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Cell Toxicology Study of RRR-Alpha-Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS).

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ABSTRACT

Cell Toxicology Study of RRR-alpha-Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS)

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This research focused on the cytotoxic properties of RRR-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) in transformed and cancerous cell lines. We used RAW264.7 macrophage and prostate cancer (LNCaP) cell lines in this study. TPGS caused cell death and decreased cell viability in a dose and time dependent manner. Cell death was evaluated fluorimetrically by employing the nucleic acid-binding fluorophore; propidium iodide. A colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate cell viability. Cell death can occur through necrosis or apoptosis. Our results suggested that TPGS triggered apoptotic cell death. Induction of apoptosis, as measured by caspase 3 enzymatic activity, was dependent upon the TPGS dose and incubation time. Caspase 8 was activated before caspase 9, suggesting the importance of the death receptor pathway in apoptosis. Our results indicated that TPGS cytotoxicity could also be due to one of its products of hydrolysis, alpha-tocopheryl succinate.

ACKNOWLEDGEMENTS

There are so many people to thank and acknowledge for their role in my life. However, this page is specifically designed to note my appreciation of those people who stand out most notably in my mind as contributing to the success of this work.

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CHAPTER 1

INTRODUCTION

RRR-alpha-tocopheryl polyethylene glycol 1000 succinate, a water-soluble form of vitamin E, was invented by Eastman Chemical Company (Kingsport, TN) in 1950. The acronym for RRR-alpha-tocopherol polyethylene glycol 1000 succinate is TPGS. TPGS is synthesized from RRR-alpha-tocopherol succinate and polyethylene glycol (PEG) 1000. The PEG 1000 with an average molecular weight of about 1000 Dalton is synthesized from fossil fuel while the d-alpha-tocopherol is derived from vegetable oils, which is esterified to succinic acid to form d-alpha-tocopherol succinate.[1] TPGS is a derivative of natural RRR-alpha-tocopherol, a member of the vitamin E family. The chemical structure of TPGS is represented below.

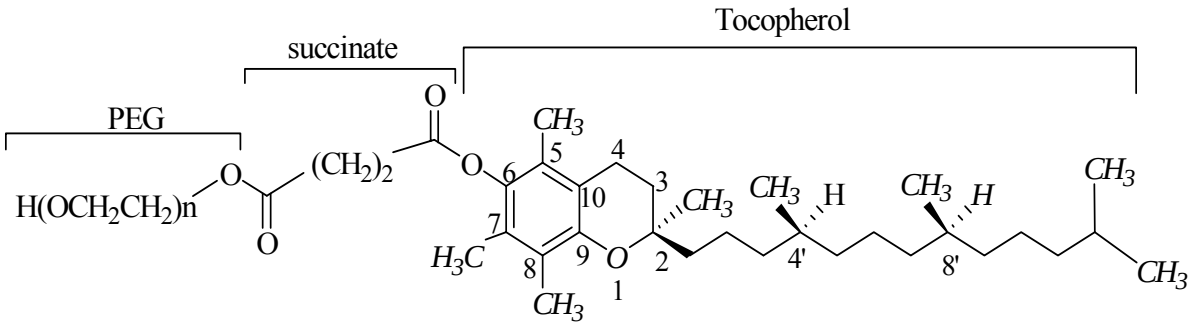
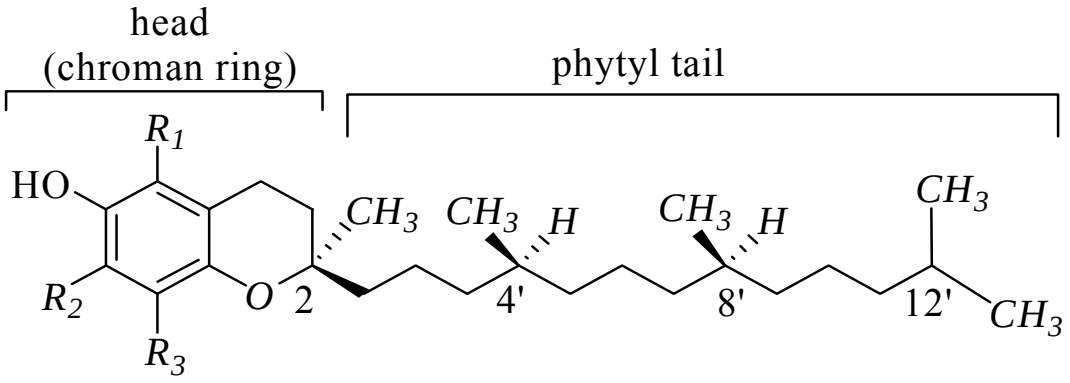


Figure 1. The chemical structure of TPGS.

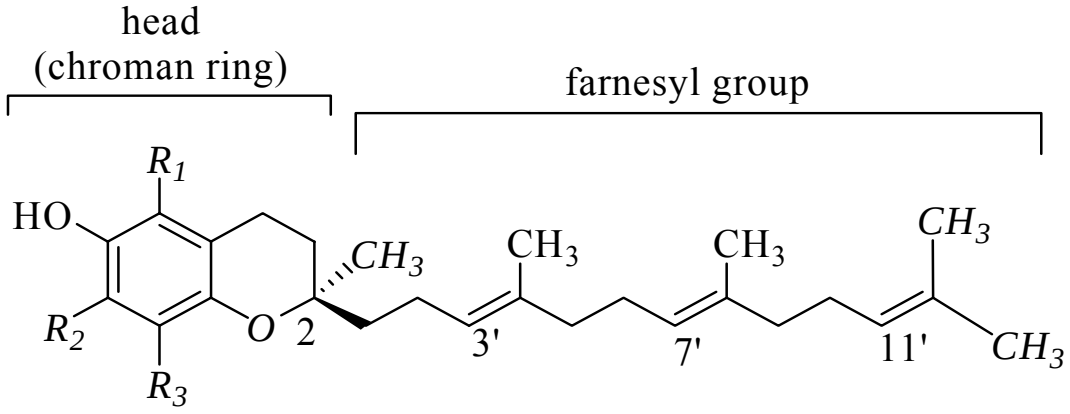
Structural Relationship between the Vitamin E Family and TPGS

At least eight different compounds make up the vitamin E family. There are four tocopherols (alpha, beta, gamma, and delta) and four tocotrienols (alpha, beta, gamma, and delta). They are fat-soluble vitamins and function as chain-breaking antioxidants by preventing the propagation of destructive free radicals. In addition, they have nonantioxidant functions such as cellular signaling and prevention of infertility.[2, 3, 4] Structurally they all have the chroman ring (head) and tocopherols have a phytyl tail while the tocotrienols have a farnesyl group. Structurally, TPGS is very similar to the tocopherols, it has a phytyl tail and the chroman ring

but the hydroxyl group on the chroman head is esterified to the polyethylene 1000 succinate moiety. Like tocopherols, TPGS has three asymmetric carbons. One of the chiral centers is located at the carbon 2 of the heterocyclic ring and the other two are located on the 4^{th} and 8^{th} carbon of the phytyl tail. There are two possible configurations for each chiral center R and S (i.e. right and left configuration respectively); therefore, the tocopherols can have 8 (2^3) stereoisomers. Naturally occurring tocopherol exists in the RRR configuration at all 3 chiral centers. Synthetic alpha-tocopherol is a mixture of approximately equal amounts of RRR, RRS, RSS, SSS, SSR, SRR, SRS, and RSR stereoisomers and is usually identified as all-rac (all-racemic) or as dl-alpha-tocopherol. TPGS which is a derivative of natural alpha-tocopherol also has the RRR configuration at the three chiral centers and its correct chemical name is RRR-alpha-tocopheryl polyethylene glycol 1000 succinate or 2R, 4′R, 8′R-alpha-tocopheryl polyethylene glycol 1000 succinate or better still, 2,5,7,8-tetramethyl-2- (4′8′12′-trimethyltridecyl)-6-chromanyl polyethylene glycol 1000 succinate. The tocotrienols have a chiral center at C2 carbon in the heterocyclic ring which can have two possible stereoisomers (i.e. R or S configuration) and three double bonds located at the 3′, 7′, and 11′ positions of the farnesyl group. The double bonds at the 3′ and 7′ positions can generate geometric isomers (E or Z isomers). Natural tocotrienols are E,E in the 3′ and 7′ positions. The chemical structures of the tocopherols and tocotrienols are shown in Figure 2 below.



tocopherol structure



tocotrienol structure

When $R_1 = R_2 = R_3 = CH_3$ alpha-tocopherol / tocotrienol

When $R_1 = R_3 = CH_3$, $R_2 = H$ beta-tocopherol / tocotrienol

When $R_2 = R_3 = CH_3$, $R_1 = H$ gamma-tocopherol / tocotrienol

When $R_1 = R_2 = H$, $R_3 = CH_3$ delta-tocopherol / tocotrienol

Figure 2. Structures of various homologs of tocopherol and tocotrienol. The four tocopherols and tocotrienols share a similar chromanol moiety. However, tocopherols have saturated phytyl tail, while tocotrienols have unsaturated farnesyl side-chain.

Properties of TPGS

TPGS is a waxy cream white solid with an average molecular weight of about 1513 Dalton. It is very stable and does not hydrolyze under normal conditions except in the presence of acids or bases where the ester linkages are hydrolyzed. Vitamin E TPGS is a water-soluble form of natural vitamin E; the polyethylene glycol (PEG) 1000, portion of the molecule is water soluble, while the d-alpha-tocopherol portion is fat-soluble.[5] It is amphipathic (has both hydrophilic and lipophilic ends) and forms its own micelles; it has a critical micelle concentration of about 20% wt/wt of water. Recent studies have shown that it can serve as a bioavailable source of natural vitamin E for people with fat malabsorption, such as childhood chronic cholestasis and congenital hepatic cholestasis.[6, 7] It melts between 37-41^{o}C and is heat stable at temperatures up to 199^{o}C; this is very important in the pharmaceutical industry because it can be processed at thermally stable temperatures without degradation. TPGS is used as an ingredient in making drugs such as amprenavir, an HIV protease inhibitor, to increase the solubility and permeability of the drug.[8, 9] Studies also indicate that TPGS functions as an emulsifier in enhancing the absorption of certain fat-soluble drugs and vitamins like cyclosporine and vitamin D.[10, 11]

Unlike members of the vitamin E family, TPGS does not possess antioxidant ability. TPGS is a diester of the well-known antioxidant alpha-tocopherol. When taken up by cells, TPGS can undergo intracellular hydrolysis to alpha-tocopheryl succinate and polyethylene glycol 1000.[12] The alpha-tocopheryl succinate can further hydrolyze to alpha-tocopherol and succinate. Alpha-tocopherol, like other members of the vitamin E family, functions as a scavenger of damaging free radicals which, if not controlled, can cause cancer and other diseases.

Free Radicals, Antioxidants, and Cancer

Cancer refers to a disease in which the body's cells become abnormal and divide without control. Cancer development involves modulation of genes and increased cell proliferation. Various endogenous and environmental factors have been linked to the development of cancer. Studies have shown that reactive oxygen species are highly involved in the development of

human cancer.[13] Sources of reactive oxygen species may be exogenous such as drugs, ozone, cigarette smoking, and ultraviolet radiation; and endogenous like free radicals produced via the respiratory chain in the mitochondria.[13, 14] Free radicals are molecules with unpaired electrons, often highly reactive once formed and can cause damage to important cellular components such as DNA, protein, or the cell membrane. The damage to these important cellular components can lead to poor functioning of cells or even cell death. To prevent free radical damage the body has a defense system of antioxidants. Antioxidants are molecules that can safely interact with free radicals and terminate potentially damaging reaction to vital biomolecules. Reactive oxygen species are formed via the single electron reduction of oxygen. The scheme for the formation of reactive oxygen species (ROS) is presented below.

