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Title: Liposomal formulation of α -tocopheryl maleamide: in vitro and in vivo toxicological profile and anticancer effect against spontaneous breast carcinomas in mice

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Keywords: Vitamin E analogues; liposome; α -tocopheryl maleamide; α -tocopheryl succinate; apoptosis; cancer; mouse cancer model; ultrasound imaging; hollow fibres; immunotoxicity

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Abstract: The vitamin E analogue α -tocopheryl succinate (α -TOS) is an efficient anti-cancer drug. Improved efficacy was achieved through the synthesis of α -tocopheryl maleamide (α -TAM), an esterase-resistant analogue of α -tocopheryl maleate. In vitro tests demonstrated significantly higher cytotoxicity of α -TAM towards cancer cells (MCF-7, B16F10) compared to α -TOS and other analogues prone to esterase-catalyzed hydrolysis. However, in vitro models demonstrated that α -TAM was cytotoxic to non-malignant cells (e.g. lymphocytes and bone marrow progenitors). Thus we developed lyophilised liposomal formulations of both α -TOS and α -TAM to solve the problem with cytotoxicity of free α -TAM (neurotoxicity and anaphylaxis), as well as the low solubility of both drugs. Remarkably, neither acute toxicity nor immunotoxicity implicated by in vitro tests was

detected in vivo after application of liposomal α -TAM, which significantly reduced the growth of cancer cells in hollow fiber implants. Moreover, liposomal formulation of α -TAM and α -TOS each prevented the growth of tumours in transgenic FVB/N c-neu mice bearing spontaneous breast carcinomas. Liposomal formulation of α -TAM demonstrated anti-cancer activity at levels 10-fold lower than those of α -TOS. Thus, the liposomal formulation of α -TAM preserved its strong anti-cancer efficacy while eliminating the in vivo toxicity found of the free drug applied in DMSO. Liposome-based targeted delivery systems for analogues of vitamin E are of interest for further development of efficient and safe drug formulations for clinical trials.

Response to reviewers:

We thank the reviewers for their insightful comments that have been dealt with as detailed below. We believe that this has significantly improved the manuscript and it is now acceptable for publication in *Toxicology and Applied Pharmacology*.

Reviewer 1:

1. The x-axis in Fig.3A and B should be log(c) μ M. It was corrected in Fig. 3
2. With respect to splenocytes and generally to lymphocytes, the MTT test has a small dynamic range (0.1-0.8 AU) in comparison to the $[H]^3$ thymidin assay (100-100,000 cpm). This assay is more sensitive to any suppression of lymphocyte proliferation and is preferred for lymphocytes. Moreover, metabolic activity of lymphocytes, even if stimulated, is roughly one order of magnitude (measured by the MTT assay) lower in comparison with cancer cell lines like B16F10 or MCF-7. Hence, the number of lymphocytes per well is 5-10 times higher to achieve comparable levels of the signal. The results of both MTT and $[H]^3$ thymidin assay were confirmed by microscopy in our laboratory. That is a reason for using $[H]^3$ thymidin assay instead of the MTT assay for the lymphocyte proliferation test.
3. Additional discussion on this topic together and several new references were incorporated in the Discussion part of the revised version of the manuscript.

Reviewer 2:

1. The original title was shortened as follows: '**Liposomal formulation of α -tocopheryl maleamide: *in vitro* and *in vivo* toxicological profile and anticancer effect against spontaneous breast carcinomas in mice**'.
2. The Abstract was rewritten to stress the elimination of the side-effects of free α -TAM when formulated into liposomes.

3. Introduction: Pg3, 2nd para.: What were the toxicities associated with these other potent VE analogues? Answer: Kogura et al. do not describe in their article the cause of mice death. Based on our experiences and according to the rapid course of death described after the first or the second dose of α -TAM, we can conclude with high probability that death occurred due to anaphylaxis or neurotoxicity.

Pg3, 2nd para: accepted and changed.

Pg4, 2nd para: accepted and changed.

4. Material and Methods: We agree with the reviewer and have changed the dosing data to mg/kg throughout the manuscript.

The conditions for characterization of liposomes by NanoSizer ZS have now been added (they were removed by mistake during reduction of the length of the text).

Pg5, 1st para: information on the filter set was added.

Pg5, 2nd para: the information was added.

Pg5, 3rd para: the information was added and the text was modified to describe the method unequivocally.

Pg6, 2nd para: Both Balb/c and C57Bl mouse strains were used for the Berlin test, as described in Material and Methods. We used female mice in this study (information added). Mice treated with PBS and empty liposomes were used as controls (added to the text). We know from our previous experiments, that α -TOS in DMSO is tolerable and α -TAM in DMSO is toxic. So these experiments were not repeated following the recommendation of the animal ethics committee. Both i.v. and i.p. applications were tested, as stated in the Material and Methods section.

Pg 6, 3rd para: The information on the treatment schedule was added as follows:

“Cumulative doses of 75 mg α -TAM per kg (three equivalent doses of 25mg/kg applied on day 1, 3 and 5 after implantation of hollow fibers) were applied”.

Pg.7, last para - α -Tocopheryl acetate was used as the internal standard. Details on HPLC analysis of - α -TOS were added.

5. Results

Pg.11, 2nd para: The data were calculated on the basis of the 3-dimesional reconstitution of the tumours, using the Vevo770 ultrasound imaging machine and its software. The tumour volume was expressed per the initial volume, to allow for its relative increase over the duration of the experiment. The average volume of the tumours at the onset of the therapy was $21 \pm 5 \text{ mm}^3$, and this information is included in the revised version of the manuscript (in the Legend to Fig.5C). We only could run the experiments for 14 d, since we had to inject the liposomal preparations via the tail vein, allowing no more than 4-5 injections per animal.

Discussion

The discussion on the toxicity of the drugs was extended significantly according to the reviewer's suggestions and with respect to our unpublished data. The fundamental outcome of this study is elimination of acute toxicity of free α -TAM by incorporation into liposomes, lacking immutoxicity. These toxicological parameters are critical for further development of this drug for future pre-clinical and, hopefully, clinical studies. The doses were selected to be effective against cancer but not to interfere with the immune system. We therefore used tolerable doses with respect to immunotoxicity or more precisely the doses that do not induce suppression of hemotopoiesis in the bone

marrow. Pharmacokinetic and pharmacodynamic studies are the follow-up to this study and will be published in a separate paper.

