Anti-Cancer Drugs 2002, **13,** pp. 797–805

Clinical report

Pharmacokinetics and antitumor activity of vincristine entrapped in vesicular phospholipid gels

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In vivo **antitumoral activity, pharmacokinetics (PK) and biodistribution of a new liposomal formulation of vincristine (VCR-Lip) were compared to VCR in aqueous solution (VCR-Conv). VCR was entrapped into a vesicular phospholipid gel (VPG) consisting of densely packed liposomes. Redispersed VCR-containing VPG (VCR-Lip) consisted of 54% liposomally entrapped and 46% free VCR.** ***In vivo*** **efficacy of VCR-Lip versus VCR-Conv was tested using the s.c. growing human small cell lung carcinoma LXFS 650 and the human mammary carcinoma MX1. PK and biodistribution were evaluated using radiolabeled drug and lipid in LXFS 650 tumor-bearing mice. VCR-Lip at a dose of 1.0 mg/kg (dose near the maximum tolerated dose) led to partial remissions in the MX1 tumor xenograft model (T/C=3.9%). VCR-Conv at an equitoxic dose of 0.6 mg/kg produced only a tumor growth inhibition (T/C=7.0%). In LXFS 650 tumor-bearing mice, VCR-Lip was highly active at doses of 0.75 (T/C=0.7%) and 1.0 (T/C=0.0%) mg/kg, and complete tumor regressions were observed. In contrast, equitoxic doses of VCR-Conv (0.6 mg/kg) resulted only in less pronounced tumor remissions (T/C=4.1%). The PK study revealed that VCR-Lip achieved an over 10-fold higher plasma AUC (22.6 μg · h/ml) than VCR-Conv (2.16 μg · h/ml). Moreover, tumor drug levels were 2.3-fold higher when VCR was injected as VCR-Lip in comparison to VCR-Conv. In some cases, however, VCR-Lip as well as blank VPG appeared to be toxic. We conclude that VCR-Lip is an effective VCR delivery system with superior antitumor activity compared to VCR-Conv. The enhanced** ***in vivo*** **efficacy can be explained by sustained release and passive tumor targeting. [© 2002 Lippincott Williams & Wilkins.]**

Supported by the government of the state Baden-Württemberg, Germany within the program 'Förderprogramm Biotechnologie Baden-Württemberg (720.430-22/17A)'.

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Key words: Drug delivery systems, drug targeting, pharmacokinetics, phospholipids, vincristine, liposomes, tissue distribution, toxicity, xenograft model antitumor assay.

Introduction

Vincristine (VCR) is a naturally occurring cytotoxic agent, isolated from the periwinkle plant *Catharantus roseus*. The mechanism by which VCR and other vinca alkaloids exert their antitumor activity is by inhibition of tubulin polymerization. The latter causes disruption of microtubules, dissolution of mitotic spindles and metaphase arrest of dividing cells.[1,2] VCR has been used as an antitumor agent since the 1960s, and it is registered for the therapy of acute leukemia, M. Hodgkin and Non-Hodgkin lymphomas, lung, and breast carcinomas.[3]

VCR has a poor bioavailability upon i.v. injection because it is rapidly bound to blood cells and tissues ($t_{1/2\alpha}$=2–6 min).[4] Attempts to overcome the rapid elimination of VCR from the blood after i.v. administration by continuous infusion with a dose escalation above 2 mg/patient/week have resulted in unacceptable neurologic toxicities.[5,6] On the other hand, cell culture studies have clearly shown that the antitumor activity of VCR increases by several orders of magnitude when the drug exposure to tumor cells is prolonged.[7] Thus, novel concepts resulting in prolonged drug exposure to tumor but not healthy tissues appear promising.

In general, tumor exposure to drugs and biodistribution can be influenced by controlled released or targeted drug formulations such as liposomes.[8,9]

Liposomal formulations of anticancer drugs appear promising due to prolonged circulation of liposomally entrapped drug in blood and therefore extended drug exposure to the tumor as a result of protection of the drug against rapid metabolic inactivation.

