d-alpha-tocopheryl polyethylene glycol succinate (TPGS) induces cell cycle arrest and apoptosis selectively in Survivin-overexpressing breast cancer cells

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ABSTRACT

d-alpha-tocopheryl polyethylene glycol succinate (TPGS) is a vitamin E derivative that has been intensively applied as a vehicle for drug delivery systems to enhance drug solubility and increase the oral bioavailability of anti-cancer drugs. Recently, it has been reported that TPGS acts as an anti-cancer agent alone or synergistically with chemotherapeutic drugs and increases the efficacy of nanoparticle formulations. In this study, we investigated the antitumor efficacy and the molecular mechanism of action of TPGS in breast cancer cell lines. Our results show that TPGS can induce G1/S cell cycle arrest and apoptosis in breast cancer cell lines (MCF-7 and MDA-MB-231) but not in “normal” (non-tumorigenic) immortalized cells (MCF-10A and MCF-12F). An investigation of the molecular mechanism of action of TPGS reveals that induction of G1/S phase cell cycle arrest is associated with upregulation of P21 and P27Kip1 proteins. Induction of apoptosis by TPGS involves the inhibition of phospho-AKT and the downregulation of the anti-apoptotic proteins Survivin and Bcl-2. Interestingly, our results also suggest that TPGS induces both caspase-dependent and -independent apoptotic signaling pathways and that this vitamin E derivative is selectively cytotoxic in breast cancer cell lines. When compared to the Survivin inhibitor YM155, TPGS was shown to be more selective for cancer cell growth inhibition. Overall our results suggest that TPGS may not only be useful as a carrier molecule for drug delivery, but may also exert intrinsic therapeutic effects suggesting that it may promote a synergistic interaction with formulated chemotherapeutic drugs.

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1. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among women [1]. Breast cancer cells commonly have increased proliferation potency and activate potent anti-apoptotic pathways to promote their survival and drug resistance [2]. Conventional chemotherapeutic agents generally do not distinguish between tumor and cancer cells and often cause toxic side effects. Therefore, there is a need for the discovery of novel anti-cancer agents that inhibit proliferation and survival pathways selectively in cancer cells.

Emerging evidence suggests that vitamin E isoforms may be useful in the treatment of breast cancer [3–5]. Vitamin E exists in nature as a group of 8 isoforms: α-, β-, γ-, and δ- tocopherols and α-, β-, γ-, and δ- tocotrienols [reviewed in [6]]. Specifically, research in the past few years has focused on synthetic derivatives of α-tocopherol (α-TOC), such as α-tocopherol succinate (α-TOS).
and α-tocopherol ether-linked acetic acid (α-TEA). These compounds have shown enhanced pro-apoptotic potency and anti-cancer action in tumorigenic cell lines and animal models [4,7–9]. Therefore, there is great interest for further evaluation of the most promising alpha-tocopherol synthetic derivatives using in vitro and in vivo systems.

d-alpha-tocopherol polyethylene glycol succinate (TPGS) is an amphiphilic, water-soluble derivative of natural vitamin E which is gaining interest in the development of drug delivery systems [reviewed in [10]]. TPGS is prepared from the esterification of d-alpha-tocopherol acid succinate (α-TOS) and PEG 1000. As such, it possesses the advantages of PEG and Vitamin E in applications of various nanocarriers for drug delivery, including the ability to extend the half-life of the drug in the plasma. The co-administration of TPGS has been shown to enhance drug solubility, inhibit P-glycoprotein mediated multi-drug resistance and increase the oral bioavailability of anti-cancer drugs [reviewed in [10]]. As an effective emulsifier, TPGS has been shown to greatly enhance the performance of nanoparticles, resulting in increased drug cellular uptake and improved in vivo pharmacokinetics [10]. Importantly, the United States’ Food and Drug Administration (FDA) as well as the European Food Safety Authority (EFSA), have already approved TPGS as a safe pharmaceutical adjuvant used in drug formulation and have estimated the safety limits for TPGS use for research purposes [11,12].

Based on the multi-functional nature of TPGS, its displayed synergistic effectiveness with anti-cancer drugs and its potent apoptotic effects in prostate cancer cell lines [13], it is important to investigate the efficacy of this compound in breast cancer cells and to determine if its effects are cancer cell specific. It is also crucial to elucidate the underlying mechanism by which TPGS mediates its anti-cancer effects so that this compound can be combined with other chemotherapeutic agents to provide maximum anti-cancer effectiveness.

Our findings clearly demonstrate that TPGS induces G1-phase cell cycle arrest and apoptotic cell death in breast cancer cells. The ability of a carrier compound to also act as an anti-cancer agent provides new insights and a rationale for the development of effective cancer-cell-selective therapies with diminished side effects.

