/kg each) (U_0, U_1 intercepts on the ordinate at time zero; k_0, k_1 elimination rate constants; $t_{1/2\alpha}$, $t_{1/2\beta}$ serum half-lives)

	U_0	k_0 (h^{-1})	U_1	k_1 (h^{-1})	$t_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)
dFdC (from GemConv)	138.2 µg/ml	4.88	–	–	0.14	–
dFdC (from GemLip)	110.6 µg/ml	2.85	98.0 µg/ml	0.05	0.15	13.2
Liposomes (from GemLip)	20.1 mg/ml	0.80	141.2 mg/ml	0.06	0.87	12.5

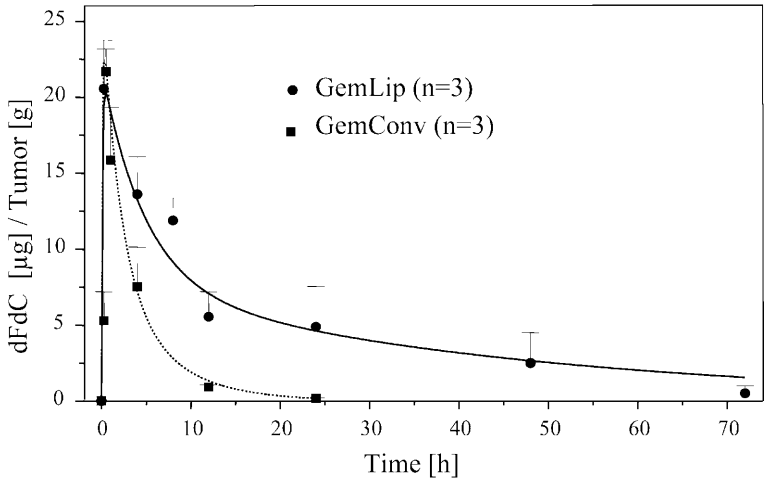

Fig. 5. Accumulation of radiolabel (presented as equivalent amounts of dFdC) in the tumors after administration of ^{14}C-dFdC given as GemLip or GemConv (6 mg dFdC/kg each)

Table 3. Accumulation of ^{14}C (presented as equivalent amounts of dFdC) in the tumor, heart, lungs, liver and spleen of SXF 1301 tumor-bearing nude mice after administration of equimolar doses of GemLip and GemConv containing ^{14}C-dFdC (6 mg dFdC/kg each). Values are means ± SD

	dFdC (µg·h/g tissue)		GemLip/ GemConv
	GemLip	GemConv	
BXF-1301 tumor	357 ± 11	89 ± 16	4.0
Heart	118 ± 21	81 ± 13	1.5
Lung	125 ± 35	72 ± 15	1.7
Liver	1525 ± 307	393 ± 26	3.9
Spleen	2143 ± 462	569 ± 63	3.8

As shown in Table 3, the fourfold increase in tumor AUC was paralleled by an approximately fourfold higher ^{14}C accumulation in organs of the reticuloendothelial system (RES) such as the liver and spleen after dFdC was given as GemLip. In contrast, the ^{14}C levels in the lungs and heart of GemLip-treated animals were only 1.5-fold higher than the corresponding levels of the GemConv-treated animals. Thus, considerable ^{14}C accumulation was observed in both tumors and RES organs.

Discussion

We demonstrated that through the VPG approach, dFdC can be stably entrapped into small liposomes with a satisfactory drug entrapment efficiency and good storage stability. The liposome formulation obtained after redispersion of dFdC-containing VPG (GemLip) is a dual drug formulation consisting of one-third of the dFdC in entrapped form and two-thirds in free form. To our knowledge, VPG technology is so far the only way to prepare a liposomal dFdC formulation with a sufficient shelf life for potential clinical use. Hence, this is the first report of the evaluation of liposomal dFdC in preclinical studies. The aims of our investigations were to compare the antitumor activity of GemLip with that of the aqueous solution of dFdC (GemConv) and describe changes in the PK and PD behavior of dFdC in human tumor xenografts upon entrapment into VPG.

