α-Tocopheryl Succinate Induces Apoptosis in Prostate Cancer Cells in Part through Inhibition of Bcl-xL/Bcl-2 Function*

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Chung-Wai Shiau‡, Jui-Wen Huang‡, Da-Sheng Wang‡, Jing-Ru Weng‡, Chih-Cheng Yang‡, Chia-Hui Lin‡, Chenglong Li‡, and Ching-Shih Chen‡†

From the ‡Division of Medicinal Chemistry, College of Pharmacy, Ohio State University, Columbus, Ohio 43210 and the †Department of Biological Science and Technology, China Medical University and Hospital, Taichung, Taiwan 404

Although the antitumor effect of α-tocopheryl succinate (vitamin E succinate) has been well demonstrated, its underlying mechanism remains elusive. This study provides evidence that inhibition of Bcl-xL/Bcl-2 function represents a major pathway whereby α-tocopheryl succinate mediates apoptosis induction in prostate cancer cells. In vitro data indicate that α-tocopheryl succinate was able to disrupt the binding of Bak BH3 peptide to Bcl-xL and Bcl-2 with IC50 of 26 μM, in line with its potency in antiproliferation. Treatment of PC-3 cells with this agent led to reduced association of Bcl-2 and Bcl-xL with Bak, leading to caspase-dependent apoptosis. Moreover, overexpression of Bcl-xL protected LNCaP cells from the apoptosis induction. This mechanistic finding provided a basis to develop potent Bcl-xL/Bcl-2 inhibitors. Docking of α-tocopheryl succinate into the Bak peptide-binding site indicates that it adopted a unique hairpin-shaped conformation for protein interactions. We rationalized that the hemiscuccinate and the two proximal isopropyl units of the side chain played a crucial role in ligand anchoring and protein-ligand complex stabilization, respectively. However, exposure of the distal isopropyl unit to a polar environment might diminish the binding affinity of α-tocopheryl succinate. This premise was corroborated by a structure–activity analysis of a series of derivatives with truncated side chains and/or altered carboxyl terminus. This computer model predicted that the removal of the distal isopropyl unit from the side chain would improve binding affinity, leading to two agents with significantly higher potency in inhibiting Bak peptide binding and in suppressing prostate cancer cell proliferation.

Although numerous epidemiological and intervention studies have failed to establish a correlation between vitamin E intake and the incidence of cancer, recent investigations have suggested the potential use of α-tocopherol succinate (vitamin E succinate) as a therapeutic agent for cancer (for reviews see Refs. 1 and 2). Evidence indicates that α-tocopheryl succinate induces apoptosis in cells with a malignant or transformed phenotype without incurring significant toxicity to normal cells (3–6). Moreover, its formed phenotype without incurring significant toxicity to normal cells (3–6). Furthermore, in vivo experiments, including suppression of breast and melanoma tumor growth (7–9), inhibition of colon cancer liver metastases of animal model experiments, including suppression of breast and melanoma tumor growth (7–9), inhibition of colon cancer liver metastases of animal model experiments, including suppression of breast and melanoma tumor growth (7–9), inhibition of colon cancer liver metastases of animal model experiments, including suppression of breast and melanoma tumor growth (7–9), inhibition of colon cancer liver metastases of animal model experiments, including suppression of breast and melanoma tumor growth (7–9), inhibition of colon cancer liver metastases of animal model experiments, including suppression of breast and melanoma tumor growth (7–9), inhibition of colon cancer liver metastases of animal model experiments, including suppression of breast and melanoma tumor growth (7–9), inhibition of colon cancer liver metastases of animal model experiments, including suppression of breast and melanoma tumor growth (7–9), inhibition of colon cancer liver metastases (3,6). Sensitization of colon tumor cells to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (11). Despite these advances, the mechanism underlying the effect of this redox-inactive vitamin E derivative on apoptosis remains elusive. Putative signaling mechanisms include inhibition of cyclin A binding to the transcription factor E2F (12), mitochondrial destabilization through sphinogomelysinase activation (13), suppression of NFκB activation (14), activation of protein kinase C α (15), and up-regulation of mitogen-activated protein kinase signaling (16, 17). Another school of thought is that α-tocopheryl succinate represents a provitamin form, of which the conversion to vitamin E occurs selectively in tumor interstitium by its acidic environment (1).

