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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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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 lyophilized 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 and Neuzil, 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 solubilizers (*e.g.* polyethylene glycols) have been tested as suitable formulations for human application. Spontaneous vesiculation of

Abbreviations: CFU-E, erythroid colony-forming unit; ConA, 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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sodium or TRIS salts of α-TOS (Jizomoto et al., 1984) and other dicarboxylic acid analogues has 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 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin, and 300 μg/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 solubilized 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 μM, 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, 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

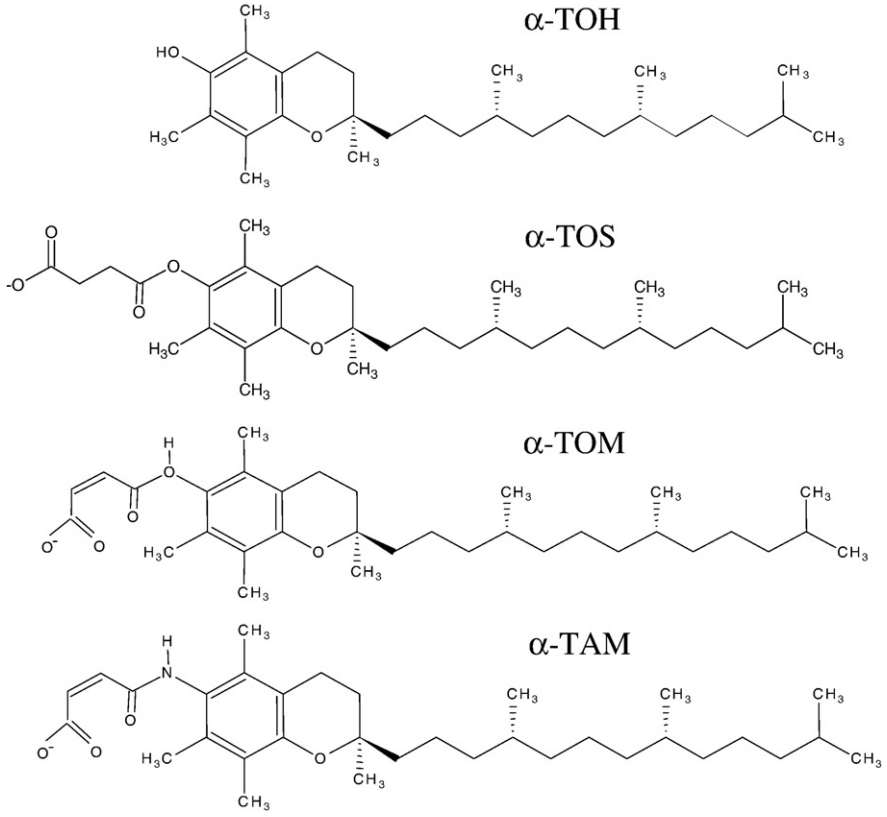

Fig. 1. Structures of VE analogues. The structures of α-tocopherol (α-TOH), α-tocopheryl succinate (α-TOS), α-tocopheryl maleate (α-TOM) and α-tocopheryl maleamide (α-TAM) are shown.