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α-Tocopheryloxyacetic acid is superior to α-tocopheryl succinate in suppressing HER2-high breast carcinomas due to its higher stability

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Breast cancer is the number one neoplastic disease of women, with the HER2-high carcinomas presenting a considerable challenge for efficient treatment. Therefore, a search for novel agents active against this type of cancer is warranted. We tested two vitamin E (VE) analogs, the ester hydrolyzable α-tocopheryl succinate (α-TOS) and the non-hydrolyzable ether α-tocopheryloxyacetic acid (α-TEA) for their effects on HER2-positive breast carcinomas using a breast tumor mouse model and breast cancer cell lines. Ultrasound imaging documented that α-TEA suppressed breast carcinomas in the transgenic animals more efficiently than found for its ester counterpart. However, both agents exerted a comparable apoptotic effect on the NeuTL breast cancer cells derived from the FVB/N c-neu mice as well as in the human MBA-MD-453 and MCF7HER2-18 cells with high level of HER2. The superior anti-tumor effect of α-TEA over α-TOS in vivo can be explained by longer persistence of the former in mice, possibly due to the enhanced plasma and hepatic processing of α-TEA in comparison to the esterase-non-cleavable α-TEA. Indeed, the stability of α-TOS in plasma was inferior to that of α-TEA. We propose that α-TEA is a promising drug efficient against breast cancer, as documented by its effect on experimental HER2-positive breast carcinomas that present a considerable problem in cancer management.

Key words: HER2, breast cancer, transgenic mouse model, vitamin E analogs, apoptosis induction

Abbreviations: HPLC: high-performance liquid chromatography; PBS: phosphate buffered saline; PE: propidium iodide; USI: ultrasound imaging; VE: vitamin E; α-TEA: α-tocopheryloxyacetic acid

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c-neu mice, and ascribe the superiority of α-TEA over α-TOS, in part, to its higher stability in vivo.

**Material and Methods**

**Cell culture and reagents**

The breast cancer cell line NeuTL was derived originally from breast carcinomas of the FVB/N c-neu transgenic mice (see below). Human breast cancer cell line MDA-MB-435 (ATCC) and MCF7HER2,18 cells24 with high level of expression of HER2 were also used. The cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics and were treated when ~70% confluent. The cells were exposed to α-TOS (Sigma-Aldrich) or α-TEA dissolved in EtOH. Control cells received identical volume of EtOH (<0.1%, v/v).

**Assessment of apoptosis induction, proliferation rate, generation of reactive oxygen species and mitochondrial potential**

Apoptosis was quantified using the annexin V-FITC method, which detects phosphatidyl serine externalization in the early phases of apoptosis. Following exposure to various compounds, floating and attached cells were collected, washed with phosphate buffered saline (PBS), resuspended in 200 µL binding buffer, incubated for 20 min at 4°C with 2 µL annexin V-FITC plus 10 µL propidium iodide (PI) (10 µg/mL) and analyzed by flow cytometry (FACSCalibur) using channel 1 for annexin V-FITC binding and channel 2 for PI staining. Proliferation was assessed using the standard MTT assay. Intracellular ROS levels were assessed using the fluorescent dye dihydroethidium. The cells were seeded in 24-well plates, treated, resuspended in PBS and supplemented with 5 µM DHEA, a cell-permeable, ROS-sensitive dye with relative specificity for superoxide. After a 30-min incubation in the dark, the cells were collected, washed and analyzed by flow cytometry. The level of ROS was expressed as mean fluorescence intensity. To assess the mitochondrial membrane potential (ΔΨm), we used the ΔΨm-sensitive fluorescent probe tetramethylrhodamine methyl ester (Sigma) followed by flow cytometry assessment of red fluorescence of the mitochondria-accumulated probe.