The MTD of GemLip (6–9 mg dFdC/kg) was 40–60 times higher than that of GemConv (360 mg dFdC/kg). Considering the fact that only one-third of the dFdC in GemLip was entrapped (corresponding to 2 mg dFdC/kg) and the other two-thirds (4 mg dFdC/kg) were free dFdC, the real increase in potency upon liposomal entrapment was even higher (approximately 120–180 times). This striking decrease in MTD after entrapment of dFdC in liposomes is in marked contrast to many other drugs which show equal or decreased MTDs and better tolerability after entrapment into liposomes, e.g. doxorubicin [27, 36, 37] and vincristine [2, 14, 35]. However, this might be explained by the fact that the MTD of dFdC is more dependent on schedule than on dose. The MTD of dFdC administered once weekly, for example, differed by a factor of 70 from the MTD of dFdC given for five consecutive days, which is in agreement with literature. It has been reported that administration of free dFdC in different schedules results in MTDs ranging from 2.5 mg/kg (given on days 1–5) to 120 mg/kg (given on days 1, 3, 5 and 7) and 240 mg/kg (given once weekly for 2 weeks) [3]. The MTD of a 24-h continuous infusion has been established as 15 mg/kg [4]. The fractioned and prolonged administration of free dFdC therefore results in increased toxicity. The sustained release formulation GemLip also conferred an increase of dFdC toxicity, but this was paralleled, or was even overcompensated for, by the increase in antitumoral activity, as demonstrated in two human tumor xenograft models, the SXF 1301 (soft tissue sarcoma) and the orthotopically growing human bladder cancer BXF 1299T.

When treating the SXF 1301 human tumor xenograft in a weekly schedule, GemLip (6 mg dFdC/kg) was much more active than GemConv at equimolar doses. The liposomal formulation resulted in complete remissions of the SXF 1301 human tumor xenograft. In a

second study however, when comparing GemLip and GemConv at equitoxic doses (9 and 360 mg dFdC/kg, respectively), GemLip showed only slightly improved anticancer activity. This might be explained by differences in the starting tumor volume of the SXF 1301: the experiment at equimolar doses was performed with early-stage tumors (< 150 mm^3), the equitoxic study with more advanced tumors (< 300 mm^3). In addition, we tested GemLip (6 mg/kg on days 1, 8 and 15) versus the optimal bolus schedule for GemConv (120 mg dFdC/kg on days 1, 5 and 9). Despite this improved schedule for GemConv, which yielded the best antitumor activity for GemConv in our studies (GD 34 days, optimal T/C 3.0%), GemLip was still found to be superior to GemConv as it resulted in complete remissions (Table 1).

When evaluated for activity in the orthotopically growing and metastasizing BXF 1299T bladder tumor xenograft, GemLip resulted in an even higher anticancer activity than Vindesine and Taxotere (given at their MTDs), both in terms of GD of the primary tumor and suppression of distant metastases.

The plasma elimination of dFdC after administration of GemLip showed a biphasic pattern with a rapid phase ($t_{1/2\alpha}$ -0.15 h), similar to the elimination of free dFdC, and a slow phase ($t_{1/2\beta}$ 13.2 h) which may be attributed to the liposome-entrapped fraction of dFdC. The observed change in PK properties resulted in a 35-fold increase in plasma AUC compared to GemConv (both administered at doses of 6 mg/kg). In consequence, dFdC liposomes, which circulate over longer periods of time compared to free dFdC, increased the exposure time of the tumor to anticancer agent. This is in agreement with the observation that prolonging the infusion time of the non-entrapped dFdC reduces the MTD (see above).

The improved activity as well as decreased tolerability of GemLip could be further explained by reduced dFdC deamination due to the protection of dFdC by the liposomes. When administering GemLip, the dFdU levels in plasma never exceeded the dFdC levels. In contrast, when giving GemConv, the dFdU levels were always higher than the dFdC levels.