Preclinical studies by several groups have previously shown that liposomal formulations of VCR can achieve increased plasma half-life and enhanced tumor exposure time as well as improved antitumor activities.[7,10–19] One of these liposomal VCR formulations is currently undergoing clinical trials.[20,21]

Whilst the so-far reported VCR liposomal formulations were shown to have significant therapeutic potential, their use appeared to be hampered by insufficient stability. Most of the recent approaches to prepare VCR liposomes employ active loading protocols (acidic pH inside the liposomes).[11,16,22] Certain drugs such as VCR can permeate liposomes membranes at neutral or alkaline pH (outside the liposomes) because they are not charged and sufficiently lipophilic. Once entrapped, an acidic milieu inside the liposomes causes protonation and then the drug no longer permeates the membranes. For VCR a pH of 4 or below is required for sufficient loading and retention.[22] As a result of the low pH, however, liposomes show limited shelf-life due to hydrolytic degradation of their main components, the phospholipids.[23]

Earlier approaches to load liposomes with VCR without using a pH gradient were not further pursued owing their poor entrapping efficiencies of 2–14% for glycerophospholipid liposomes.[24]

Here we present an approach to generate liposomes loaded with VCR that enables entrapment at moderate pH values (pH 5.4) and guarantees sufficient encapsulation efficiencies. It is based on the vesicular phospholipid gel (VPG) concept, a novel liposome technology.[25] VPG consist of very densely packed liposomes.[26] The latter allows for efficient and stable entrapment of hydrophilic drugs. Due to their high lipid concentrations, these formulations are semisolid or gel-like. Nevertheless, they can be transferred into homogeneous liposome dispersions suitable for systemic use by simple dilution.[27] Furthermore, after preparation and autoclaving, VPG can be loaded with hydrophilic drugs by a passive loading technique.[25] This VPG approach has recently been successfully applied for liposomal delivery of another low-molecular weight drug, i.e. gemcitabine.[28]

The aim of this study was to evaluate the pharmacokinetics, toxicity as well as antitumor efficacy of the VCR-loaded VPG versus conventional VCR formulation (VCR-Conv; aqueous solution of VCR) in two human tumor xenograft models—the small cell lung tumor xenograft LXFS 650 and the human breast tumor xenograft MX1.

Materials and methods

Materials

Hydrogenated egg phosphatidylcholine (EPC-3) was a kind gift of Lipoid (Ludwigshafen, Germany). Vincristine sulphate was a kind gift of Hexal (Holzkirchen, Germany). Cholesteryl[1-^{14}C]oleyl ether and [^{3}H]VCR sulfate were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). H_2O_2 (30%), cholesterol (Chol) and HPLC solvents were obtained from Merck (Darmstadt, Germany). Scintillation cocktail Hionic-Fluor and tissue solubilizer Soluene-350 were obtained from Canberra-Packard (Dreieich, Germany).

Preparation of VCR-containing VPG

Firstly, blank VPG consisting of EPC-3 and Chol (1:1 molar ratio) at a total lipid concentration of 40% (m/m) (660 mM lipid) was prepared as described before.[27] In brief, EPC-3 and Chol were dissolved in chloroform:methanol (2:1 v/v). Solvents were removed under reduced pressure at 40°C resulting in a lipid film. Solvent traces were removed under vacuum for 24 h. The dry lipid film was hydrated using citric buffer (20 mM, 135 mM NaCl, pH 5.6) and the resulting semisolid lipid dispersion was treated by a high-pressure homogenizer Micron Lab 40 (APV Gaulin, Lübeck, Germany) at 70 MPa for 10 cycles. The resulting VPG was autoclaved[29] and stored at 4–8°C. Then, VCR was loaded into the blank VPG using the recently described *passive loading* technique.[25,28] VCR solution was added to the VPG and the mixture incubated at 60°C for 16 h in order to achieve equilibration.