In this study, we obtained several lines of evidence that Bcl-xL represented a major target by which α-tocopheryl succinate mediated antiapoptotic activities in prostate cancer cells. This finding not only shed light onto the mode of action of this redox-inactive vitamin E analogue but also provided a molecular basis to carry out structure-based optimization to generate potent Bcl-xL inhibitors.

EXPERIMENTAL PROCEDURES

Cell Culture—LNCaP androgen-dependent (p53+/−) and PC-3 androgen-nonresponsive (p53−/−) prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA). The preparation of the stable Bcl-xL-overexpressing LNCaP clone B3 (LNCaP/B3) was previously described (18). PC-3, LNCaP, and LNCaP/B3 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% carbon dioxide. Normal human prostate epithelial cells (PrEC) were purchased from Cambrex Bio Science Walkersville, Inc. (East Tutherford, NJ). Cells were maintained in prostate epithelial cell medium with growth supplements at 37 °C in a humidified incubator containing 5% carbon dioxide. The recommended seeding density for subculture is 2,500 cells/cm2. It takes 6–9 days from subculture to attain confluency.

Reagents—α-Tocopherol, α-tocopheryl succinate, 2,2,5,7,8-pentamethyl-6-chromanol, and other chemical reagents required for the synthesis of various analogues were purchased from Sigma Aldrich unless otherwise indicated. Synthesis of TS-1 (succinic acid mono-[2-(4,8-dimethyl-nonyl)-2,5,7,8-tetramethyl-chroman-6-yl] ester), TS-2 (succinic acid mono-[2,5,7,8-tetramethyl-2-(4-methyl-pentyl)-chroman-6-yl] ester), TS-3 (succinic acid mono-[2,5,7,8-pentamethyl-chroman-6-yl] ester), TS-4 (2-(4,8-dimethyl-nonyl)-2,5,7,8-tetramethyl-chroman-6-yl) ester), TS-5 (3-[2,5,7,8-tetramethyl-2-(4,8-dimethyl-nonyl)]-chroman-6-yl)propionic acid) will be published elsewhere. The identity and purity (≥99%) of these

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1 To whom correspondence should be addressed: College of Pharmacy, 336 Parks Hall, The Ohio State University, 500 West 12th Ave., Columbus, OH 43210. Tel.: 614-688-4008; Fax: 614-688-8556; E-mail: chen.844@osu.edu.

2 The abbreviations used are: FBS, fetal bovine serum; PrEC, prostate epithelial cell; TS-1, succinic acid mono-[2-(4,8-dimethyl-nonyl)-2,5,7,8-tetramethyl-chroman-6-yl] ester; TS-2, succinic acid mono-[2,5,7,8-tetramethyl-2-(4-methyl-pentyl)-chroman-6-yl] ester; TS-3, succinic acid mono-[2,5,7,8-pentamethyl-chroman-6-yl] ester; TS-4, (2-(4,8-dimethyl-nonyl)-2,5,7,8-tetramethyl-chroman-6-yl) propanoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; PARP, poly(ADP-ribose) polymerase; PIPES, 1,4-piperazinediethanesulfonic acid.
synthetic derivatives were verified by proton nuclear magnetic resonance, high resolution mass spectrometry, and elemental analysis. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)) for cell viability assay were purchased from TCI America, Inc. (Portland, OR). The Cell Death Detection ELISA kit was purchased from Roche Diagnostics. Rabbit antibodies against Bcl-xL, Bax, Bak, Bid, PARP, and cleaved caspases-9 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit antibodies against Bad, cytochrome c, and mouse anti-Bcl-2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-actin was from ICN Biomedicals, Inc. (Costa Mesa, CA).