$O_2 + e + H^+ \rightarrow HO^{\cdot}_2$	Hydroperoxyl radical	(1)
$HO^{\cdot}_2 \rightarrow H^+ + O^{-\cdot}_2$	superoxide radical	(2)
$O^{-\cdot}_2 + 2H^+ + e \rightarrow H_2O_2$	hydrogen peroxide	(3)
$H_2O_2 + e \rightarrow OH^- + {}^{\cdot}OH$	Hydroxyl radical	(4)
$O^{-\cdot}_2 + NO \rightarrow ONOO$	peroxynitrite	(5)
${}^{\cdot}OH + e + H^+ \rightarrow H_2O$		(6)

Although the body has several chemical antioxidants such as urate, glutathione, lipoic acid, pro-cysteine, lipid-soluble flavonoids, and retinoids that scavenge destructive ROS, there is need for supplementation with antioxidants such as vitamin E, beta-carotene, vitamin C, and selenium which is a trace element that is required for proper function of glutathione peroxidase which metabolizes lipid hydroperoxides formed from polyunsaturated fatty acids or H_20_2. When there is a deficiency of antioxidants, the rates of production of damaging free radicals can exceed the ability of antioxidants to fight them; this physiological condition is known as oxidative stress. Oxidative stress has been shown to contribute to aging and cancer by causing lipid peroxidation, cross-linking of proteins and DNA damage.[15]

Lipid peroxidation has received considerable attention in recent years because of its possible role in aging and cancer.[16, 17] During lipid peroxidation, polyunsaturated fatty acids (PUFA) are attacked by free radicals and converted to lipid peroxides. This follows a free

radical chain mechanism proceeding in three steps; chain initiation, chain propagation, and chain termination. In the initiation step, a free radical $X^{\bullet}$ such as a hydroxyl radical, the most damaging free radical in biological systems, abstracts a proton from a bisallylic methylene group of a polyunsaturated fatty acid (LH) molecule to form a carbon-centered lipid radical, ($L^{\bullet}$). This radical is very unstable and rearranges to a conjugated diene radical.

$$X^{\bullet} + LH \rightarrow XH + L^{\bullet} \qquad (7)$$

In the propagation step, the conjugated diene radical reacts with an oxygen molecule to form a lipid peroxyl radical. The lipid peroxyl radical formed reacts with another molecule of lipid to form lipid hydroperoxide and another carbon-centered lipid radical. This chain propagation continues over and over and one molecule of chain-initiating radical may cause the oxidation of thousands of lipid molecules.

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \qquad (8)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \qquad (9)$$

The scheme is presented in Figure 3 below.

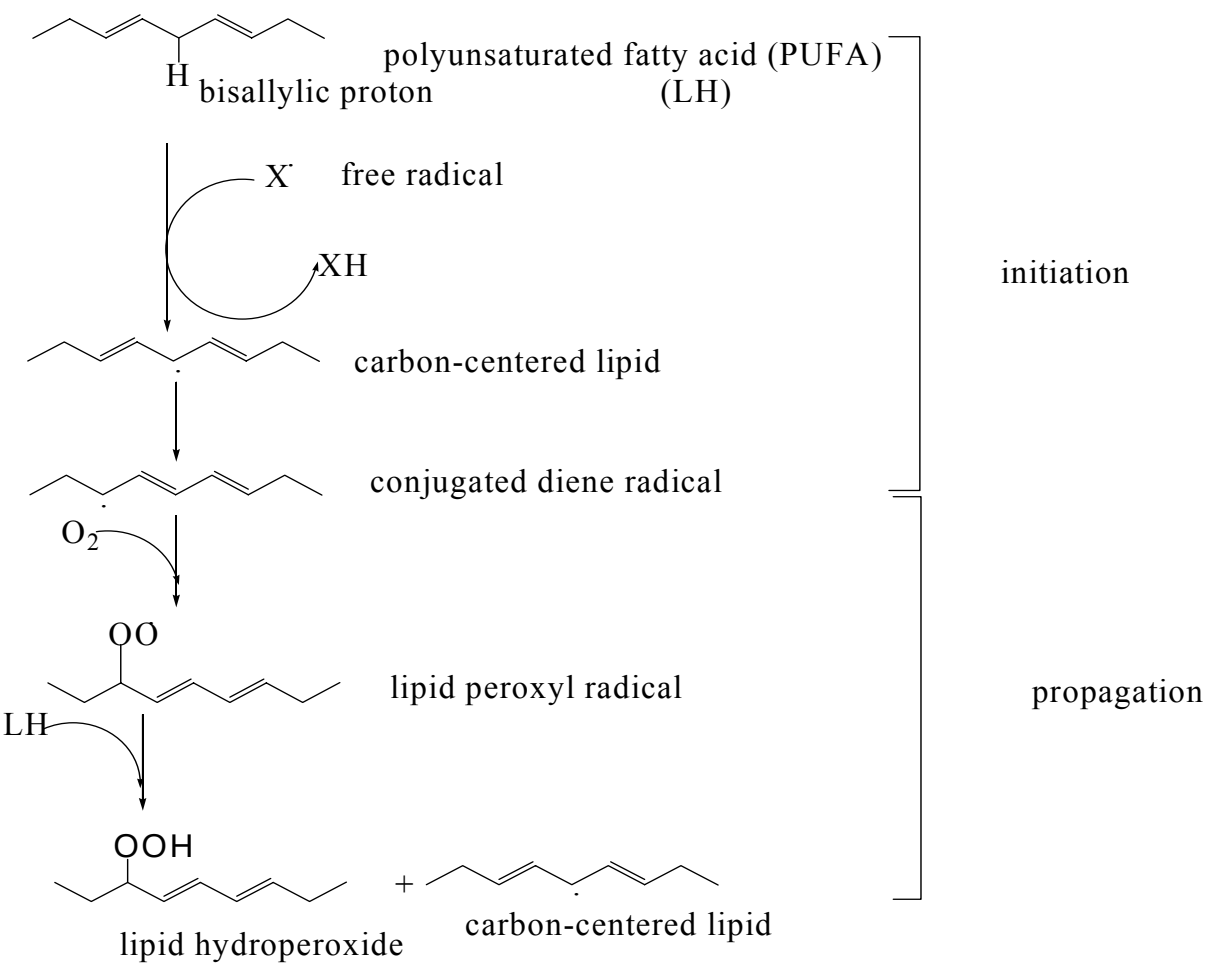


Figure 3. Scheme for lipid peroxidation.

The last step is the termination in which the free radicals are quenched by other free radicals, proteins, or compounds that act as free radical trap like vitamin E and ascorbate. The radicals may be reduced to non-radical species like hydroperoxyl fatty acid (LOOH) or may undergo cyclization to produce cyclic endoperoxides.[18] Scheme for quenching of free radicals is shown below.

$LOO^{\cdot} + LOO^{\cdot} \rightarrow$ Non-radical products (10)

$L^{\cdot} + LOO^{\cdot} \rightarrow$ Non-radical products (11)

$L^{\cdot} + L^{\cdot} \rightarrow$ Non-radical products (12)

The main role of vitamin E is to prevent oxidative stress by keeping the concentration of free radicals during lipid peroxidation at a minimum. Vitamin E, as a chain-breaking antioxidant, effectively traps these destructive free radicals thereby protecting membranes from being damaged. Vitamin E owes it antioxidant power to the hydroxyl group in the phenolic ring of the chroman head. Vitamin E donates its phenolic proton to a free radical thus making it less reactive. The chain breaking reaction of vitamin E (TOH,) can be represented as follows:

$LOO^{\cdot} + TOH \rightarrow LOOH + TO^{\cdot}$ (13)

$LOO^{\cdot} + TO^{\cdot} \rightarrow$ non-radical products (14)

$TO^{\cdot} + TO^{\cdot} \rightarrow$ non-radical products (15)

The damaging action of the lipid peroxyl radical can be prevented via the coupling reaction with vitamin E and vitamin E radical (equations 13 and 14 respectively), to generate non-radical species like the lipid hydroperoxide. The vitamin E radical ($TO^{\cdot}$) is stabilized by resonance by the pi electrons in the benzene ring. The lipid peroxyl radicals ($LOO^{\cdot}$) are chain carriers in the chain reaction of lipid peroxidation. Their removal in reactions (13) and (14) disrupts the further propagation of the reaction. The action of vitamin E as a chain breaker is very unique; it anchors itself in biological membranes with its phytyl side chain embedded deep in the hydrophobic domain. Its hydrophilic head extends outward, closer to the surface. This gives it a good position to fight free radicals and protect fatty acids.[19] Vitamin C and other antioxidants regenerate the reduced vitamin E form to the required antioxidant form. Equation for the regeneration of alpha-tocopherol by ascorbate (AH) is shown below.[20]

$TO^{\cdot} + AH \rightarrow TOH + A^{\cdot}$ (16)

Antioxidants may help prevent cancer by preventing damage to DNA that could cause mutations. It has been suggested, therefore, that antioxidants can be useful as chemopreventive agents. In contrast, chemotherapeutic agents are used to treat preexisting cancer. Studies have shown that alpha-tocopheryl succinate one of the products of hydrolysis of TPGS is highly cytotoxic to various cancer cells compared to normal cells and hence may be useful as a chemotherapeutic agent.[21, 22] Alpha-tocopheryl succinate has been proven to induce apoptotic cell death thereby limiting inflammatory effects.[23] The terminal carboxylic moiety is important for the apoptogenic property of alpha-tocopheryl succinate.[24,25] The *in vitro* cytotoxicity of TPGS is an important factor in evaluating its potential role as an anticancer drug.

Cytotoxicity

Cytotoxicity simply refers to the cell-killing property of a chemical compound. An objective of this research was to study the cytotoxicity of TPGS. Various assays have been designed to measure cytotoxicity. In our research, we used the propidium iodide assay to measure dead cells in a population of cells, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used to measure metabolically active cells, and the caspase enzymatic activity was used to assay for apoptosis (apoptosis is a tightly regulated form of cell death).

Propidium iodide is a nucleic acid-binding fluorophore. It is excluded from viable cells but is membrane permeable to dying or dead cells. It is commonly used for identifying dead cells in a population of cells. Propidium iodide intercalates into double stranded nucleic acid by interacting with four or five of its base pairs (adenine, thymine, cytosine, uracil, or guanine). This binding greatly enhances its fluorescence.

MTT measures metabolically active cells. The up take of tetrazolium salt (MTT) by cells leads to intracellular cleavage of the yellow tetrazolium salt MTT by the succinate-tetrazolium reductase system to form purple formazan crystals in the respiratory chain of mitochondria. These purple formazan crystals are membrane impermeable and are insoluble in aqueous solution, but upon addition of solubilizing agents like DMSO, they become soluble and are liberated into the assay buffer.

Caspases are a family of cysteine proteases which become activated when a cell is about to undergo apoptosis. More than 10 caspases have been identified. Caspase 2, 8, 9, and 10 are involved in initiation of apoptosis, while caspase 3, 6, and 7 execute the death order by destroying essential proteins in the cell. Other caspases that have nothing to do with apoptosis such as caspases 1, 4, 5, and 11 are primarily involved in cytokine processing. Before an apoptosis-triggered event, caspases exist as inactive proenzymes which become activated during apoptosis.

Necrosis and Apoptosis

Cytotoxic agents induce cell death to affected cells. Two mechanisms of cell death have been identified; necrosis and apoptosis. Exposure of cells to cytotoxic drugs makes them lose the integrity of their plasma membrane and become permeable irrespective of whether the form of cell death is necrosis or apoptosis. Assays such as caspase enzymatic study distinguish between necrosis and apoptosis.

Necrosis is an accidental form of cell death caused by adverse conditions in the environment. It is characterized by the swelling of the cell and its organelles and a subsequent rupture and release of intracellular contents causing damage to surrounding cells leading to inflammation.

Apoptosis is a tightly regulated form of cell death also called programmed cell death (PCD). It is characterized by the early activation of caspases, cell shrinkage, membrane blebbing, and the hallmark of apoptosis is the fragmentation of DNA. Apoptotic bodies formed during apoptosis are engulfed by phagocytes like the macrophages and dendritic cells. These phagocytes secrete cytokines that regulate inflammation. Apoptosis is very important for growth and development. It is also involved in aging and various diseases such as cancer, AIDS,

Alzheimer's disease, and Parkinson's disease.[26, 27] Radiation and most drugs used to treat cancer induce apoptosis in the cancer cells.[28]

Apoptotic Pathway

Two apoptotic pathways have been identified: the death receptor-mediated (extrinsic) pathway and the mitochondria (intrinsic) pathway. The two pathways are shown in detailed in Figure 4 below.