Preparation of VCR-Lip (diluted VCR-containing VPG)

The resulting VCR-containing VPG was redispersed as described[27] by adding 5 glass beads (2 mm diameter) and citric buffer in one step to a final total lipid concentration of 220 mM.[27] After addition of buffer, the dispersion was shaken in a Mikro-Dismembrator

U (Braun, Melsungen, Germany) for 2 min at 2000 r.p.m.

Radiolabeled VCR-Lip

$^{3}H/^{14}C$-labeled VCR-Lip was prepared as described above except that the lipid composition contained additional cholesteryl[1-^{14}C]oleyl ether (43.1 kBq/mmol lipid) and the stock solution of VCR sulfate contained additional [^{3}H]VCR (298 kBq/μmol VCR sulfate).

In vitro release studies

VCR-Lip was incubated with fresh pooled human plasma (1:10), and the released VCR was quantified at 0, 1,2, 5, 8, 24 and 48 h time points by HPLC upon gel chromatographic separation on Sephadex G25 M. For quantification of VCR from human plasma, VCR was extracted from human plasma using chloroform and hypertonic phosphate buffer pH 5.0,[30] and than assayed by HPLC: column, Nova-Pak CN HP 4.6 × 20 mm; eluent, phosphate buffer pH 3.0:acetonitrile (35:65); UV detection at 268 nm.

Animal experiments

All animal experiments were performed in accordance to German Animal License Regulations (Tierschutzgesetz) identical to UKCCCR Guidelines for the welfare of animals in experimental neoplasia.[31] Male and female outbred thymus aplastic nude mice (8–12 weeks old) with a body weight between 28 and 40 g were used. The animals were bred in our own facility and are of NMRI genetic background.

The maximum tolerated dose (MTD) of VCR-Lip and free VCR was determined in non-tumor-bearing mice. Both preparations were administered into the tail vein on days 1, 8 and 15. Drug-related toxic effects in terms of body weight changes and abnormalities in animal behavior were monitored twice weekly over 21 days.

Assessment of in vivo anticancer activity in human tumor xenograft models. Fragments (3–5 mm) of the human tumors kept in serial passage in our tumor bank were transplanted between the hind- and fore-flanks of thymus aplastic nude mice (LXFS 650 lung carcinoma: male animals; MX-1 breast carcinoma: female animals).

Treatment was initiated when median tumor volumes reached 80–180 mm^3. Tumor growth was followed by serial caliper measurements (tumor volume = length × width^2/2).[32]

VCR-Lip was given at doses of 0.25, 0.75 and 1.0 mg/kg (MX1) and 0.25, 0.75, 1.0 and 1.25 mg/kg (LXFS-650), all at a constant total lipid dose of 2.28 mmol/kg (1.144 g/kg). Free VCR was administered at a dose of 0.6 mg/kg (dose around the MTD for both tumor models). The preparations were administered i.v. on days 1, 8 and 15 (MX1) and on days 1 and 8 (LXFS-650). Control groups received blank (drug-free) redispersed VPG (2.28 mmol/kg). Differences in median tumor size were compared to control (T/C, in %). In addition, tumor doubling time and growth delay (GD, in days) were determined.

Pharmacokinetic studies. $^{3}H/^{14}C$-labeled VCR-Lip or ^{3}H-labeled free VCR was injected i.v. at equitoxic doses of 1.0 mg/kg VCR (VCR-Lip) or 0.6 mg/kg VCR (free VCR). Three or four mice of each treatment group were sacrificed at t 0 and 10 min, and 1, 2.5, 4, 6 and 24 h post drug injection. Blood was collected in vials containing NaEDTA. After centrifugation plasma was stored at −80°C until analysis. Tumors, livers, spleens, hearts, lungs and kidneys were rapidly excised, rinsed in Ringer's solution, weighed and stored at −80°C until analysis.

