A Peptide Conjugate of Vitamin E Succinate Targets Breast Cancer Cells with High ErbB2 Expression

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Abstract

Overexpression of erbB2 is associated with resistance to apoptosis. We explored whether high level of erbB2 expression by cancer cells allows their targeting using an erbB2-binding peptide (LTVSPWY) attached to the proapoptotic α-tocopheryl succinate (α-TOS). Treating erbB2-low or erbB2-high cells with α-TOS induced similar levels of apoptosis, whereas α-TOS-LTVSPWY induced greater levels of apoptosis in erbB2-high cells. α-TOS rapidly accumulated in erbB2-high cells exposed to α-TOS-LTVSPWY. The extent of apoptosis induced in erbB2-high cells by α-TOS-LTVSPWY was suppressed by erbB2 RNA interference as well as by inhibition of either endocytotic or lysosomal function. α-TOS-LTVSPWY reduced erbB2-high breast carcinomas in FVB/N c-neu transgenic mice. We conclude that a conjugate of a peptide targeting α-TOS to erbB2-overexpressing cancer cells induces rapid apoptosis and efficiently suppresses erbB2-positive breast tumors.

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Introduction

Receptor tyrosine kinases, such as erbB2/HER2 (a product of the c-neu gene), render cancer cells resistant to apoptosis treatment and make patients with erbB2-positive carcinomas a therapeutic challenge (1, 2). The major problem associated with high expression of erbB2 is autophosphorylation of the receptor and the ensuing activation of growth signaling pathways and proangiogenic and antiapoptotic mechanisms.

ErbB2 is overexpressed in ~30% of breast cancers. A major complication associated with its high expression is linked to activation of Akt (3, 4), a serine/threonine kinase that promotes cellular survival (5) due to phosphorylation of proteins like caspase-9 (6), Bad (7), or Bax (8). Akt also causes activation of nuclear factor-κB (9) that controls expression of prosurvival genes, such as the inhibitor of apoptosis proteins family members (10, 11) and the caspase-8 inhibitor FLIP (12).

We have studied the molecular mechanism of the proapoptotic and antineoplastic activity of vitamin E analogues, epitomized by the redox-silent α-tocopheryl succinate (α-TOS; refs. 13, 14). This agent has been proven efficient in selective induction of apoptosis in cancer cells (15) and in suppression of experimental cancer (16–20). We showed that α-TOS can bypass mutations or deficiencies in important tumor suppressor genes such as p53 or p21Waf1/Cip1 (18). Recently, our results documented that α-TOS can also kill breast cancer cells overexpressing erbB2 (21).

A limiting point in efficient delivery of anticancer drugs is their uptake by malignant cells. Uptake of α-TOS is relatively slow because it, most likely, relies on passive diffusion. This follows from experiments, in which acidification of media enhanced uptake by breast cancer cells, suppressing the ensiing activation of growth signaling pathways and proangiogenic and antiapoptotic mechanisms.

Materials and Methods

Cell culture. ErbB2-low MCF-7 and erbB2-high MDA-MB-453 cells as well as the MCF-7/HER2-18 cells stably transfected with an erbB2 vector (24, 25) were cultured in DMEM supplemented with 10% FCS and antibiotics. To inhibit lysosomal function, culture medium was supplemented with 20 mmol/L NH₄Cl. In some cases, cells were cotreated with 25 μmol/L each of the pan-caspase, caspase-8, or caspase-9 inhibitors Z-VAD-FMK, Z-IETD-FMK, or Z-LEHD-FMK, respectively (Calbiochem, La Jolla, CA).