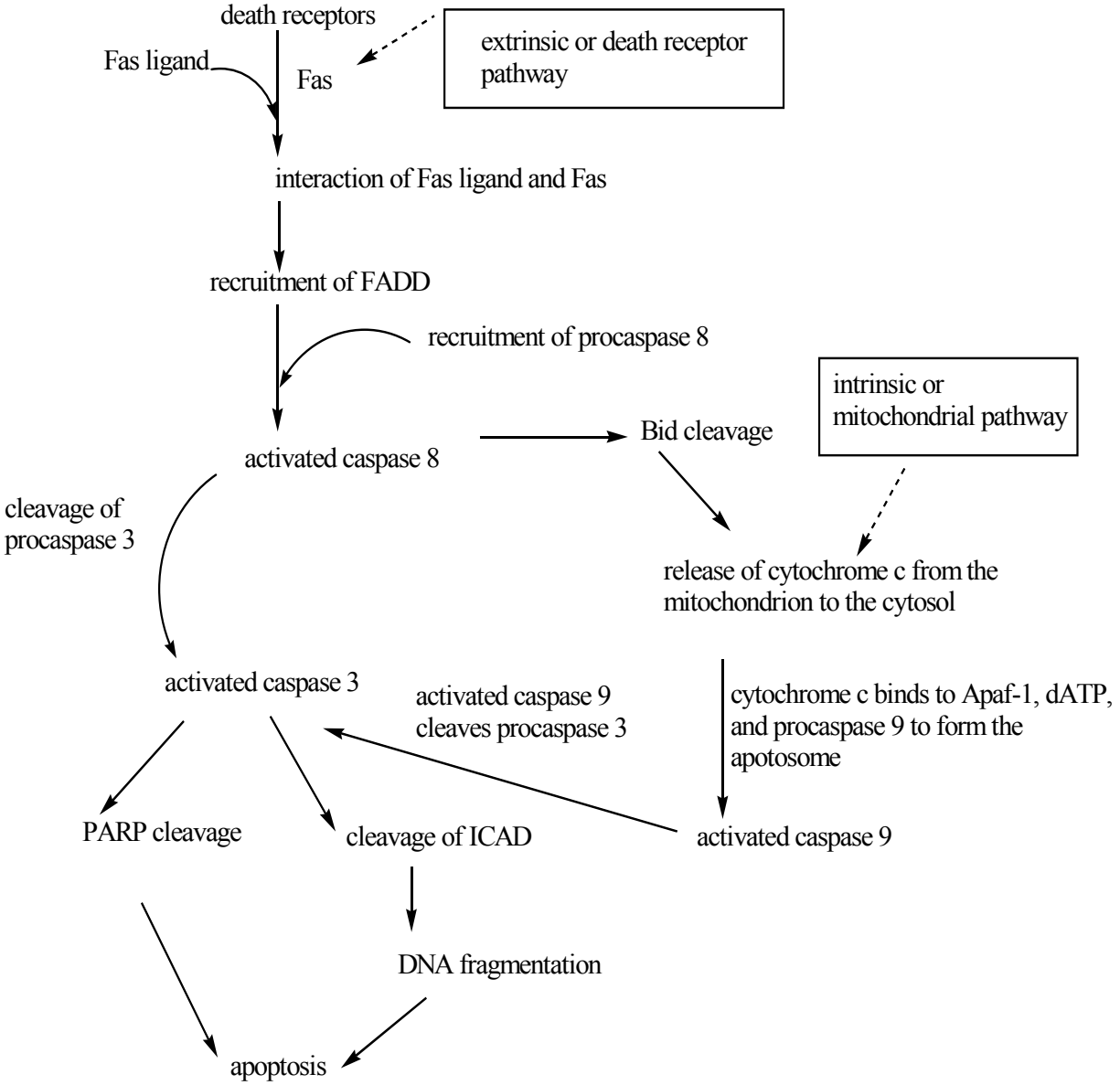


Figure 4. Apoptotic pathways induced by Fas and mitochondria.

The extrinsic or death receptor pathway involves death receptors belonging to the tumor necrosis factor (TNF) and nerve growth factor (NGF) family such as CD95/Fas/APO-1, TNFR1, DR3/WSL-1/TRAMP, DR4/TRAIL-R1, DR5/TRAIL-R2, and DR6. When a cell receives apoptotic signals such as oxidative stress, toxic substances, or byproducts of cellular metabolism, a death receptor ligand such as Fas ligand binds with its death receptor Fas. This interaction leads to the activation of death receptors and a subsequent recruitment of an adapter protein FADD (Fas-associated death domain) which is a TNF-receptor-associated death domain. This recruits procaspase 8 to the receptor complex, leading to autocatalytic activation, releasing the active caspase 8 heterotetrameric enzyme. Activated caspase 8 subsequently activates the 'executioner' caspase 3.[29]

The intrinsic or mitochondria pathway involves the release of the loosely attached cytochrome c protein to the cytosol in response to apoptotic stimuli. The proapoptotic protein Bax is though to trigger the release of cytochrome c from the mitochondria.[30] Released cytochrome c forms a complex with apoptotic protease activating factor-1 (Apaf-1) in the presence of dATP/ ATP and procaspase 9. This leads to a subsequent activation of caspase 9 which in turn cleaves procaspase 3 with a resultant activation of 'executioner' caspase 3. Activated executioner caspases leads to the proteolytic cleavage of cellular substrates such as poly (ADP-ribose) polymerase (PARP) and inhibitor of caspase-activated DNase (ICAD),[31] and subsequent fragmentation of DNA then apoptosis. The activation of caspase 8/ caspase 3 or caspase 9/ caspase 3 cascades ensures the irreversibility of the apoptotic process. Studies have shown that the death receptor and mitochondrial pathway can be interconnected at some point. Activated caspase 8 can cleave Bcl-2 interacting protein (Bid), the carboxyl terminal part of the cleaved Bid translocates to the mitochondria causing the release of cytochrome c to the cytosol.[32]

The goal of the present study was to investigate the cell toxicity of TPGS in RAW264.7 macrophage cells; study the mechanism through which cells die when treated with TPGS and also look at the role of the products of hydrolysis of TPGS in triggering cell death. The susceptibility of cancerous cell lines to TPGS was also investigated. This study is important for the potential use of TPGS as an anticancer drug. There has been no previous work done on the cell toxicity of TPGS.

CHAPTER 2
METHODOLOGY

Materials

Reagents and Materials

RPMI 1640 medium without phenol red, fetal bovine serum (FBS), penicillin (10,000 units/ml), streptomycin (10,000 μg/mL), phosphate buffer saline (PBS, pH 7.4), and 0.4% trypan blue were purchased from Invitrogen Corporation (Grand Island, NY). Vitamin E TPGS was donated by Eastman Chemical Company (Kingsport, TN). Succinic acid, propidium iodide, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), digitonin, dimethylsulfoxide (DMSO), polyethylene glycol 1000, Phenylmethylsulphonyl fluoride (PMSF 100 mM), and staurosporine were purchased from SIGMA Chemical Co. (St. Louis, MO). Ethanol was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY); dl-alpha-tocopheryl succinate was purchased from Fluka, and dl-alpha-tocopherol was purchased from United States Biochemical Coorporation (Cleveland, OH). The bicinochoninic acid (BCA) protein assay reagent kit was purchased from PIERCE (Rockford, IL). Caspase kit was purchased from Molecular Probes (Eugene, OR). Corning 25 cm^2 and 75 cm^2 tissue culture flasks (sterile), corning centrifuge tube (15.0 mL, 50.0 mL), and cell culture plates (sterile) were purchased from Corning Co. (Corning, NY). Cell scrapers (25.0 cm, sterile) were purchased from Fisher Scientific (Fair Lawn, NJ). Syringe filters (0.2 μm and 0.45 μm) were purchased from Gellman Science (Ann; Arbor, MI).

Preparation of Reagents

Deionized water. Distilled water was filtered through a Sep-Pak Cartridge (Atlanta, GA) and stored at 4^{o}C.

Propidium iodide (PI, 1 mg/mL). Propidium iodide (10 mg) was dissolved in 10 mL of 1x phosphate buffer saline (PBS, pH 7.4). The bottle was protected from light by wrapping in aluminum foil and refrigerated at 4^{o}C.

Digitonin (40 mg/mL). Digitonin (100 mg) was dissolved in 2.5 mL of 1x phosphate buffer saline (PBS, pH=7.4). The bottle was gently warmed for 10 minutes to dissolve all the digitonin. It was stored at 4°C.

Staurosporine (50 μM). A 50.0 μL aliquot of 1 mM stock staurosporine was diluted to 1000 μL with Dimethylsulfoxide (DMSO).

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), 0.5 mg/mL. MTT (2.5 mg) was dissolved in 5 mL of RPMI 1640 medium without phenol red, enriched with 10% FBS and 50 IU penicillin/streptomycin.

Trypan blue (0.04%). A 1.0 mL aliquot of 0.4% Trypan blue in 0.85% saline was diluted to 10.0 mL with deionized water.

Culture medium enriched with 10% FBS and 50 IU penicillin and streptomycin. RPMI 1640 (450.0 ml) without phenol red was supplemented with 50.0 mL fetal bovine serum (FBS) and 2.5 mL penicillin (10,000 units/mL) / streptomycin (10,000 μg/mL).

TPGS (5 mM) solution. TPGS (151.3 mg) was melted by gently heating and 20 mL of boiling 1x phosphate buffer saline (PBS, pH 7.4) was added and stirred for 2 hours. The solution was filtered through a 0.2 μm filter and stored refrigerated at 4°C in a glass bottle.

Alpha-tocopheryl succinate. dl-alpha-tocopheryl succinate (10 mg) was dissolved in 1884 μl ethanol to give a concentration of 10 mM. The solution was stored at 4°C until used.

Alpha-tocopherol. dl-alpha-tocopherol (12 mg) was dissolved in 2786 μL ethanol to give a concentration of 10 mM. The solution was stored at 4°C until used.

Polyethylene glycol (PEG) 1000 (5 mM). PEG 1000 (100 mg) was dissolved in 20 mL of 1x phosphate buffer saline (pH 7.4) and stored at 4°C until used.

Succinate (5 mM). Succinic acid (0.0118 g) was dissolved in 1x phosphate buffer saline (pH 7.4) and the pH of the solution was adjusted to 7.4 with sodium hydroxide solution. This was stored at 4°C until used.

Ac-DEVD-AFC (5 mM) substrate solution. Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (25 mg) was dissolved in 264 μL of DMSO. The substrate was heated gently (approximately 50°C) to completely dissolve it. The solution was stored desiccated at -20°C protected from light.

Ac-IETD-AFC (5 mM) substrate solution. Acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin (25 mg) was dissolved in 264 μL of DMSO. The substrate was heated gently (approximately 50°C) to completely dissolve it. The solution was stored desiccated at -20°C protected from light.

Ac-LEHD-AFC (5 mM) substrate solution. Acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (25 mg) was dissolved in 264 μL of DMSO. The substrate was heated gently (approximately 50°C) to completely dissolve it. The solution was stored desiccated at -20°C protected from light.

Dithiothreitol (DTT) (1mM) solution. DTT (100 mg) was dissolved in 650 μL of deionized water and stored desiccated at -20°C.

Ac-DEVD-CHO (1 mM) solution. Ac-Asp-Glu-Val-Asp-CHO (0.2 mg) was dissolved in 400 μL of DMSO and stored desiccated at -20°C.

Rhodamine 110 (R110) (5 mM). R11O (0.5 mg) was dissolved in 273 μL of DMSO and stored desiccated at -20°C, protected from light.

1 x cell lysis buffer. A 400 μl aliquot of 20 x cell lysis buffer (1.5 mL of 200 mM TRIS, pH 7.5, 2 M NaCl, 20 mM EDTA, 0.2% TRITON TMX-100) was mixed with 3600 μL deionized water and vortexed for 1 minute.

2 x reaction buffer. A 600 μL aliquot of 5 x reaction buffer (50 mM PIPES, 10 mM EDTA, 0.5% CHAPS, pH 7.4) and 15 μL of 1 M DTT were mixed with 885 μL of deionized water.

2 x Ac-DEVD-AFC substrate working solution. A 15 μL aliquot of 5 mM Ac-DEVD-AFC substrate was mixed with 1485 μL of 2 x reaction buffer.

Bicinochoninic acid (BCA) working solution. BCA protein assay reagent A (containing sodium carbonate, sodium bicarbonate, bicinochoninic acid reagent, and sodium tartrate in 0.1 N sodium hydroxide) and BCA protein assay reagent B (4% cupric sulfate) were mixed in the ratio of 50:1, prepared freshly as required.

Bovine serum albumin (BSA) (1 mg/mL). A 1 mL aliquot of 2 mg/mL BSA (in 0.9% NaCl solution containing sodium azide) was mixed with 1 mL of 2 x cell lysis buffer.

Experimental Design

Two types of cell lines were used in this study; RAW264.7 macrophage and prostate cancer (LNCaP) cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured at 37^{o}C in a humidified incubator (95% air and 5% CO_2). Confluent cells were incubated with TPGS, alpha-tocopheryl succinate, alpha-tocopherol, polyethylene glycol 1000, or succinate enriched cell culture medium and the following measurements were carried out:

1- Concentration-dependent propidium iodide assay to measure dead cells.
2- Concentration-dependent MTT assay to measure metabolically active cells.
3- Digitonin plus the PI assay to determine total number of cells.
4- Concentration-dependent Caspase 3 enzymatic assay to measure apoptosis.
5- Time-dependent caspase 3, 8, and 9 enzymatic assays to study apoptotic pathway.
6- BCA protein assay.

All fluorescence measurements were done using FLUOstar* GALAXY BMG TECHNOLOGY Microplate Reader (Durham, NC). BCA protein assay and MTT assay measurements were carried out using UVmax kinetic microplate reader (Molecular Device; Sunnyvale, CA). Diagram of experimental design is shown in Figure 5 below.