Determination of ^{3}H and ^{14}C in plasma and tissues. For the determination of ^{3}H and ^{14}C in plasma, tissues and tumors, the livers were homogenized in 1 ml Ringer's solution using an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany). Then, 300 μl aliquots of the liver homogenate, the whole tumors, hearts, kidneys and spleens were incubated in 1 ml Soluene 350 at 55°C for 2 h. The samples were bleached twice by addition of 100 μl H_2O_2 (30%) and heating to 55°C for 2 h. ^{3}H and ^{14}C activities were measured using a Tricarb 1900 CA analyzer using Hionic Fluor as scintillation cocktail (both Canberra-Packard, Dreieich, Germany). Organs and plasma of untreated animals were used as negative control.

Results

Preparation of VCR-Lip (diluted VCR-containing VPG)

Blank VPG were prepared by high-pressure homogenization (660 mM total lipid) and subsequently autoclaved to assure sterility.[27,29] In a second step,

different amounts of VCR were entrapped into the blank VPG by diffusion of VCR into the liposomes at increased temperature (60°C for 16 h; passive loading procedure).[25] VCR-containing VPG can be stored at 4°C for at least 6 months without any change in phosphatidylcholine content, VCR content, VCR encapsulation efficiency and particle size (data not shown).

Prior to i.v. administration, VCR-containing VPG were diluted with buffer to a final lipid concentration of 228 mM (VCR-Lip). The mean diameter of the liposomes as determined by quasi-elastic light scattering was either 50–60 nm (monomodal size distribution; Gaussian model) or around 100 nm with a minor contaminant (below 4%) of larger particles (bimodal size distribution; Nicomp-model). In any case, no particles larger than 1 μm could be detected. The proportion of VCR entrapped in the liposomes (entrapping efficiency) was predetermined by the core:external phase ratio of the VPG and was thus constant. Here, 54.0 $\pm$ 3.3% (n=23) of VCR was present in liposomal form irrespective of the drug concentration. VCR-Lip therefore represents a dual drug formulation, containing free and liposomal VCR.

The totally injected dose of liposomal lipid was the same for all animal experiments, corresponding to a lipid concentration of 2.28 mmol/kg, independent of the VCR dose.

In vitro release of VCR from VCR-Lip in the presence of human plasma

Upon incubation of VCR-Lip with the 9-fold volume of fresh pooled human plasma, a slow release of the entrapped VCR from the liposomes was observed (Figure 1). Retention values at 1, 8, 24 and 48 h were 75.0 $\pm$ 0.2, 36.9 $\pm$ 6.9, 32.7 $\pm$ 5.7 and 28.1 $\pm$ 6.3%, respectively, of the originally liposome-associated VCR.

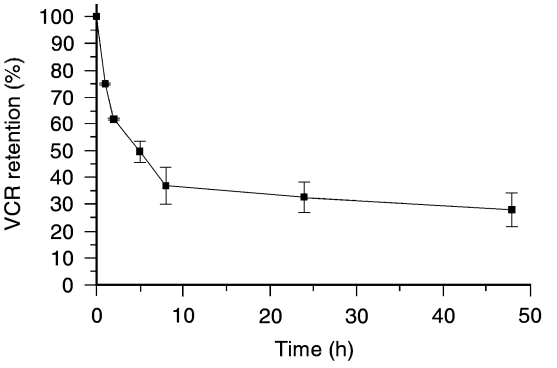

Figure 1. Retention of VCR within VCR-Lip at 37°C in human plasma given as percentage of initially entrapped VCR.

In vivo studies

Determination of MTD for VCR-Lip and VCR-Conv was performed in non-tumor-bearing nude mice. For a VCR-Lip, a dose of around 1.0 mg/kg VCR administered once weekly for 3 weeks was identified as close to the MTD, resulting in a weight loss of up to 20%. MTD of the VCR-Conv solution was determined to range between 0.6 and 0.8 mg VCR/kg (data not shown).