Synthesis of α-TOS-peptide conjugate. The H-Tyr(t-Bu)-Trp(t-Boc)-Pro-Ser(t-Bu)-Val-Thr(t-Bu)-Leu-Rink amide was prepared according to the standard Fmoc protocol on a LIPS Vario Peptide synthesizer. All acylation reactions were carried out for 1 h using a 10-fold excess of Fmoc-amino acids activated with TBTE (1 equivalent) in the presence of DipeA (2 equivalents) and HOBr (1 equivalent). The NH2-terminal conjugation was carried out by activation of α-TOS with 1 equivalent of PyBOP in the presence of HOBr (1 equivalent) and DiPEA (2 equivalents). The conjugated peptide was cleaved from the resin using trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5) for 2 h. The highly lipophilic peptide conjugate was extracted...
Transfections were carried out as described previously (26). In brief, wild-type (WT) and dominant-negative (DN; K44A) dynamin-2 (30). Rockford, IL). the blots were developed using the ECL kit (Pierce, Cruz Biotechnology) was used as a loading control. After incubation with 2.5 mol/L mitochondrially targeted coenzyme Q (MitoQ), a triphenylphosphonium adduct of coenzyme Q that accumulates in the mitochondrial inner membrane and acts as an efficient scavenger of mitochondrial ROS (28).

Western blotting. Cells were lysed and stored at −80°C until analysis. Protein levels were quantified using the bicinchoninic assay. Protein samples (80 µg per lane) were denatured, resolved using 12.5% SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was blocked and incubated overnight with anti-dynamin-2 or anti-erbB2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-α-actin IgG (Santa Cruz Biotechnology) was used as a loading control. After incubation with a horseradish peroxidase–conjugated secondary IgG (Amersham, Arlington Heights, IL), the blots were developed using the ECL kit (Pierce, Rockford, IL).

Cell transfection. The plasmids used were Bcl-xL-EGFP (29) and wild-type (WT) and dominant-negative (DN; K44A) dynamin-2 (30). Transfections were carried out as described previously (26). In brief, MDA-MB-453 cells at 50% to 70% confluence were supplemented with 5 µg DNA preincubated for 10 min with 10 µL LipofectAMINE 2000 (Life Technologies, Gaithersburg, MD) and 1 mL Opti-MEM. After 3 to 4 h, cells were washed and supplemented with complete DMEM and left for 24 h, at which stage the cells were incubated with complete DMEM supplemented with G418. The cells were maintained in the selection medium for at least five passages and assessed for protein expression by fluorescence microscopy (Bcl-xL) or Western blotting (dynamin-2).

RNA interference. ErbB2-specific and validated short interfering RNA (siRNA) oligonucleotides and nonspecific scrambled siRNA were purchased from Ambion (Austin, TX). Transfection of MDA-MB-453 cells with siRNA was done as reported elsewhere (27). Briefly, cells were allowed to reach ~50% confluence and supplemented with 60 µmol/L siRNA preincubated with Oligofectamine (Invitrogen, San Diego, CA) and overlayed with Opti-MEM. Cells were washed 24 h later with PBS, overlayed with complete DMEM, and cultured for additional 24 h, and transgene expression was confirmed before using the cells for further experiments.

Endocytosis assay. Low-density lipoprotein (LDL) uptake, mediated by endocytosis dependent on the LDL receptor, was assessed as reported (31). LDL was labeled with the fluorescent dye 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI) for 8 h at 37°C. MDA-MB-453 parental cells, and the WT and DN dynamin-2 transfectants at ~50% confluence were incubated with DiI-labeled LDL for 2 h at 37°C, and the level of internalized DiI-LDL estimated by flow cytometry. Endocytosis was also assessed using FITC-conjugated transferrin (FITC-Tr). In brief, FITC-Tr (Molecular Probes) at 0.1 µg protein per mL was added to cells at ~50% confluence; the cells were incubated in serum-free DMEM for 1 h at 37°C and assessed by flow cytometry.

Mouse tumor experiments. A colony of transgenic FVB/N202 c-neu mice carrying the rat HER-2/neu proto-oncogene driven by the mouse mammary tumor virus promoter on the H-2q FVB/N background (32) was established at the Griffith University Animal Facility and maintained.