Cell Viability Analysis—The effect of individual test agents on cell viability was assessed by using the MTT assay in 6–12 replicates. PC3, LNCaP, and B3-LNCaP cells were seeded and incubated in poly-p-lysine-coated, 96-well, flat-bottomed plates in RPMI 1640 medium supplemented with 10% FBS medium for 24 h. PrEC cells were seeded at the recommended density in 96-well, flat-bottomed plates in prostate epithelial cell medium with growth supplements for 3 days. All cells were exposed to various concentrations of test agents dissolved in ethanol (for α-tocopherol, α-tocopheryl succinate, and TS-3) or MeSO (all other test agents used) with a final concentration of 0.1% in serum-free RPMI 1640 medium for PC3, LNCaP, and LNCaP/B3 cells or in prostate epithelial cell basal medium with growth supplements for PrEC cells. Controls received MeSO or ethanol vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200 μl of 0.5 mM MTT in 10% FBS-containing RPMI 1640 medium, and cells were incubated in the CO2 incubator at 37 °C for 2 h. Supernatants were removed from the wells, and the reduced MTT dye was solubilized in 200 μl/well of MeSO. Absorbance at 570 nm was determined on a plate reader.

Apoptosis Detection by ELISA—Induction of apoptosis was assessed with a cell death detection ELISA kit (Roche Diagnostics) by following the manufacturer’s instruction. This test is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes and oligonucleosomes after induced apoptotic death. In brief, 5 × 10⁶ cells were cultured in a T-25 flask in 10% FBS-containing medium for 24 h and were treated with the test agents at various concentrations in serum-free medium for 24 h. Both floating and adherent cells were collected; cell lysates equivalent to 5 × 10⁵ cells were used in the ELISA.

Western Blot Analysis of Cytochrome c Release into the Cytoplasm—Cytoxic-specific, mitochondria-free lysates were prepared according to an established procedure (18). In brief, after individual treatments for 24 h, both the incubation medium and adherent cells in T-75 flasks were collected and centrifuged at 600 × g for 5 min. The pellet fraction was recovered, placed on ice, and triturated with 100 μl of a chilled hypotonic lysis solution (50 mM PIPES-KOH (pH 7.4) containing 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EDTA, 2 mM MgCl₂, 1 mM dithiothreitol, and a mixture of protease inhibitors including 100 μM aprotonin, 100 μM bestatin, 1.5 μM E-64 protease inhibitor, 2 μM leupeptin, and 1 μM pepstatin A). After a 45-min incubation on ice, the mixture was centrifuged at 600 × g for 10 min. The supernatant was collected in a microcentrifuge tube and centrifuged at 14,000 rpm for 30 min. An equivalent amount of protein (50 μg) from each supernatant was resolved in 15% SDS-polyacrylamide gel. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-cytochrome c antibodies as described below.

Bcl-xL Knockdown by siRNA—To validate the role of Bcl-xL in α-tocopheryl succinate-mediated apoptosis, we used siRNA against Bcl-xL from Santa Cruz Biotechnology (Santa Cruz, CA) to attenuate Bcl-xL expression in PC-3 cells. In brief, PC-3 cells were seeded in 6-well plates at the density of 4 × 10⁵ cells/well in 2 ml of 10% FBS-supplemented RPMI 1640 medium and, after a 24-h incubation, were washed with 2 ml of antibiotic-free normal growth medium supplemented with 10% FBS. For each transfection, the indicated amount of siRNA duplex and 6 μl of siRNA transfection reagent (Santa Cruz Biotechnology) were each added into 100 μl of siRNA transfection medium (Santa Cruz Biotechnology), incubated for 5 min separately, mixed, incubated for additional 30 min, and added to 800 μl of siRNA transfection medium. This mixture was added to PC-3 cells in each well, and the transfected cells were incubated at 37 °C for 5 h, and an equal volume of 20% FBS-containing RPMI 1640 medium was added without removing the transfection medium. The transfected cells were incubated with the medium for another 36 h before cells were lysed for Western blot analysis of Bcl-xL expression.