Activity against s.c. growing MX1. Table 1 summarizes the antitumor activity of VCR-Lip at doses of 0.25, 0.75 and 1.0 mg/kg (MTD), and VCR-Conv at a dose of 0.6 mg/kg. VCR-Lip 0.25 mg/kg was inactive (T/C=52.5%). VCR-Lip 0.75 mg/kg led to tumor growth inhibition of 89.4% (T/C=10.6%). VCR-Conv at a dose of 0.6 mg/kg led also to tumor growth inhibition of 93% (T/C=7.0%). However, more prominent effects were seen upon administration of VCR-Lip 1.0 mg/kg (T/C=3.9%), where partial tumor regressions were observed.

Activity against s.c. growing LXFS 650. Table 2 summarizes the antitumor activity of VCR-Lip at doses of 0.25, 0.75 and 1.0 (MTD) given at days 1 and 8, and 1.25 mg/kg (single dose MTD) and VCR-Conv at a dose of 0.6 mg/kg. VCR-Lip 0.25 mg/kg was inactive (T/C=43.0%). After administration of VCR-Lip at a dose of 0.75 mg/kg complete tumor remissions (two of seven; T/C=0.7%) were observed 28 days after initiation of treatment. In the case of VCR-Lip 1.0 mg/kg, complete tumor remission (six of seven and five of five; T/C=0.0%) were seen already 17 days after initiation of treatment and lasted until termination of the experiment (49 days). VCR-Lip 1.25 mg/kg could only be given once due to severe weight loss at the time of the second injection. Nevertheless, good antitumor activity was observed with 96% growth inhibition versus control (T/C=4.0%). In marked contrast to VCR-Lip therapy, application of VCR-Conv led to a temporary tumor remission only (complete remission in one of four tumors tested, T/C: 0.9%). Moreover, on day 14 the tumors regrew again.

Formulation and drug toxicity

In some mice unexpected lethal toxicities were observed upon application of both, redispersed

Table 1. Antitumor efficacy of VCR-Lip versus VCR-Conv in the human breast cancer xenograft MX1

Group	VCR dose (mg/kg)	Schedule (days)	Optimal T/C (%)	GD (days)	DT (days)	Activity rating	Drug-related deaths	BWL (%)
Control (blank VPG)	–	1,8,15	100		5.4	−	1/4	±0
VCR-Lip	0.25	1,8,15	52.5	−0.7	4.6	−	1/3	−1.8
VCR-Lip	0.75	1,8,15	10.6	15.6	21.0	+	0/3	−11.1
VCR-Lip	1.0	1,8,15	3.9	NR	NR	+ + +	1/4	−8.8
VCR-Lip	0.75	1	35.8	8.1	11.2	+	0/3	⩾20
VCR-Lip	1.0	1	22.9	10.0	13.2	+ + +	0/4	⩾20
VCR-Lip	1.25	1	16	15.6	18.8	+	0/5	⩾20
VCR-Conv	0.6	1,8,15	7.0	19.2	24.6	+	0/3	±0

GD = growth delay [from 100% (d0) to 200%]; DT = tumor doubling time [from 100% (d0) to 200%]; Activity rating: + + + +, 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 < x < 50%, tumor inhibition; −, T/C < 50%, inactive; BWL, maximal median body weight loss (%) compared to BW at time of start of experiment; NR = not reached due to tumor regression.

Table 2. Antitumor efficacy of VCR-Lip versus VCR-Conv in the human small cell lung cancer xenograft LXFS 650

Group	VCR dose (mg/kg)	Schedule (days)	Optimal T/C (%)	GD (days)	DT (days)	Activity rating	Drug-related deaths	BWL (%)
Control (blank VPG)	0	1,8	–		5.1	−	1/3	−12.1
Control (blank VPG)	0	1,8	–		4.5	−	0/5	−4.0
VCR-Lip	0.25	1,8	43.0	4.4	9.5	+	2/3	−14.9
VCR-Lip	0.75	1,8	0.7	CR (2/7)	NR	+ + + +	1/5	−11.5
VCR-Lip	1.0	1,8	0.0	CR (6/7)	NR	+ + + +	0/5	−16.0
VCR-Lip	1.0	1,8	0.9	CR (5/5)	NR	+ + +	0/3	−22.5
VCR-Lip	1.25	1[a]	4.0	39.1	43.7	+ + +	0/6	−20.7
VCR-Conv	0.6	1,8	4.1	CR (1/4)	NR	+ + +	0/3	−9.2