Data

Fig.2: The DLS method is mentioned in the legend. Average size was expressed in volume (see y-axes). It was added again to the text for clarity.

Fig. 3: The media control data are presented in Figure 3, the zero point is at -1 owing to the log scale. This information was added to the legend to the figure. Both normalised and AU-based cytotoxicity curves are used in literature. We prefer the AU-based curves to show quality of data and vitality of the cells used. Normalised curves can conceal differences in vitality of cells and blank differences in different microplates, etc. We are showing that media control data are consistent for each experiment and hence the cells are at the same level of vitality for each drug tested.

6. Fig. 4 was corrected as required.

Figure 2

Figure 3

Figure 4

Figure 5

Liposomal formulation of α -tocopheryl maleamide: *in vitro* and *in vivo* toxicological profile and anticancer effect against spontaneous breast carcinomas in mice

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Keywords: Vitamin E analogues; liposome; α -tocopheryl maleamide; α -tocopheryl succinate; apoptosis; cancer; mouse cancer model; ultrasound imaging; hollow fibers; immunotoxicity;

Abbreviations: CFU-E, erythroid colony-forming unit; Con A, concanavaline A; EPC, egg phosphatidylcholine; GM-CFC, granulocyte-macrophage colony-forming cell; PVDF, polyvinylidene difluoride; α -TAM, α -tocopheryl maleamide; α -TOH, α -tocopherol; α -TOM, α -tocopheryl maleate; α -TOS, α -tocopheryl succinate; USI, ultrasound imaging; VE, vitamin E

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Abstract

The vitamin E analogue α -tocopheryl succinate (α -TOS) is an efficient anti-cancer drug. Improved efficacy was achieved through the synthesis of α -tocopheryl maleamide (α -TAM), an esterase-resistant analogue of α -tocopheryl maleate. *In vitro* tests demonstrated significantly higher cytotoxicity of α -TAM towards cancer cells (MCF-7, B16F10) compared to α -TOS and other analogues prone to esterase-catalyzed hydrolysis. However, *in vitro* models demonstrated that α -TAM was cytotoxic to non-malignant cells (e.g. lymphocytes and bone marrow progenitors). Thus we developed lyophilised liposomal formulations of both α -TOS and α -TAM to solve the problem with cytotoxicity of free α -TAM (neurotoxicity and anaphylaxis), as well as the low solubility of both drugs. Remarkably, neither acute toxicity nor immunotoxicity implicated by *in vitro* tests was detected *in vivo* after application of liposomal α -TAM, which significantly reduced the growth of cancer cells in hollow fiber implants. Moreover, liposomal formulation of α -TAM and α -TOS each prevented the growth of tumours in transgenic FVB/N *c-neu* mice bearing spontaneous breast carcinomas. Liposomal formulation of α -TAM demonstrated anti-cancer activity at levels 10-fold lower than those of α -TOS. Thus, the liposomal formulation of α -TAM preserved its strong anti-cancer efficacy while eliminating the *in vivo* toxicity found of the free drug applied in DMSO. Liposome-based targeted delivery systems for analogues of vitamin E are of interest for further development of efficient and safe drug formulations for clinical trials.

Introduction

α -Tocopheryl succinate (α -TOS) is a semi-synthetic analogue of vitamin E (VE) with selective toxicity for cancer cells (Neuzil *et al.*, 2001a) and anti-cancer efficacy *in vivo* (Prasad *et al.*, 2003). A new derivative, α -tocopheryl maleamide (α -TAM), represents a novel class of apoptogenic VE analogues with a non-cleavable amide bond, endowing them with higher pro-apoptotic effects *in vitro* (Tomic-Vatic *et al.*, 2005). α -TOS is a potent inducer of apoptosis in a wide range of human and murine cancer cells (Dalen *et al.*, 2003; Weber *et al.*, 2002; Neuzil *et al.*, 2001b; Israel *et al.*, 2000; Yu *et al.*, 1997, 1999), while showing limited or no toxicity toward non-malignant cells (Neuzil *et al.*, 2001a; Israel *et al.*, 2000; Yu *et al.*, 1999). In experimental models, α -TOS and its derivatives have been demonstrated to inhibit a variety of cancers (Wang *et al.*, 2006), including breast (Wang *et al.*, 2006; Hahn *et al.*, 2006; Dong *et al.*, 2008) lung (Ramanathapuram *et al.*, 2004) and colon cancer (Barnett *et al.*, 2002; Weber *et al.*, 2002; Neuzil *et al.*, 2001b; Prasad *et al.*, 2003), as well as melanomas (Malafa *et al.*, 2002) and mesotheliomas (Stapelberg *et al.*, 2005; Tomasetti *et al.*, 2004).

A significant limitation of using α -TOS and other VE derivatives is their low solubility in aqueous solvents. Hydrophobic character and low solubility of α -tocopherol and its derivatives pre-determine their drug formulations. Applications of α -TOS in ethanol, DMSO or vegetable oil emulsions by intravenous or intraperitoneal routes are largely restricted to mouse tumour models, with little clinical relevance. Vesiculated forms of α -TOS and various surfactants and solubilisers (e.g. polyethylene glycols) have been tested as suitable formulations for human application. Spontaneous vesiculation of sodium or TRIS salts of α -TOS (Jizomoto *et al.*, 1994) and other dicarboxylic acid analogues have been utilized for drug formulations that are suitable for *i.v.* applications. Anti-cancer effects of vesiculated α -TOS were proven in mouse tumour models, but this formulation does not eliminate *in vivo* toxicity

of some potent analogues of VE, such as α -tocopheryl oxalate (Kogure *et al.*, 2005). Some practical problems with long-term stability during storage were also not addressed.