Furthermore, marked differences in tissue distribution were seen between the two formulations. The injection of GemLip resulted in a fourfold higher dFdC accumulation in the tumors. This enhancement might be attributed to significant extravasation and selective accumulation of the liposomes in the primary tumor. This is in agreement with the hypothesis of Matsumura and Maeda that solid tumors show enhanced permeability of their vasculature which leads to a passive tumor targeting effect of macromolecules as well as liposomes. Moreover, due to the absence of functional lymphatic drainage in tumors, liposomes can remain longer in tumors than in normal tissues. This may lead to prolonged exposure and enhanced activity, termed the enhanced permeability and retention (EPR) effect [21].

The accumulation of dFdC in the tumors was paralleled by an equivalent fourfold increase in dFdC in the RES organs liver and spleen. It is remarkable that the increase in dFdC in the RES organs after liposomal entrapment was not higher than the increase in the tumors, since macrophages of the liver and spleen are known to eliminate non-protected liposomes very rapidly. An explanation for this effect could be the high number of liposomes used in our experiments (always 2.17 mmol lipid/kg). Thus, we assume that the macrophages from the liver and spleen, as well as circulating macrophages, are rapidly saturated after phagocytosis of a small proportion of the total amount of liposomes administered. As a consequence, the remaining liposomes can circulate for a time sufficient to accumulate in the tumors via the EPR effect. This hypothesis is supported by the elimination kinetics of the dFdC liposomes seen in plasma. The liposomes were eliminated biphasically with a low elimination rate constant in the second phase. Therefore, after 24 h a reasonable amount of liposomes were still detectable in the plasma. Furthermore, Mauk and Gamble [22] have found that within 1 h of administration of 0.05 mg lipid/g body weight the RES system of mice was saturated. In our studies, the total lipid dose (1.3 mg/g body weight) was 25-fold higher than that used by Mauk and Gamble. Therefore, the biphasic elimination of the dFdC liposomes can be explained by the rapid uptake of a fraction of the liposomes by the RES system, followed by a considerably slower elimination of the remaining liposomes.

Nevertheless, dFdC accumulation in tumors and RES organs was twice as high as in normal tissues such as the heart and lungs following administration of GemLip than of GemConv. The limited accumulation of liposomal dFdC in heart and lung might contribute to the fact that GemLip has a safe therapeutic window and thus that the increase in antitumor activity of dFdC after entrapment into liposomes is more pronounced than the increase in toxicity.

In summary, we showed that VPG is a promising approach to the generation of a stable liposomal formulation of the hydrophilic anticancer agent dFdC. Liposomal entrapment was demonstrated to positively change the PK and PD of dFdC and hence enhance its antitumor activity. There are three possible reasons for this effect: (1) prolonged circulation of the liposomally entrapped dFdC in blood and therefore prolonged drug exposure to the tumor, (2) protection of the drug against rapid metabolic inactivation, and (3) enhanced uptake and accumulation of the drug within the tumor by the EPR effect [21, 39]. In general, VPGs have proven to be a useful approach to the efficient and stable entrapment of hydrophilic low molecular weight drugs into small liposomes. Such liposomal formulations have a good potential for enhancing the anticancer effect of cytotoxic drugs.

