

Triphenylmethyl Derivatives Enhances the Anticancer Effect of Immunotoxins

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Summary: The combined use of several drugs targeting different cellular functions is one approach to achieve tumor control in cancer. We studied the effects of *Pseudomonas* exotoxin A (PE)-based immunotoxins (ITs), the 9.2.27PE and the 425.3PE, together with 2 different triphenylmethyl derivatives, triphenylmethyl phosphonates and phosphonochloridates (TPMP)-I-2 and 4BI. Combining the triphenylmethyl derivatives with ITs enhanced the cytotoxic effect of the ITs, with TPMP-I-2 in combination with the 425.3PE (targeting the epidermal growth factor receptor) being the most promising combination. The cytotoxicity involving signs of apoptosis was observed in cancer cells from different origins in vitro. It is interesting to note that treatment with IT, TPMP-I-2, or 4BI alone or in combination resulted in strongly decreased protein levels of stearoyl-CoA desaturase. Stearoyl-CoA desaturase is the rate-limiting enzyme for converting saturated fatty acids into monounsaturated fatty acids needed for membrane genesis. Furthermore, the combination of 425.3PE and TPMP-I-2 prolonged the survival time of nude rats in a simulated micrometastatic cervical cancer model. In addition, we demonstrate that a combination of the 425.3PE and 4BI was more effective in reducing tumor growth in a breast cancer model in nude mice compared with either agent alone.

Key Words: immunotoxin, triphenylmethyl derivatives, apoptosis, protein synthesis, stearoyl-CoA desaturase

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An immunotoxin (IT) consists of an antibody linked to a toxin. ITs are designed to specifically kill tumor cells, as the antibody targets cell-associated antigens or antigens overexpressed on cancer cells, theoretically leaving normal cells unaffected. ITs are taken up through endocytosis,

processed within the cell, and cell death is caused by inhibition of protein synthesis through adenosine diphosphate-ribosylation of elongation factor 2 and induction of apoptosis.^{1–4} Apoptosis is commonly associated with activation of caspases, inactivation of the DNA repair enzyme poly-(adenosine diphosphate-ribose) polymerase (PARP), chromatin condensation, and fragmentation of DNA. Our ITs consist of the *Pseudomonas* exotoxin A (PE) linked to antibodies targeting the epidermal growth factor receptor or the high molecular weight-melanoma associated antigen, the 425.3 and the 9.2.27 antibody, respectively. Both the 425.3PE and 9.2.27PE have antitumor activity in vitro and in vivo in different cancer types.^{1,3–6} We hypothesized that by combining ITs with agents that target different signaling pathways in cancer cells, enhanced cytotoxic effect of the ITs would be achieved.

Triphenylmethylamides (TPMAs) were first identified as compounds that arrest cells in the G1-phase of the cell cycle and induce apoptosis in melanoma cell lines in culture.⁷ One of the TPMAs developed, 4BI (Fig. 1), has been shown to reduce the level of active nuclear factor κ -B (NF κ B) and is relatively nontoxic against bone marrow cells from healthy human donors.⁷ Clotrimazole, another compound containing the triphenylmethyl motif, elicits anticancer properties through inhibition of cell growth both in vitro and in vivo, affecting intracellular Ca²⁺ levels, thereby inhibiting translation.^{8,9} In addition, clotrimazole inhibits glycolysis by inducing the detachment of mitochondrial-bound hexokinase.^{8–10} Other agents containing the triphenylmethyl motif also show antiproliferative effects in cancer cells, indicating that triphenylmethyl-containing compounds have potential as anticancer agents.^{11,12} Recently, a panel of triphenylmethyl phosphonates and phosphonochloridates (TPMPs) have been synthesized, of which, several are able to induce cell death in cancer cell lines in culture.¹⁰ However, the precise mechanism by which the TPMAs and TPMPs exert their anticancer effect remains unknown.

Here, we report enhanced cytotoxic effect of ITs in vitro by combining ITs with triphenylmethyl derivatives, shown by inhibition of protein synthesis, induction of apoptosis, and decreased stearoyl-CoA desaturase (SCD) protein levels. In vitro results further indicate a cytotoxic effect by monotreatment of the triphenylmethyl derivative, TPMP-I-2, involving decreased protein expression level of SCD. SCD is necessary for cancer cell growth,¹³ and is now for the first time, to the best of our knowledge, linked to the function of a triphenylmethyl derivative. By combining triphenylmethyl derivatives and ITs, reduced tumor growth in a breast cancer model in nude mice was obtained. In addition, combination treatment significantly prolonged the survival time in nude rats' intracardially administered cervical carcinoma cells.

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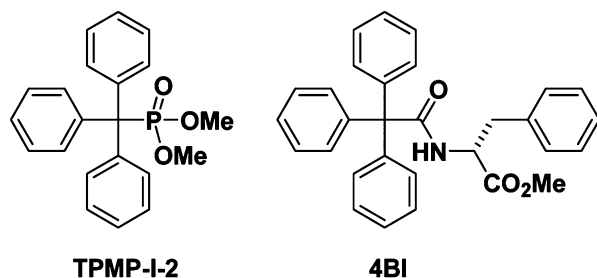


FIGURE 1. Structure of TPMP-I-2 and 4BI.

MATERIALS AND METHODS

ITs and chemicals

The epidermal growth factor receptor antibody 425.3 and the high molecular weight-melanoma associated antigen antibody 9.2.27 were both gifts from Dr Ralph Reisfeld (Scripps Institute, La Jolla, CA). The antibodies were conjugated to PE obtained from Dr Darrel Galloway (University of Ohio, Columbus, OH) by a thioether bond formed with the reagent sulfo-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Pierce, Rockford, IL) as described earlier.¹⁴ The triphenylmethyl derivatives, 4BI and TPMP-I-2, were developed and synthesized as described earlier.^{7,10} The ITs were diluted in phosphate-buffered saline (PBS) 0.1% human serum albumin (Octapharma, Stockholm, Sweden), and the triphenylmethyl derivatives were dissolved in ethanol. In control cells, PBS 0.1% human serum albumin and ethanol were used as vehicles. For the animal studies, the triphenylmethyl derivatives were dissolved in corn oil (Sigma-Aldrich, Schnellendorf, Germany).

Antibodies

Anti-PARP and anti- α -tubulin were purchased from Calbiochem (La Jolla, CA), anti-scaffold attachment factor B (SAF-B) was from Abcam (Cambridge, MA), anti-caspase-3 was from R&D Systems (Minneapolis, MN), anti-heat shock protein 27 (HSP27) was from Cell Signaling Technology (Danvers, MA), and anti-SCD was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Establishment and characterization of the breast carcinoma cell line MA-11 and the malignant melanoma cell line FEMX have been described earlier.^{15–17} The origin of the HeLa* cell line is described in a recent study.⁴ The cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 8% heat-inactivated fetal calf serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and GlutaMAX (Gibco, Paisley, UK) at 37°C. All cell lines were routinely tested and found to be free from contamination with *Mycoplasma* species.