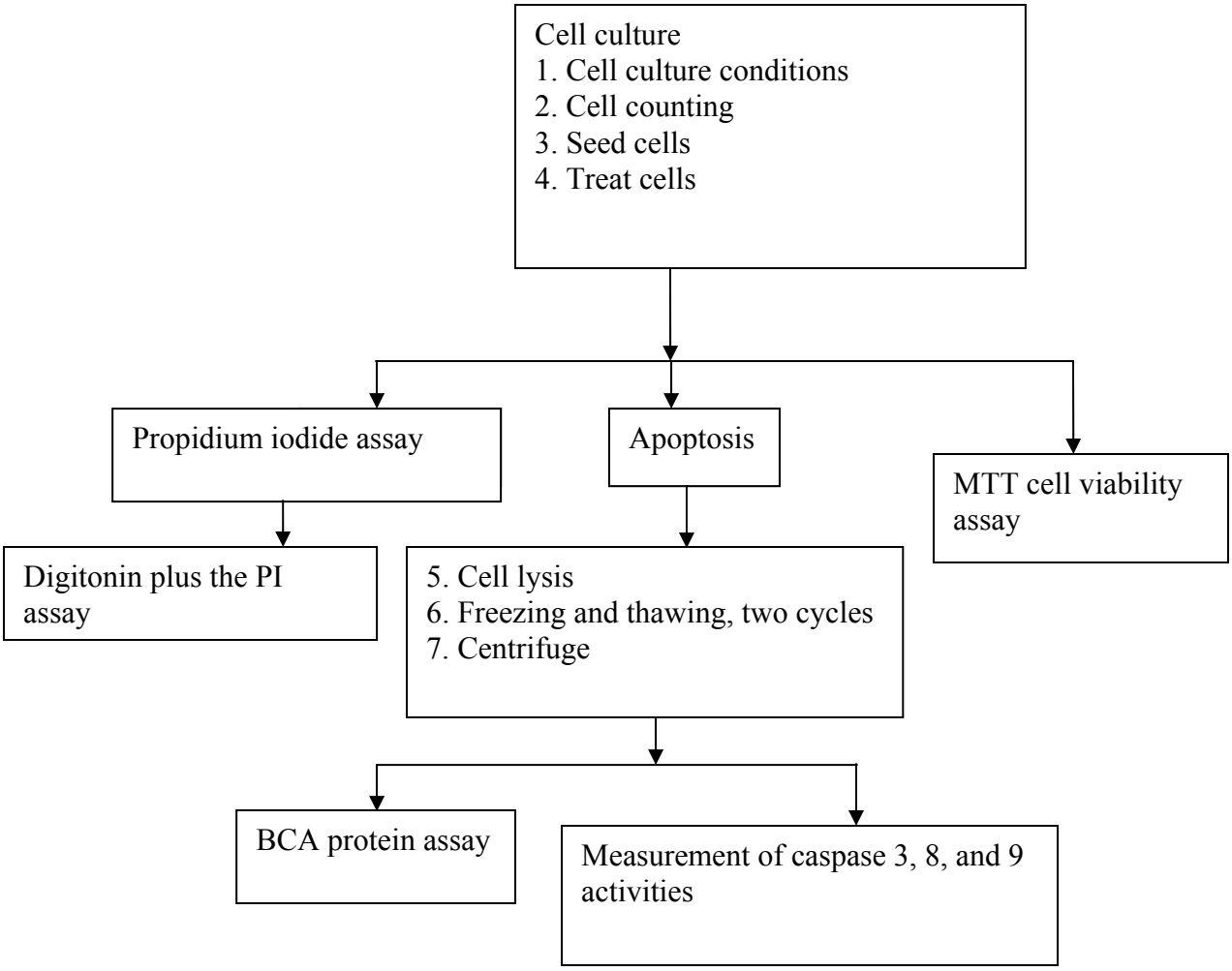


Figure 5. Diagram of experimental design.

Principles of Fluorescence Techniques

Fluorescence is a phenomenon in which the absorption of light by a fluorophore at a shorter wavelength is followed by emission at longer wavelength. Fluorescence methods are of increasing use in many research laboratories especially in biomedical, medical and chemical labs. This is due to the high sensitivity and selectivity of the detection techniques. Fluorescence is a three stage process that occurs mainly in polyaromatic hydrocarbons or heterocycles called fluorophore or fluorescent dyes. A fluorescence detection system is made up of the following components:

- An excitation source (high-energy xenon flash lamp).
- A fluorophore (The chemical moiety of a dye molecule which absorbs light and emits it through fluorescence).
- Wavelength filters or monochromator to isolate emission photons from excitation photons.
- A detector that registers emission photons and produces a recordable output.

The first stage in a fluorescence process is called the excitation stage in which a photon of high energy is supplied by the xenon flash lamp, and this energy is absorbed by the fluorophore, creating an excited electronic singlet state. The second stage is known as the excited-state lifetime which is usually very short, during which the fluorophore undergoes conformational changes and may also interact with its molecular environment. There are some consequences to these processes. The energy of excited electronic singlet state is partially dissipated resulting to a relaxed singlet excited state giving rise to fluorescence emission, also some molecules may undergo processes such as collisional quenching, fluorescence resonance energy transfer and intersystem crossing. The third stage is known as fluorescence emission, whereby a photon of lower energy is emitted bringing the fluorophore to its ground state. The emission energy is lower because some of the energy was dissipated during the excited-state lifetime.

In our fluorescence measurements, we used the FLUOstar* Galaxy BMG Technology Microplate Reader Instrument (Durham, NC). This instrument can measure concentrations as low as 50 amolar (50×10^{-18} M) and it measures the average property of bulk sample,

therefore a single fluorophore can generate thousands of detectable photons thus increasing its sensitivity.

Description of Cell Lines

RAW264.7 Macrophage Cell Line (ATCC Catalog No. TIB-71)

Macrophages are involved at all stages of the immune system to protect against foreign particles such as parasitic bacteria, pathogenic protozoa, fungi and helminthes as well as against tumors. The RAW264.7 macrophage cell line is not a normal macrophage cell line; it is a transformed male mouse macrophage cell line. Functional macrophage cell lines were transformed by abelson leukemia virus (A-MuLV)) so that the cells can grow well *in vitro*. We chose this cell line for our studies because the cells grow rapidly, give reproducible results, and this cell line has high esterase activity to hydrolyze TPGS.

LNCaP Cell Line (ATCC CRL-1740)

This cell line is a human prostate carcinoma. It was established in 1977 from the left supraclavicular lymph node metastasis from a 50-year-old Caucasian man with prostate carcinoma. These cells were described to be androgen-dependent.

Cell Culture

RAW264.7 macrophage cells and LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 50 IU penicillin/streptomycin. Cells were incubated at 37^{o}C in a humidified incubator (95% air with 5% $CO_{2.}$

Cell Counting

Cells were suspended in culture medium; a 100 μL aliquot of the suspension was mixed with 100 μL of 0.04% trypan blue for one minute. An aliquot was taken for cell counting using Nikon Invented Phase Contrast Microscope (American Optical Corporation; Buffalo, NY) and a Hemocytometer.

Rationale for Propidium Iodide (PI) Assay [33]

Propidium iodide is a nucleic acid-binding fluorophore. It is excluded by viable cells but membrane permeable to dying or dead cells. Propidium iodide intercalates into double stranded nucleic acid by interacting with its base pairs (adenine, guanine, cytosine, uracil, or thymine). The binding occurs stoichiometrically with one propidium iodide molecule binding with 4 to 5 base pairs of DNA or RNA, enhancing its fluorescence about 20 to 30 folds. Excitation of the propidium iodide-DNA complex at 485 nm gives rise to maximum emission at 650 nm. The increase in fluorescence intensity of treated cells with respect to the control (untreated cells) represents an increase in the quantity of dead cells. Figure 6 illustrates the PI assay.

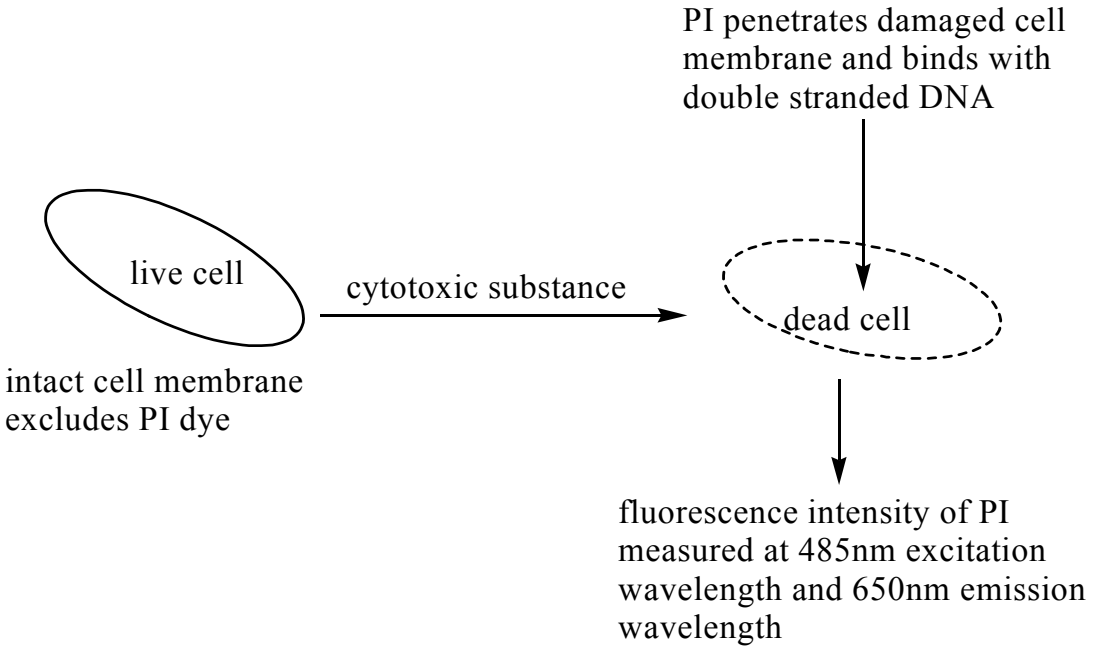


Figure 6. Diagrammatic representation of the rationale of propidium iodide assay.

The Rationale of Digitonin plus Propidium Iodide Assay

Digitonin is a surfactant that permeabilizes cells. The digitonin plus PI assay is such that after treating cells with cytotoxic substance, the initial fluorescence intensity of dead cells are measured and then digitonin added to permeabilize the live cells which are then labeled with PI. After 30 minutes of incubation of the plate, the fluorescence intensity of PI is remeasured which corresponds to the total number of cells (live plus dead cells). The diagrammatic representation of the assay is shown in Figure 7 below.

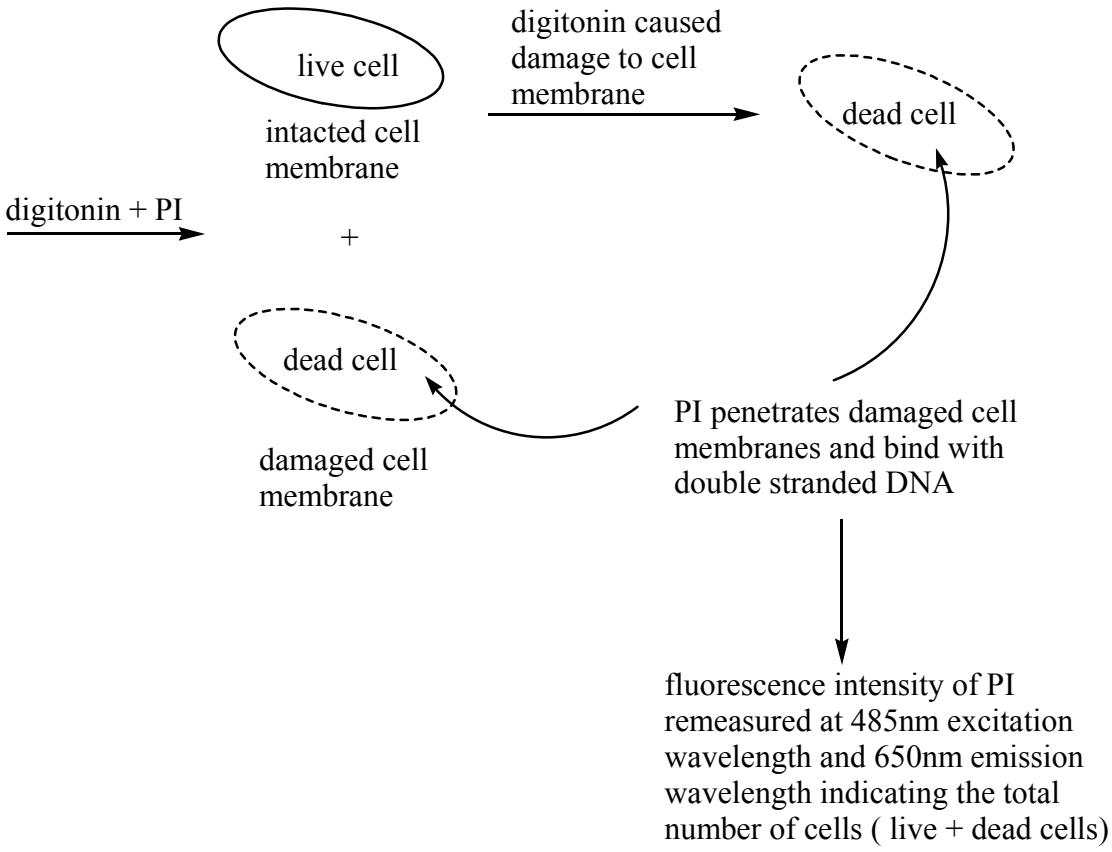


Figure 7. Diagrammatic representation of the rationale of digitonin plus propidium iodide assay.