Liposomes represent an advanced and versatile nanodelivery system for drug formulation that can eliminate or suppress organ-specific toxic side-effects of various drugs (Allen, 1997). α -TOS and other vitamin E analogues could be easily incorporated into the lipid bilayers to produce liposomes of various size distribution and surface modification, affecting their half-life, organ distribution and targeting to cancer cells. Recently, successful experimental treatment of pre-established tumours of the highly metastatic murine mammary cancer cell line 4T1 was demonstrated, using combination of chemotherapy with vesiculated α -TOS and dendritic cell-based immunotherapy (Ramanathapuram *et al.*, 2005). Low toxicity and, especially, immunotoxicity of anticancer drugs and their formulations are important requirements for successful combination of chemo- and immunotherapy.

In this paper we present data showing that both α -TOS and the new, highly potent analogue α -TAM, when formulated in liposomes, efficiently induced apoptosis in cancer cells *in vitro* and suppressed tumours in mouse models without secondary toxicity and immunotoxicity.

Materials and Methods

Cell culture and treatment. B16F10 mouse melanoma and MCF-7 human breast cancer cell lines were obtained from the European Collection of Cell Cultures. The cells were grown in the RPMI-1640 medium supplemented with 10% of fetal calf serum, 50 μ g/ml penicillin, 50 μ g/ml streptomycin, 100 μ g/ml neomycin, and 300 μ g/ml L-glutamine, and were treated with α -tocopherol (α -TOH), α -TOS (both Sigma), α -tocopheryl maleate (α -TOM) (Birringer *et al.*, 2003) or α -TAM (Tomic-Vatic *et al.*, 2005) (see Fig. 1 for structures of VE and its analogues). Drugs were solubilised in small volume of DMSO and then in PBS (residual

concentration of DMSO in medium was below 1%). The concentration range was 0.6 – 300 μ M, exposure time 24 h.

Preparation of liposomes. Liposomes containing VE analogues in combination with EPC (egg phosphatidylcholine, 99%; Avanti Polar Lipids), were prepared using the proliposome-liposome method, or hydration of a lipid film, followed by extrusion through polycarbonate filters with different pore size in an analogous way to that described earlier.(Turanek *et al.*, 2003; Turanek, 1994). The hand-operated mini-extruder (Avanti Polar Lipids) was used for preparation of small volumes of liposomes (up to 1 ml). Large-volume liposomes were extruded using a high-pressure cell attached to the FPLC instrument (GE Healthcare) (Turanek *et al.*, 1994).

Size and zeta-potential measurements. DLS (dynamic light scattering) and micro-electrophoresis were performed using a NanoSizer SZ (Malvern, UK) to measure the size and zeta-potential of liposome preparations, using phospholipids at 1 mg/ml in PBS and temperature of 25°C. Disposable cells were used for zeta-potential measurements. The size of the liposomal preparation was expressed as volume distributions (% in class).

MTT-based cytotoxicity assay. The MTT viability assay was used as described (Mosmann, 1983; Bank *et al.*, 1991) to assess the cytotoxicity of VE analogues. The results of the test were confirmed by Hoffman modulation contrast and fluorescent microscopy (Nikon T200 microscope equipped with G2B filter set) to visualize morphological changes of treated cells. Propidium iodide (PI; 5 μ M) and YO-PRO-1 (5 μ M) (Molecular Probes) were used to distinguish dead or apoptotic cells from living cells (Idziorek *et al.*, 1995). The exposure time was 24 h.

Cytotoxic effects of VE analogues on ConA-stimulated mouse spleenocytes. Standard ^3H -thymidine incorporation test was used to study *in vitro* cytotoxic effects of α -TAM and α -TOS on mouse spleenocytes stimulated with concanavalin A (ConA; 10 $\mu\text{g/ml}$). Briefly, spleenocytes were prepared from of Balb/c mouse spleen by its homogenization through a fine nylon mesh. Cells density was adjusted to 10^6 cells per ml of the RPMI-1640 medium supplemented with 10% pre-colostral calf serum and antibiotics. Cells were seeded in 96-well plates in triplicate and exposed to α -TOH, α -TOS or α -TAM. The tested drugs were solubilized in DMSO and the final concentration of DMSO in the medium was 0.5% (v/v) or lower. The cells were supplemented with ^3H -thymidine (50 μl , 5 $\mu\text{Ci/ml}$) 24 h before harvesting), and assessed for ^3H -thymidine incorporation using the TopCount NXT β -counter (Packard Bioscience). Exposure times 24, 48, and 72 h were used to follow the toxic effect on lymphocytes in various stages of activation and proliferation.

In vitro cytotoxic effect of VE analogues on mouse bone marrow GM-CFC. For granulocyte-macrophage colony-forming cell (GM-CFC) determination, bone marrow cells from Balb/c mice were drawn by flushing the femoral bone with IMDM, counted (Model ZN, Coulter Electronics), and kept on ice until used. Femoral marrow cells (10^5 per ml) were plated in triplicate in a semi-solid environment created by a plasma clot containing IMDM plus 20% fetal calf serum, and 3% Il-3-containing conditioned medium (Vacek *et al.*, 1990). Tested compounds (α -TOS or α -TAM) were added into medium and the cultures were incubated for next 7 d in semisolid medium. Colonies of at least 50 cells were scored using a microscope (40 x magnification).

In vivo cytotoxic effect of α -TOS and α -TAM on GM-CFC from mouse bone marrow and on peripheral blood leucocytes. Balb/c mice (female, 8 per group) were injected *i.v.* with 100 μ l of PBS (control), empty liposomes (liposomal control, 1.3 g of PC/kg), liposomal α -TOS (100 or 10 mg/kg) and liposomal α -TAM (25 mg/kg). Four animals from each group were sacrificed by cervical dislocation 48 h later and the number of GM progenitors in bone marrow counted as described (Vacek *et al.*, 1990). The other 4 mice in each group were used to assess the effect of VE analogues on peripheral blood count during 3 weeks after drug administration. To do this, 20 μ l of blood was taken every week from the tail vein and the number of leucocytes counted (Model ZN, Coulter Electronics).