Protein Synthesis Inhibition assay

MA-11 and HeLa* cells were treated with 425.3PE and FEMX cells with 9.2.27PE, either as monotherapy or in combination with TPMP-I-2. Protein synthesis inhibition was evaluated by a (³H)-leucine incorporation assay as described earlier.³ In brief, 3 to 5 × 10⁴ cells were seeded per well, depending on the cell type, in 48-well plates (Costar, Corning, NY) and incubated at 37°C overnight. The cells were pretreated for 1 hour with TPMP-I-2 (50 μ M). MA-11 and HeLa* were subsequently treated for 24 hours with

425.3PE (0.1 and 1 ng/mL, respectively), and the FEMX cells with 9.2.27PE (10 ng/mL). The experiment was repeated 3 times. Furthermore, MA-11, HeLa*, and FEMX cells were treated with 0.05 to 5 μ M 4BI for 24 hours before subjected to protein synthesis inhibition assay as shown earlier. The experiment was repeated 3 times for MA-11 and FEMX, and twice for HeLa*. In addition, MA-11 cells were treated with low doses of TPMP-I-2 as single agent (0.5 and 5 μ M) for 24 hours. The experiment was repeated twice. The results for treated cells were normalized against the respective control value and expressed as percentage leucine incorporation. All control values were set to 100%.

Cell Viability

MA-11, HeLa*, and FEMX cells were treated with 425.3PE or 9.2.27PE alone or in combination with TPMP-I-2 or 4BI, and cell viability was measured using CellTiter 96AQ_{ueous} One Solution Cell Proliferation Assay [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), assay; Promega, Madison, WI] as described earlier.³ In brief, cells were seeded in 96-well plates (Costar, Corning, NY) at 8000 to 10,000 cells per well, depending on the cell line, and kept at 37°C overnight. The cells were pretreated for 1 hour with different concentrations of TPMP-I-2 (0.5 to 50 μ M) or 4BI (0.05 to 5 μ M). Subsequently, 425.3PE (0.1 to 100 ng/mL) was added to the MA-11 and HeLa* cells, and 9.2.27PE (0.1 to 100 ng/mL) was added to the FEMX cells for 24 hours. CellTiter reagent was subsequently added to the culture and the assay plates were incubated at 37°C for approximately 2 hours before absorbance was measured at 490 nm (Wallac Victor2 TM 1420 Multilabel Counter). The values were corrected for background absorbance, and the values for treated cells were compared with values for untreated cells. The experiment was repeated 3 times. In addition, FEMX cells were pretreated with TPMP-I-2 (50 μ M) for 1 hour, and subsequently treated with 9.2.27PE (1 to 100 ng/mL) for 30 hours before cell viability was determined using the MTS assay. The experiment was repeated 3 times. Furthermore, MA-11 cells were pretreated with TPMP-I-2 (50 μ M) for 1 hour, and subsequently treated with 425.3PE (0.1 ng/mL) for 48 hours before cell viability was determined using the MTS assay. This experiment was repeated twice.

Microarray

MA-11 cells were treated with 425.3PE (0.1 ng/mL) and/or TPMP-I-2 (50 μ M) and/or 4BI (5 μ M) for 24 hours. The cells were washed twice in cold PBS before total RNA was extracted using GenEluteTM Mammalian Total RNA Miniprep Kit from Sigma-Aldrich according to the manufacturer's instructions. Gene expression analysis through microarray CodeLink UniSet Human 20K Oligo Bioarray (GE Healthcare/Amersham Biosciences, Piscataway, NJ) containing less than 20,000 gene probes was used to generate gene expression profiles for all the samples examined. All reagents and protocols were provided by GE Healthcare/Amersham Biosciences. The procedure has been described earlier in detail.¹⁸ The slides were scanned by Axon GenePix Professional 4200A microarray scanner using Genepix Pro 5.1 software. The images were grided by Codelink 4.1 software (GE-Healthcare/Amersham Biosciences) and exported to GeneSpring Software 7.2 (Agilent, Palo Alto, CA). Under Cross-Gene Error Model, a normalization step was performed in 2 steps: (A) "per chip normalization," in which each measurement was

divided by the 50th percentile of all measurements in its array, and (B) "per gene normalization," in which all the samples were normalized against the specific samples (controls). The results were filtered by flags. The expression profiles were compared using 1-way analysis of variance analysis with *P* value less than 0.05.

Quantitative Real-time RT-PCR

The total RNA used for the microarray analysis was also used for the quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. Two additional experiments were performed, where total RNA was extracted from 425.3PE (0.1 ng/mL) and/or TPMP-I-2 (50 μ M)-treated MA-11 cells (24 h). Furthermore, total RNA was extracted from MA-11 cells treated with low concentration TPMP-I-2 (0.5 and 5 μ M, 24 h). The latter experiment was repeated twice. Total RNA was reverse transcribed using the High Capacity RNA-to-DNA Master Mix from Applied Biosystems (Foster City, CA) according to the manufacturer's manual. Quantitative real-time RT-PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems). The expression level was determined for the SCD gene (assay ID Hs01682761_m1), sterol regulatory element binding transcription factor 1 (SREBF1; assay ID Hs01088691_m1), glyceraldehyde 3-phosphate dehydrogenase (GADPH; assay ID Hs99999905_m1), and TATA binding protein (TBP; assay ID Hs99999910_m1). GADPH and TBP were used as endogenous controls for normalization. The PCR amplification was performed in duplicate series using the ABI 7700 Sequence Detection System (Applied Biosystems). The expression levels were determined using the comparative CT method as described by the manufacturer, and the expression level of the mRNA was normalized against the average expression of GADPH and TBP. In our analysis, the untreated samples were set to the given value 1, and the values for the treated samples were calculated accordingly.

DNA Fragmentation Analysis

MA-11 and HeLa* cells were treated with 425.3PE (0.1 and 1 ng/mL, respectively) \pm TPMP-I-2 (50 μ M) or 4BI (5 μ M), and FEMX cells were treated with 9.2.27PE (100 ng/mL) \pm TPMP-I-2 (50 μ M) or 4BI (5 μ M) for 24 hours. To determine DNA fragmentation in the MA-11, HeLa*, and FEMX cells, both floating and adherent cells were collected and washed twice with PBS before pellets were fixed in ice-cold methanol and kept at -20°C . The terminal deoxynucleotidyl transferase dUTP nick end labeling assay was performed as described earlier.³

Western Blot Analysis

MA-11 and HeLa* cells were treated with 425.3PE (1 and 10 ng/mL, respectively) \pm TPMP-I-2 (50 μ M) or \pm 4BI (5 μ M), and FEMX cells were treated with 9.2.27PE (100 ng/mL) \pm TPMP-I-2 (50 μ M) or \pm 4BI (5 μ M) for 24 hours. In addition, MA-11 cells were treated with increasing doses of 425.3PE (0.1 to 10 ng/mL) and TPMP-I-2 (0.5 to 50 μ M) for 24 hours. The cells were lysed using a sodium dodecyl sulphate boiling method as described earlier.¹ Protein concentration was measured using bicinchoninic acid protein (BCA) assay from Pierce (Rockford, IL). Equal amounts of protein (15 μ g) were separated by NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA), and subsequently transferred by electrophoresis to Immobilon membrane (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dry milk for 1 hour at room

temperature followed by incubation with primary antibodies at 4°C overnight. The membranes were washed before incubation with appropriate horseradish peroxidase-coupled secondary antibodies. After several washes, the peroxidase activity was visualized with Electrochemiluminescence (Pierce) on an AGFA Curix 60.