Immunoct blotting—Cells were seeded in 10% FBS-containing RPMI 1640 medium for 24 h and treated with various agents as aforementioned. After individual treatments for 24 h, the adherent cells in T-25 or T-75 flasks were scraped, combined with the medium, and centrifuged at 2,200 rpm for 10 min. The supernatants were recovered, placed on ice, and triturated with 20–50 μl of a chilled lysis buffer (M-PER, mammalian protein extraction reagent, Pierce), to which was added 1% protease inhibitor mixture (set III, EMD Biosciences, Inc., San Diego, CA). After a 30-min incubation on ice, the mixture was centrifuged at 16,100 × g for 3 min. Two μl of the suspension was taken for protein analysis using the Bradford assay kit (Bio-Rad); to the remaining solution the same volume of 2 × SDS-polyacrylamide gel electrophoresis sample loading buffer (100 μl Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.1% bromphenol blue) was added. The mixture was boiled for 10 min. Equal amounts of proteins were loaded onto 8–12% SDS-polyacrylamide gels. After electrophoresis, protein bands were transferred to nitrocellulose membranes in a semidy transfer cell. The transblotted membrane was blocked with Tris-buffered saline, 0.1% Tween 20 (TBST) containing 5% nonfat milk for 90 min, and the membrane was incubated with the appropriate primary antibody in TBST, 5% nonfat milk at 4 °C overnight. After washing three times with TBST for a total of 45 min, the transblotted membrane was incubated with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (diluted 1:1,000) for 1 h at room temperature and washed four times with TBST for a total of 1 h. The immunoblots were visualized by enhanced chemiluminescence.

Competitive Fluorescence Polarization Assay—The binding affinity of the test agent to Bcl-Xₙ was analyzed by a competitive fluorescence polarization assay in which the ability of the agent to displace the binding of a Bak BH3-domain peptide to either Bcl-2 or Bcl-Xₙ was determined. Flu-BakBH3, a Bak-BH3 peptide labeled at the NH₂ terminus with fluorescein, was purchased from Genemed Synthesis (San Francisco, CA). COOH-terminus-truncated, His-tagged Bcl-Xₙ was purchased from EMD Biosciences (San Diego, CA) and soluble glutathione S-transferase-fused Bcl-2 was obtained from Santa Cruz Biotechnology. The binding analysis was carried out in a dual path length quartz cell with readings taken at 480 nm and 530 nm at room temperature using a luminescence spectrometer according to an established procedure (19).

Determination of IC₅₀ Values—Data from cell viability and fluorescence polarization assays were analyzed by using the CalcuSyn software (Biosoft, Ferguson, MO) to determine IC₅₀ values, in which the calculation was based on the medium effect equation i.e. \( \log(\text{fa/fix}) = \log(D) - \log(D_{\text{m}}) \), where fa and fix denote fraction affected and unaffected, respectively, m represents the Hill-type coefficient signify-
ing the sigmoidicity of the dose-effect curve, and $D$ and $D_\text{ed}$ are the dose used and $IC_{50}$, respectively.

Co-immunoprecipitation—PC3 cells treated with 40 $\mu$M $\alpha$-tocopheryl succinate or 10 $\mu$M TS-1 for 24 h were scraped off the flask, transferred into centrifuge tubes, and centrifuged at 2,200 rpm for 10 min to pellet the cells. The pellet was resuspended in ice-cold 0.5 ml of radioimmunoprecipitation assay buffer ($50 \text{ mM Tris-Cl, pH 7.4, 1\% Nonidet P-40, 0.25\% sodium deoxycholate, 150 \text{ mM NaCl, 1\ mM EDTA, and 1\% protease inhibitor mixture}$) and gently mixed on an orbital shaker at 4 $^\circ\text{C}$ for 15 min, followed by centrifugation at 14,000 $\times$ g for 15 min to yield cell lysates. These cell lysates were treated with 100 $\mu$l of protein A-agarose bead slurry followed by brief centrifugation to remove nonspecific binding proteins. Equal amounts of proteins from these lysates, as determined by the Bradford assay, were mixed with anti-Bcl-2 or anti Bcl-X$_L$ antibodies in an orbital shaker at 23 $^\circ\text{C}$ for 2 h, followed by 100 $\mu$l of protein A-agarose bead slurry at 4 $^\circ\text{C}$ for 12 h. The immunocomplex was collected by brief centrifugation, washed four times with 800 $\mu$l of ice-cold radioimmunoprecipitation assay buffer, and suspended in 50 $\mu$l of 2 $\times$ SDS sample loading buffer. The suspension was boiled for 10 min, cooled, and briefly centrifuged to remove the beads. Western blot analysis with antibodies against Bak as described above.