Rationale of the MTT Assay [34]

MTT measures metabolically active cells. Cells take up MTT and the yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals. These crystals are solubilized by the addition of a solvent such as DMSO, followed by incubating the plate for at least 30 minutes at 37^{o}C. During the incubation period they become soluble in the medium. This assay is useful for quantitative determination of cellular proliferation and activation, it occurs only in metabolically intact cells. The soluble formazan product can be quantified colorimetrically by measuring the absorbance at 570nm. An increase in the number of live cells indicates an increase of total metabolic activity giving rise to a stronger color. A schematic diagram of the MTT assay is shown in Figure 8 below.

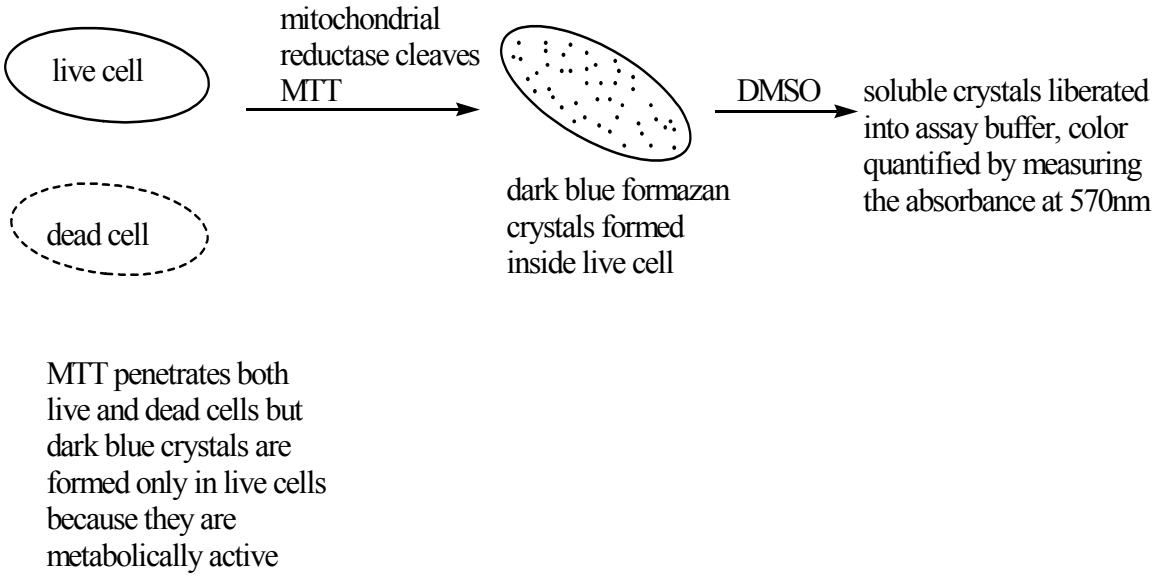


Figure 8. Diagrammatic representation of the rationale of the MTT assay.

Rationale of Caspase Enzymatic Assay [35]

Caspases are a family of cysteine proteases which become activated when a cell undergoes apoptosis. Caspase substrates are synthesized such that they mimic the cleavage site of the various caspase substrates *in vivo* like poly (ADP-ribose) polymerase (PARP). In our assays we used acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC for caspase 3), acetyl-Ile-Glu-Thr-Asp-7-amino-4- trifluoromethyl coumarin (Ac-IETD-AFC for caspase 8) and acetyl-Leu-Glu-His-Asp-7-amino-4- trifluoromethyl coumarin (Ac-LEHD-AFC for caspase 9). When a caspase enzyme is activated during apoptosis, it cleaves its specific substrate at the aspartate residue to release the fluorogenic substance, 7-amino-4-trifluoromethyl coumarin (AFC) which can be detected fluorimetrically. When conjugated, AFC emits blue light at $\lambda = 390$ nm but in the free form, AFC emits a yellow-green fluorescence at 500 nm. A schematic diagram of the caspase assays is shown in Figure 9.

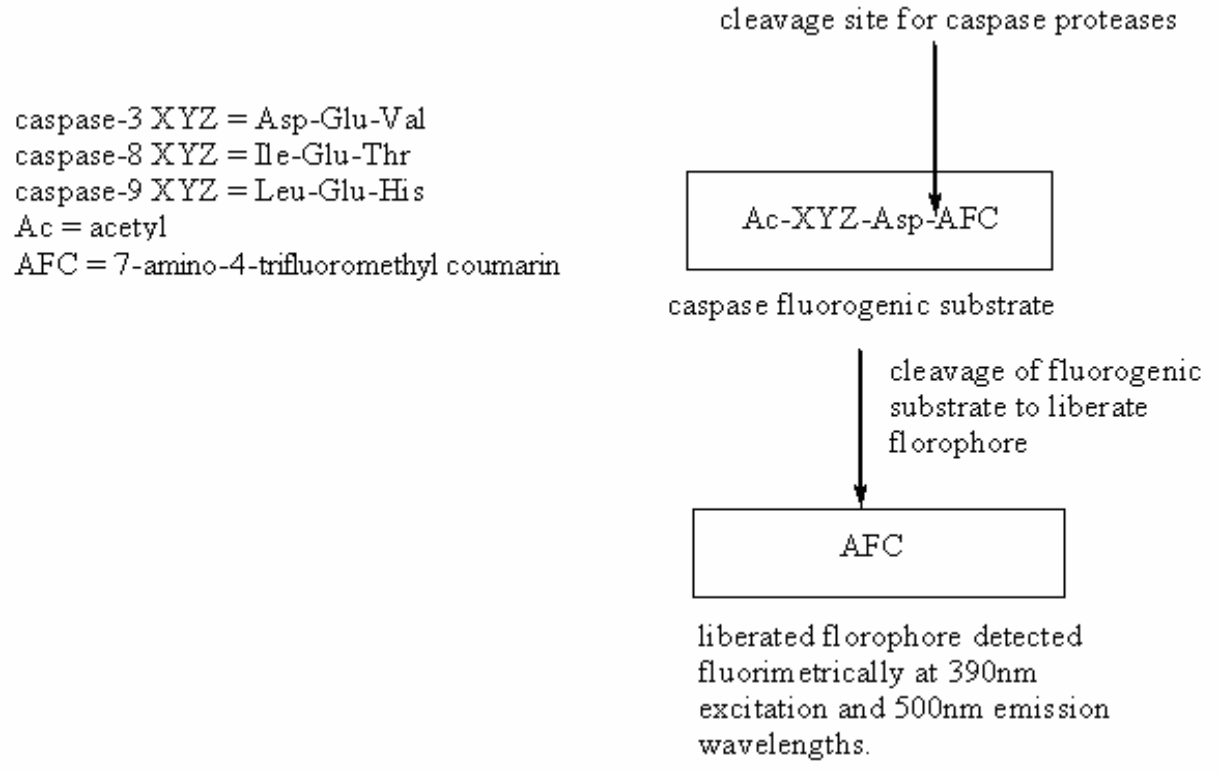
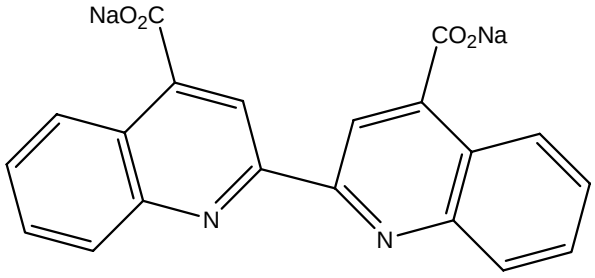


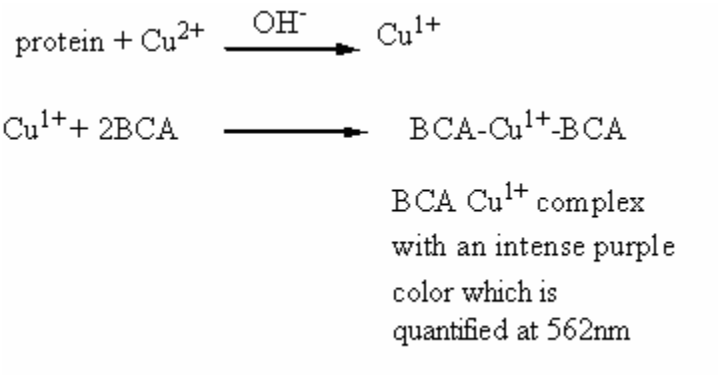
Figure 9. Diagrammatic representation of the rationale of caspase activity.

The Rationale for the BCA Protein Assay [36]

Bicinochoninic acid (BCA) is a sensitive, stable, and highly specific reagent for the detection of cuprous ion (Cu^{+}). When incubated with protein, a Cu^{2+}-protein complex is formed under alkaline conditions. The molecular structure of BCA is shown below:



The presence of cysteine, cystine, tryptophan, tyrosine and the peptide bond in protein are thought to reduce Cu^{2+} to Cu^{+}. This reaction depends on the amount of protein present. More protein means a greater amount of Cu^{+} produced. Chelation of two BCA molecules with one Cu^{+} ion gives purple coloration in basic medium. This complex is water-soluble and exhibits a strong absorbance at 562 nm.



Cell Viability Determination by Propidium Iodide and Digitonin Assays

Adherent cells (1 x 10^5 cells) were subcultured in a 96 well Costar tissue culture plate for 12 hours to let cells adhere to the bottom of the wells. Confluent cells were treated with TPGS, alpha-tocopheryl succinate, alpha-tocopherol, polyethylene glycol 1000, or succinate enriched culture medium and incubated at 37 ^oC in humidified incubator (95% air and 5% CO_2) for the required time point. Positive control was 0.5 μM staurosporine and negative control was untreated cells. Cell death was evaluated using a 96 well plate by a modified protocol of Zhang et al.[37] A 1 μL aliquot of 1.5 μM PI was added into each well giving a final concentration of 7.5 nM. The blank was cell culture medium in each case. The plate was incubated for 5 minutes at 37^{o}C, the initial fluorescence intensity from the dead cells was measured using a fluorescence microplate reader (FLUOstar * Galaxy, BMG Technology), with an excitation wavelength of 485 nm and an emission wavelength of 650 nm. After this initial measurement, a 4 μL aliquot of 32.5 mM digitonin was added to permeabilize all live cells in each well and label all nuclei with PI. The plate was incubated for 30 minutes at 37^{o}C and the fluorescence intensity remeasured to obtain a value corresponding to the total number of cells. The percentage of dead cells was calculated by taking the ratio of the fluorescence intensity of dead cells (PI assay) to that of total number of cells (digitonin plus PI assay).

MTT Cell Proliferation Assay

Adherent cells (1 x 10^5 cells) were subcultured in a 96 well Costar tissue culture plate for 12 hours when the cells were confluent. Cells were then treated with TPGS, alpha-tocopheryl succinate, alpha-tocopherol, polyethylene glycol 1000, or succinate enriched medium and incubated for the required time point at 37 ^oC in humidified incubator (95% air and 5% CO_2). Positive and negative controls were 0.5 μM staurosporine and untreated cells, respectively. At the end of the incubation period, the treated medium was removed and 200 μL of 0.5 mg/mL MTT solution was added to each well and the plate was incubated for 30 minutes. Then the MTT medium was replaced with 200 μL of DMSO and the plate incubated for 30 minutes to solubilize the purple formazan crystals and absorbance was measured at 570 nm wavelength with UVmax kinetic microplate reader (Molecular Device; Sunnyvale, CA).