2. Materials and methods

2.1. Cell cultures and reagents

MCF-7, MDA-MB-231, BT-474, SKBR3, MDA-MB-361, MCF-10A and MCF-12F cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). MCF-7 and MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic, BT-474 and MDA-MB-361 cells in DMEM-F12 supplemented with 10% FBS, 1% antibiotic/antimycotic and 4 mM l-glutamine, SKBR3 in McCoys media supplemented with 5% FBS and 1% antibiotic/antimycotic, MCF-10A and MCF-12F in DMEM-F12 supplemented with 20 ng/ml EGF, 100 ng/ml Cholera Toxin, 500 ng/ml hydrocortisone, 10 μg/ml insulin, 5% Horse Serum (HS) and 1% antibiotic, antimycotic. DMEM, McCoys, FBS, HS, antibiotic/antimycotic and trypsin used in cell culture were purchased from Gibco, Invitrogen (Carlsbad, California, USA). The caspase inhibitor z.vad.fmk was purchased from Calbiochem (Darmstadt, Germany). PARP-1, α-Fodrin, Bax, AIF, Cytochrome-C, p-AKT Ser473, p-AKT Thr308, total-AKT, Caspase -3, -6, -7, -8, -9 and Survivin antibodies and LY294002 inhibitor were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). α-Tubulin was purchased from Sigma (St. Louis, Missouri, USA). Bcl-2, p-Survivin Thr34, p21, p27Kip1, GAPDH and Endo-G antibodies were purchased from Santa Cruz Biotechnology Inc. YM155 was obtained from Selleck chemicals (Houston, TX, USA). TPGS was purchased from Eastman Chemical Company (Kingsport, Tennessee, USA). All other reagents were purchased from Sigma (St. Louis, Missouri, USA).

2.2. MTT assay

A total of 1 × 10⁴ cells were seeded per well of a 96-well plate and incubated for 24 h. At the end of the incubation period, cells were treated with different concentrations of TPGS or (and where stated) in the presence of 20 μM z.vad.fmk, for the time periods described in the figure legends. The assay was preformed as described elsewhere [14].

2.3. Cell cycle analysis

Cells were seeded at a concentration of 1 × 10⁶ cells per well of a 10 mm plate. Following incubation, samples were prepared as previously described [14] and analyzed for DNA content using the Guava EasyCyte™ flow cytometer and the GuavaSoft analysis software (Millipore, Watford, UK).

2.4. Cell death detection ELISA

Cells were seeded at a concentration of 1 × 10⁴ cells per well of a 96-well plate and incubated for 24 h. Cells were treated with TPGS or z.vad.fmk as indicated. The quantification of mono- and oligonucleosomes present in the cytoplasm of apoptotic cells was performed using the Cell Death ELisaPLUS Apoptosis Kit according to the manufacturer’s instructions (Roche, Indianapolis, IN).

2.5. Talim™ apoptosis kit

Cells were seeded at a concentration of 1 × 10⁵ cells per well of a 60-mm plate and treated with TPGS or Etoposide as indicated. Cells were harvested and stained using annexin V Alexa Fluor® 488/PI (propidium iodide), as described by the Talim™ apoptosis kit (Life Technologies, Carlsbad, CA). Cell viability, death and apoptosis were evaluated using the Talim™ image-based Cytometer (Life Technologies, Carlsbad, CA). The annexin-V positive/PI negative cells were recognized as early apoptotic cells by the cytometer software whereas the annexin V positive/PI positive cells were identified as late apoptotic/dead cells. Similarly, the annexin V negative/PI negative cells were identified as viable cells.

2.6. Western blot analysis

To determine protein levels we performed Western blot analysis as described previously [13]. For preparation of mitochondrial and cytosolic extracts, the Mitochondria/Cytosol Fractionation Kit (ab65320) was used according to the manufacturer’s instructions (Abcam, Cambridge, MA). The intensity values from the densitometry analysis of Western blots were normalized against GAPDH or α-Tubulin using ImageJ analysis software (NIH). Intensity values were expressed as fold change compared to control.