[a] Single dose MTD of VCR-Lip. CR = complete remission (related to number of individual tumors, some animals bear two tumors).
For other abbreviations see Table 1 footnote.

blank VPG as well as of VCR-Lip within 7 days of treatment independently of the VCR dose. Within the MX1 study three of 26 mice and within the LXFS 650-study four of 30 mice died after application of liposomes, a phenomenon which cannot be explained so far.

Plasma elimination kinetics

The elimination of VCR from plasma was determined by measurement of the [^{3}H]VCR concentration in plasma by scintillation counting. Values are given as VCR concentrations. Figure 2 shows the plasma elimination curve after i.v. injection of VCR-Conv (0.6 mg/kg) or VCR-Lip (1.0 mg/kg). A very fast elimination from plasma after injection of VCR-Conv was observed. The VCR concentration decreased rapidly during the first 10 min after injection from 20.6 to 0.9 μg/ml. One hour after injection no detectable VCR was present in plasma.

In contrast, after administration of VCR-Lip, the VCR concentration in plasma initially decreased also in a rapid manner but the decrease was retarded after a few minutes (0 h: 32.2 μg/ml; 10 min: 13.0 μg/ml). VCR reached detection limit between 6 and 24 h. Hence, the area under the plasma concentration curve (AUC, 0–6 h) of VCR after application of VCR-Lip was 70.1 μg · h/ml compared to 6.67 μg · h/ml after injection of VCR-Conv. At equitoxic doses, 10-times higher AUC could be reached with VCR-Lip.

Elimination of the liposomes from plasma. The elimination of the liposomes from plasma was determined by measurement of [^{14}C]lipid concentration in plasma by scintillation counting. We could observe that about 40% of the injected liposomes were rapidly cleared from plasma. However, the elimination rate decreased markedly with time and after 24 h, 50% (SD=11.4%, n=3) of the administered radiolabel was found retained in plasma (Figure 2).

Tumor elimination kinetics of VCR and lipids

After application of VCR-Conv, the drug reached a maximum concentration in tumor tissue 1 h after injection (Figure 3). The following clearance was slow, but 24 h after application VCR reached the detection limit. In contrast, after application of VCR-Lip the maximum of VCR concentration in tumor tissue could be observed 2.5 h after injection, followed by a sustained drug elimination. After 24 h 40.0±3.5% (mean±SD, n=3) of the maximum amount of ^{3}H (VCR) concentration could still be

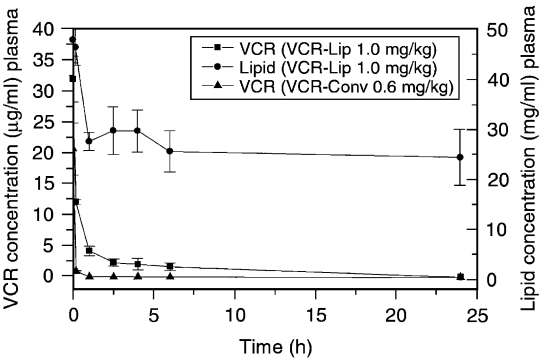

Figure 2. Plasma concentrations of VCR and lipids after i.v. administration of VCR–Lip 1.0 mg/kg and VCR-Conv 0.6 mg/kg, respectively, in LXFS 650-bearing nude mice.