Animals

All procedures and experiments that involved animals were approved by The National Animal Research Authority and carried out according to the European Convention for the Protection of Vertebrates used for Scientific Purposes. Nude rats (Han: rnu/rnu Rowett) and nude female mice [BALB/c (nu/nu)] were bred in our nude rodent facility. The animals were kept in a specific pathogen-free environment, in positive pressure rooms with filtered and humidified air. The animals were kept under standard conditions, and food and water were supplied *ad libitum*.

In Vivo Studies

Four to 5-week-old nude rats of both sexes were used and injected on day 1 with 106 HeLa* cells into the left cardiac ventricle as described earlier.⁴ TPMP-I-2 (26 mg/kg diluted in 300 μ L corn oil) was administered on days 0 and 6 by intraperitoneal injection in animals anesthetized with halothane and N_2O mixed with O_2 . The 425.3PE (2.5 μ g diluted in 100 μ L PBS) was administered intravenous 3 to 4 hours after each TPMP-I-2 treatment. The animals were divided into 4 groups: control (n = 8), 425.3PE (n = 7), TPMP-I-2 (n = 4), and TPMP-I-2 + 425.3PE (n = 9). The animals were inspected daily with respect to symptoms of tumor-related disease and general condition, and sacrificed when symptoms of metastatic disease appeared, at the latest on day 60. Disease related to growth of metastasis caused neurologic symptoms (paresis) and cachexia. No other adverse effects were observed. For each group, the time from inoculation of the tumor cells to the appearance of symptoms of metastasis was recorded as the mean latency time. Survival analysis was performed according to the Kaplan-Meier method, and survival was compared using the log rank test. Analysis was based on results pooled from 3 different experiments. Statistical analyses were performed using Statistical Package for Social Sciences, version 16.0 (SPSS Inc, Chicago).

Nude female mice with MA-11 xenografts in the right and left flanks were used. Treatment commenced when the mean tumor volume reached 130 to 150 mm^3 /group and the mean body weight was ≤ 25 g. The mice were randomized to 8 to 12 tumors per treatment group. The antibody 4BI (120 mg/kg diluted in 300 μ L corn oil) was administered on days 0 and 6 by intraperitoneal injection and 425.3PE (1.25 μ g/mouse diluted in 200 μ L PBS) was administered 3 to 4 hours after 4BI treatment. Tumor size was measured twice weakly by caliper measurements and tumor volume was calculated (tumor volume = $L \times W^2/2$). The mice were sacrificed when the tumor reached a volume of approximately 1700 mm^3 . No other adverse effects were observed during the treatment period. The relative tumor volume was calculated and set to 1 on day 0. Relative tumor volume was plotted against time (days). Analysis was based on results pooled from 2 different experiments.

RESULTS

IT-mediated Cytotoxicity in MA-11 and HeLa* Cells was Enhanced by TPMP-I-2

TPMP-I-2 at 0.5 to 5 μ M and 4BI at 0.05 to 0.5 μ M had no significant effect on cell viability after 24 hours

treatment on either of the cell lines used in this study (data not shown). TPMP-I-2 and 4BI at concentrations higher than 50 and 5 μ M, respectively, strongly decreased cell viability in all 3 cell lines after 24 and 30 hours as determined by the MTS assay (data not shown). Therefore, the cell lines were treated with TPMP-I-2 and 4BI at doses from 0.5 to 50 μ M and 0.05 to 5 μ M, respectively.

We know from earlier studies that 425.3PE and 9.2.27PE cause inhibition of protein synthesis in cancer cells,^{1,3} and minor inhibition of protein synthesis was achieved with low doses of 425.3PE in MA-11 cells (0.1 ng/mL), HeLa* cells (1 ng/mL 425.3PE), and with a low dose of 9.2.27PE in FEMX cells (10 ng/mL) after 24 hours treatment (Fig. 2A). Furthermore, we assessed whether the triphenylmethyl derivatives, TPMP-I-2 and 4BI, were able to inhibit the protein synthesis in MA-11, HeLa*, and FEMX cells. TPMP-I-2 (50 μ M, 24 h) caused minor inhibition of protein synthesis in the MA-11 and HeLa* cells. In the FEMX cells, TPMP-I-2 (50 μ M, 24 h) was relatively ineffective in inhibiting the protein synthesis. Combining the ITs with TPMP-I-2 markedly enhanced the inhibition of protein synthesis in the 3 cell lines. Furthermore, the 3 cell lines were subjected to 4BI (0.05 to 5 μ M, 24 h) treatment. In contrast to TPMP-I-2, the highest concentration of 4BI (5 μ M) caused inhibition of protein synthesis in HeLa* and FEMX by approximately 60%, and approximately 20% in the MA-11 cells (Fig. 2B). In parallel, this dose caused none to minor decrease in cell viability after 24 hours (Fig. 2C). Through an unknown mechanism, 4BI inhibited the protein synthesis especially in HeLa* and FEMX cells and the inhibition was dose-dependent. TPMP-I-2, in contrast, showed little effect on protein synthesis in these cells (Fig. 2A).

We further wanted to evaluate the cytotoxic effect of 425.3PE or 9.2.27PE in combination with TPMP-I-2 or 4BI in MA-11, HeLa*, and FEMX cells. The effect of increasing doses of 425.3PE in MA-11 and HeLa* cells, and 9.2.27PE in FEMX cells as well as TPMP-I-2 (50 μ M) and 4BI (5 μ M), was determined using the MTS assay. As shown in Figure 2C, TPMP-I-2 strongly enhanced the cytotoxic effect of 425.3PE-treated MA-11 and HeLa* cells after 24-hour treatment. In the FEMX cells, TPMP-I-2 was not able to significantly enhance the cytotoxic effect of 9.2.27PE after 24 hours (Fig. 2C) or 30 hours (Supplementary Figure 1, Supplemental Digital Content 1, <http://links.lww.com/JIT/A110>). Furthermore, 4BI did neither significantly enhance the cytotoxic effect of 425.3PE in MA-11 or HeLa* cells nor in 9.2.27PE-treated FEMX cells (Fig. 2C). In terms of obtaining increased cytotoxicity, these results indicate that especially TPMP-I-2 is promising in combination with ITs. To emphasize the effectiveness of the combination regime, the treatment period of 425.3PE (0.1 ng/mL) and TPMP-I-2 (50 μ M) was extended from 24 to 48 hours in MA-11 cells. Cell viability was reduced by approximately 70% with the combination treatment, compared with approximately 15% for each monotherapy (data not shown).