**Analysis of VE analogs**

Serum samples were analyzed for VE analogs by high-performance liquid chromatography (HPLC). The system consisted of a Prostar 240 Solvent Delivery Module, Prostar 430 Autosampler and Prostar 335 Photodiode Array Detector (Varian). Samples for α-TEA and α-TOS analysis, spiked with the internal standard DL-α-tocopheryl acetate (α-TOA), were prepared by methanol precipitation and hexane extraction, respectively. An injection volume of 25 µL was used for each analysis. The stationary phase consisted of a Phenomenex Gemini C18 column (250 × 4.6 mm, 5-µm particle size) with temperature at 30 ± 1°C, the mobile phase consisted of acidified (0.25% v/v glacial acetic acid) methanol (A) and acetonitrile (B). Chromatographic separation of α-TEA and α-TOS from other VE analogs was achieved by linear gradient from 0–25% B and 0–31% B over 16 min, respectively. VE analogs were detected at the dual wavelength of 225 and 287 nm. Calibration curves (from 1.5 to 150 µM) were generated by least-squares quadratic curve-linear regression and had a correlation coefficient of ≥0.99.

**Animal tumor experiments**

The transgenic female FVB/N c-neu mice with spontaneous, HER2-positive ductal breast carcinomas in situ were used. The animals present with tumors at 6–8, with 7 of 10 animals developing carcinomas. The animals with small tumors were subjected to therapy using α-TOS or α-TEA, both administered by intraperitoneal injection at 8.2 µmol per animal per dose (4.35 mg or 4 mg, respectively), with two doses given per week. The agents were dissolved in corn oil after presolubilization in EtOH (at 4% v/v). Control animals received corn oil/EtOH only. The appearance of tumors and their progression were visualized and quantified using the Vevo770 USI device equipped with the RMV708 scan-head (VisualSonics) operating at the frequency of 80 MHz and with the resolution of 30 µm.

In vivo disposition studies

To assess the level of drugs in the mouse circulation, the animals developing carcinomas. The animals with small tumors were subjected to therapy using α-TOS or α-TEA, both administered by intraperitoneal injection at 8.2 µmol per animal per dose (4.35 mg or 4 mg, respectively), with two doses given per week. The agents were dissolved in corn oil after presolubilization in EtOH (at 4% v/v). Control animals received corn oil/EtOH only. The appearance of tumors and their progression were visualized and quantified using the Vevo770 USI device equipped with the RMV708 scan-head (VisualSonics) operating at the frequency of 80 MHz and with the resolution of 30 µm.

**Assessment of lipid peroxidation in vivo**

NeuTL cells (5 × 10⁶) were injected subcutaneously under the front arm to 3-month old FVB/N c-neu female mice. When solid tumors reached the size of ~50 mm³ (about 2 weeks), mice were injected intraperitoneally with either 100 µL solution of α-TOS (43.5 mg/mL) or α-TEA (40 mg/mL) every 3 days, while the control were injected with the same volume of the excipient. After 3 weeks of treatment, the mice were sacrificed and the tumor and liver tissue excised. The animals present with tumors at 6–8, with 7 of 10 animals developing carcinomas. The animals with small tumors were subjected to therapy using α-TOS or α-TEA, both administered by intraperitoneal injection at 8.2 µmol per animal per dose (4.35 mg or 4 mg, respectively), with two doses given per week. The agents were dissolved in corn oil after presolubilization in EtOH (at 4% v/v). Control animals received corn oil/EtOH only. The appearance of tumors and their progression were visualized and quantified using the Vevo770 USI device equipped with the RMV708 scan-head (VisualSonics) operating at the frequency of 80 MHz and with the resolution of 30 µm.
read at 540 nm. The lipid peroxide levels were calculated using the calibration curve and related to the protein concentration in the samples. The experiment was performed in triplicate.

**VE analog stability analysis**

α-TEA or α-TOS (25 μM each) was incubated in freshly prepared human plasma (prepared by a standard procedure form blood obtained from healthy volunteers) at 37°C for 1, 2, 6, 12 and 24 hr, and the level of the VE analog assessed by HPLC as described above, with α-tocopheryl acetate as the internal control. Esterase activity of plasma samples was confirmed by adapted Ellman's method.26 The experiment was performed in triplicate on two separate days.