Berlin test for toxicity of liposomal VE analogues in mice. The Berlin test of general toxicity was used as the method for evaluation of potential toxic effects of VE analogues on Balb/c or C57Bl mice (females). Individual animals (10 per group) treated with given agents were weighed and their behavior monitored for the next 10 days. Mice treated with PBS or empty liposomes were used as control groups. Liposomal preparations were administered *i.v.* or *i.p.* as a single dose (100 mg/kg α -TOS, 25 mg/kg α -TAM) or as 4 repeated doses within 6 d (25 mg/kg per dose α -TOS, 5 mg/kg per dose α -TAM). Typical symptoms of toxicity based on the Berlin test, including motoric disorder, respiratory problems, apathy, horrent fur, behavioral changes and loss of body mass, were monitored daily. The test was complemented by dissection of the sacrificed animals and inspection of their organs (weighing, microscopic observation of morphological changes).

Hollow fiber assay for anti-tumour efficacy of VE analogues. Polyvinylidene difluoride (PVDF) hollow fibers (molecular mass cut-off 500 kDa, internal diameter 1 mm) (CELMAX[®] Implant Membrane, Spectrum Laboratories) were processed according to the manufacturer's

instructions. Loading of fibers with cells and their surgical implantation were done according to the published protocol.(Hall *et al.*, 2000; Hollingshead *et al.*, 1995)A 5 mm incision on the *linea alba* exposed the peritoneum which was then incised (2 mm) to allow passage of hollow fibers into the abdomen. Three fibers per mouse were inserted in the craniocaudal direction on the right side of the peritoneal cavity.

Balb/c or C57Bl mice (3 per group) with hollow fiber implants containing B16F10 or MCF-7 cancer cell were treated i.v. with liposomal α -TAM by tail vein injection. Cumulative doses of 75 mg α -TAM per kg (three equivalent doses of 25mg/kg applied on day 1, 3 and 5 after implantation of hollow fibers) were applied. The mice in the control group were treated with the same dose of empty liposomes.

On day 6, the hollow fibers were retrieved from the mice and the samples assessed for viable cells using the MTT viability assay. The fibers were incubated in 6-well plates (3 fibers/well) in 3 ml of the complete RPMI 1640 medium, containing 1 mg/ml MTT, for 4 h at 37°C. The MTT solution was then aspirated and the fibers washed with 2 ml saline containing 2.5% protamine sulfate overnight at 4°C. A second wash was performed with 2 ml saline containing 2.5% protamine sulfate, and the fibers maintained at 4°C for 4 h. The fibers were then placed in a 24-well plate (1 fiber/well), SDS (0.2 ml of 10% solution) added to each well to extract the formazan crystals, and the plates rotated for 4 h at room temperature. The extracted samples were transferred to 96-well plates, and the absorbance was recorded at 540 nm.

Mouse breast cancer experiments. The transgenic FVB/N *c-neu* mice with spontaneous breast carcinomas(Guy *et al.*, 1992) were treated with α -TOS solubilized in DMSO or formulated in liposomes. From 3 months of age, the mice were regularly scanned for tumours using the Vevo770 ultrasound imaging (USI) system (VisualSonics) equipped with the 60 MHz (40 μ m

resolution) RMV704 scan-head (VisualSonics), essentially as detailed elsewhere (Dong *et al.*, 2007; Wang *et al.*, 2007; Dong *et al.*, 2008). After tumours were detected (typically 10-20 mm³), treatment with α -TOS in DMSO, or liposomal α -TOS or α -TAM commenced.

Therapy with α -TAM in DMSO was not attempted since in preliminary toxicity studies, 1 or 2 injections of α -TAM in DMSO at the dose of 0.8 mg per animal (16 mg/kg) killed the test (Balb/c) mice, *i.e.* at levels 10-fold lower than those of α -TOS used in therapy of mice with experimental tumours and showing minimum toxicity. Mice were injected with α -TOS in DMSO intraperitoneally and with the liposomally formulated agents via the tail vein, with 5 total injections on days 1, 4, 7, 10 and 13. α -TOS in DMSO was injected at 400 mg/kg.

Liposomal preparations of α -TOS and α -TAM, comprising 10% (w/w) of the VE analogue and 90% (w/w) EPC, were reconstituted in water so that the preparation contained 2.5% (w/v) liposomes. The average size of the liposomes was 140-145 nm, their polydispersity index in the range of 0.1-0.13. The volume of the liposomal preparation injected per animal was such that every animal received 400 mg/kg α -TOS or 40 mg/kg α -TAM per dose.

Analysis of VE analogues. VE analogues were extracted from liposomes by Bligh-Dyer two-phase extraction (Bligh *et al.*, 1959) chloroform was removed on a rotary evaporator and the dry residuum dissolved in the mobile phase (methanol-water, 99:1, acidified with 3 % acetic acid), and subjected to HPLC analysis. The Eclipse XDB-C18 HPLC column (4.6x15 mm, 5 μ m) (Agilent) was eluted at 1.2 ml/min using the Beckman System Gold HPLC system.

Injected volume: 10 μ l. UV detection: 205 nm. α -Tocopheryl acetate (Sigma) was used as the internal standard (20 μ g α -tocopheryl acetate per 1 mg of α -TOS). Calibration curve for vitamin E and the tested analogues was linear in the concentration range 1-100 μ g/ml. Elution times: α -TOS, 6.1 min; α -TOH, 7.8 min; α -tocopheryl acetate, 11.2 min.

Statistics. The program GraphPad PRISM was used for the calculation of cytotoxicity curves and IC₅₀. Newman-Keuls Multiple Comparison Test was used for statistical analysis of haemotoxicity and the hollow fiber studies. For the pre-clinical tumour model, differences in the mean relative tumour size ($\pm$ S.D.) were examined using analysis of covariance (ANCOVA) with days as the covariate. Statistical analyses were performed using the SPSS[®] 10.0 analytical software. Statistical significance was accepted at $p < 0.05$.

Animal safety. Mice were housed and handled according to the rules of the Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic, and according to the guidelines of the Australian and New Zealand Council for the Care and Use of Animals in Research and Teaching, and were approved by the local Animal Ethics Committees.