425.3PE in Combination With TPMP-I-2 Increases DNA Fragmentation in MA-11 Cells

MA-11 cells showed some DNA fragmentation when treated with 425.3PE, TPMP-I-2, or 4BI (12%, 25%, and 32%, respectively) compared with untreated cells (8%). Combining 425.3PE with either TPMP-I-2 or 4BI increased this percentage markedly to 59% and 44%, respectively. In

contrast, limited DNA fragmentation was detected when treating HeLa* and FEMX cells with IT \pm TPMP-I-2 or \pm 4BI (Fig. 3A).

ITs in Combination With TPMP-I-2 Result in Inactivation of PARP

PARP and caspase-3 are commonly used markers for apoptosis. Treatment of MA-11, HeLa*, and FEMX with their respective ITs resulted in partial inactivation of PARP after 24 hours, as reported earlier,^{1,3} whereas TPMP-I-2 (50 μ M) as single-agent had no effect on this DNA repair enzyme (Fig. 3B). Cotreatment of IT and TPMP-I-2 increased the level of inactive PARP (the lower band) in all 3 cell lines. In addition, active caspase-3 was induced upon cotreatment in MA-11 cells, whereas very long-exposure times were needed to visualize this band in HeLa* and FEMX cells, indicating minor induction of active caspase-3 in these cells (Fig. 3B). Furthermore, cleavage of SAF-B, as indicated by the decreased protein level of the 130 kd band and appearance of a approximately 85 kd band, was induced upon cotreatment with IT and TPMP-I-2 in MA-11 cells (Fig. 3B, left panel). SAF-B is a nuclear matrix-associated factor inactivated by active caspase-3 and involved in the regulation of HSP27.^{19,20} Decreased level of HSP27 was observed in cotreated MA-11 cells (Fig. 3B, left panel). The protein levels of SAF-B and HSP27 were not significantly affected in IT \pm TPMP-I-2-treated HeLa* and FEMX cells, supporting our findings of marginal caspase-3 activation in these cells. In addition, a slightly smaller SAF-B band appeared in 9.2.27PE-treated FEMX cells, and also with the combination of 9.2.27PE and TPMP-I-2 (Fig. 3B, right panel). This modified SAF-B protein was not induced by TPMP-I-2 treatment alone.

Cotreatment of IT and TPMP-I-2 Decreased the Protein Level of SCD Responsible for Biosynthesis of Monounsaturated Fatty Acids

Seventy-four genes were differentially expressed with the 425.3PE + 4BI combination (independent of 425.3PE or 4BI treatment alone) compared with untreated MA-11 cells according to our microarray results (Fig. 4A). Of these, 82% (61 of 74) were down-regulated ($P < 0.05$). In the 425.3PE + TPMP-I-2 combination, 35 genes were differentially expressed compared with untreated cells (independent of 425.3PE or TPMP-I-2 treatment alone), whereas 71% (25 of 35) of the genes were up-regulated ($P < 0.05$). Hence, TPMP-I-2 and 4BI in combination with 425.3PE regulate a different diversity of genes (Supplementary Table 1, Supplemental Digital Content 2, <http://links.lww.com/JIT/A111>). Only 5 of these were common for both combination treatments; chemokine (C-X-C motif) ligand 2, interleukin 7 receptor, Kelch-like ECH-associated protein 1, SCD, and MGC9629 (the latter is recently linked to the histone family) (Fig. 4A). Chemokine (C-X-C motif) ligand 2, interleukin 7 receptor, and Kelch-like ECH-associated protein 1 have been found to be involved in cell proliferation and exert antiapoptotic activity.²¹⁻²⁴ The microarray results suggest that these genes are up-regulated by the combination compared with untreated cells. SCD, the rate limiting enzyme of the biosynthesis of mono-unsaturated fatty acids (MUFA), was down-regulated in both combination groups. The quantitative real-time RT-PCR results confirmed the microarray data of 425.3PE + TPMP-I-2-treated MA-11 cells, illustrated in Table 1 as fold differences between treated and untreated cells. In