**Animal ethics**

Animal studies were performed 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 Committee.

**Statistical analysis**

All data shown are mean values of three independent experiments (unless stated otherwise) ± S.D. Statistical significance was assessed using Student’s t-test and differences were considered significant at p < 0.05.

**Results and Discussion**

Ductal breast carcinomas with high expression of the oncogenic HER2 present a considerable problem in cancer management.27 VE analogs epitomize an intriguing group of novel selective anti-cancer agents with promise to act across the landscape of solid tumors, most likely due to their effect on the mitochondria of cancer cells.28 The remarkable efficacy of these agents stems from their effect on the mitochondrial redox chain, more specifically on complex II (CII).29 We have shown that both α-TOS and α-TEA,30,31 as well as, more recently, mitochondrially targeted VE succinate,32 act via CII and functional CII is required for their anti-cancer activity in vivo.33 CII, now classified as a tumor suppressor,34 is an excellent target for anti-cancer agents, since it only mutates in rare cancers, such as paragangliomas and phaeochromocytomas, while being intact in frequent cancers, with only one in one million breast cancer patients featuring mutated CII.35,36

The idea of this project was to document whether the esterase-stable α-TEA is more efficient in suppressing HER2-positive breast carcinomas than the prototypic VE analog α-TOS. We anticipated this to be the case since the ether form of the agent would be expected to be more stable (therefore exerting longer half-life) in the circulation of the experimental animal than the ester α-TOS, a paradigm documented earlier for another ether analog, α-tocopherylxybutyric acid.37 Further, we showed before that α-TOS efficiently kills cultured HER2-positive breast cancer cells by causing mitochondrial-dependent apoptosis.17

We first assessed the effect of α-TOS and α-TEA on several cell lines with high level of HER2, i.e., the human lines MCF7HER2-18 and MDA-MB-453, and the NeuTL murine breast cancer cells, originally derived from spontaneous breast carcinomas of the transgenic FVB/N c-neu mice. We tested the two VE analogs for their efficacy in the inhibition of the cellular viability, induction of apoptosis, generation of ROS and the level of dissipation of the mitochondrial potential. Figure 1 reveals that the effect of α-TOS and α-TEA was similar for all three cell lines. Although the ether analog was slightly more efficient, it did not reach statistically significant difference compared to the effect of α-TOS. Similarly, we found slightly while not significantly increased apoptosis in the three HER2-positive breast carcinoma cell lines induced by α-TEA compared to α-TOS (Fig. 2). Finally, since α-TOS and α-TEA act by targeting the mitochondrial CII, we tested the two drugs for their ability to cause ROS generation in the three cell lines as well as their propensity to cause dissipation of ΔΨm.3 Again, we observed the same trend in the activities of α-TEA and α-TOS with no significant difference (Fig. 3). The relatively small variation in the effect of α-TOS and α-TEA in vitro can be ascribed to the fact that cancer cells possess low levels of esterases. In support of this premise, we
observed virtually no hydrolysis of α-TOS in different cancer cell lines.28

We next evaluated the effect of α-TOS and α-TEA on the progression of HER2-positive tumors using the transgenic FVB/N c-neu mice.22 This is an excellent model for testing the efficacy of prospective drugs against breast cancer, since the transgenic animals develop spontaneous breast carcinomas within the context of an intact immune system. Moreover, being morphologically ductal in situ tumors, they represent one of the most frequent types of breast carcinomas found in human patients. Further, this is an ideal experimental tumor model, since it allows for testing potential anti-cancer agents for their propensity to suppress breast tumors with high level expression of the oncogene HER2. Administration of the VE analogs in FVB/N c-neu mice commenced immediately after tumors were detected by ultrasound imaging (USI), at their size of ∼50 mm³. We administered both drugs intraperitoneally in corn oil, following their presolubilization in a small volume of EtOH. Although α-TEA can be given orally since it is resistant to esterases and, unlike α-TOS, will not be hydrolyzed upon uptake by the colon epithelial cells,21,22 we administered it intraperitoneally to compare its effects with those of α-TOS that is subject to esterase hydrolysis.19 Figure 4 clearly documents that α-TEA was significantly more efficient in suppression of breast carcinomas in the transgenic animals compared to α-TOS. Importantly, even during this relatively short experiment, we observed tumor disappearance in 20% (2 of 10) of the mice treated with α-TEA, which did not occur when the corresponding tumors were treated with α-TOS, or the ester counterpart.