Caspase Activity

We measured caspase 3, 8, and 9 activities in the cell lines fluorimetrically. A 2.5 mL aliquot of cells ($1x10^6$ cells) was cultured in a Falcon 6 well plate for 24 hours and incubated with 2.5 mL TPGS or alpha-tocopheryl succinate enriched medium for various time periods. The negative control was untreated cells and the positive control was 0.5 μM staurosporine. At the end of the incubation period, the cells were collected, resuspended in lysis buffer, and frozen for 30 minutes at -80^{o}C. Two cycles of freezing and thawing were carried out. The samples were then centrifuged at 2000 x g for 5 minutes. A 10 μL aliquot of cell lysate from each sample was reserved for the BCA protein assay. For caspase 3, 8, or 9 assays, a 40 μL aliquot of fluorogenic substrate Ac-DEVD-AFC (caspase 3), Ac-IETD-AFC (caspase 8), and Ac-LEHD-AFC (caspase 9) in 5x reaction buffer (10 mM HEPES pH 7.5, containing 50 mM NaCl and 2.5 mM Dithiothreitol) respectively, was added to 40 μL cell lysate and incubated in the FLUOstar* Galaxy microplate reader at 37^{o}C while measurements were been made. The released AFC (7-amino-4-trifluormethyl coumarin) was measured at 390 nm for excitation and 500 nm for emission using a FLUOstar* Galaxy fluorescence microplate reader.

BCA Protein Assay

Protein levels were measured by mixing 10 μL aliquot of cell lysate (from caspase assay) with 200 μL of BCA working solution (made freshly) and after incubating for 30 minutes, the absorbance was measured at 562 nm using UVmax kinetic microplate reader (Molecular Device; Sunnyvale, CA). A 10 μL aliquot of 1x cell lysis buffer was used as a blank.

Bovine Serum Albumin (BSA) Standard Curve

BSA standard curve for BCA protein assay was determined by mixing 10 μL of 0 μM (1x cell lysis buffer), 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM, 0.5 μM, 0.6 μM, 0.7 μM, 0.8 μM, 0.9 μM, and 1.0 μM BSA with 200 μL of BCA working solution. The plate was incubated for 30 minutes and the absorbance measured at 562 nm using UVmax kinetic microplate reader (Molecular Device; Sunnyvale, CA).

CHAPTER 3

RESULTS

Evaluation of Dead RAW264.7 Macrophage Cells Treated with TPGS Using Propidium Iodide Staining and Digitonin

Dead RAW264.7 cells were assessed with propidium iodide after 18 hours treatment of cells with 10 μM, 20 μM, 30 μM, and 40 μM TPGS. The fluorescence intensity of PI increased in a concentration dependent manner as shown in Figure 10 below. The number of dead cells increased gradually with a significant increase occurring at 30 μM TPGS compared to untreated cells. The fluorescence intensity of PI after permeabilizing live cells with digitonin is shown in Figure 11 and the intensity remained fairly constant showing that total number of cells (live plus dead cells) did not substantially change throughout the experiment. The percentage of dead cells was calculated as the ratio of fluorescence intensity of dead cells to that of total cells (live plus dead cells) as shown in the equation below.

$$\text{percentage of dead cells} = \frac{\text{fluorescence intensity of the PI assay}}{\text{fluorescence intensity of digitonin plus the PI assay}} \times 100$$

The results are shown in Figure 12 and indicated that cell death increased with increasing concentration of TPGS.

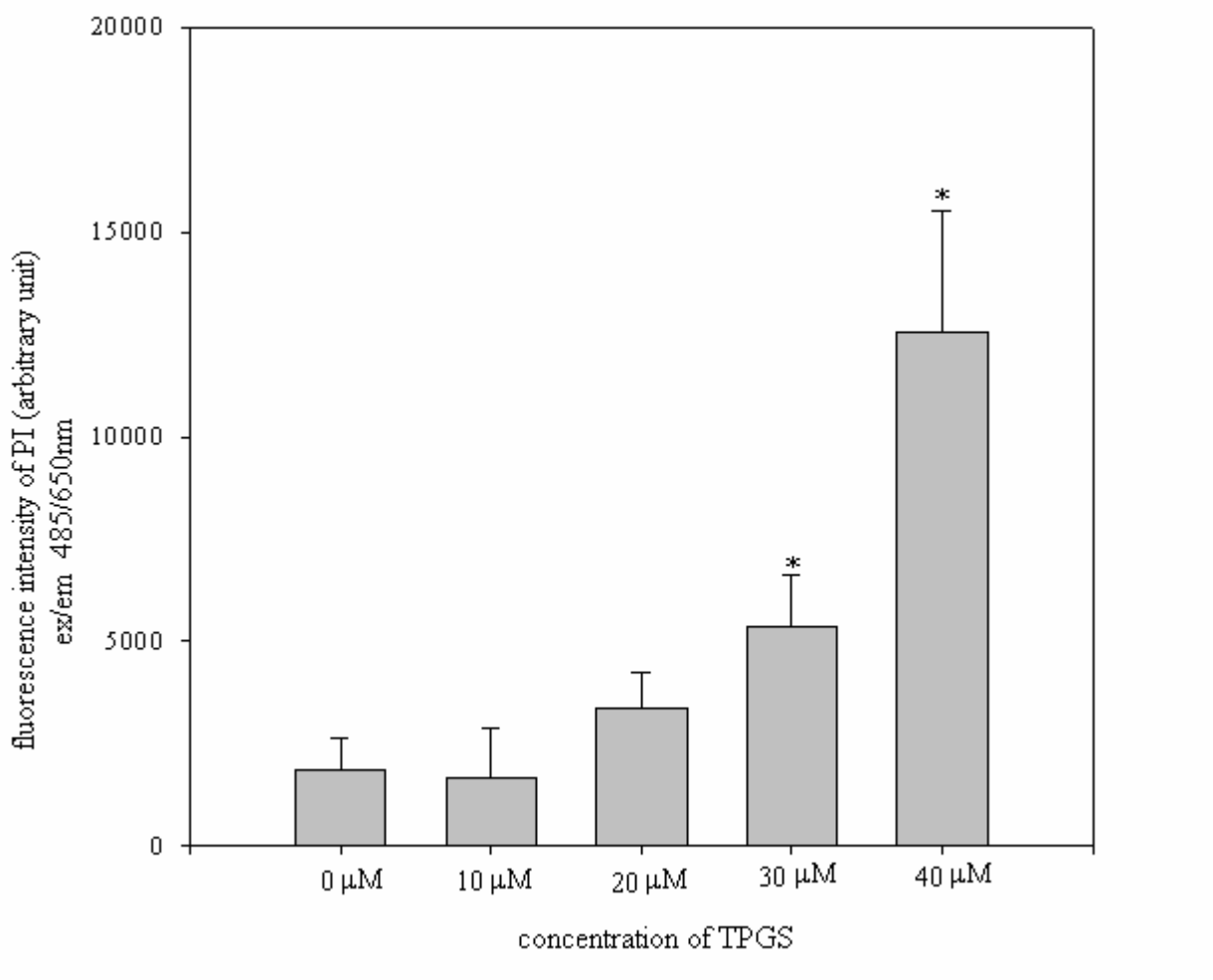


Figure 10. Measurement of cell killing by utilizing PI after exposing RAW264.7 cells to TPGS. Cells were incubated with 10 μM, 20 μM, 30 μM, and 40 μM TPGS for 18 hours at 37^{o}C. Propidium iodide (7.4 nM) was added into each well after treatment with TPGS and the initial fluorescence intensities from the dead cells measured using a fluorescence microplate reader. The figure represents a typical result of four independent experiments. '*' $P < 0.05$ compared with untreated cells (0 μM TPGS). Data are expressed as means of five wells ± SEM. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

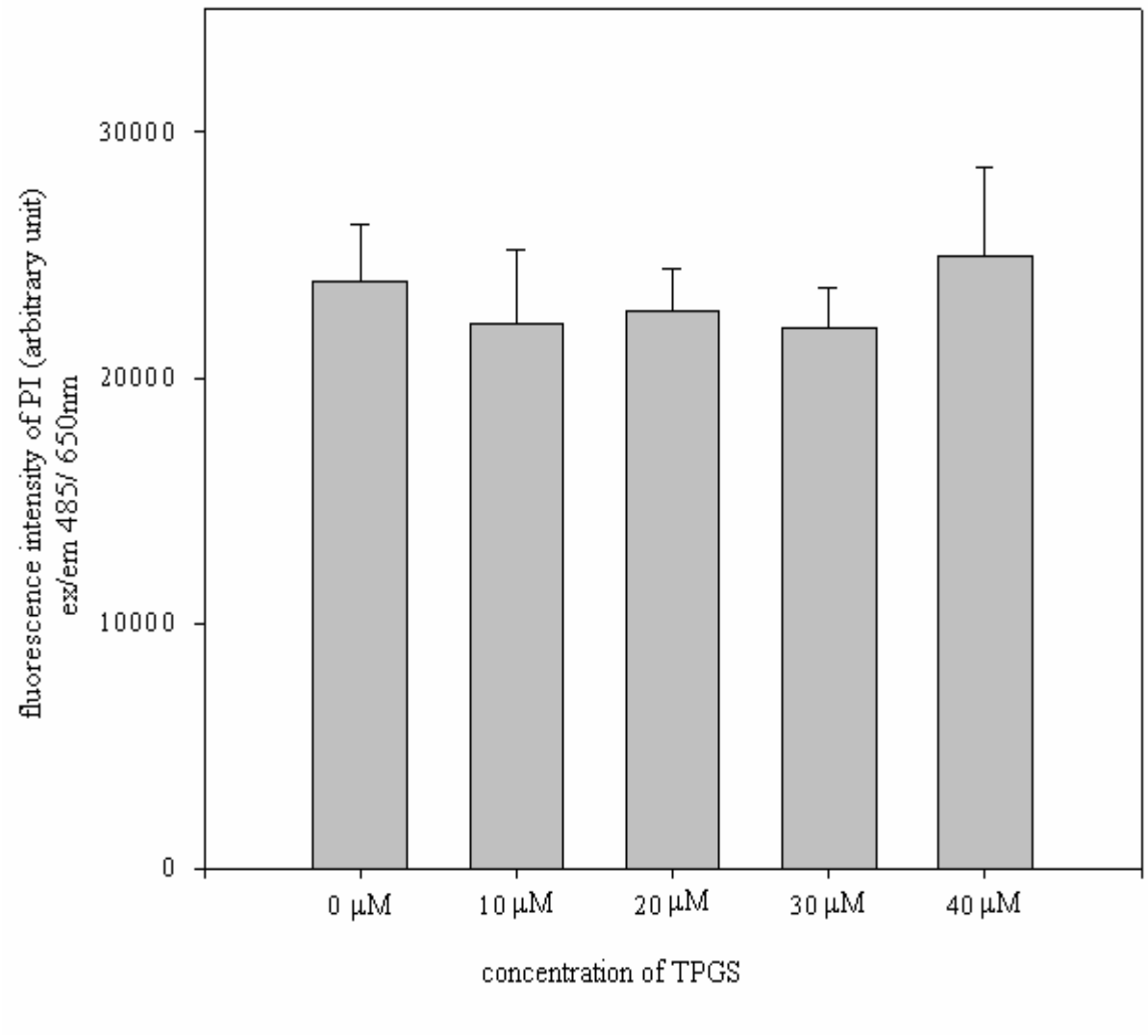


Figure 11. Total number of cells (live plus dead cells) determined by digitonin plus PI assay. Cells were permeabilized with 635 μM digitonin solution. Fluorescence intensity of PI corresponding to total number of cells was remeasured. This represents typical result of four independent experiments. Data are expressed as mean of five wells ± SEM.