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References

1. Abratt RP, Bezwoda WR, Falkson G, Goedhals L, Hacking D, Rugg TA (1994) Efficacy and safety profile of gemcitabine in non-small-cell lung cancer: a phase II study. J Clin Oncol 12:1535
2. Boman NL, Tron VA, Bally MB, Cullis PR (1996) Vincristine-induced dermal toxicity is significantly reduced when the drug is given in liposomes. Cancer Chemother Pharmacol 37:351
3. Boven E, Schipper H, Erkelens CA, Hatty SA, Pinedo HM (1993) The influence of the schedule and the dose of gemcitabine on the anti-tumour efficacy in experimental human cancer. Br J Cancer 68:52
4. Braakhuis BJ, Ruiz van Haperen VW, Boven E, Veerman G, Peters GJ (1995) Schedule-dependent antitumor effect of gemcitabine in in vivo model system. Semin Oncol 22:42
5. Braakhuis BJ, Ruiz van Haperen VW, Welters MJ, Peters GJ (1995) Schedule-dependent therapeutic efficacy of the combination of gemcitabine and cisplatin in head and neck cancer xenografts. Eur J Cancer 31A:2335
6. Brandl M, Bachmann D, Drechsler M, Bauer KH (1990) Liposome preparation by a new high-pressure homogenizer Gaulin Micron Lab 40. Drug Dev Ind Pharm 16:2167
7. Brandl M, Bachmann D, Drechsler M, Bauer KH (1993) Liposome preparation using high-pressure homogenizers. In: Liposome technology. CRC, Boca Raton, p 49
8. Brandl M, Tardi C, Drechsler M, Bachmann D, Reszka R, Bauer KH, Schubert R (1997) Three-dimensional liposome networks: freeze fracture electron microscopical evaluation of their structure and in-vitro analysis of release of hydrophilic markers. Adv Drug Del Rev 24:161
9. Brandl M, Drechsler M, Bachmann D, Bauer KH (1997) Morphology of semisolid aqueous phosphatidylcholine dispersions, a freeze fracture electron microscopy study. Chem Phys Lipids 87:65
10. Brandl M, Drechsler M, Bachmann D, Tardi C, Schmidtgen M, Bauer KH (1998) Preparation and characterisation of semisolid phospholipid dispersions and dilutions thereof. Int J Pharm 170:187
11. Carmichael J, Possinger K, Phillip P, Beykirch M, Kerr H, Walling J, Harris AL (1995) Advanced breast cancer: a phase II trial with gemcitabine. J Clin Oncol 13:2731
12. Freeman KB, Anliker S, Hamilton M, Osborne D, Dhahir PH, Nelson R, Allerheiligen SR (1995) Validated assays for the determination of gemcitabine in human plasma and urine using high-performance liquid chromatography with ultraviolet detection. J Chromatogr B Biomed Appl 665:171
13. Gatzemeier U, Shepherd FA, Le Chevalier T, Weynants P, Cottier B, Groen HJ, Rosso R, Mattson K, Cortes-Funes H, Tonato M, Burkes RL, Gottfried M, Voi M (1996) Activity of gemcitabine in patients with non-small cell lung cancer: a multicentre, extended phase II study. Eur J Cancer 32A:243
14. Gelmon KA, Tolcher A, Diab AR, Bally MB, Embree L, Hudon N, Dedhar C, Ayers D, Eisen A, Melosky B, Burge C, Logan P, Mayer LD (1999) Phase I study of liposomal vincristine. J Clin Oncol 17:697
15. Geran RI, Greenbaum NH, Macdonald MM, Abbott BJ (1977) Modified protocol for the testing of new synthetics in the lymphoid leukemia murine model in the DR&D program, DCT, NCI. Natl Cancer Inst Monogr 45:151
16. Israel VP, Garcia AA, Roman L, Muderspach L, Burnett A, Jeffers S, Muggia FM (2000) Phase II study of liposomal doxorubicin in advanced gynecologic cancers. Gynecol Oncol 78:143
17. Lee NC, Bouvet M, Nardin S, Jiang P, Baranov E, Rashidi B, Yang M, Wang X, Moossa AR, Hoffma RM (2000) Antimetastatic efficacy of adjuvant gemcitabine in a pancreatic cancer orthotopic model. Clin Exp Metastasis 18:379
18. Lyass O, Uziely B, Ben-Yosef R, Tzemach D, Heshing NI, Lotem M, Brufman G, Gabizon A (2000) Correlation of toxicity with pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in metastatic breast carcinoma. Cancer 89:1037