Results

Preparation of liposomal VE analogues. We first attempted to prepare various types of liposomes encapsulating the VE analogues α -TOS and α -TAM. Because α -TOS, unlike α -TAM, is commercially available, we optimized the liposomal preparation using the succinyl analogue, and the results were then applied to preparation of liposomal α -TAM. Liposomes composed of EPC could accept up to 15 molar % of α -TOS without affecting the stability of the bilayer. Phase separation was observed by transmission electron microscopy when the concentration of α -TOS in liposomal composition was 20 molar % of total lipid (results not shown). This observation was confirmed by zeta-potential measurements. Two peaks with zeta-potential of -48 and -18 mV (assessed in PBS) were observed confirming non-liposomal and liposomal α -TOS containing structures, respectively. The composition of 90% EPC and 10% α -TOS was chosen for further experiments as optimal with respect to morphological

stability of liposomes. Liposomes with well defined size distribution were prepared by application of extrusion through polycarbonate filters with various pore sizes. Small-scale preparations (up to 1 ml) were performed using a hand-operated mini-extruder. Large-scale preparations (10-20 ml) were achieved by application of a high-pressure extrusion cell and the FPLC chromatographic system as the source of high pressure.

Fig. 2A shows fine-tuning of the size distribution with respect to the applied filter pore size. Both techniques used for extrusion produced liposomal preparations of similar quality, therefore the high-pressure extrusion cell linked to the FPLC apparatus was used for the large-scale process (grams of phospholipids). Polycarbonate filters with 0.2 μm pore size were used to produce monodispersive liposomal preparation (size of 140-145 nm and polydispersity within 0.1-0.13) suitable for i.v. application. The extrusion process was also running at relatively low pressure (~ 1 MPa). Zeta-potential of liposomal α -TOS (10%) in PBS was -12 ± 2 mV.

For long term storage and transport, liposomes were stabilized by lyophilization in the presence of sucrose. The lipid:sucrose molar ratio of 1:5 was found sufficient to preserve the physical (size, polydispersity) and chemical (content of α -TAM or α -TOS) properties of the liposomal preparations. Physical stability of lyophilized liposomal α -TOS after rehydration is shown in Fig. 2B, which demonstrates no changes in the physical stability of the liposomes. Also, there was virtually no difference in the content of α -TAM and α -TOS (as assessed by HPLC) in the lyophilized liposomal samples stored for up to 12 months at 4°C (data not shown).

In vitro cytotoxicity of VE analogues against cancer cell lines. The human breast cancer cell line MCF-7 and the murine melanoma cell line B16F10 were used for testing the effects of α -TOH and its analogues α -TOS, α -TOM and α -TAM. The cytotoxic potential of the four

agents is presented in Fig. 3A,B, with the order of α -TAM> α -TOM> α -TOS>> α -TOH. No toxicity was observed for the redox-active α -TOH. Similarly, the IC₅₀ values followed the same trend, with the lowest ones for α -TAM for both cell lines (Table I). The high level of toxicity of α -TAM to cancer cells was confirmed by fluorescence microscopy of B16F10 cells exposed to the VE analogue using the marker of apoptosis YO-PRO-1 and necrosis PI, showing profound appearance of apoptotic morphology of the cells (Fig. 3C).

Cytotoxic effects of VE analogues on ConA-stimulated mouse spleenocytes. Mouse spleenocytes were stimulated with ConA and exposed for increasing periods of time to α -TOS or α -TAM, and assessed for inhibition of their proliferation. The dose response curves in Fig. 4A demonstrate that the inhibitory effect of α -TAM, was stronger than that of α -TOS. This data is consistent with the toxicity of the agents to cancer cells.

In vitro and in vivo cytotoxic effects of VE analogues on GM-CFC from mice bone marrow. We next studied toxicity of free α -TOS and α -TAM to the GM-CFC progenitors *in vitro*. Our results show that α -TOS was non-toxic when applied at doses of up to 50 μ M, while α -TAM, in contrast, was highly toxic at 50 μ M and was also toxic when applied at 10 μ M (Fig. 4B). *In vivo* application of both derivatives as liposomal preparations did not cause any suppression of bone marrow proliferation and decrease in GM-CFC progenitors. On the contrary, we observed 1.5-2.5-fold stimulation of bone marrow proliferation reflected by an increase in GM-CFC progenitors after application of liposomal formulation of both derivatives (Fig. 4B).

Effect of liposomal VE analogues on leukocyte count. Application of neither liposomal α -TOS nor α -TAM affected the leukocyte count in peripheral blood within 3 weeks following their administration in mice, when applied as a single dose at 100 mg/kg α -TOS, or 25 or 10 mg/kg

α -TAM (Fig. 4C). Our results demonstrate that the leukocyte counts in mice treated with the two agents administered as liposomal preparations were well within the range of normal leukocyte numbers in mice. Thus, there is a good correlation between *in vivo* effects of liposomal preparations of both VE analogues on bone marrow proliferation (*cf.* Fig. 4B) and leukocyte count in peripheral blood after *i.v.* application of the agents.

Berlin toxicity test. We studied the general toxicity of liposomal preparations of both α -TOS and α -TAM in mice. Untreated mice and mice treated with empty liposomes were used as controls. Neither of the typical symptoms of toxicity used by the Berlin test, including motoric disorder, respiratory problems, apathy, horrent fur, behavioral changes, anorexia and loss of body mass, were observed immediately after application of the drugs or within the following 10 d in either Balb/c or C57Bl mice. No morphological changes of inner organs were observed after dissection and microscopic examination. Contrary to the liposomal formulation, free α -TAM applied in DMSO at 5 mg/kg caused rapid death of the animals. Symptoms like spasms and heavy breathing pointed to neurotoxicity or anaphylactic reaction. Therefore, we discontinued experiments that involved the use of non-liposomal formulations of α -TAM.