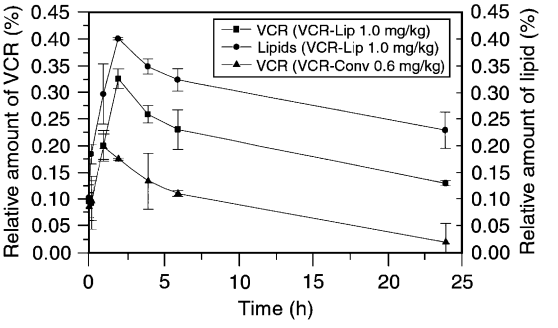

Figure 3. Amount of VCR and lipids detected in the lung tumors LXFS 650 after i.v. administration of VCR-Lip 1.0 mg/kg and VCR-Conv 0.6 mg/kg, expressed as percent of total administered dose.

detected in the tumors. Because of the different elimination behavior, the AUC of VCR after administration of VCR-Lip was 4.73% · h and, thus, about 2.3-fold higher compared to 2.08% · h observed for VCR-Conv.

The tumor uptake and elimination kinetics of lipids after treatment with VCR-Lip were parallel to the VCR uptake and elimination, with maximum concentrations after 2.5 h and an amount of 56.0±8.5% (mean±SD, n=3) of the maximum lipid concentration after 24 h (Figure 3).

Tissue distribution of VCR

Biodistributions of VCR after application of VCR-Conv and VCR-Lip are summarized in Table 3. The VCR tissue AUC show accumulations of VCR in liver and lung after treatment with VCR-Lip compared to VCR-Conv. The accumulations in spleen and kidney were similar after treatment with VCR-Lip and VCR-Conv, whereas the level of VCR in the heart was

Table 3. Tissue distribution AUC (0–24 h) after application of VCR-Lip 1.0 mg/kg or VCR-Conv 0.6 mg/kg

Organs	VCR-AUC (% · h): VCR Conv	VCR-AUC (% · h): VCR-Lip	Ratio VCR-AUC for VCR-Lip: VCR-Conv
Heart	19.3	2.31	0.1
Liver	21.9	47.8	2.2
Lung	5.33	9.47	1.8
Spleen	27.7	26.2	0.9
Kidneys	6.56	7.52	1.1
Tumors	2.08	4.73	2.3

reduced to one-tenth after application of VCR-Lip compared to VCR-Conv.

Discussion

Our studies have shown that VCR-Lip is an efficient drug delivery system for VCR to solid tumors. Using the VPG concept and passive loading technology, VCR was passively entrapped in small liposomes to an extent (54%) which allowed application without removing non-entrapped (free) drug. Other passive entrapment efforts reached only 14% entrapment in glycerophospholipid liposomes.[24] Active entrapment efforts achieved almost 100% entrapment, but with the drawback of acidic pH values which markedly limit the shelf-life of the liposomes.[18]

The MTD of VCR-Lip was higher than for VCR-Conv. Previous reports on VCR-liposomes described different results with respect to tolerability. Allen *et al.*,[11] for example, observed roughly equal lethalities for free VCR and their VCR encapsulated in sterically stabilized liposomes after i.v. injection in ICR mice (LD_{50} around 2.5 mg/kg).[11] In contrast, Mayer observed a 2.5-fold increase in lethal dose for VCR entrapped in 200 nm DSPC/Chol liposomes as compared to free VCR upon i.v. injection in CD-1 mice (LD_{50}: 1.9 and 4.8 mg/kg, respectively).[13] Our VCR-Lip resulted in a 1.6-fold increase of MTD compared to VCR-Conv, despite the fact that it contained 46% free VCR. It can therefore be postulated that our liposomal entrapment of VCR caused a reduction in toxicity or increase in tolerability.

Following determination of MTD, two clinically relevant tumor xenograft models have been chosen to examine the antitumor activity of VCR-Lip in comparison to VCR-Conv—the breast carcinoma model MX1[33] and the small cell lung carcinoma model LXFS 650,[34] which are both sensitive to VCR.

In the MX1 model, when comparing equitoxic doses, VCR-Lip (1 mg/kg) resulted in partial tumor regressions, whereas VCR-Conv (0.6 mg/kg) showed only tumor growth inhibition. Antitumor effects in the small cell lung model LXFS 650 were even more pronounced. VCR-Lip 1.0 mg/kg led to complete tumor regression whilst VCR-Conv induced only a partial and transient reduction in tumor volume for most of the treated tumors.