19. Massing U, Fuxius S (2000) Liposomal formulations of anticancer drugs: selectivity and effectiveness. Drug Resistance Updates 3:171
20. Massing U, Unger C, Moog R (1998) Verfahren zur Herstellung von liposomalen Wirkstoffformulierungen (“Passive Loading”-Technik). International patent application (PCT/EP99/01992)
21. Matsumura Y, Maeda H (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46:6387
22. Mauk MR, Gamble RC (1979) Stability of lipid vesicles in tissues of the mouse: a gamma-ray perturbed angular correlation study. Proc Natl Acad Sci U S A 76:765
23. Merimsky O, Meller I, Flusser G, Kollender Y, Issakov J, Weil-Ben-Arush M, Fenig E, Neuman G, Sapir D, Ariad S, Inbar M (2000) Gemcitabine in soft tissue or bone sarcoma resistant to standard chemotherapy: a phase II study. Cancer Chemother Pharmacol 45:177
24. Moog R (1998) Einschuß von Gemcitabine (dFdC) in vesikuläre Phospholipidgele: in vivo und in vitro – Untersuchungen zur Stabilität, Pharmakokinetik und antitumoralen Wirksamkeit. Department Pharmaceutical Technology, Albert-Ludwigs-University of Freiburg, Freiburg
25. Moog R, Brandl M, Schubert R, Unger C, Massing U (2000) Effect of nucleoside analogues and oligonucleotides on hydrolysis of liposomal phospholipids. Int J Pharm 206:43
26. Noble S, Goa KL (1997) Gemcitabine. A review of its pharmacology and clinical potential in non-small cell lung cancer and pancreatic cancer. Drugs 54:447
27. Olson F, Mayhew E, Maslow D, Rustum Y, Szoka F (1982) Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. Eur J Cancer Clin Oncol 18:167
28. Poplin EA, Corbett T, Flaherty L, Tarasoff P, Redman BG, Valdivieso M, Baker L (1992) Difluorodeoxycytidine (dFdC)-gemcitabine: a phase I study. Invest New Drugs 10:165
29. Ruiz van Haperen VW, Veerman G, Boven E, Noordhuis P, Vermorken JB, Peters GJ (1994) Schedule dependence of sensitivity to 2′,2′-difluorodeoxycytidine (Gemcitabine) in relation to accumulation and retention of its triphosphate in solid tumour cell lines and solid tumours. Biochem Pharmacol 48:1327
30. Schueler J (1998) Entwicklung und Charakterisierung humaner Tumormodelle durch orthotope Implantation. Institut für Veterinärpathologie. Freie Universität, Berlin
31. Simmons SP, Kramer PA (1977) Liposomal entrapment of floxuridine. J Pharm Sci 66:984
32. Stadler WM, Kuzel TM, Raghavoan D (1996) A phase II study of gemcitabine (GEM) in bladder cancer (BC) (abstract 272P). Ann Oncol 7:58
33. Tardi C (1999) Vesikuläre Phospholipidgele: in vitro Charakterisierung, Autoklavierbarkeit, Anwendung als Depotarzneiform. Department of Pharmaceutical Technology, Albert-Ludwigs-University of Freiburg, Freiburg
34. van Borssum Waalkes M, van Galen M, Morselt H, Sternberg B, Scherphof GL (1993) In-vitro stability and cytostatic activity of liposomal formulations of 5-fluoro-2′-deoxyuridine and its diacylated derivatives. Biochim Biophys Acta 1148:161
35. Webb MS, Logan P, Kanter PM, St-Onge G, Gelmon K, Harasym T, Mayer LD, Bally MB (1998) Preclinical pharmacology, toxicology and efficacy of sphingomyelin/cholesterol liposomal vincristine for therapeutic treatment of cancer. Cancer Chemother Pharmacol 42:461
36. Working PK, Dayan AD (1996) Pharmacological-toxicological expert report. CAELYX (Stealth liposomal doxorubicin HCl). Hum Exp Toxicol 15:751
37. Working PK, Newman MS, Sullivan T, Yarrington J (1999) Reduction of the cardiotoxicity of doxorubicin in rabbits and dogs by encapsulation in long-circulating, pegylated liposomes. J Pharmacol Exp Ther 289:1128

38. Workman P, Balmain A, Hickman JA, McNally NJ, Rohas AM, Mitchison NA, Pierrepoint CG, Raymond R, Rowlatt C, Stephens TC, et al (1988) UKCCCR guidelines for the welfare of animals in experimental neoplasia. Lab Anim 22:195

39. Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, Jain RK (1995) Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. Cancer Res 55:3752