Molecular Modeling—Human Bcl-xL crystal structure, obtained from the Brookhaven Protein Data bank (entry code of 1R2D) (20), was subject to the deletion of water molecules, the addition of all hydrogens, and the assignment of Gasteiger charges (21) and then nonpolar hydrogens were merged. Three-dimensional affinity grids centered on the Bak peptide-binding site with 0.375-Å spacing were calculated for each of the following atom types: (a) protein, A (aromatic C), C, HD, N, NA, OA, SA; (b) ligands, C, A, N, NA, OA, S, SA, HD, Br, e (electrostatic), and d (desolvation) using Autogrid4. AutoDock version 4.0.0 was used for the docking simulation. We selected the Lamarckian genetic algorithm for ligand conformational searching because it has enhanced performance relative to simulated annealing or the simple genetic algorithm. The translation, rotation, and internal torsions of the ligand are defined as its state variables, and each gene represents a state variable. The Lamarckian genetic algorithm adds local minimization to the genetic algorithm, enabling modification of the gene population. For each compound, the docking parameters were as follows: trials of 100 dockings, population size of 150, random starting position, and conformation, translation step ranges of 2.0 Å, rotation step ranges of 50°, elitism of 1, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06, and 100 million energy evaluations. Final docked conformations were clustered using a tolerance of 2.0 Å root mean square deviation.

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**FIGURE 1.** Differential sensitivity of PC-3, LNCaP, and Bcl-xL-overexpressing LNCaP (LNCaP/B3) cells to $\alpha$-tocopheryl succinate-induced apoptosis. A, dose-dependent effects of $\alpha$-tocopheryl succinate on the viability of PC-3 (curve a), LNCaP (curve b), and LNCaP/B3 (curve c) cells after 24 h exposure in serum-free RPMI 1640 medium. Points, mean; bars, S.D. (n = 6). Inset, expression levels of Bcl-XL in PC-3 (a), LNCaP (b), and LNCaP/B3 (c) cells. B, evidence of apoptotic death in $\alpha$-tocopheryl succinate-treated PC-3 cells. Top panel, formation of nucleosomal DNA in PC-3 cells that were treated with $\alpha$-tocopheryl succinate at the indicated concentrations for 24 h. DNA fragmentation was quantitatively measured by a cell death detection ELISA kit. Columns, mean; bars, S.D. (n = 3). Bottom panel, cytoplasm and PARP proteolysis to the apoptosis-specific 85-kDa fragment induced by different doses of $\alpha$-tocopheryl succinate in PC-3 cells. PC-3 cells were treated with the drug at the indicated doses for 24 h in serum-free RPMI 1640 medium. Equivalent amounts of proteins from mitochondrial-free cell lysates were electrophoresed and probed by Western blotting with the respective antibodies. C, dose-dependent effects of $\alpha$-tocopheryl succinate on the viability of PC-3 and LNCaP cells after 24-h exposure in 5% FBS-containing RPMI 1640 medium. Points, mean; bars, S.D. (n = 6). Inset, expression levels of Bcl-xL in serum-free and 5% FBS-supplemented medium in PC-3 and LNCaP cells. D, dose-dependent effects of $\alpha$-tocopheryl succinate on PARP cleavage in LNCaP and PC-3 cells after 24-h exposure in 5% FBS-containing RPMI 1640 medium.
RESULTS