Effect of liposomal α -TAM on hollow fiber model of cancer cell proliferation. The hollow fiber model was used for pre-screening of potential anti-cancer activity of α -TAM *in vivo*. Because of the surgical intervention required for insertion of the hollow fiber implants, it was of interest to follow the potential effects of liposomal α -TAM on healing of surgical wounds. We did not observe any adverse effects (inflammation, ulceration, abnormal cicatrisation or re-opening of cut) of liposomal α -TAM on wound healing which proceeded in a normal and comparable manner in both control Balb/c or C57Bl mice and in animals treated with

liposomal α -TAM. We next evaluated the effect of liposomal α -TAM on proliferation of MCF-7 and B16F10 cell lines in Balb/c mice. Application of liposomal α -TAM significantly reduced the growth of both cell lines in the implants (Fig. 5A). In the second experiment, we focused on B16F10 cells implanted in young (3-4 months) and old (8-9 months) C57Bl mice. In both groups of animals, liposomal α -TAM suppressed growth of cancer cells in the hollow fiber implants by 50-70%, with somewhat higher efficacy in the older mice (Fig. 5B). No symptoms of toxicity after liposomal α -TAM application were observed in either group of mice.

Anti-cancer effects of liposomal VE analogues in experimental breast cancer. We next assessed the effect of liposomal α -TOS and α -TAM on breast carcinomas in the transgenic FVB/N *c-neu* mice with spontaneous ductal HER2-high breast carcinomas. Liposomal preparations of both α -TOS and α -TAM suppressed breast carcinomas in the *c-neu* mice by 90-100%, when applied in amounts corresponding to approximately 15 μ mol α -TOS and 1.5 μ mol α -TAM per dose (Fig. 5C). Importantly, neither of the two liposomally formulated analogs was toxic to the *c-neu* mice. This is especially encouraging in the case of α -TAM, which is extremely toxic when applied as solution in DMSO.

Discussion

We and others have recently focused on a novel class of anti-cancer agents, redox-silent VE analogues. Of these, the ester α -TOS has been studied most, due to its relatively high apoptogenic activity and selectivity for cancer cells, as well as anti-cancer efficacy in animal models of neoplasia (for review see Neuzil *et al.*, 2007a). In the quest for more efficient pro-apoptotic drugs, over 60 compounds analogous to the prototypic α -TOS have been synthesized and tested for their apoptogenic activity towards cancer cells, some showing IC₅₀

values in the low micromolar range (for review see Neuzil *et al.*, 2007b for review). A serious problem encountered when maximizing the apoptogenic activity of compounds by modifying their structure is loss of selectivity for cancer cells *in vitro* or tumour tissue in pre-clinical models. For example, the highly apoptogenic α -tocopheryl oxalate is toxic to immunocompromized mice (Kogure *et al.*, 2005).

We have recently developed a novel group of VE analogues, in which the functional domain is linked to the tocopheryl head group via an amide bond that is not prone to esterase-catalyzed hydrolysis. Furthermore, since peptidases are in general far more discriminating and less promiscuous than esterases, we reasoned that the apoptogenic activity of tocopheryl amides may be more pronounced *in vivo*, thereby increasing the anti-cancer efficacy of the amides *vs.* the esters (Tomic-Vatic *et al.*, 2005). The IC_{50} value for α -TAM was $\sim 2 \mu M$ for the Jurkat T lymphoma or the Meso-2 mesothelioma cells, while it was about 10-times higher for the corresponding α -TOM. On the other hand, the non-malignant fibroblasts were rather resistant to α -TAM (Tomic-Vatic *et al.*, 2005). However, when we tested α -TAM *in vivo*, in an experiment in which 40 mg/kg (1.5 μmol) of the agent, *i.e.* ~ 10 -times less than the efficient dose used in mouse models of cancer for α -TOS (Dong *et al.*, 2007; Stapelberg *et al.*, 2005; Malafa *et al.*, 2002; Barnett *et al.*, 2002; Weber *et al.*, 2002; Dong *et al.*, 2008), the amide analogue showed extreme toxicity. Thus, one or two injections of α -TAM in DMSO in Balb/c, C57Bl or FVB/N *c-neu* mice caused death of the animals, associated with severe neurotoxicity and anaphylactic shock. We therefore were not able to proceed in testing this highly apoptogenic amide analogue of VE in pre-clinical models of cancer. Application of liposomal preparations of α -TAM was not accompanied by any sign of toxicity, as assessed using the Berlin toxicity test and gross pathological examination of the animals. This fact is supportive to application of liposomes as carriers for this drug.

Liposomal formulations have been used to administer hydrophobic drugs into the bloodstream (Allen, 1997). This approach offers several advantages. For example, the drug is embedded in the liposomal particles that form an emulsion in the bloodstream, while the ‘naked’ drug would (at least to some extent) precipitate at the site of administration, possibly resulting in severe local and/or systemic inflammation, activation of the complement and general toxicity. Liposomal preparations also allow for longer half-life of the drug in the bloodstream and better uptake by cancer cells, in particular when the drug is embedded in long-circulating liposomes (Karanth *et al.*, 2007; Gabizon, 1992). An interesting report described the use of pH-sensitive liposomes comprised of α -TOS and phosphatidyl ethanolamine for drug delivery (Jizomoto *et al.*, 1994). Such liposomes are more selective for cancer cells with an acidic pH gradient in the proximity of the plasma membrane (Gerweck *et al.*, 1996; Karanth *et al.*, 2007), and we reported that α -TOS, a weak acid, is taken up considerably faster by tumour cells than their non-malignant counterparts (Neuzil *et al.*, 2002). Most importantly, perhaps, liposomes have been used as a delivery vehicle to bypass the frequent generic toxicity of anti-cancer agents (Tattersall *et al.*, 2003; Krishna *et al.*, 1997).

For the above reasons, in particular the last one, we decided to investigate whether VE analogues, represented by α -TOS and α -TAM, can be used as anti-cancer agents following their liposomal formulation. For this, the method of extrusion was used for liposomal preparation, applying filters of a defined pore size. We chose for further application liposomes containing 10% of the VE analogue prepared by extrusion through a 200-nm filter, with appropriate physical properties including the size distribution and zeta-potential (*c.f.* Fig. 2A). These liposomes also showed very good physico-chemical stability, such as little or no change in the size distribution following lyophilization and reconstitution after prolonged storage (*c.f.* Fig. 2B), and no change in the contents of either of the two VE analogues (see the

Results section). Therefore, we exploited the use of liposomes comprising α -TOS and α -TAM mouse models of cancer.