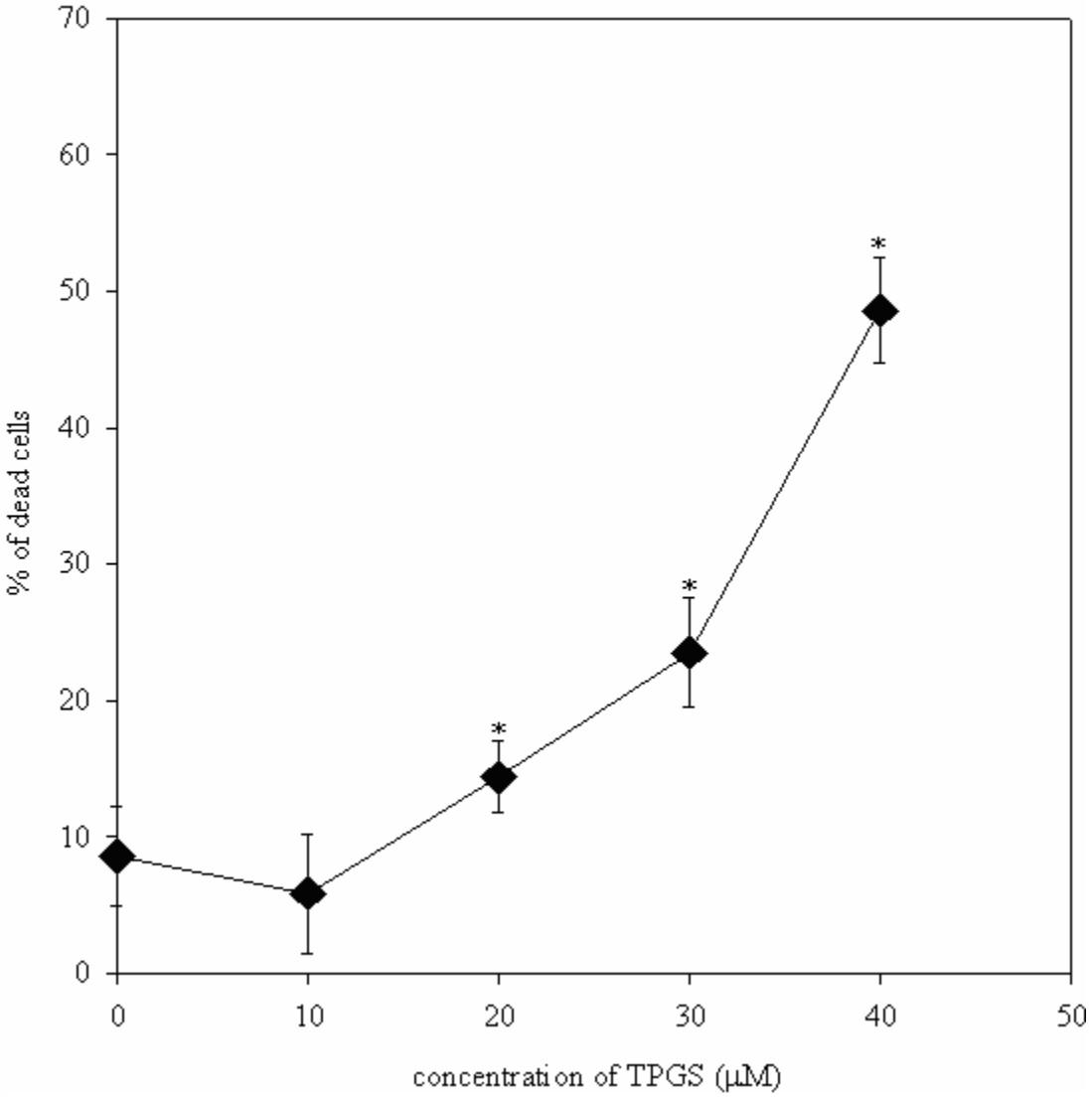


Figure 12. Percentage of dead cells after exposure of RAW264.7 cells to TPGS. Cells were incubated with 10 μM, 20 μM, 30 μM, and 40 μM concentrations of TPGS for 18 hours. '*' ($p < 0.05$), means statistically different from untreated cells (0 μM). Each value is a mean of five wells ± SEM. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

Effect of TPGS on the Viability of RAW264.7 Macrophage Cells

To substantiate the above results, cell proliferation assays were carried out to determine if the population of viable cells decreased with increasing concentration of TPGS. Figure 13 shows the viability of RAW264.7 cells treated for 18 hours with TPGS enriched cell culture medium. Compared with the untreated cells, the viability of RAW264.7 macrophage cells was significantly suppressed at concentration of TPGS greater than 20 μM. Cell viability was measured using the MTT assay.

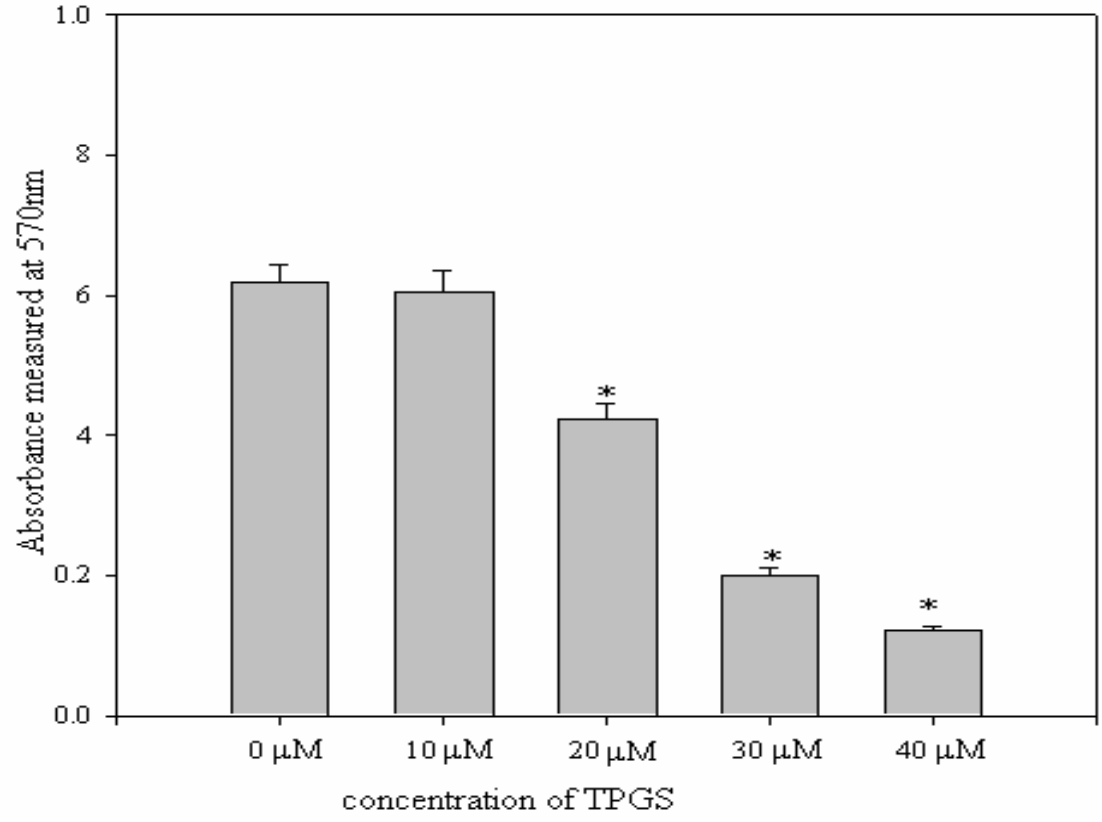


Figure 13. Effect of TPGS on RAW264.7 cell viability. Cells were exposed to 10 μM, 20 μM, 30 μM, and 40 μM TPGS for 18hours at 37^{o}C. Viability was analyzed by the MTT assay as described in Material and Method Section. Data are expressed as means of eight wells ± SEM. * $p < 0.05$ compared with the untreated cells (0 μM). Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

Evaluation of Cell Death Triggered by TPGS in RAW264.7 Macrophage Cells

Apoptosis is generally accompanied by the activation of caspases when cells are still intact. This parameter was used to discriminate between apoptotic and necrotic cell death. The involvement of caspase 3 was evaluated because it has been shown to be one of the most important executioners of apoptosis (programmed cell death). The cell permeable fluorogenic substrate (Ac-DEVD-AFC), which becomes fluorescent upon cleavage by caspase 3, was employed to measure activity of caspase 3. Treatment of RAW264.7 cells for 18 hours with various concentrations of TPGS enriched medium led to a significant increase in caspase 3 enzymatic activity beginning at 30 μM concentration (see Figure 14). These results indicated that cell death induced by TPGS was apoptotic and not necrotic.

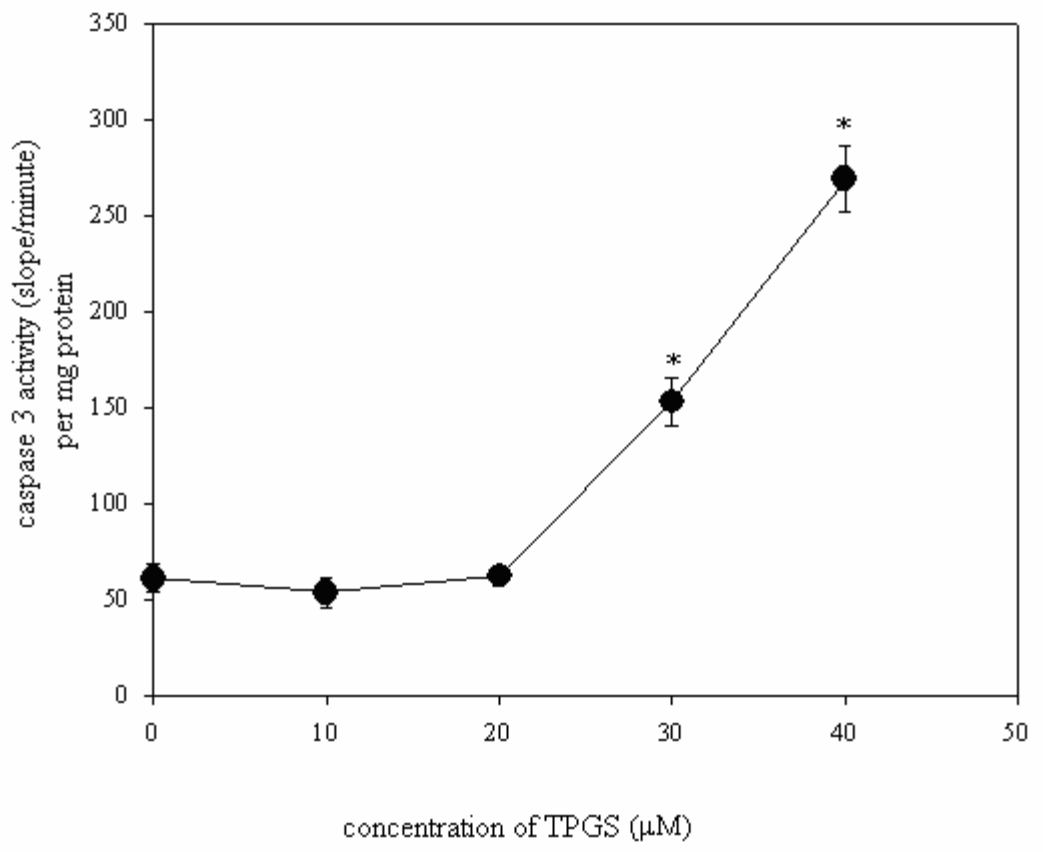


Figure 14. Apoptosis in RAW264.7 cells induced by TPGS involves caspase 3 activation. Cells (1×10^6 cells per well) were treated with 10 μM, 20 μM, 30 μM, and 40 μM TPGS for 18 hours. The activation was measured as the increase in activity with respect to the control (untreated cells) per mg of protein. '*' $p<0.05$ compared with untreated cells. The data shown are mean of five wells ± SEM. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

Study of the Apoptotic Pathway Triggered by TPGS in RAW264.7 Macrophage Cells

We studied the involvement of caspase 3, 8, and 9 in triggering apoptotic cell death. Caspase 8 and 9 represent the initiating caspases, while caspase 3 is an important executioner caspase during apoptosis.[38] Specifically, we studied caspase 8 enzymatic activity because it represents the initiating caspase in the death receptor (extrinsic) pathway and caspase 9 because it represents the apical caspase in the mitochondrial (intrinsic) pathway. RAW264.7 cells were treated with 40 μM TPGS enriched medium for 0 hour, 4 hours, 6 hours, 8 hours, 16 hours, and 24 hours. The cleavage of specific fluorogenic substrates (Ac-DEVD-AFC, Ac-IETD-AFC and Ac-LEHD-AFC for caspase 3, 8 and 9, respectively) by activated caspase 3, 8, and 9 enzymes, respectively, released the fluorophore AFC (7-amino-4-trifluoromethyl coumarin). An increased fluorescence intensity of the released AFC corresponded to an increased caspase activity. The results are shown in Figure 15 and indicated a marked increase in the activities of caspase 3, 8, and 9 in a time-dependent manner. The maximum caspase activities were observed after 8 hours of incubation with TPGS. Caspase 8 was activated before the fourth hour of treatment of cells with TPGS. Significant caspase 3 and 9 activities were visible only after 4 hours of treatment with TPGS. The early activation of caspse 8 suggested the importance of the death receptor pathway in apoptosis.