Differential Susceptibility of LNCaP and PC-3 Prostate Cancer Cell Lines to α-Tocopheryl Succinate—As part of our effort to understand the mode of action of α-tocopheryl succinate, we examined its antiproliferative effect in two human prostate cancer cell lines, LNCaP and PC-3. Of these two types of cells, LNCaP cells were more susceptible to the proliferation inhibition than PC-3 cells, with IC_{50} values of 15 and 40 μM, respectively, in serum-free medium (Fig. 1A, curves a and b, respectively). This reduction in cell viability was, at least in part, resulting from mitochondria-dependent apoptosis induction, as evidenced by DNA fragmentation, cytchrome c release, and PARP cleavage (Fig. 1B). As both LNCaP and PC-3 cells exhibit up-regulated phosphatidylinositol 3-kinase/Akt signaling due to loss of PTEN function, this differential sensitivity might be attributable to differences in the respective ability to maintain mitochondrial integrity in response to apoptotic signals. Data from this and other laboratories have demonstrated that PC-3 cells were resistant to the apoptosis-inducing effect of many therapeutic agents because of Bcl-xL overexpression (18, 22). This premise is supported by the inverse relationship between increased expression levels of Bcl-xL and susceptibility to the apoptosis-inducing effects of α-tocopheryl succinate in these three cell lines. For example, in the presence of 5% FBS, the IC_{50} values of α-tocopheryl succinate in inducing apoptosis in LNCaP and PC-3 cells were 25 and 53 μM, respectively (Fig. 1, inset). This decrease in sensitivity was associated with serum-stimulated increases in Bcl-xL expression in both cell lines (Fig. C, inset).

Ectopic Bcl-xL Expression Protects LNCaP Cells from α-Tocopheryl Succinate-induced Apoptosis—To establish a mechanistic link between Bcl-xL and α-tocopheryl succinate-induced cell death, we assessed the effect of enforced Bcl-xL expression in a stably transfected LNCaP clone (LNCaP/B3). The expression level of ectopic Bcl-xL in B3 cells was ∼5-fold of that of the endogenous counterpart in PC-3 cells (Fig. 1A, inset), whereas that of Bcl-2 was slightly lower in the LNCaP/B3 cells (18). The high level of ectopic Bcl-xL expression in LNCaP/B3 cells substantially increased the resistance of LNCaP cells to α-tocopheryl succinate-induced cell death, to a degree greater than that of PC-3 cells (Fig. 1A).

α-Tocopheryl Succinate Is an Inhibitor of Bcl-xL Function—The above finding suggested that α-tocopheryl succinate-mediated apoptosis might involve the modulation of the function of Bcl-xL and/or other Bcl-2 members. Accordingly, we examined this putative link at both transcriptional and posttranscriptional levels. First, we assessed the dose-dependent effect of α-tocopheryl succinate on the expression of different Bcl-2 family members in PC-3 cells, including Bcl-xL, Bcl-2, Bak, Bak, Bad, and Bid by Western blotting. Fig. 2A indicates that with the exception of a decrease in Bad expression, the exposure to α-tocopheryl succinate did not cause appreciable changes in the expression level of these Bcl-2 members. Second, we used a competitive fluorescence polarization analysis to investigate the effect of α-tocopheryl succinate on the binding of a fluorescein-labeled Bak BH3 domain peptide to Bcl-xL and Bcl-2. Fig. 2B depicts the ability of α-tocopheryl succinate to disrupt the BH3 domain-mediated interactions with Bcl-xL and Bcl-2 with equal potency, with IC_{50} of 26 ± 2 μM.

To confirm the mode of action of α-tocopheryl succinate, we assessed the intracellular effects on the dynamics of Bcl-xL/Bak and Bcl-2/Bak interactions in PC-3 cells. Lysates from PC-3 cells treated with α-tocopheryl succinate at 20 μM for 12 h were immunoprecipitated with antibodies against Bcl-xL or Bcl-2. Probing of the immunoprecipitates with anti-Bak antibodies by Western blotting indicates that the level of Bak associated with Bcl-xL and Bcl-2 was significantly reduced compared with the Me_{2}SO control (Fig. 2C, upper panel). This decrease in intracellular association bode out the above in vitro binding data. As Bcl-xL and Bcl-2 abrogated the effects of Bak and other proapoptotic Bcl-2 members through BH3 domain-mediated heterodimerization, we also showed that this decrease in Bak binding was accompanied by caspase-9 activation in a dose-dependent manner in drug-treated cells (Fig. 2C, lower panel).