2.7. Total RNA preparation and real-time quantitative PCR (q-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. cDNA was synthesized with random primers using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Primer sequences were designed using Primer3 and are as follows: human survivin, 5’-GACGACCCCATAGAGCACA-3’ (forward) and 5’-GACGAAAGAAAGGCGCAAAC-3’ (reverse); human Bcl-2,
5'-ATGTCGTTGGAGCGCTCA-3' (forward) and 5'-ACAGTTCAACAAGCGTACTC-3' (reverse); human GAPDH, 5'-TGGATCCCTG- GAGAGTCACCTCCATGGTTT-3' (reverse), human p21, 5'-GGCTGTTTGGTCTGGTCAAACA-3' (forward) and 5'-GGTGAGTTGC- CTTCTATT-3' (reverse), human Bcl-xL, 5'-GTAAATTGCTGTCGATC- TGGT-3' (forward) and 5'-TGGATCCAGGCTCTAGGTG-3' (reverse), human XIAP, 5'-TGGGGTCTAGTTTACCGAC-3' (forward) and 5'-TGCAACACAGCTACTGGT-3' (reverse), human Livin, 5'-TGGCCTCCTTATGACTGG-3' (forward) and 5'-ACCCAGCTGTTTGTGGTG-3' (reverse), c-IAP-1, 5'-CCGGTTCACAGCCATGCTC-3' (forward) and 5'-ATTGGAGCTGATGCTCTG-3' (reverse), c-IAP-2, 5'-GGATTTCCTCAACCTGTGC-3' (forward) and 5'-ATTTCACACCAAGCAGG-3' (reverse), human Cyclin D1, 5'-GATTCAATTGAGCCGACT-3' (forward) and 5'-TCTCTCTCTCTCCTCT-3' (reverse). Real-Time PCR was performed using the BioRad CFX96 Real-Time System and the SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The PCR products were normalized to those obtained from GAPDH mRNA amplification.

2.8. Survivin-targeted siRNA transfection

The siRNA sequences used for targeted silencing of human survivin [survivin siRNA (h): sc-29499] were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA) as ready-to-use pools of 3–5 target-specific 19–25 nt double-stranded siRNAs designed to efficiently knock down the expression of BIRC5/survivin gene. siRNA A (sc-37007) was employed as negative control. Transfections were performed as described in Santa Cruz technical bulletin.

2.9. Plasmid construction lentiviral mediated stable gene knockdown

PLKO-puro.1 lentiviral vector expressing shRNAs against Survivin was constructed as described before [15] using the following 54 bp nucleotide oligos: F1:CGGCGGCTTTCTCTTGTCAACTTGAGGTGACAGAAAGGCAAAGGCTTTCG, R1:AAACAAAAGCGGCTTCTCTGCTCAACTTGAGGTGACAGAAAGGCAAAGGCTTTCG, R2:AAACAAAAGCGGCTTCTCTGCTCAACTTGAGGTGACAGAAAGGCAAAGGCTTTCG. Lentivirus was generated by cotransfection of 5 μg plkO-shSurvivin or plkO-shScrambled [16] along with psPAX2 (packaging plasmid) and pMD2.G (envelope plasmid) in a 3:1 ratio in 293T cells using Fugene 9.0 (Roche, Indianapolis, IN). After 48 h, target cells were infected with virus-containing medium in the presence of 10 μg/ml polybrene, selected with 2 μg/ml puromycin for 7–10 days, pooled and used for further assays.

2.10. Statistical analysis

Results for continuous variables were presented as Mean ± Standard Error. Two-group differences in continuous variables were assessed by the unpaired T-test. P-values are two-tailed with confidence intervals 95%. Statistical analysis was performed by comparing treated samples with untreated control. All statistical tests were conducted using Prism software version 5.0 (Graphpad, San Diego, California, USA).

3. Results

3.1. TPGS causes induction of cell cycle arrest and apoptosis in breast cancer cell lines

The effects of TPGS on cell viability were tested on five different breast cancer cell lines (SKBR-3, BT-474, MDA-MB-361, MCF-7 and MDA-MB-231) and two “normal” immortalized cell lines (MCF-10A and MCF-12F). TPGS caused a decrease in cell viability in all breast cancer lines tested at 24 and 48 h (Table 1). Specifically, the order of cell line sensitivity to the drug after 48 h of treatment was as follows: MCF-12F < MCF-10A < BT-474 < MDA-MB-361 < MCF-7 < MCF-MB-231 < SKBR3.

The response of the “normal” immortalized MCF-12F and the breast cancer MDA-MB-231 cells to increasing TPGS concentrations at 48 h was also investigated and is shown in Fig. 1A. These results show that at concentrations ranging from 11 to 33 μM TPGS there was a reduction in cell viability in MDA-MB-231 cells (Fig. 1A (ii)) but no decrease in the viability of “normal” immortalized breast cell line MCF-12F (Fig. 1A (i)). The hydrolysis products of TPGS, alpha-tocopherol succinate (α-TOS), alphatocopherol (α-TOC) and succinate did not reduce the viability of either cell line (Fig. 1A (i) and (ii)). To further investigate the mechanism of action of TPGS, we chose the most sensitive triple negative (ER−, PR−, HER2−) cell line, MDA-MB-231, and the most sensitive, double positive (ER+, PR+, HER2+) cell line, MCF-7. Cell viability was determined by flow cytometry at 24 h of incubation in all cell lines (Fig. 1B). Furthermore, exposure to TPGS led to a significant increase of the sub-G1 phase starting at 24 h in MCF-7 and 48 h in MDA-MB-231 cells, suggesting that the drug-induced reduction in cell viability may be attributed to apoptosis. The cell cycle profile of MCF-12F cells was not affected when treated with 20 μM TPGS at several time points (Fig. 1B (iii)). MDA-MB-231 cells were treated with increasing concentrations of TPGS for 3, 6, 12, 24, or 48 h followed by a post-treatment recovery period in drug free medium for up to 72 h. Cell viability was fully restored when TPGS was removed after 3 h of treatment, and partially restored for treatments ranging from 6 to 12 h. Treatments for 24 and 48 h produced an irreversible effect (Fig. 1C). Thus, the effect of TPGS on cell viability is irreversible after a minimum of 24 h of treatment.