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Figure 1. Induction of apoptosis in breast cancer cells exposed to α-TOS or α-TOS-LTVPWY. MCF-7 (A and B), MDA-MB-453 (C and D), and MCF-7/HER2-18 cells (E and F) were incubated with α-TOS (A, C, and E) or α-TOS-LTVPWY (B, D, and F) at 25 or 50 µmol/L for 6, 12, and 24 h and assessed for apoptosis using the Annexin V method. E, inset, level of expression of erbB2 in MCF-7, MDA-MB-453, and MCF-7/HER2-18 cells using Western blotting. Points, mean (n = 3); bars, SD. Representative Western blotting of three independent experiments. * and **, significantly different apoptosis data in erbB2-low and erbB2-high cells exposed to α-TOS or α-TOS-peptide at 25 and 50 µmol/L, respectively.
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under strict inbreeding conditions. The presence of the transgene was routinely confirmed by PCR. Some 70% of the female mice developed spontaneous mammary carcinomas with a mean latency time of >7 months. Female transgenic FVB/N c-neu mice bearing progressively growing tumors with mean volume of 25 mm^3 were randomly assigned to control or treatment groups. Tumor size was quantified by ultrasound imaging using the Vevo770 instrument and the 40-MHz RMV704 scan-head (both Visualsonics, Toronto, Ontario, Canada) allowing 40-μm resolution of individual scans as well as by digital calipers. Mice received treatment with the excipient (corn oil/4% ethanol) alone (control), 15 or 5 μmol α-TOS, or 5 or 2.5 μmol α-TOS-LTVSPWY given by i.p. injection every 3 to 4 days.

Animal experiments were done 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 Griffith University Animal Ethics Committee.

α-TOS analysis. Intracellular levels of α-TOS were quantified using an HPLC method (33).

Statistics. Data were obtained from at least three different experiments, and the results are presented as mean ± SD. Significance between data points was assessed using the Student’s t test, and data were considered significantly different when P < 0.05.

Results

We first tested whether conjugating α-TOS to a peptide recognized by erbB2 would target the vitamin E analogue to cells overexpressing the receptor. To do this, we prepared a conjugate of α-TOS with the heptapeptide LTVSPWY by its addition to the free carboxylate on the succinyl moiety of the vitamin E ester. We found that erbB2-low (MCF-7) and erbB2-high cells (MCF-7/HER2-18 and MDA-MB-453) were all similarly sensitive to α-TOS, whereas erbB2-low cells were relatively resistant to α-TOS-LTVSPWY (Fig. 1). However, the peptide conjugate of α-TOS was significantly more effective in inducing apoptosis in erbB2-high cells than was α-TOS (Fig. 1). The LTVSPWY peptide itself did not induce any detectable apoptosis (data not shown).

Previous reports showed that α-TOS induced apoptosis by the intrinsic pathway (26, 27, 34, 35). We tested whether mitochondria and the ensuing events are also involved in killing of MDA-MB-453 cells exposed to α-TOS-LTVSPWY. We first studied the effect of α-TOS-LTVSPWY on generation of ROS and dissipation of ∆ψ_m as early indicators of apoptosis preceding the onset of the commitment phase. Figure 2A and B documents that the peptide conjugate caused a more rapid accumulation of ROS and ∆ψ_m dissipation in MDA-MB-453 cells than did α-TOS. MitoQ is a potent antioxidant that is targeted to the mitochondrial inner membrane (27), and using this agent revealed that the effect of α-TOS-LTVSPWY on mitochondrial destabilization was caused by mitochondrial derived ROS because inhibition of ROS accumulation and dissipation of ∆ψ_m by the addition of MitoQ also inhibited the peptide conjugate–induced apoptosis (data not shown). We next tested whether apoptosis induced by α-TOS-LTVSPWY was dependent on caspases. Figure 2C shows that the pan-caspase inhibitor and the caspase-9 inhibitor suppressed apoptosis induction in MDA-MB-453 cells exposed to both α-TOS-LTVSPWY and α-TOS, whereas this was not observed in case of the caspase-8 inhibitor. We also studied whether overexpression of the mitochondrial antiapoptotic protein Bcl-xL protects MDA-MB-453 cells from apoptosis induced by α-TOS-LTVSPWY. Figure 2D shows that apoptosis induced by α-TOS-LTVSPWY was inhibited in erbB2-overexpressing cells transfected with Bcl-xL. Collectively, these data confirm that mitochondria are important