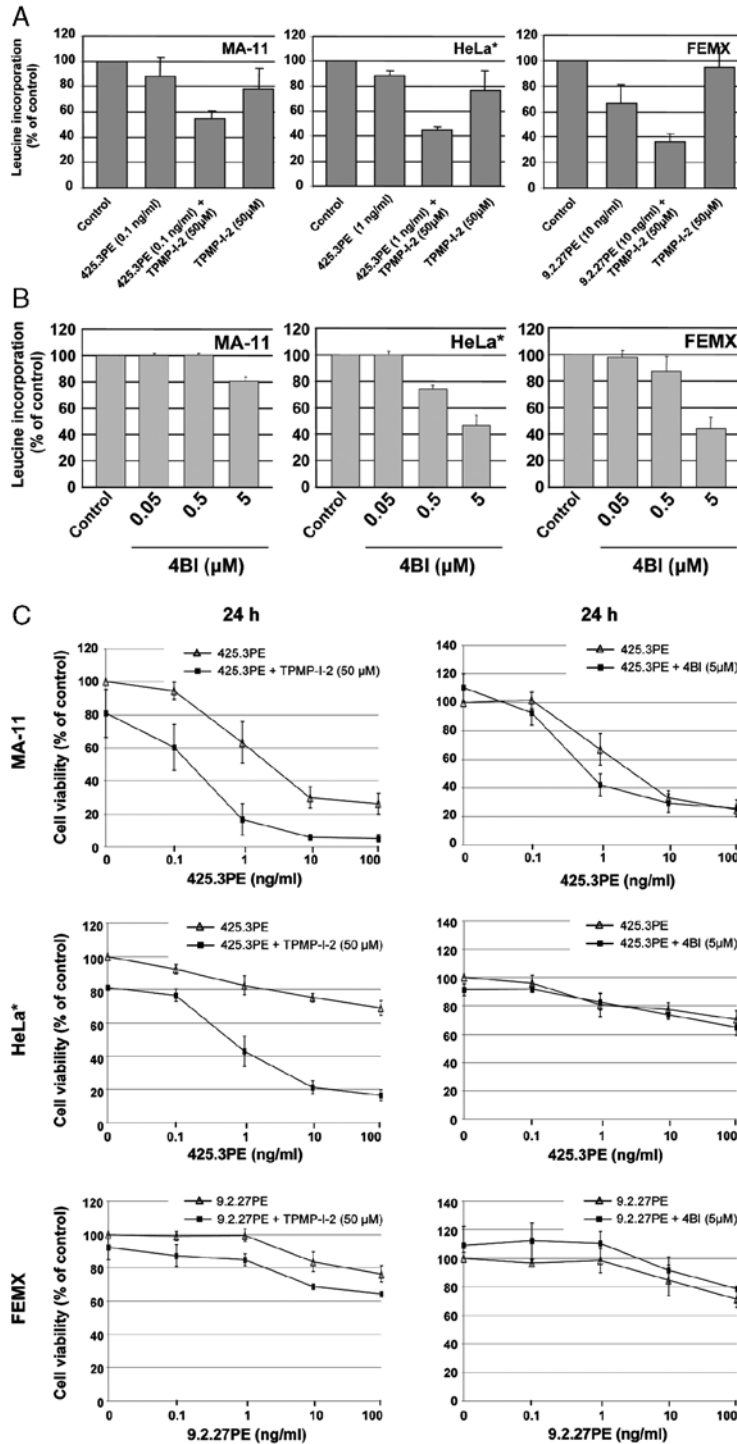


FIGURE 2. A, MA-11, HeLa* cells treated with 425.3PE (0.1 and 1 ng/mL, respectively), and FEMX cells treated with 9.2.27PE (10 ng/mL) ±TPMP-I-2 (50 μM) for 24 hours as described in Materials and Methods. Single treatments of either IT or triphenylmethyl derivatives caused limited inhibition of protein synthesis, whereas a combination of IT and TPMP-I-2 caused substantial inhibition of protein synthesis in all cell lines. B, The 3 cell lines were subjected to 4BI (0.05 to 5 μM, 24h) treatment and protein synthesis inhibition was measured. The highest concentration of 4BI (5 μM) caused inhibition of protein synthesis in HeLa* and FEMX by approximately 60%, and approximately 20% in the MA-11 cells. C, Cytotoxic effect of ITs in combination with either TPMP-I-2 (50 μM) or 4BI (5 μM) in MA-11, HeLa*, and FEMX cells assessed by the MTS assay. TPMP-I-2 strongly enhanced the cytotoxic effect of 425.3PE-treated MA-11 and HeLa* cells after 24 hours treatment. TPMP-I-2 was not able to significantly enhance the cytotoxic effect of 9.2.27PE in the FEMX cells. The 4BI did neither enhance the cytotoxic effect of 425.3PE in MA-11 or HeLa* cells, nor in 9.2.27PE-treated FEMX cells. The experiments were repeated 3 times. IT indicates immunotoxins; PE, *Pseudomonas* exotoxin A.

addition, quantitative real-time RT-PCR data of TPMP-I-2-treated MA-11 cells showed down-regulation of SCD in a dose-dependent manner (Fig. 4D). Furthermore, the SCD protein level was assessed by using Western Blot analysis. Upon treatment with TPMP-I-2 at a concentration which caused no/minor decrease in cell viability (50 μ M), the protein level of SCD was decreased compared with untreated cells in all 3 cell lines (Fig. 4B).

In comparison, the SCD protein was not detectable after monotreatment with 9.2.27PE and 425.3PE in FEMX

and HeLa* cells, respectively, whereas in MA-11 cells, the SCD protein was still clearly visible. However, this effect was dose dependent, as higher concentrations of 425.3PE (1 and 10 ng/mL) decreased the SCD protein level in these cells (Fig. 4C). Similar results were obtained when combining 425.3PE and 4BI in MA-11 cells (Supplementary Fig. 2, Supplemental Digital Content 3, <http://links.lww.com/JIT/A112>). To show that the decreased SCD protein level was not caused by a direct effect of TPMP-I-2 on protein synthesis, MA-11 cells were treated with TPMP-I-2 (0.5 to 5 μ M) and no significant inhibition of protein synthesis was observed (Supplementary Figure 3, Supplemental Digital Content 4, <http://links.lww.com/JIT/A113>). At these low TPMP-I-2 concentrations, the protein expression of SCD was not detectable by Western Blot, indicating a link between the triphenylmethyl derivative TPMP-I-2 and SCD (Fig. 4C). We further investigated the effect of TPMP-I-2 on SREBF1, a transcription factor of SCD. SREBF1 was down-regulated by TPMP-I-2 in a dose-dependent manner as shown in Figure 4D, indicating a link between TPMP-I-2 and SCDs transcription factor SREBF1.

Effects of Combination of 425.3PE and the Triphenylmethyl Derivatives In Vivo

Our in vitro data showed that 425.3PE in combination with TPMP-I-2 enhanced the cytotoxic effects in HeLa* cells. To determine whether these effects could be validated in vivo, nude rats injected intracardially with HeLa* cells were treated with 425.3PE and TPMP-I-2. The combination was more effective than each drug administered individually (Fig. 5A). Thus, the mean latency time was 21 days for the control and the TPMP-I-2 animals, whereas the surviving fraction of rats in the combination group was significantly higher than for the 425.3PE group, with a mean latency time of 44 days versus 37 days. Moreover, 1 rat in the combination group was sacrificed at the end of the experiment (day 60), not showing any signs of disease (Fig. 5B).