To further investigate the mechanism of loss of cell viability by TPGS, we examined the ability of TPGS to induce apoptosis. TPGS-induced substantial DNA fragmentation in MDA-MB-231 cells (16.3-fold compared to control) and lesser fragmentation in MCF-7 cells (4.7-fold). DNA fragmentation in both cell lines was greatly reduced in the presence of the caspase inhibitor z.vad.fmk (Fig. 2A). The latter indicates the activation of caspase-dependent pathways by TPGS in these cell lines. However, even though co-incubation of TPGS with z.vad.fmk restored DNA fragmentation (Fig. 2A) it did not fully restore the viability of cells (Fig. 2B). MCF-7 viability was increased from 43% to 53% and MDA-MB-231 viability was...
increased from 17% to 24% in the presence of z.vad.fmk suggesting that caspase-independent pathways of cell death may also be activated.

The induction of caspase-independent cell death by TPGS required further examination. Towards this objective we determined the localization of AIF and Endo-G endonucleases which have previously been shown to be involved in the induction of caspase-independent pathways of apoptosis [17]. Our results have showed that TPGS causes the translocation of AIF and the 25 kDa form of Endo G from the mitochondria to the cytosol in the presence of the drug in MCF-7 cells (Fig. 2C). In MDA-MB-231 cells however, cytosolic levels of AIF and Endo G were not increased in response to TPGS treatment (data not shown). It seems that the translocation of AIF and the 25 kDa form of Endo G from the mitochondria to the cytosol is cell type specific and therefore other molecules must be involved in the induction of caspase-independent cell death. Furthermore, the localization of Bax, a pro-apoptotic member of the Bcl-2 family, and Cytochrome-C, an

Fig. 1. TPGS decreases the viability of breast cancer cells lines but not immortalized “normal” cell lines. (A) MCF-12F (i) and MDA-MB-231 (ii) cells were exposed to increasing concentrations of TPGS, alpha-tocopherol succinate (α-TOS), alpha-tocopherol (α-TOC) and succinate for 48 h. Cell survival was determined with the MTT cell viability assay and is expressed as percentage of survival in untreated cells. (B) TPGS induces cell cycle arrest and apoptosis in MCF-7 (i) and MDA-MB-231 (ii) breast cancer cells but not in MCF-12F (iii) “normal” cells treated with 20 μM TPGS for 0–96 h prior to cell cycle analysis. (C) The effects of TPGS are irreversible after 24 h of treatment. MDA-MB-231 cells were exposed to increasing concentrations of TPGS for 0–72 h followed by removal of the agent after 3, 6, 12, 24, or 48 h as shown, and a recovery period in drug free medium for up to 72 h. A positive control, where TPGS was not removed for 72 h is shown for comparison. Cell survival was determined with the MTT cell viability assay. The results represent the mean ± SEM of three different replicates and are representative of at least three different experiments. *P value < 0.05, **P value < 0.01, ***P value < 0.001.
integrated protein of the Apoptosome complex that subsequently activates caspase-9 were not affected in the presence of the drug (Fig. 2C).

Apoptotic induction was also analyzed using the Tali™ Apoptosis Kit, where Annexin V and PI are used for determination of apoptotic cell death. Treatment with TPGS for 72 h resulted in about 37% of MCF-7 cells (Fig. 2D(i)) and 28% of MDA-MB-231 (Fig. 2D(ii)) cells staining positive for Annexin V (early apoptotic).

To further investigate the involvement of caspases in the apoptotic process induced by TPGS, we determined its ability to induce PARP-1 and α-Fodrin cleavage, which are known substrates of effector caspases -3 and -7 [18]. TPGS-induced cleavage of PARP-1 and α-Fodrin in both cell lines examined (Fig. 2E). Treatment with TPGS led to the appearance of the active form of caspase-7 (c-Casp-7) in MCF-7 cells and of the active forms of caspases-3 (c-Casp-3) and -7 in MDA-MB-231 cells, reconfirming that the compound induces caspase-mediated apoptosis. However, TPGS did not produce an increase in the active form of initiator caspases-8 or -9 (data not shown). Caspase-3 was undetectable in MCF-7 cells as previously reported [19].