Figure 2. α-TOS-LTVSPWY–induced apoptosis is mediated by the intrinsic pathway. MCF-7 and MDA-MB-453 cells were exposed to 50 μmol/L α-TOS or α-TOS-LTVSPWY for the periods (h) shown. The cells were evaluated for ROS generation using dihydroethidium (A) and for ∆ψ_m using JC-1 (B), C, effect of the pan-caspase, caspase-8 (Casp8), or caspase-9 (Casp9) inhibitors (all 25 μmol/L) on apoptosis induced by 50 μmol/L α-TOS or α-TOS-LTVSPWY, as evaluated by the Annexin V assay. D, effect of overexpression of Bcl-xL on apoptosis induction by 50 μmol/L α-TOS or α-TOS-LTVSPWY in MDA-MB-453 cells. Columns, mean (n = 3); bars, SD. *, significantly different values obtained in MCF-7 and MDA-MB-453 cells (A and B). **, significantly different data from control values (C and D). MFI, mean fluorescence intensity.
for apoptosis induction by the peptide conjugate in erbB2-overexpressing cells.

To find out whether rapid killing of MDA-MB-453 cells by α-TOS-LTVSPWY was due to high levels of erbB2, we knocked down the receptor using erbB2 siRNA. Figure 3A shows that siRNA treatment for 48 h suppressed expression of erbB2. MDA-MB-453 cells pretreated with erbB2 siRNA were significantly more resistant to α-TOS-LTVSPWY but not to α-TOS alone (Fig. 3B).

A plausible explanation for the greater efficacy of apoptosis induced in erbB2-overexpressing cells by α-TOS-LTVSPWY is that the conjugate is rapidly endocytosed. To address this, we inhibited endocytosis of MDA-MB-453 cells by overexpression of DN dynamin-2 (30). Figure 4A reveals that the transfected cells expressed higher levels of either the WT or DN dynamin-2, and that the resulting inhibition of endocytosis suppressed apoptosis induced in MDA-MB-453 cell by the α-TOS-peptide conjugate, whereas levels of apoptosis triggered by α-TOS alone in either the control or the endocytosis-inhibited cells were not affected. Figure 4A (inset) documents that DN dynamin-2 inhibits endocytosis because overexpression of DN dynamin-2 inhibited the uptake of both LDL and transferrin.

An important feature of the peptide conjugate of α-TOS is that the bond between the heptapeptide and the vitamin E analogue can be hydrolyzed once inside cells, liberating the apoptogenic, free α-TOS. This follows from our previous work, in which we showed that masking the free carboxylate of vitamin E analogues by methylation negated their ability to kill cancer cells (36, 37). We tested whether lysosomal function was important for apoptosis induction by α-TOS-LTVSPWY due to presence of peptidases in the organelles. Addition of ammonium chloride to cells, which buffers the acidic pH of lysosomes, suppressed apoptosis induction in MDA-MB-453 cells by α-TOS-LTVSPWY, whereas apoptosis induced by α-TOS alone was not affected (Fig. 4B). This suggests involvement of lysosomes in conversion of the α-TOS-peptide conjugate into the proapoptotic α-TOS.