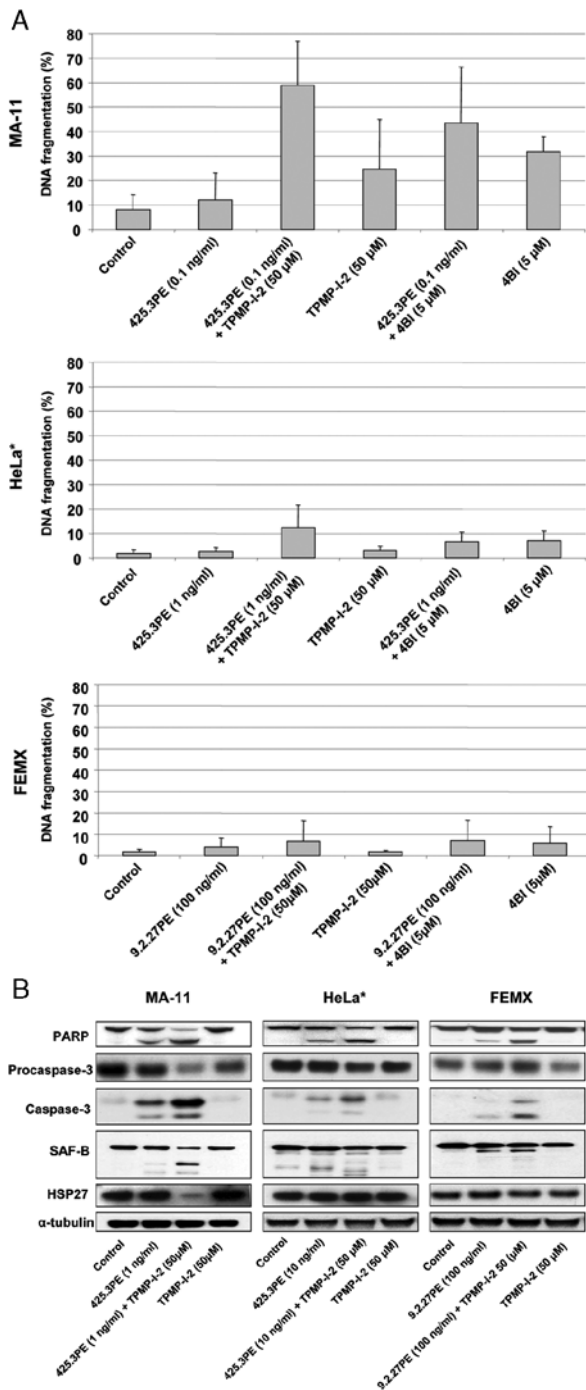


FIGURE 3. A, DNA fragmentation in IT±TPMP-I-2 or 4BI-treated cells. MA-11, HeLa*, and FEMX cells were treated with IT (0.1, 1, and 100 ng/mL, respectively), TPMP-I-2 (50 μ M), 4BI (5 μ M), or a combination of IT±TPMP-I-2 or 4BI as described in Materials and Methods. Increased DNA fragmentation was obtained in MA-11 cells when treated with a combination of IT and TPMP-I-2 or 4BI compared with IT, TPMP-I-2, and 4BI alone. Combining IT and TPMP-I-2 in HeLa* cells increased DNA fragmentation somewhat compared with IT and TPMP-I-2 treatment alone. Minimal DNA fragmentation was obtained with any treatment in FEMX cells. B, Effects on the apoptotic markers PARP and caspase-3, the nuclear matrix-associated factor SAF-B, and HSP27 by combination therapy with IT and TPMP-I-2. MA-11, HeLa*, and FEMX cells were treated with 425.3PE (1 ng/mL for MA-11 and 10 ng/mL for HeLa*) or 9.2.27PE (100 ng/mL for FEMX)±TPMP-I-2 (50 μ M) for 24 hours as described in Materials and Methods. All cell lines showed inactivation of PARP upon IT treatment, indicated by the lower 89 kd band, and activation of caspase-3 was primarily detected in MA-11 cells. In addition, cotreatment leads to cleavage of SAF-B and decreased levels of HSP27 in MA-11 cells. In the HeLa* and FEMX cells, very little active caspase-3 was detected, and SAF-B was not significantly inactivated. * indicates SAF-B cleaved by 9.2.27PE only. The data shown is a representative example of 3 experiments. HSP27 indicates heat shock protein 27; IT, immunotoxins; PARP, poly(adenosine diphosphate-ribose) polymerase; PE, *Pseudomonas* exotoxin A; SAF-B, scaffold attachment factor B.

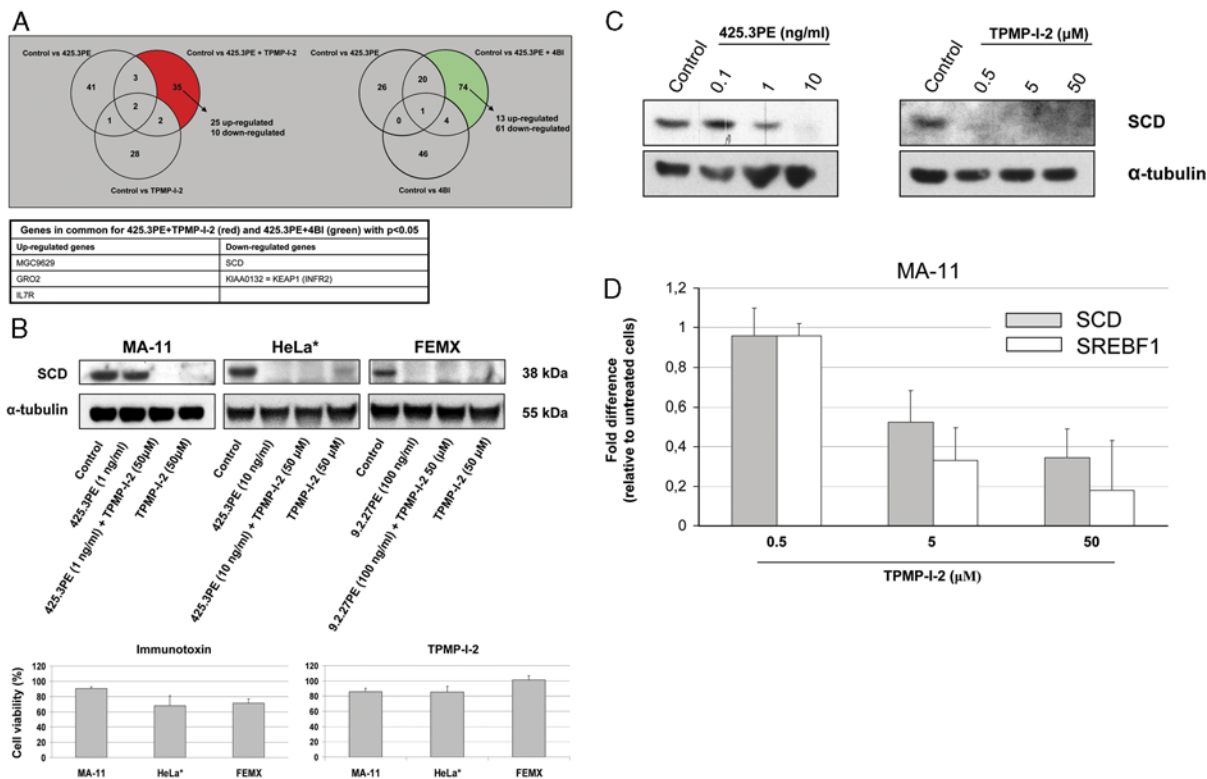


FIGURE 4. A, Venn diagram of control versus 425.3PE, control versus TPMP-I-2, and control versus 425.3PE+TPMP-I-2; and of control versus 425.3PE, control vs. 4BI, and control versus 425.3PE+4BI after 24 hours treatment. For the combination 425.3PE (0.1 ng/mL)+TPMP-I-2 (50 μM), 35 genes were regulated compared with the control (independent of 425.3PE or TPMP-I-2 treatment alone), where 25 were up-regulated and 10 were down-regulated. For the combination 425.3PE (0.1 ng/mL)+4BI (5 μM), 74 genes were regulated compared with the control (independent of 425.3PE or 4BI treatment alone), 13 up-regulated and 61 down-regulated. B, SCD protein expression levels in IT+TPMP-I-2 (50 μM)-treated MA-11, HeLa*, and FEMX cells. TPMP-I-2 strongly decreased the protein level of SCD in all 3 cell lines. In parallel, cell viability was only slightly decreased in the MA-11 and the HeLa* cells by the same concentration TPMP-I-2 (50 μM). No decrease in cell viability was observed in the FEMX cells. MA-11 (425.3PE, 1 ng/mL), HeLa* (425.3PE, 10 ng/mL), and FEMX cells (9.2.27PE, 100 ng/mL), caused some decreased cell viability, in parallel, the protein level of SCD was strongly decreased in HeLa* and FEMX cells. C, SCD protein expression levels in 425.3PE and TPMP-I-2-treated MA-11 cells. MA-11 cells were treated with increasing doses 425.3PE (0.1 to 10 ng/mL) and TPMP-I-2 (0.5 to 50 μM) for 24 hours as described in Materials and Methods. There was a dose-dependent effect on SCD levels in 425.3PE-treated cells, with no detectable SCD protein levels at 10 ng/mL. In TPMP-I-2-treated MA-11 cells, a SCD protein level was strongly decreased at 0.5 μM and not detectable at concentrations of 5 and 50 μM. D, Quantitative real-time reverse transcription-polymerase chain reaction results in TPMP-I-2-treated MA-11 cells. MA-11 cells were treated with increasing concentrations of TPMP-I-2 for 24 hours as described in Materials and Methods. The mRNA level of untreated cells were set to 1, and mRNA levels of treated cells were calculated accordingly. A dose-dependent decrease of mRNA levels of SCD and its transcription factor sterol regulatory element binding transcription factor 1 (SREBF1) levels was observed. The experiment was repeated 3 times. IL7R indicates interleukin 7 receptor; IT, immunotoxins; PE, *Pseudomonas* exotoxin A; SCD, stearyl-CoA desaturase.