3.2. TPGS reduces the levels of anti-apoptotic proteins Survivin and Bcl-2

The underlying mechanism of TPGS action was further evaluated by monitoring its effects on the mRNA levels of genes implicated in apoptosis and cell cycle regulation. Treatment with TPGS resulted in a significant decrease in the mRNA levels of Survivin and Bcl-2 in all cell lines tested with the exception of MCF-10A, MCF-12F and BT-474 (that we found to be resistant to the agent) (Fig. 3A(i) and (ii)). The mRNA levels of other IAP members XIAP, Livin, c-IAP-1 and c-IAP-2, of anti-apoptotic Bcl-xl and of the G1/S transition regulatory protein Cyclin D1 were not reduced by TPGS (data not shown).

Survivin and Bcl-2 protein levels were also evaluated in both cell lines. In MCF-7 cells, a decrease in the levels of Survivin and Bcl-2 was evident as early as 3 h post TPGS-treatment and the levels of both proteins continued to decline for up to 48 h. A similar but somewhat delayed response was evident in MDA-MB-231 cells (Fig. 3B). The effects of TPGS on Thr34 p-Survivin, which has been shown to maintain Survivin stability in cancer cells [20,21], mirrored those of Survivin. Thus, TPGS treatment effectively reduced the levels of p-Survivin Thr34 in MDA-MB-231 and MCF-7 cells (Fig. 3C). Co-incubation of cells with TPGS and z.vad.fmk did not restore Survivin or Bcl-2 levels, indicating that the TPGS-induced reduction was not caspase-dependent (Fig. 3C). The total levels of Bax and AIF remained unaffected in both cell lines under these treatment conditions (Fig. 3C).

3.3. TPGS inhibits phosphorylation of AKT at Ser473 and Thr308

Survivin expression can be deregulated in cancer by increased upstream signaling in the PI3K pathway [22]. In order to investigate whether the effects of TPGS are mediated via the PI3K/AKT pathway we determined its effects on the phosphorylation levels of AKT at Thr308 and Ser473. Phosphorylation of Thr308 partially activates AKT/PKB, while phosphorylation of both sites is required for full activation [23]. In MCF-7 cells, insulin treatment increased by 38-fold the levels of Ser473 (compared to the untreated control) and by 3.6-fold the Thr308 levels (Fig. 4A). The effects of insulin where more robust in MDA-MB-231 cells where it increased the levels of Ser473 by 52-fold and those of Thr308 by 17.9-fold compared to the untreated control. TPGS inhibited insulin-induced phosphorylation of AKT at Ser473 in both cell lines with a more prominent effect in MDA-MB-231 cells, where p-AKT levels were completely abolished by the drug. TPGS treatment inhibited the insulin-induced phosphorylation of AKT at Thr308 in MDA-MB-231 cells but not in MCF-7 cells (Fig. 4A). Furthermore, TPGS decreased p-AKT levels in non-tumorigenic MCF-12F cells following insulin-induced upregulation of phosphorylation of AKT (Fig. 4B). In order to confirm the implication of the AKT/Survivin pathway in the observed mechanism of action, MDA-MB-231 cells were incubated with LY294002 that is known to downregulate phosphorylated AKT levels. Results show that treatment with LY294002 led to the downregulation of p-AKT (Ser473) and Survivin and upregulation of P21 and P27Kip1 protein levels (Fig. 4C).

3.4. Sensitivity to TPGS treatment is associated with Survivin levels

According to previous reports, Survivin is often upregulated in breast tumors and acts by inhibiting apoptosis and regulating mitosis, conferring resistance to common chemotherapeutic drugs [24,25]. To examine the role of Survivin in TPGS-mediated cytotoxicity, we determined the basal mRNA levels of the protein in all cell lines using qPCR. With the exception of BT-474, Survivin mRNA levels were higher in all cancer cell lines tested in comparison to non-cancerous cells (Fig. 5A). Thus, SKBR-3 breast cancer cells express up to ~20-fold higher Survivin levels compared to MCF-12F cells. Interestingly, we found that the level of Survivin expression in the breast cancer cell lines is proportional to the degree of sensitivity to TPGS treatment. Furthermore, all cancer cell lines, with the exception of SKBR-3 displayed higher mRNA levels of Bcl-2 compared to MCF-12F cells (Fig. 5B).