Together, these data demonstrate that the effect of α-tocopheryl succinate on apoptosis in prostate cancer cells was, at least in part, mediated through the inhibition of Bcl-xL function by disrupting BH3 domain-mediated heterodimerization. We also showed that this decrease in Bak binding was accompanied by caspase-9 activation in a dose-dependent manner in drug-treated cells (Fig. 2C, lower panel).

Validation of the Involvement of Bcl-xL in α-Tocopheryl Succinate-mediated Apoptotic Death—To validate the role of Bcl-xL in α-tocopheryl succinate-induced apoptosis, we used siRNA to knockdown the expression of Bcl-xL. This silencing led to enhanced apoptotic death in a dose-dependent manner, as evidenced by increases in cytochrome c release, PARP cleavage, and nucleosome formation.

Molecular Docking of α-Tocopheryl Succinate into the Bak Peptide-binding Site of Bcl-xL—α-Tocopheryl succinate was docked into the Bak peptide-binding site that is located in a hydrophobic cleft bound by the BH1, BH2, and BH3 regions of Bcl-xL (20, 23–25). A docking analysis indicates that α-tocopheryl succinate adopted a unique hairpin-
shaped conformation in interacting with this hydrophobic pocket (Fig. 4A). As shown, the carboxyl terminus of the hemisuccinate formed electrostatic interactions and hydrogen bonding with the guanidino side chain of Arg-100. Although the chroman aromatic ring interacted with Tyr-101 and Phe-105 through π-π interactions, the phytyl chain coiled back to gain access to the hydrophobic side chain of Leu-108, Leu-130, and Ala-142. However, the terminal isopranyl unit of the aliphatic long chain overhung into a polar region that consisted of Asn-136, the amide backbone of Trp-137, Gly-138, Arg-130 located at the beginning end of a large helical dipole, and solvent.

α-Tocopheryl Succinate Derivatives with Truncated Side Chains Exhibit Higher Potency in Bcl-xL Inhibition—This computer model shed light onto the mode of binding of α-tocopheryl succinate to Bcl-xL and provided a molecular basis for structural optimization. We rationalized that the hemisuccinate and the two proximal isopranyl units of the side chain play a crucial role in ligand anchoring and stabilization of the protein-ligand complex, respectively. However, exposure of the distal isopranyl unit to a polar environment might diminish the binding affinity of α-tocopheryl succinate. This premise was corroborated by docking TS-1, an analogue with one isopranyl unit removed from the phytyl side chain, into the Bcl-xL binding domain (Fig. 4B). The mode of binding of this truncated analogue was analogous to that of α-tocopheryl succinate, however, without the unfavorable interaction with the protein. Theoretical ΔGbinding values were calculated to be −7.5 kcal/mol and −8.1 kcal/mol for α-tocopheryl succinate and TS-1, respectively, of which the discrepancy would give rise to a 3-fold difference in binding affinity.

To validate the above modeling data, we carried out structural modifications of α-tocopheryl succinate by gradually removing the isopranyl unit from its phytyl side chain, yielding TS-1, TS-2, and TS-3 (Fig. 5A). In addition, TS-4 and TS-5 were synthesized to verify the role of the terminal carboxylic function in ligand anchoring, which represented TS-1 analogues with the hemisuccinate removed and replaced with an ether-linked propionate, respectively.

Functional assays indicate that the potency of these derivatives vis-à-vis α-tocopheryl succinate in inhibiting Bak peptide-Bcl-xL binding paralleled that of suppressing cell viability (Fig. 5B). The potency was in the order of TS-1, TS-5 > TS-2 > α-tocopheryl succinate, whereas TS-3 and TS-4 lacked appreciable activity even at 100 μM. There existed a 3-fold difference in IC50 between TS-1 and α-tocopheryl succinate in blocking Bak peptide binding to Bcl-xL, which is consistent with that of the theoretical calculation.