Survival was compared using the log-rank test and a *P* value of less than 0.0001 was obtained, indicating a significant difference in the survival of the groups. A significant difference between the control and the 425.3PE group

(*P* < 0.0001), and the 425.3PE and the TPMP-I-2 + 425.3PE group was also obtained using the log-rank test (*P* = 0.004).

Nude mice with subcutaneous MA-11 xenografts were then treated with 425.3PE and 4BI. The combination regime increased tumor growth delay for a 2-fold tumor size increase compared with untreated control animals (TGD2) to 10 days compared with mice treated with each of the drugs individually (TGD2 on day 7) and the control animals (TGD2 on day 3) as shown in Figure 5C. One animal each in the control and the 425.3PE group, were sacrificed on days 10 and 14, respectively, when the maximal tumor volume limitation of 1700 mm³ was reached.

TABLE 1. Fold Difference in Expression Levels of Stearyl-CoA Desaturase (SCD) in Treated Versus Untreated MA-11 Cells

| Treatment | Fold Difference (Treated vs. Untreated) | |
|--------------------|---|-------------|
| | Microarray | qRT-PCR |
| 425.3PE + TPMP-I-2 | 0.3 | 0.26 ± 0.07 |
| 425.3PE + 4BI | 0.1 | 0.19* |

*qRT-PCR performed once only
 Comparison of microarray and quantitative real-time RT-PCR results (qRT-PCR ± SEM).
 PE indicates *Pseudomonas* exotoxin A.

DISCUSSION

Combinations of different drugs are frequently used in cancer therapy, as various cellular functions can be targeted

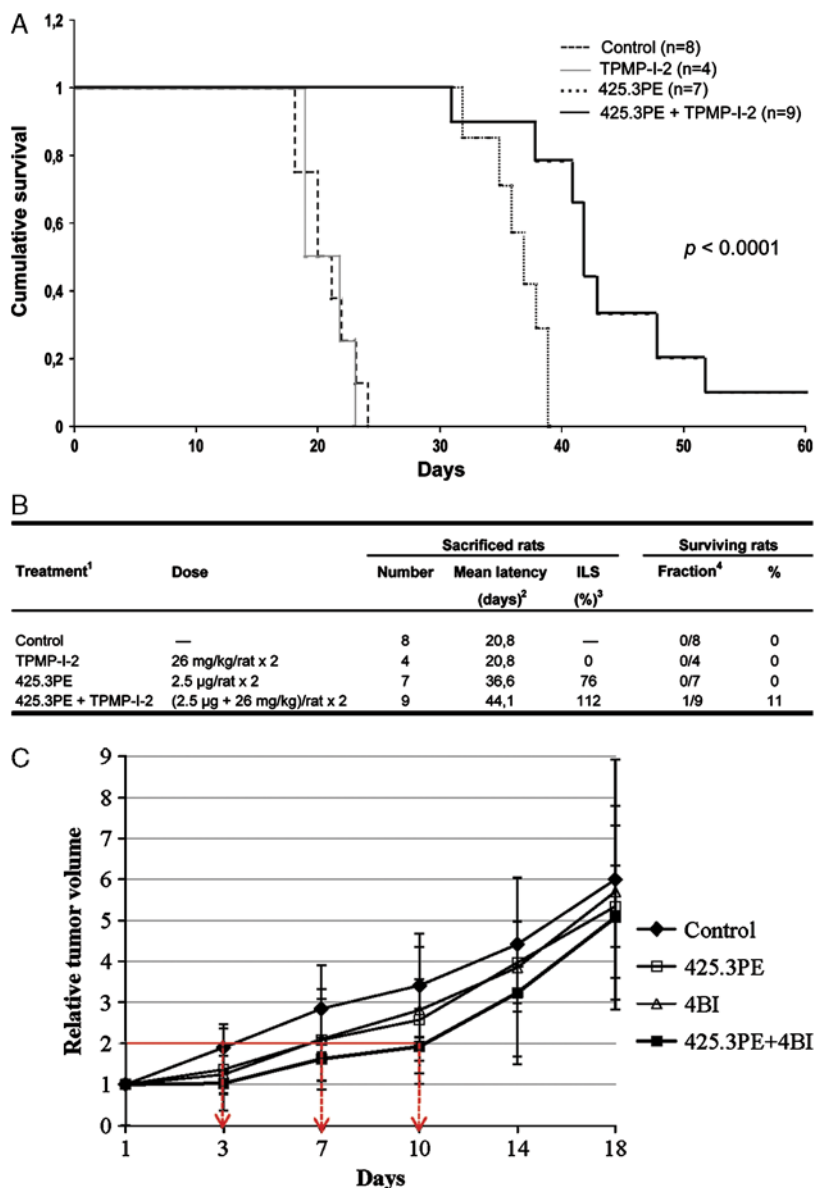


FIGURE 5. Survival curves (A) of nude rats injected with HeLa* cells in left cardiac ventricle. The rats were treated with 425.3PE +/- TPMP-I-2 or control vehicle on day 0 and 6. This graph illustrates the tumor-free survival of the animals, ie, the time from the injection of the tumor cells to the appearance of symptoms of progressive disease. Survival was compared using the log rank test and a P -value of < 0.0001 was obtained, indicating a significant difference in the survival of the groups. A significant difference between the control and the 425.3PE group ($P < 0.0001$), and the 425.3PE and the TPMP-I-2 + 425.3PE group was also obtained using the log rank test ($P = 0.004$). B, Anti-metastatic effect of 425.3PE and TPMP-I-2 administered in nude rats injected with 1×10^6 HeLa* cells into the left cardiac ventricle. ¹Cells injected on day 0, treatment given on day 1 and 7. ²Values calculated for the deceased animals in the various treatment groups. ³ILS – Increase in Life Span, calculated from the survival in the control group compared with survival in the respective treatment group. ⁴Values given as the number of animals surviving/total number of animals in each treatment arm. The follow-up period was 60 days. C, The 425.3PE in combination with 4BI is efficacious in MA-11 xenograft model. TGD₂ was increased to day 10 for the 425.3PE + 4BI group compared to days 3 for the control group as indicated by the red lines. Filled diamond = vehicle, open circle = 4BI (120 mg/kg), open square = 425.3PE (1.25 µg/mouse), filled square = 425.3PE (1.25 µg/mouse) + 4BI (120 mg/kg). PE indicates *Pseudomonas* exotoxin A; TPMP, triphenylmethyl phosphonates and phosphonochloridates.

simultaneously. The aim of this study was to further enhance the efficacy of our ITs by combining the ITs with two different triphenylmethyl derivatives, TPMP-I-2 and 4BI, shown to induce apoptosis in melanoma cell lines in culture.⁷ Our ITs have previously been found to induce cell death through inhibition of protein synthesis and induction of apoptosis in various cancer cell lines.^{1,3,4}

Enhanced cytotoxic effects were obtained in MA-11 breast cancer cells and HeLa* cervical cancer cells by combining the 425.3PE with the triphenylmethyl derivative TPMP-I-2. Combining 425.3PE with 4BI did not show significant enhanced cytotoxicity in either of the cell lines used, Ma-11, HeLa*, or the FEMX melanoma cell line. However, the combination of 423.5PE and 4BI showed a

tendency to slow down tumor growth in the MA-11 breast cancer xenograft model, compared with monotreatments of 425.3PE and 4BI. This result emphasizes that the *in vitro* data can not directly be translated to an *in vivo* situation, and treatment with 425.3PE and 4BI in combination might be useful for the treatment of solid tumors.