Consistent with qPCR results, MDA-MB-231 and MCF-7 cells express high basal protein levels of Survivin and Bcl-2, which were downregulated by TPGS while both proteins were almost undetectable in MCF-12F and MCF-10A cells (Fig. 5C). These data suggest that high Survivin levels confer sensitivity to TPGS and that the cytotoxic effects of the agent may be mediated through the downregulation of Survivin. Furthermore, we compared the effectiveness of TPGS on breast cancer cells with the established small-molecule Survivin inhibitor YM155 that induces apoptosis in various human cancer models [26]. We compared TPGS and YM155 cancer cell selectivity by using the effective concentration of the compounds (Table 2). As expected from our previous results (Table 1 and Fig. 1A) TPGS decreased the viability of cancer cell lines without affecting the viability of non-cancerous cells (Fig. 5D). Contrary to that, YM155 decreased MCF-12F and MCF-10A cell viability more effectively than that of the cancer cells (Fig. 5D).

3.5. P21 and P27Kip1 proteins are upregulated in response to TPGS treatment or Survivin knockdown

To investigate the effect of TPGS treatment on the levels of proteins involved in cell cycle regulation, we measured P21 and P27Kip1 levels in TPGS-treated cells. TPGS treatment produced 20–40-fold increase of p21 mRNA levels in MDA-MB-231, MCF-7 and SKBR-3 cells compared to the untreated controls (Fig. 6A(i)). These
TPGS induces caspase-dependent and -independent apoptosis in breast cancer cells. (A) Tumor cells were treated with 20 μM TPGS in the presence or absence of 20 μM z.vad.fmk as shown for 48 h and the presence of nucleosomes was detected using the Cell Death Detection ELISA. (B) Tumor cells were treated with 20 μM TPGS in the presence or absence of 20 μM z.vad.fmk as shown for 48 h. Cell viability was assessed with the MTT assay. (C) TPGS induces Endo G and AIF release from the mitochondria to the cytosol in MCF-7 cells. Cells were incubated with 20 μM TPGS for 48 h and cytosolic and mitochondrial protein extracts were prepared. Samples were run by SDS–PAGE and immunoblotted with the indicated antibodies. The relative expression levels (below) were analyzed by Image J software. Intensity values were expressed as fold change compared to mitochondrial or cytosolic control. (D) Cells were treated with 20 μM TPGS for 24, 48 or 72 h and apoptosis was assessed with the Annexin-V Alexa Fluor® 488/PI.
are the same cell lines that displayed the greatest reduction in cell proliferation. The levels of P27Kip1 were affected in a similar manner, although the TPGS-induced increase was not as robust (Fig. 6A (ii)).

Since MDA-MB-231 cells displayed the greatest changes in P21 and P27Kip1 mRNA levels in the presence of TPGS, this cell line was used to further investigate the underlying cell cycle delay mechanism. Indeed, robust increases were evident in the P21 and P27Kip1 protein levels accompanied by a decrease in Survivin protein levels (Fig. 6B). In order to investigate whether cell cycle inhibition is directly linked to Survivin downregulation, we used shRNA to induce stable knockdown of Survivin in MDA-MB-231 cells. mRNA and protein levels of Survivin were measured in order to confirm stable knockdown (Fig. 6C (i), (iii)). Specific targeting of Survivin led to an increase in the G1 population from 43.4% in the control cells treated with shScrable to 62% in the ShSurvivin knockdown cells (Fig. 6C (iii)). Survivin knockdown was accompanied by the upregulation of P21 and P27Kip1 mRNA and protein levels (Fig. 6D (i), (ii)). These results substantiate the involvement of Survivin downregulation in the mode of action of TPGS and provide new insights on the mechanism by which Survivin regulates the G1 checkpoint.

4. Discussion

Vitamin E natural isoforms and synthetic derivatives are showing great promise as anti-cancer agents [6]. The FDA- and EFSA-approved use of TPGS as an adjuvant in drug formulations and delivery systems (either as coated liposomes or micellar formulation) increases the need to know exactly how it works at the molecular level.

In the present study we evaluated the molecular mechanism of action of TPGS in breast cancer cells. We show here that TPGS induces G1-phase cell cycle arrest and caspase-dependent and independent apoptotic cell death selectively in breast cancer cells while the viability of “normal” cells is not significantly affected. Compared to the potent Survivin inhibitor YM155, TPGS shows greater sensitivity between “normal” and tumor cells.