The differential inhibitory activity among α-tocopheryl succinate and its truncated analogues underlies the subtle impact of the length of the hydrophobic side chain on Bcl-xL binding. However, complete removal of the side chain in TS-3 abrogated the binding affinity, supporting the role of this hydrophobic interaction in the stabilization of protein-ligand complexes. In addition, replacement of the succinate with an ether-linked propionate had no effect on the Bcl-xL binding and anti-proliferative activities. This finding suggests that the carbonyl group of the hemisuccinate ester linkage was not involved in ligand binding, consistent with the modeled recognition mode (Fig. 4).

Evidence indicates that TS-1 mediated antiproliferative effects in PC-3 cells through the same mechanism as α-tocopheryl succinate. TS-1-induced apoptotic death was evidenced by DNA fragmentation,


**DISCUSSION**

Although the antitumor effect of α-tocopheryl succinate has been the focus of many recent investigations, the underlying mechanism has not yet been fully characterized. Here, we obtained several lines of evidence that inhibition of Bcl-xL/Bcl-2 function represents a major mechanism by which α-tocopheryl succinate mediates antitumor effect in prostate cancer cells. First, high expression levels of Bcl-xL render PC-3 and LNCaP/B3 cells resistant to α-tocopheryl succinate-induced apoptosis. Second, fluorescence polarization analysis demonstrates that α-tocopheryl succinate disrupted the binding of the Bak BH3 peptide to Bcl-xL. Third, the immunoprecipitation study demonstrates that the level of Bak associated with Bcl-xL and Bcl-2 was greatly reduced in drug-treated cells compared with Me2SO control. Fourth, the docking analysis of α-tocopheryl succinate into the Bak peptide-binding domain of Bcl-xL led to the development of more potent Bcl-xL inhibitors. α-Tocopheryl succinate exhibited equal potency in blocking Bak BH3 peptide binding to Bcl-xL and Bcl-2. Considering the pivotal role of Bcl-xL and Bcl-2 in regulating mitochondrial integrity, this mode of action provides a molecular framework to account for the effect of α-tocopheryl succinate on apoptotic death in cancer cells. More importantly, it constituted a mechanistic basis for the structural optimization of α-tocopheryl succinate to generate more potent Bcl-xL inhibitors such as TS-1 and TS-5. The ability of these inhibitors to disrupt the cytochrome c release, and PARP cleavage (Fig. 6A). Moreover, 10 μM TS-1 abolished the intracellular binding of Bak to Bcl-xL and Bcl-2 in PC-3 cells (Fig. 6B). As shown in Fig. 6C, normal PrECs were resistant to the antiproliferative effect of α-tocopheryl succinate, TS-1, and TS-5 even at high doses, whereas PC-3 cells exhibited differential susceptibility to these agents under the same conditions. This differential sensitivity between PrECs and PC-3 cells indicates that the effect of TS-1 and TS-3 on apoptosis was tumor cell-specific.
dynamics in the interactions between antiapoptotic and proapoptotic Bcl-2 members leads to the release of cytochrome c, thereby activating the apoptosis machinery. Previously, we obtained evidence that Bcl-xL overexpression provides a distinct survival mechanism independent of phosphatidylinositol 3-kinase/Akt signaling in prostate cancer cells (18), which underlies the resistance to apoptotic signals emanating from withdrawal of trophic factors and/or exposure to chemotherapeutic agents. Consequently, these α-tocopheryl succinate-derived Bcl-xL inhibitors have translational relevance to be developed into antitumor agents for the prevention and/or therapy of cancer alone or in combination with other treatment. For example, α-tocopheryl succinate has been reported to sensitize Jurkat T lymphocytes to TRAIL-induced apoptosis (14). Consequently, validation of the involvement of Bcl-xL/Bcl-2 inhibition in α-tocopheryl succinate-mediated chemosensitization and further structure-based optimization of TS-1 are currently under way.

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