Apoptosis is associated with certain biological and morphologic features, and caspase-3 and PARP are commonly used as biochemical markers for detection of apoptosis. In addition, increased DNA fragmentation is often a result of caspase-3 activation.²⁵ Our Western Blot results show that increased inactivation of PARP was observed by combining ITs with TPMP-I-2 in all 3 cell lines. In addition, active caspase-3 and DNA fragmentation was observed, primarily in the MA-11 breast cancer cells. The difference in active caspase-3 protein levels and DNA fragmentation is likely dependent on the cell line used. IT-treated MA-11 breast cancer cells has previously been linked to strong apoptotic features,¹ whereas the FEMX malignant melanoma cells only shown some apoptotic features after IT treatment.³ Malignant melanoma cells are highly resistant to induction of apoptosis,²⁶ possibly explaining minor activation of caspase-3 and limited DNA fragmentation in these cells upon treatment. The results indicate that TPMP-I-2 enhance the cytotoxic effect of the ITs, as increased inactivation of PARP and increased cell death is observed in all 3 cell lines used in this study.

Continuous proliferation of cancer cells depends on biosynthesis of MUFA to sustain the increased need of phospholipids for membrane biogenesis, metabolic energy, and important signaling molecules. The rate limiting enzyme in this synthesis from saturated fatty acids to MUFA is SCD.^{27,28} Expression of SCD is increased in cancers,^{28–31} and ablation of SCD expression has been shown to decrease cancer cell proliferation and *in vitro* invasiveness *in vitro* and to impair tumor formation and growth *in vivo*.^{13,28,32} Upon treatment with IT, TPMP-I-2, and 4BI as single agents or in combination, the protein level of SCD was decreased in the 3 cancer cell lines used in this study. The decreased protein level of SCD caused by the ITs is likely an effect of inhibited protein synthesis, as SCD has a relatively short half-life of approximately 3 hours.³³ To exclude that the decreased SCD protein level was a result of inhibited protein synthesis caused by TPMP-I-2, very low concentrations of this triphenylmethyl derivative was used for treatment of MA-11 cells. It is interesting to note that the SCD protein level was decreased by TPMP-I-2 concentrations not affecting general protein synthesis. In addition, the quantitative real-time RT-PCR results show that TPMP-I-2 ± 425.3PE down-regulate the gene expression of SCD and its transcription factor SREBF1 in MA-11 cells, suggesting a role of these triphenylmethyl derivatives on a transcriptional level in the cells. Preliminary quantitative real-time RT-PCR results indicate that the triphenylmethyl derivative 4BI down-regulates SCD gene expression (data not shown). Taken together, our results indicate an association between several triphenylmethyl derivatives and decreased mRNA levels of SCD. The decreased SCD levels observed when treating cancer cells with IT ± TPMP-I-2 or ± 4BI is possibly a contributing factor to the observed increased cytotoxicity, as it has been shown that inhibition of SCD expression in human lung adenocarcinoma cells impairs cell proliferation and increases apoptosis, in addition to significantly delaying tumor formation and reducing the growth rate of tumor xenografts in mice.¹³ In a

rat model simulating micrometastatic disease, we examined the effect of 425.3PE, TPMP-I-2, and 425.3PE + TPMP-I-2. Although 425.3PE alone showed prolonged survival of the rats compared with the control group, TPMP-I-2 did not have an effect as a single-entity agent at the dose used. Importantly, TPMP-I-2 enhanced the effect of 425.3PE resulting in further prolongation in survival time.

Several potent specific inhibitors of SCD have been synthesized and they show positive anticancer activity in *in vitro* and *in vivo* models.^{27,34–36} At least one of them, the BZ36, has been shown to inhibit the AKT pathway,³¹ a pathway which functions to promote tumor growth in cancer cells. Furthermore, inhibition of NFκB is linked to inhibition of AKT,³⁷ and the triphenylmethyl derivative 4A has been shown to reduce the level of active NFκB,⁷ which indicates that triphenylmethyl derivatives may exert their effect through inhibition of SCD involving inhibition of the AKT/NFκB cell survival pathway. Evidence indicates that (long-term) endoplasmic reticulum (ER) stress negatively regulates the PI-3K/AKT pathway.³⁸ As no activation of AKT was seen in IT ± TPMP-treated MA-11 cells after 24 hours (data not shown), ER stress cannot be ruled out as a cause for the enhanced cytotoxic effect observed by the combination treatment. Prolonged ER stress can induce apoptosis through caspase-3 activation.³⁵ Further investigations are needed to elucidate the effect of IT ± TPMP on the ER stress machinery. In a large siRNA screening, 3700 genes examined, SCD was shown to be 1 of the 3 main promising anticancer targets, further strengthening the importance of inhibited SCD activity in anticancer therapy.³⁹ Afterwards, several studies on knockdown of SCD protein in tumor cells by siRNA have implicated that an accumulation in saturated fatty acids is lipocytotoxic and that a lack of endogenous synthesis of MUFA decrease cancer cell survival.^{27,28,36} The induction of cancer cell death could be linked to ER stress and induction of apoptosis through caspase-3 activation.³⁵ It is interesting to note that the siRNA ablation of SCD in noncancer cells was shown to be nontoxic.³⁵ This is supported by our results showing that our TPM-derivates ± IT are nontoxic to human umbilical vein endothelial cells (data not shown). Further investigation is needed to elucidate the effect of TPMP-I-2 and 4BI on a molecular level. In summary, our studies show that TPMP-I-2 or 4BI (in low concentrations), which as single agents only have minor effect on decreased cell viability, enhances the cytotoxic effect of ITs which results in increased apoptosis in different cancer cells. In addition, expression of SCD, an important enzyme in the biosynthesis of MUFA needed for membrane genesis, was reduced by cotreatment of IT and TPMP-I-2 in all cell lines used, as well as by monotreatment of IT and TPMP-I-2. The prolonged survival and slower tumor growth observed in animal models using IT in combination with either TPMP-I-2 or 4BI, may involve several essential mechanisms impeded by the combination of ITs and triphenylmethyl derivatives, making such combinations promising as a new anticancer treatment strategy.

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