α -TAM, however, strongly suppressed *in vitro* proliferation of ConA-primed mouse spleenocytes (*c.f.* Fig. 4A) as well as formation of GM-CSF colonies (*c.f.* Fig. 4B). On the other hand, when injected into the bloodstream of the experimental animals, liposomal α -TOS and α -TAM did not induce suppression of GM-progenitors in the femur. In fact, both agents stimulated proliferation of progenitors by 50-150% (*c.f.* Fig. 4B). This is indicative of the promotion of hematopoiesis by liposomal formulations of VE analogues at doses that have been used to suppress tumours in animal models (Dong *et al.*, 2007; Stapelberg *et al.*, 2005; Malafa *et al.*, 2002; Barnett *et al.*, 2002; Weber *et al.*, 2002; Dong *et al.*, 2008). Elimination of the potential *in vivo* adverse effect on GM-progenitors in the femur by α -TAM, which was indicated by cytotoxic effect found for the 'naked' compound *in vitro*, could be explained by favourable redistribution of the drug incorporated in liposomes, so that the concentration of α -TAM in bone marrow did not reach the toxic levels. Elimination of bone marrow toxicity of some antiviral drugs, *e.g.* azidothymidine, entrapped in liposomes was well documented in literature (Phillip and Tsoukas, 1992; Garg and Jain, 2006). Moreover, it was found by us and others that liposomes themselves exert slight immunostimulating activity and can also induce stimulation of bone marrow GM-progenitors (Turanek *et al.* 1997; Kasna *et al.* 2004). These data can be reconciled with an earlier report, documenting promotion of erythropoiesis by α -TOS (Gogu *et al.*, 1991) as well as toxicity of α -TOS to leukemic cells while not affecting normal hematopoiesis (Freitas *et al.*, 2008). The authors showed that α -TOS, when added to bone marrow cells at $\sim 10 \mu\text{M}$, stimulated an increase in the number of the erythroid colony-forming unit (CFU-E)-derived colonies, comparable to the effects of 50 mU of erythropoietin or 200 U of IL-3. When applied at 50 mg/kg, α -TOS caused $\sim 75\%$ increase in CFU-E-derived colonies in femur bone marrow cells (Gogu *et al.*, 1991). Collectively, these data indicate that

liposomal formulations of both α -TOS and α -TAM can be used safely in experimental mouse models of cancer.

Before assessing the anti-cancer activity of liposomal analogues in a relevant mouse cancer model, we first tested the effect of liposomal α -TAM on the growth of cancer cells in hollow fiber implants in mice (Hall *et al.*, 2000; Hollingshead *et al.*, 1995). Our data reveal a very good response to the VE analogue applied via the tail vein, with up to 90% inhibition of cancer cell proliferation (*c.f.* Fig. 5A,B). Importantly, too, liposomal α -TAM did not exert any discernible toxicity in these animals, as assessed using the Berlin test. This is in sharp contrast to the pilot experiment, in which we injected mice with α -TAM dissolved in DMSO, resulting in rapid death of the animals (see the Results section). The lack of general toxicity of liposomal α -TAM in mice allowed us to move to the ultimate experiment of this project, testing the liposomal formulations of α -TOS and α -TAM in a relevant model of neoplastic disease. We used the transgenic FVB/N *c-neu* mice carrying the rat *HER-2/neu* proto-oncogene driven by the MMTV promoter on the H-2^d FVB/N background, with spontaneous formation of ductal breast carcinomas (Guy *et al.*, 1992). Both liposomal α -TOS and α -TAM reduced the tumour growth by almost 90% (*c.f.* Fig. 5C,D), similarly as shown before for α -TOS solubilized in corn oil and injected in the *c-neu* mice with breast carcinomas (Dong *et al.*, 2007; Wang *et al.*, 2007, 2008). In accord with the *in vitro* data, some 10-times lower doses of liposomal α -TAM were needed in comparison to α -TOS to induce comparable anti-tumour effects. Again, and consistent with previous experiments, there was no adverse reaction of the transgenic mice to the liposomal VE analogues during the course of the therapy. These results clearly demonstrate that liposomal formulations eliminate the deleterious generic toxicity inherent to non-liposomal α -TAM. This is in agreement with previously published data for other anti-cancer agents (Tattersall *et al.*, 2003; Krishna *et al.*, 1997).

There are scant reports on pharmacokinetics and tissue distribution of α -TOS and other analogues after *i.v.* administration in vesiculated or liposomal forms. One study showed that α -TOS applied to rats had a half-life of approximately 10 h with a small volume of distribution and low clearance. α -TOS was preferentially accumulated in the lung and liver tissue and associated with cell membrane lipids, especially within the microsomal and mitochondrial membranes (Teng *et al.*, 2005). Considering the toxicity, pharmacokinetics and pharmacodynamics of vitamin E analogues, the physical nature of the applied drugs is an important factor. *In vivo* toxicity of some derivatives of vitamin E could be ascribed to their toxicity toward certain somatic cells when applied at higher concentrations, but, more probably, the rapid onset of acute toxicity is due to the physical nature of the vitamin E derivatives. These hydrophobic compounds dissolved in the organic phase (e.g. DMSO or ethanol) can form nano- and microparticles after injection into water phase. The structure of these particles has not been described in an adequate manner in the literature. According to our experience, after injection of α -TOS ethanolic solution into water phase, the VE analogue forms microparticles and metastable nanoparticles (30-60 nm), which tend to precipitate. The high density of the surface negative charge of these particles could be responsible for activation of the complement and rapid precipitation of the drugs can lead to formation of microparticles inducing embolization in the lungs and the heart. We observed these symptoms in mice that died after *i.v.* application of α -TAM dissolved in DMSO. Intraperitoneal application of the DMSO-dissolved derivatives appeared to have caused toxic effects due to their precipitation on the surface of inner organs and induction of inflammation in the peritoneum. Fusion of inner organs after *i.p.* application of α -TOS is a good evidence of inflammation in peritoneum and injury to pellicia of inner organs. This supports our view that suitable drug formulation such as using liposomes or other nanoparticulate carriers is useful for getting relevant pre-clinical data. In this study we were not focused on finding the

maximal tolerated doses. Instead, the applied doses of liposomal preparations were selected with respect to be effective against cancer but not harmful to the immune system. In other words, we used doses tolerable with respect to immunotoxicity or, more precisely, doses that did not interfere with hematopoiesis. This is also important for the development of future combined immuno-chemotherapy based on co-application of autologous dendritic cells (Ramanathapuram *et al.* 2004; Ramanathapuram *et al.* 2005) or co-application of various immunomodulators (e.g. CpG oligonucleotides, MDP analogues, monophosphoryl Lipid A, bacterial extracts etc.).