In order to examine the molecular mechanism of induction of apoptosis we investigated whether TPGS induces the activation of caspases in the different breast cancer cell lines. Our results have shown that TPGS activates a caspase-mediated apoptotic pathway i.e. caspases-7 and -3 are activated in MDA-MB-231 cells (as shown in Fig. 2) whereas in MCF-7 only caspase-7 is activated since these cells are deficient in caspase-3 [19,27]. However, cell viability is not completely restored in the presence of the pan-caspase inhibitor, suggesting that caspase-independent mechanisms may also be engaged. This prediction is supported by the release of Endo G and AIF which are mediators of caspase-independent death [17] from the mitochondria to the cytosol in MCF-7 cells after exposure to TPGS. However, we did not observe this effect in MDA-MB-231 cells suggesting that translocation of AIF or Endo G from the mitochondria to the cytosol is cell type specific and therefore other molecules may be involved in the induction of caspase-independent cell death. This finding gives additional support to our earlier reports that, based on tissue context, vitamin E natural isoforms and their synthetic derivatives may produce caspase-dependent or -independent programmed cell death [13,28]. In addition to AIF and Endo G, other mediators of CI-PCD include proteases Calpains (calcium activated neutral proteases) and Cathespins that are regulated by lysosomes and the ER respectively. Cathespins are lysosomal proteins that take part in apoptotic signaling by inducing translocation of Bax to the mitochondria as well as cleavage and translocation of Bid, thereby increasing the permeability of the outer mitochondrial membrane [29,30]. In the absence of apoptotic signals, Calpains normally reside in the cytosol as inactivezymogens. Following ER stress, Calpains activate pro-apoptotic members of the Bcl-2 family such as Bax and Bid but also induce the release of lysosomal cathespins [17].

Aberrant activation of AKT signaling has been linked to the progression of many types of cancer [31]. TPGS produced an early and substantial reduction in the phosphorylation of both Ser473 and Thr308 in MDA-MB-231 cells and a smaller reduction in Ser473 in MCF-7 cells (Fig. 4A). Interestingly, p-AKT levels were downregulated by TPGS in MCF-12F cells that were not growth inhibited by the drug. Activated AKT promotes cell survival by inducing the transcription of important anti-apoptotic proteins such as Survivin and Bcl-2 [32,33]. TPGS reduced substantially the mRNA and protein levels of both Bcl-2 and Survivin in the cancer cell lines but not in the normal immortalized MCF-12F cells or MCF-10A cells, where basal levels of both proteins were very low. This suggests that the effect of the TPGS is mediated via the AKT/Survivin/Bcl-2 axis and may account for the differential response of normal cells to the drug. Furthermore, activated AKT decreases the expression of P27Kip and phosphorylates P21 resulting in its cytoplasmic localization [34,35]. In addition, activated AKT decreases the expression of P27Kip and P21 by directly phosphorylating FoXO transcription factors [36]. P27Kip and P21 act as cyclin dependent kinase inhibitors that bind and inhibit the activity of cyclin/CDK2 complexes, thereby arresting the cell cycle at the G1 phase [37,38]. We have found that the TPGS-induced cell cycle delay in the G1 phase is accompanied by a robust increase in P21 protein levels and to a lesser extent in P27Kip1 levels. A stable knockdown of Survivin also reproduced the effects of TPGS resulting in cell cycle block at G1 and the upregulation of P21 and P27Kip1 mRNA and protein levels (see Fig. 6C, D). Thus, the results presented in this paper, provide compelling evidence that the induction of cell cycle arrest by TPGS involves inhibition of AKT phosphorylation and downregulation of Survivin mRNA and protein levels and that the latter are tightly linked to the upregulation of P21 and P27Kip1.

In Survivin-expressing cancer cell lines TPGS down-regulates Survivin mRNA and protein levels. Survivin, as a member of the IAP family, has been implicated in both cancer cell survival and regulation of mitosis [39,40]. Survivin forms a complex with XIAP, enhancing its stability against ubiquitin-dependent degradation and increasing its activity as a caspase inhibitor [41]. Furthermore, Survivin acts cytoprotectively by sequestering pro-apoptotic Smac/DIABLO away from XIAP [42], or by preventing its release from mitochondria [43]. The caspase-independent cell death that we observed in MCF-7 cells may implicate Survivin signaling, as the inhibition of Survivin precedes the translocation of AIF from the mitochondria to the nucleus, resulting in caspase-independent DNA fragmentation [44].

In order to further investigate the potential use of TPGS as a chemotherapeutic agent we compared its effects to those of the Survivin inhibitor YM155. Even though the Survivin inhibitor YM155 is more potent than TPGS, YM155 does not show selectivity between “normal” and tumor cells. On the contrary, TPGS induces
**Fig. 3.** TPGS down-regulates anti-apoptotic proteins Survivin and Bcl-2. (A) The effects of TPGS on (i) Survivin and (ii) Bcl-2 levels were evaluated in a panel of breast cancer and “normal” cell lines. Cells were incubated in the presence of 20 μM TPGS for 48 h. mRNA levels were measured by qPCR. (B) TPGS decreases Survivin protein levels in a time-dependent manner. (i) MCF-7 and (ii) MDA-MB-231 were exposed to 20 μM TPGS for 0–72 h. Samples were run by SDS–PAGE and immunoblotted with the indicated antibodies. The relative expression levels (below) were analyzed by Image J software. Intensity values were expressed as fold change compared to control. (C) TPGS induces a time- and dose-dependent manner decrease of Survivin and Bcl-2 protein levels. (i) MCF-7 and (ii) MDA-MB-231 cells were incubated with TPGS in the presence or absence of z.vad.fmk as indicated. Samples were run by SDS–PAGE and immunoblotted with the indicated antibodies. The relative expression levels (right panel) were analyzed by Image J software. Intensity values were expressed as fold change compared to control. The results represent the mean ± SEM of three different replicates and are representative of at least three different experiments. *P value < 0.05, **P value < 0.01, ***P value < 0.001.
G1-phase cell cycle arrest and apoptotic cell death selectively in breast cancer cells while the viability of “normal” cells is not significantly affected by TPGS treatment (Fig. 5D). The latter observation justifies the investigation of TPGS as a promising anti-cancer agent.