The above results support a role for overexpression of erbB2 in rapid uptake of α-TOS-LTVSPWY and in liberating the apoptogenic α-TOS. We therefore evaluated whether intracellular levels of free α-TOS in MCF-7 and MDA-MB-453 cells were altered after exposure to α-TOS-LTVSPWY or α-TOS. Figure 5 shows that MCF-7 cells accumulated free α-TOS more rapidly when exposed to the vitamin E analogue than when incubated with α-TOS-LTVSPWY. On the other hand, MDA-MB-453 cells accumulated free α-TOS fast when exposed to α-TOS-LTVSPWY, while showing slow α-TOS accumulation when exposed to the free vitamin E analogue. Very slow kinetics of α-TOS accumulation was observed when MDA-MB-453K44A cells were exposed to α-TOS-LTVSPWY (Fig. 5B), or when MDA-MB-453 cells were exposed to the peptide conjugate of the vitamin E analogue in the presence of ammonium chloride (Fig. 5C).

To determine the anticancer efficacy of the peptide conjugate of α-TOS, transgenic mice were used that spontaneously form ductal breast carcinomas due to overexpression of erbB2 in the mammary epithelial cells (32). Female mice that developed breast tumors were injected with α-TOS (15 or 5 μmol per mouse per dose) or α-TOS-LTVSPWY (5 or 2.5 μmol per mouse per dose), and the volume of the tumors was measured by ultrasound imaging, allowing precise quantification of tumors, as well as by calipers. Figure 6 shows that treatment with the peptide conjugate at 5 μmol reduced the initial volume of breast carcinomas by some 70% at a 3-fold lower dose than that of α-TOS, which reduced the tumors by about 50%. The effect of the α-TOS-peptide conjugate at 2.5 μmol was initially similar to that of α-TOS at 15 μmol but failed to reduce the tumor size, as observed also for α-TOS at 5 μmol. The effect of reduction in tumor size was apparent after one dose of the peptide-α-TOS conjugate at 5 μmol, whereas this was delayed when α-TOS at 15 μmol was used. We also compared the use of ultrasound...
imaging and calipers for monitoring tumor volume. The data shown in Fig. 6A and B clearly document the superiority of ultrasound imaging over calipers. In many cases, majority of the tumor is embedded in the body of the animal with as little as 10% of the tumor accessible for mechanical measurement.

Discussion

We present here a novel paradigm for cancer therapy based on conjugation of anticancer agents with peptides recognized by cell surface receptors preferentially expressed by malignant cells. The evidence we have provided for this strategy here involves modification of an apoptogenic compound from the group of vitamin E analogues (α-TOS) with a peptide recognized by the tyrosine receptor kinase erbB2. We show that (a) the conjugate of α-TOS and the heptapeptide LTVSPWY caused greater levels of apoptosis in erbB2-high than erbB2-low cells; (b) down-regulation of erbB2 partially inhibited α-TOS-LTVSPWY–induced apoptosis in erbB2-high cells; (c) apoptosis induced by α-TOS-LTVSPWY in erbB2-high cells was dependent on endocytosis and lysosomal function; and (d) that α-TOS-LTVSPWY efficiently suppressed the growth of breast carcinomas in c-neu transgenic mice.

Our hypothesis was based on a study by Shadidi and Sioud (23), showing that addition of specific peptides to small molecules rendered them selective for breast cancer cells. Using the phage display approach, they identified a specific heptapeptide (LTVSPWY) whose attachment to the green fluorescent protein caused rapid internalization of the fluorescent protein by breast cancer cells expressing erbB2. We document here that α-TOS-LTVSPWY was more rapidly endocytosed by erbB2-overexpressing cells than by erbB2-low cells.

Shadidi and Sioud (23) presented data from experiments using the peptide targeting the erbB2 antisense oligodeoxynucleotides, which resulted in specific down-regulation of the receptor. Supported by our results, we present an intriguing paradigm for cancer therapy because this approach can be used for targeting of divergent molecules to cells overexpressing specific surface receptors. Molecules delivered by this approach include antisense oligodeoxynucleotides, ribozymes, and siRNAs, the targeting of which may be used in treatment of different pathologies without adversely affecting healthy tissue (38–42). There are reports that describe targeting of cytotoxic drugs to tumor cells encapsulated in carriers, including peptide-coated liposomes, as documented in both in vitro and in vivo models (43, 44).