Since VE analogs induce ROS in HER2-high cell lines (cf. Fig. 3), we tested whether this translates to lipid generation also in vivo. To do this, we exposed mice with NeuTL cell-
derived xenografts with α-TOS and α-TEA and tested the tumor and liver tissue for lipid peroxidation using a TBARS assay (see Material and Methods). Figure 4c documents that there was no difference in the level of lipid peroxides in the tissue from control and treated mice. This indicates that the levels of ROS that are needed to induce apoptosis in cancer cells are relatively low and that they can be considered signaling molecules that trigger apoptosis rather than oxidizing tissue biomolecules in random manner. This is consistent with our findings that VE analogs cause generation of ROS by targeting the ubiquinone-binding site of CII,31-34 which then trigger Mst1/FoxO1-dependent transcription of the Noxa protein33,39,40 with ensuing generation of the mitochondrial Bak channel and cytosolic translocation of cytochrome.40,41

We then tested the hypothesis that α-TEA is more efficient in tumor suppression than α-TOS due to its better in vivo stability by studying pharmacokinetics of the two compounds. To do this, we injected a cohort of FVB/N c-neu animals with a single dose (4.35 or 4 mg/mouse) of α-TOS or α-TEA at a level similar to that used in the cancer treatment experiments above. After this, blood was taken from the tail vein of the mice on days 1, 3, 7, 9 and 11 post-drug administration and the plasma analyzed, as relevant, for the levels of α-TOS, α-TEA and α-TOH. As documented in Figures 5a and 5b, α-TEA was more stable in the system than α-TOS, i.e., the FVB/N c-neu mice exerted faster disposition of α-TOS than of its ether counterpart. More specifically, there was a rapid decline of α-TOS in the mouse circulation by Day 4, while there was little change in the level of α-TEA, and on Day 9, when there was still ~30% of the original level of α-TEA in the blood, α-TOS was barely detectable. Importantly, the longer half-life of α-TEA in the circulation of the experimental mice was not accompanied by an adverse reaction of the animals, as judged from no change in their behavioral pattern.

We previously observed that α-TOS, a compound that is unstable in the presence of esterases,42 is cleaved in the liver of mice. This could explain the faster disappearance of α-TOS from the circulation of mice compared to its ether counterpart. To test this possibility, we injected FVB/N c-neu mice with a single dose of α-TEA or α-TOS and analyzed their levels plus the level of α-TOH in systemic and hepatic blood. We collected blood by cannulating the vena cava and the hepatic vein, making sure that the hepatic blood did not mix with systemic blood. Figures 5c and 5d shows that the level of α-TEA was comparable in the systemic and hepatic blood, while the level of α-TOS was 3-4 times lower in the hepatic blood when compared to the generic blood. This suggests that α-TOS is hydrolyzed in the liver and only a fraction of the total pool of the drug is returned into circulation. On the other hand, α-TEA, which cannot be hydrolyzed, is efficiently recycled, so that its half-life in the blood plasma is longer. Rather surprisingly, the level of α-TOH in both the systemic and hepatic blood in the α-TOS-injected animals was found to be about half that in the α-TEA-injected animals. We would expect the opposite due to hydrolysis of α-TOS to α-TOH. It is possible, although, that under this setting, α-TOS is hydrolyzed to α-TOH and/or processed to other metabolites of the compound,43,44 or that in the presence of α-TEA, α-TOH is retained more efficiently. Notwithstanding, the lower level of α-TOS in the hepatic blood explains its lower half-life in the circulation of the animals, a reason behind its shorter half-life in vivo compared