Based on our findings, we propose a potential mechanism of action of TPGS in breast cancer cells. TPGS acts by inhibiting the phosphorylation of AKT thereby leading to the downregulation of Survivin and Bcl-2 mRNA and protein levels. Downregulation of Survivin and Bcl-2 induce activation of caspases -3 and -7 that induce Caspase-dependent programmed cell death. These proteins may also be involved in the induction of caspase-independent cell death. Downregulation of Survivin also causes an increase in P21 and P27Kip1 levels that induce cell cycle arrest at G1.

The results presented in this paper show conclusively that TPGS promotes cell cycle arrest and induces apoptosis in breast cancer cell lines expressing high levels of Survivin. TPGS-induced alterations in two AKT phosphorylation sites may be an initiating event and an important component of the underlying molecular mechanism leading to cell cycle arrest and apoptosis. The FDA- and EFSA-approved use of TPGS as an adjuvant in drug formulations and delivery systems (either as coated liposomes or micellar
Fig. 5. Cancer cells exhibit higher Survivin and Bcl-2 levels compared to "normal" cells. RNA was extracted from each cell line as described in Section 2. Basal (A) Survivin and (B) Bcl-2 mRNA levels were measured by qPCR. (C) MCF-7, MDA-MB-231, MCF-10A and MCF-12F cells were treated with 20 μM TPGS for 48 h followed by protein extraction. Samples were run by SDS–PAGE and immunoblotted with the indicated antibodies. The relative expression levels (right panel) were analyzed by Image J software. Intensity values were expressed as fold change compared to control. (D) TPGS is more selective for cancer cell growth inhibition compared to YM155. Cells were treated with 10 nM YM155 or 20 μM TPGS for 48 h. At the end of the incubation period, MTT assays were performed. The results represent the mean ± SEM of three different replicates and are representative of at least three different experiments. *P value < 0.05, **P value < 0.01, ***P value < 0.001.
Fig. 6. TPGS upregulates the levels of P21 and P27Kip1. (A) Cells were exposed to 20 μM TPGS for 48 h followed by qPCR in order to measure mRNA levels of (i) P21 and (ii) P27Kip1. (B) Cells were incubated with 20 μM TPGS for 3, 6 and 24 h followed by Western blot to detect Survivin, P21 and P27Kip1 protein levels. The relative expression levels (below) were analyzed by Image J software. Intensity values were expressed as fold change compared to control. (C) Survivin knockdown induces P21 and P27Kip1 upregulation in MDA-MB-231 cells. Cells were stably transfected with shRNA Scrambled and shRNA Survivin followed by (i) qPCR in order to measure Survivin mRNA levels and (ii) Western blotting for the detection of Survivin protein levels. The relative expression levels (below) were analyzed by Image J software. Intensity values were expressed as fold change compared to shSCR. (iii) Cell cycle analysis was performed on stably transfected cells. (D) MDA-MB-231 cells were transiently transfected with shRNA Scrambled and shRNA Survivin followed by (i) qPCR in order to measure P21 and P27Kip1 levels and (ii) Western blot for the detection of Survivin, P21 and P27Kip1 levels. The relative expression levels (right panel) were analyzed by Image J software. Intensity values were expressed as fold change compared to shSCR. The results represent the mean ± SEM of three different replicates and are representative of at least three different experiments. *P value < 0.05, **P value < 0.01, ***P value < 0.001.
formulation) increases the need to know exactly how it works at the molecular level.

Based on our results we anticipate that the combination of TPGS with conventional chemotherapeutic agents may lower the levels of tumor resistance and provide new treatment options to patients whose breast cancer cells overexpress Survivin. The synthesis of TPGS-based nanocarriers formulated with compounds that target-specific cancer markers for the development of personalized treatments, is under extensive investigation. Our work represents a new concept in the design of drug delivery systems – the carrier materials of the drug delivery system can also have therapeutic effects, which either modulate the side effects of, or promote a synergistic interaction with other drugs.

Conflict of interest

The authors declare no conflict of interest.

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