Our results are consistent with those of Song et al. (41), who showed that an erbB2 single-chain antibody fused with protamine targeted erbB2 siRNA preferentially to erbB2-overexpressing breast carcinoma cells. We present data showing that erbB2-high breast cancer cells are more prone to killing by α-TOS-LTVSPWY.
than their erbB2-low counterparts. Thus, the difference in the levels of the receptor determines, at least to some extent, the susceptibility of the cells to the peptide conjugate of α-TOS. The erbB2-low MCF-7 underwent apoptosis when challenged with α-TOS-LTVSPWY, albeit at a lower rate than did erbB2-high cells, most probably reflecting the greatly reduced levels of the receptor in these cells (45), which results in reduced binding and uptake of the peptide conjugate. Hence, the level of expression of erbB2 was shown to provide selective killing by the peptide conjugate, and we can expect that normal cells, with low erbB2 expression, will not be killed by the peptide-vitamin E analogue conjugate.

Our results with cells in which endocytosis was blocked by overexpression of DN dynamin-2 suggest that internalization of α-TOS-LTVSPWY by breast cancer cells is dependent on endocytosis via clathrin-coated vesicles because dynamin-2 is essential for the formation and release of these vesicles and their conversion into endosomes (29). Therefore, it can be postulated that after binding to the erbB2 receptor, the peptide with the drug is endocytosed. In the mildly acidic late endosome, the receptor is detached from the α-TOS-peptide conjugate, which travels to the lysosome, where acidic peptidases liberate free α-TOS from the peptide conjugate. This is an important event in the peptide-α-TOS processing by the cell because free carboxylate is required for the proapoptotic activity of α-TOS (36, 37). Free vitamin E succinate then induces apoptosis by targeting mitochondria, leading to ROS generation and dissipation of ΔΨm followed by activation of caspases and entry of the cell to the commitment phase of apoptosis (cf. Fig. 2; refs. 26, 35).

Importantly, efficient uptake of the peptide conjugate of α-TOS results in pronounced suppression of breast carcinomas, as documented by experiments with c-neu transgenic mice expressing high levels of erbB2 in the mammary epithelial cells (32). Data in Fig. 6 reveal that the peptide conjugate was more efficient than when α-TOS alone was used at thrice higher doses. The superiority of the peptide conjugate may be due to both greater selective uptake by the erbB2-high cancer cells and greater efficacy of the conjugate to deliver free α-TOS inside the cells, resulting in rapid apoptosis.

In summary, we present in this report an intriguing paradigm for cancer therapy, according to which an inducer of apoptosis, epitomized by α-TOS, is modified by addition of the peptide LTSPWY. The conjugate is rapidly endocytosed by erbB2-overexpressing cells and internalized within endosomes. Their maturation into lysosomes results in hydrolysis of α-TOS-LTVSPWY by the action of acidic peptidases. The liberated α-TOS, a potent mitocan (46, 47), then compromises the mitochondrial function via generation of ROS and dissipation of ΔΨm. This leads to activation of the mitochondria-specific caspase-9 and entry of the cell into the apoptosis commitment phase. This approach, based on targeting of anticancer drugs to malignant cell, may be of clinical relevance.

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Figure 6. α-TOS-LTVSPWY suppresses breast carcinomas. FVB/N c-neu female mice at 7 to 10 months of age with breast carcinomas, as detected by ultrasound imaging, were treated by i.p injection of the vehicle, 15 or 5 μmol per mouse per dose of α-TOS, or 5 or 2.5 μmol per mouse per dose of α-TOS-LTVSPWY (α-TOS-P). The tumors were visualized and quantified using ultrasound imaging (A) or calipers (B) and are expressed relative to their volume at the onset of the treatment. C, representative images of a tumor from an animal treated with 5 μmol of α-TOS-LTVSPWY at times shown. Points, mean (n = 4–6); bars, SD. * significant differences between the control animals and animals treated with α-TOS-peptide at 5 μmol per dose per mouse.

References
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