Comparable good effects have earlier been reported with ammonium-gradient-loaded sterically stabilized VCR-Lip.[11,17] At equitoxic doses, the liposomal formulations were significantly more effective against different solid tumors (C26 colon carcinoma and MC2 mammary carcinoma), which where of murine origin. Here, we could show improved antitumor effects against human tumors.

The improved tolerability and antitumor effects of VCR-Lip are supported by increased *in vitro* plasma retention and improved plasma and tumor pharmacokinetics.

Firstly, our *in vitro* release experiments showed that VCR is retained within the liposomal carrier to an extent of 75 and 35% in human plasma after 1 and 24 h, respectively. In comparison, Kirby found for cholesterol-rich egg phosphatidylcholine liposomes upon passive entrapment a retention of about 40% after 1 h.[24] For liposomes exhibiting a pH gradient (pH 4 inside), Mayer *et al.* reported a retention of 80 and 50% after 1 and 24 h, respectively.[7] Uster described an almost complete retention for sterically stabilized ammonium-loaded liposomes after 24 h.[35]

Secondly, VCR was rapidly eliminated when administered as free drug (VCR-Conv), whereas VCR-Lip, consisting of free and entrapped VCR, showed much slower and biphasic elimination with VCR levels in blood reaching the detection limit after 24 h only. It may be assumed that the free VCR fraction in VCR-Lip is rapidly eliminated, whereas the liposome-associated fraction shows a retarded elimination kinetic. In our studies, liposomes were slowly eliminated from circulation, with about 50% of the liposomal lipid remaining in circulation after 24 h. This might be explained by the saturation of the RES system.[36]

Taken together, the *in vivo* retention and the plasma elimination data, it appears that the liposomal carrier circulates long enough in the blood stream and retains its drug load good enough to allow for a reasonable increase in tumor exposure

against VCR. The 10-fold increased AUC of VCR after VCR-Lip application in plasma (0–6 h) resulting from prolonged circulation of VCR in blood induced a 2-fold increase of tumor AUC (0–24 h).

Since the increase in tumor AUC significantly exceeds the values of other tissues (heart, spleen and kidney), a passive targeting effect is assumed for VCR-Lip. This result could be explained by a high vascular permeability of the tumors as compared to normal tissue. In this study average liposome size was 100–200 nm. Yuan *et al.*[37] demonstrated the cut-off size of tumor vessels being about 400–600 nm. Thus, all particles smaller than this cut-off size (e.g. liposomes of the redispersed VPG) can cross the vessels and penetrate into the tumors, where they are retained. Furthermore, the uptake and elimination kinetics of VCR and lipids after i.v. application of VCR-Lip were parallel, indicating liposomal uptake of VCR into the tumors.

Despite the very favorable increase in antitumor efficacy of VCR-Lip versus VCR-Conv formulations, in some cases we could see a lethal effect of empty VPG as well as of VCR-Lip, independent of the VCR dose. The animals died 3–7 days after injection. This effect could not be explained so far. The biodistribution studies, however, revealed lung toxicities (hemorrhages) not related to embolism. The possibility exists that the toxicity is a result of the high lipid dose applied of 1.3 mg/g body weight. Mauk and Gamble investigated a saturation of the RES system in mice within 1 h when 0.05 mg lipid/g body weight was injected.[36] In line with these findings, the death of some animals after the treatment with VPG could be an effect of the high lipid dose administered and is subject of ongoing investigations.

Taken together, our data demonstrate that the newly developed VPGs allow for efficient entrapment and good retention of VCR without using deleterious acidic pH values. Furthermore, such a liposomal formulation provides an improved tolerability and antitumor activity.

Acknowledgments

The authors would like to thank V Ziroli for his excellent analytical support, and C Welsch and C Steidle for their excellent technical assistance.

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(Received 15 May 2002; accepted 18 June 2002)