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**Figure 5.** α-TEA is more stable in the circulation than α-TOS. (a, b) FVB/N c-neu mice were injected with a single dose of 4 mg α-TEA (a) or 4.35 mg α-TOS (b) at 4 mg each. Blood taken from the tail vein on the days shown was analyzed for α-TEA and α-TOS and α-TOH, as relevant, by HPLC and expressed in μM. (c, d) FVB/N c-neu mice were injected with a single dose of α-TEA (c) or α-TOS (d). After 24 hr, hepatic blood and systemic blood was taken by cannulation of the hepatic vein and vena cava, respectively, and analyzed for the levels of α-TEA and α-TOS, as relevant. (e) α-TOS and α-TEA (25 μM) were incubated in fresh human plasma at 37°C. At the time points shown, the level of the vitamin E analog was assessed by HPLC. (f) NeuTTL cells were seeded in 6-well plates, allowed to reach 60% confluency and 24 hr later, the medium was replaced and cells extracted at times shown and the level of α-TEA and α-TEA assessed by HPLC. The data are related to the controls (0 hr). The data in panels a-d and f are mean values ± S.D. derived from at least six animals per group, data in panel e are mean values ± S.E.M derived from three independent experiments. The symbol ‘**’ in panels a and b denotes statistically significant differences between levels of α-TEA (a) and α-TOS (b), with p < 0.05, the symbol ‘***’ in panel d indicates statistically significant differences between levels of α-TOS in the systemic and hepatic blood, with p < 0.05.
to α-TEA, which is more efficient in suppression of the HER2-positive breast carcinomas.

Since it is possible that the VE analogs may be also hydrolyzed, at least to some extent, by components of blood plasma, we tested their stability by incubating in freshly prepared plasma for time points up to 24 hr. The results in Figure 5e document that while we observed a drop of α-TOS from the original 25 μM to 12–13 μM within 24 hr, α-TEA was much more stable with the drop from 25 μM to only ~20 μM. This corroborates the above data showing much higher stability of α-TEA compared to its ester analog.

It was reported earlier for an ovarian cancer cell line that α-TEA induces more apoptosis than α-TOS due to esterase-mediated hydrolysis of the latter by the cell. To resolve this issue here, we tested the stability of α-TOS and α-TEA in NeuTL cells. In brief, the cells were incubated for 2 hr with 50 μM α-TOS or α-TEA, washed and incubated in fresh medium without the VE analogs. Figure 5f documents that there was <10% loss of the agents within 24 hr, which documents that the cells do not hydrolyze α-TOS, a finding consistent with our earlier report showing high stability of α-TOS in mesothelioma cells.38

One potential problem with increased levels of α-TOH may be its interference with the proapoptotic activity of VE analogs like α-TOS.41 However, to jeopardize the toxicity of the proapoptotic agents, the level of the redox-active α-TOH needs to be similar to that of its apopoptogenic counterparts.41 This is not the case in vivo, since the levels of α-TOH are 5-10-fold lower than that of α-TOS or α-TEA (c.f. Ref. 46 and Fig. 5 in this article).

We conclude that the ether analog of VE, α-TEA, is more efficient than α-TOS in suppression of breast carcinomas in a mouse model of breast tumors over-expressing the receptor tyrosine kinase erbB2 (HER2), due to its greater stability in the experimental animals. This report justifies further testing of α-TEA as an efficient drug against HER2-positive breast carcinomas. We propose that VE analogs, such as α-TEA, may become in the future an alternative for Herceptin, exerting considerable cardiotoxicity,46 in the treatment of HER2-positive breast cancer patients.

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