Studies on pharmacokinetics and pharmacodynamics of liposomal α -TOS and α -TAM are the follow-up to this study will be focused on the role of liposomes and low-density lipoproteins in redistribution of the drugs. Cancer cells express high levels of receptors for very low-density lipoproteins, which represent the best carriers for hydrophobic anti-cancer drugs. After reaching the circulation, α -TOS associates with circulating lipoproteins, which deliver it to the microvasculature of tumour tissue, where it is taken up by cancer cells (Pussinen *et al.*, 2000). Liposomes designed for selective transfer of α -TOS and other anti-cancer VE analogues to serum lipoproteins after *i.v.* application could improve the anti-cancer effect and selectivity of these drugs.

Conclusion

The fundamental outcome of this study is the elimination of acute toxicity of free α -TAM by its incorporation into liposomes. We report here that liposomal formulations of VE analogues α -TOS and α -TAM preserve their anti-cancer efficacy while eliminating any secondary toxicity. The formulation of anti-cancer agents like VE analogues in liposomes makes these drugs selective for cancer cells in pre-clinical models. This is intriguing clinically, since it

suggests a feasible approach to the preparation of anti-cancer drugs for application in human patients.

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Legend to figures

Figure 1. Structures of VE analogues. The structures of α -tocopherol (α -TOH), α -tocopheryl succinate (α -TOS), α -tocopheryl maleate (α -TOM) and α -tocopheryl maleyl amide (α -TAM) are shown.

Figure 2. Preparation of liposomes by extrusion. A. Liposomes with α -TOS were prepared by extrusion through polycarbonate filters of different pore size and their size distribution was assessed by DLS. B. α -TOS-containing liposomes prepared by extrusion through a 200 nm filter were assessed for size distribution before lyophilization and after rehydration following a 6-month storage in the refrigerator. Size distribution is expressed in volume (% in class).

Figure 3. Cytotoxic effect of VE analogues on cancer cell lines. Human breast carcinoma MCF-7 (A) and mouse melanoma B16F10 cells (B) were exposed for 24 h to α -TOH, α -TOS, α -TOM or α -TAM at the concentrations shown and assessed for MTT activity as detailed in Materials and Methods. Media control is set at -1 because of the log(c) scale. (C) The mouse melanoma B16F10 cells were treated with the vehicle (control cells), or for 8 h with α -TAM at 5 or 8 μ M, and observed under epifluorescence microscope. The fluorescent markers Yo-

Pro-1 (green) and PI (red/yellow) were used for visualization of early apoptotic changes and post-apoptotic, secondary necrosis, respectively.

Figure 4. Liposomal VE analogues are non-toxic to normal cells *in vivo*. A. Mouse spleenocytes, prepared from Balb/c mice and stimulated with 10 µg/ml ConA as detailed in Materials and Methods, were exposed to α -TOS or α -TAM for the time periods and concentration shown, and their proliferation was assessed using the ^3H -thymidin incorporation method, expressed in counts per minute. B (left panel). GM progenitors were isolated from Balb/c mouse bone marrow and treated with α -TOS or α -TAM at the concentrations shown, the number of colonies was counted and expressed per individual well. B (right panel). Balb/c mice were injected i.p. with the vehicle, empty liposomes or liposomal α -TOS or α -TAM at the doses shown, and GM-CFC colonies in bone marrow counted 48 h later and expressed per individual femur (* denotes statistically significant differences with $p < 0.05$ in comparison to control). C. Balb/c mice were injected *i.v.* with the vehicle, empty liposomes or liposomal α -TOS or α -TAM at the doses shown. Small volume of blood (20 µl) was drawn from each animal 1, 2 or 3 weeks later, and the number of leukocytes counted. The dashed horizontal line shows the normal leukocyte blood count in healthy, untreated Balb/c mice, with standard deviation indicated by the dotted lines. No statistical significant differences were found between control and treated groups.

Figure 5. Liposomal VE analogues inhibit proliferation of cancer cells *in vivo* and suppress breast carcinomas. Hollow fibers were seeded with MCF-7 or B16F10 cells and implanted in the peritoneum of Balb/c mice (A), or with B16F10 cells and implanted in the peritoneum of 3-4 or 8-9 month old C57Bl mice (B), as detailed in Materials and Methods. The mice were then injected with 3 doses of liposomal α -TAM at 25 mg/kg of the VE analogue each at 48 h

intervals, the hollow fibers removed and proliferation of the cells evaluated using the MTT assay as detailed in Materials and Methods. Results of two independent experiments are shown in B (* denotes statistically significant differences with $p < 0.05$ in comparison to control). C. Transgenic FVB/N *c-neu* transgenic mice with spontaneous breast carcinomas were treated by injection of liposomal α -TOS and α -TAM at doses corresponding to 15 and 1.5 μmol of α -TOS and α -TAM, respectively, administered on days 0, 4, 7 and 13, as detailed in Materials and Methods. The tumour volume was quantified non-invasively by USI and expressed relative to the initial volume, which was $21 \pm 5 \text{ mm}^3$. Panel D shows representative images of a control mouse at the onset of the experiment and on day 13, and a mouse treated with liposomal α -TOS on day 13. The data shown are mean values $\pm$ SD ($n=5$), the symbol “*” denotes statistically significant differences with $p < 0.05$.