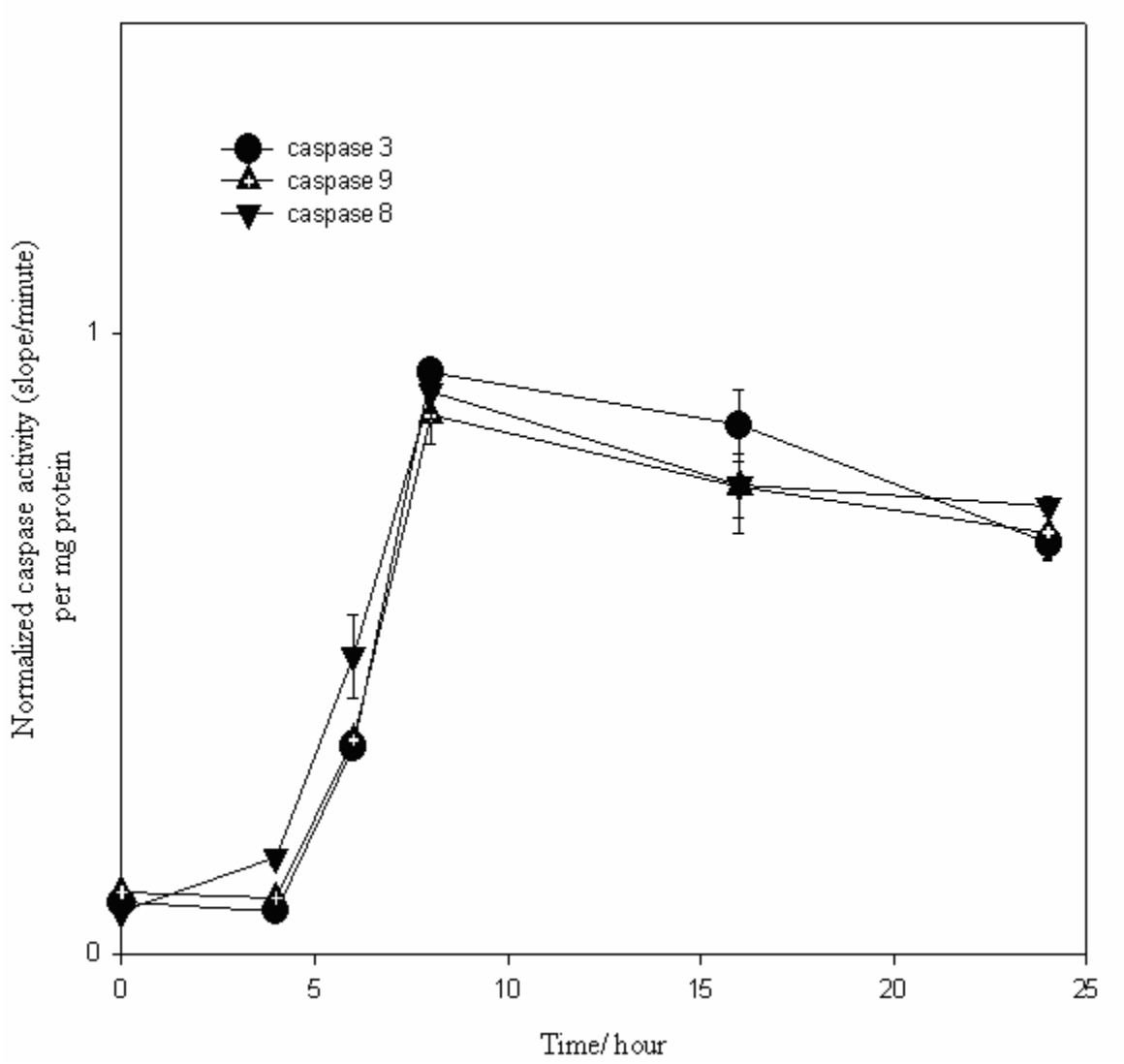


Figure 15. Time-dependent activation of caspase 3, 8, and 9 by TPGS in RAW264.7 cells. Cells were incubated with 40 μM TPGS for the indicated time periods. Activation was measured as the increase in activity with respect to control (untreated cell) per mg of protein. The results were normalized by dividing the value of each caspase activity by the largest value in each assay to get a maximum caspase activity of 1. Values are the mean ± SEM of four wells.

Role of the Products of Hydrolysis of TPGS in Triggering Apoptotic Cell Death in RAW264.7 Macrophage Cells

TPGS is a diester and can hydrolyze intracellularly when taken up by cells as follows:

TPGS $\rightarrow$ alpha-tocopheryl succinate + polyethylene glycol 1000

Alpha-tocopheryl succinate $\rightarrow$ alpha-tocopherol + succinate

We studied the cytotoxicity of alpha-tocopheryl succinate, alpha-tocopherol, polyethylene glycol 1000, and succinate to determine if they also contribute to the cytotoxicity of TPGS observed in RAW264.7 macrophage cells.

Study of the Cytotoxicity of Alpha-Tocopheryl Succinate and Alpha-Tocopherol

Evaluation of Dead RAW264.7 Macrophage Cells Treated with Alpha-Tocopheryl Succinate Using the PI Staining. Figure 16 shows the result of propidium iodide assays after incubating RAW264.7 cells with 10 μM, 20 μM, 30 μM, 40 μM, and 50 μM concentrations of alpha-tocopheryl succinate for 18 hours. There was a significant increase in the population of dead cells, beginning at 30 μM concentration. The increased fluorescence intensity of PI corresponded to an increase in the population of dead cells.

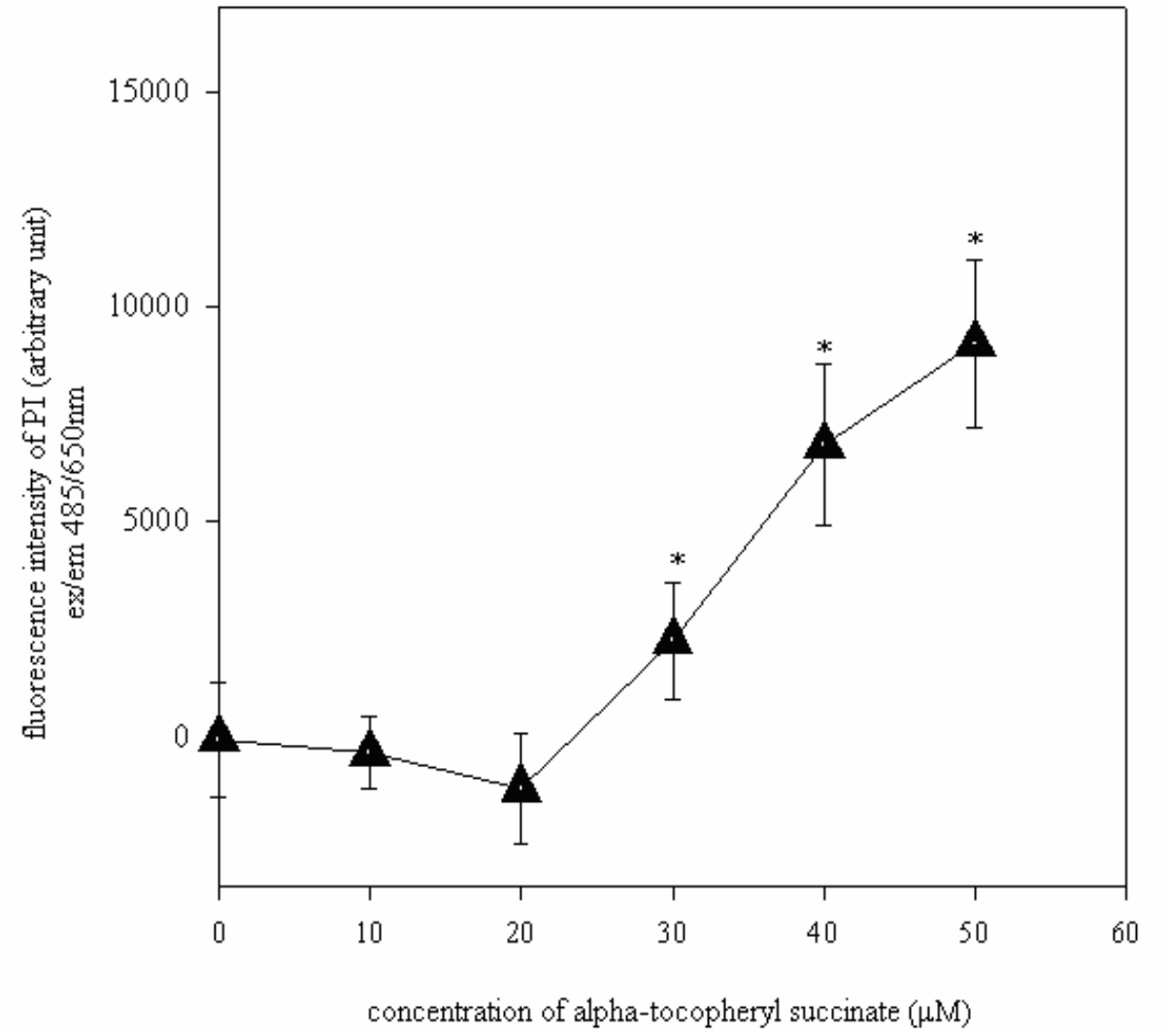


Figure 16. Measurement of cell killing in RAW264.7 cells after treating cells with alpha-tocopheryl succinate. Cells were incubated with 10 μM, 20 μM, 30 μM, 40 μM, and 50 μM alpha-tocopheryl succinate for 18 hours. Dead cells were evaluated using the PI assay as described in Material and Method Section. '*' means statistically different from untreated cells (0 μM), $P < 0.05$. Values are the mean of eight wells ± SEM. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

Effect of Alpha-Tocopheryl Succinate on the Viability of RAW264.7 Macrophage Cells. Viability of RAW264.7 cells treated with alpha-tocopheryl succinate for 18 hours was studied using the MTT assay, and the results shown in Figure 17. There was a concentration-dependent decrease in the population of viable cells.

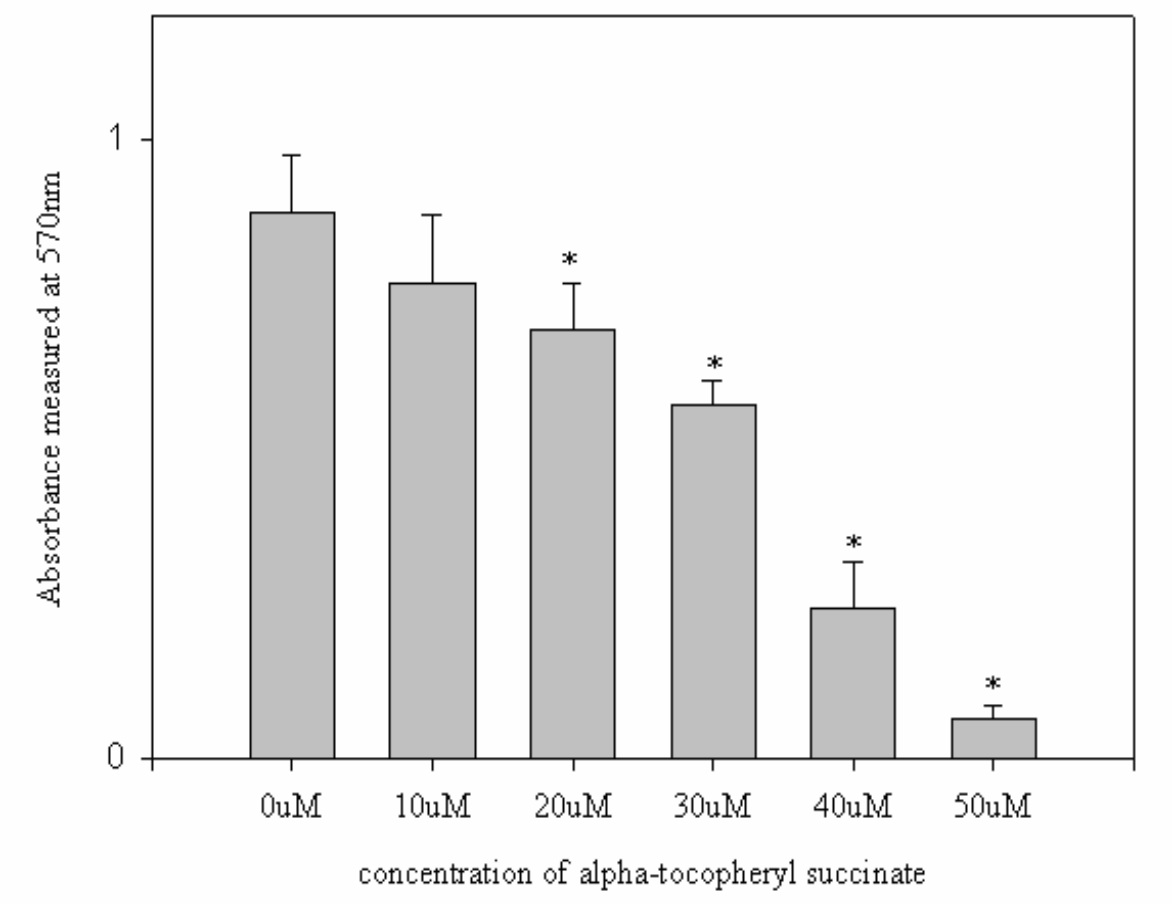


Figure 17. Effect of alpha-tocopheryl succinate on the viability of RAW264.7 macrophage cells. Cell viability was evaluated by MTT assay as described in Material and Method Section. Values are the mean ± SEM of eight wells. * $p<0.05$ compared with the untreated cells. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

A Comparative Study of the Cytotoxicity of TPGS, Alpha-Tocopheryl Succinate and Alpha-Tocopherol in RAW264.7 Macrophage Cells Using the PI Staining. Figure 18 confirms the cell killing properties of TPGS and alpha-tocopheryl succinate. In marked contrast, the results did not show any cytotoxicity for alpha-tocopherol.

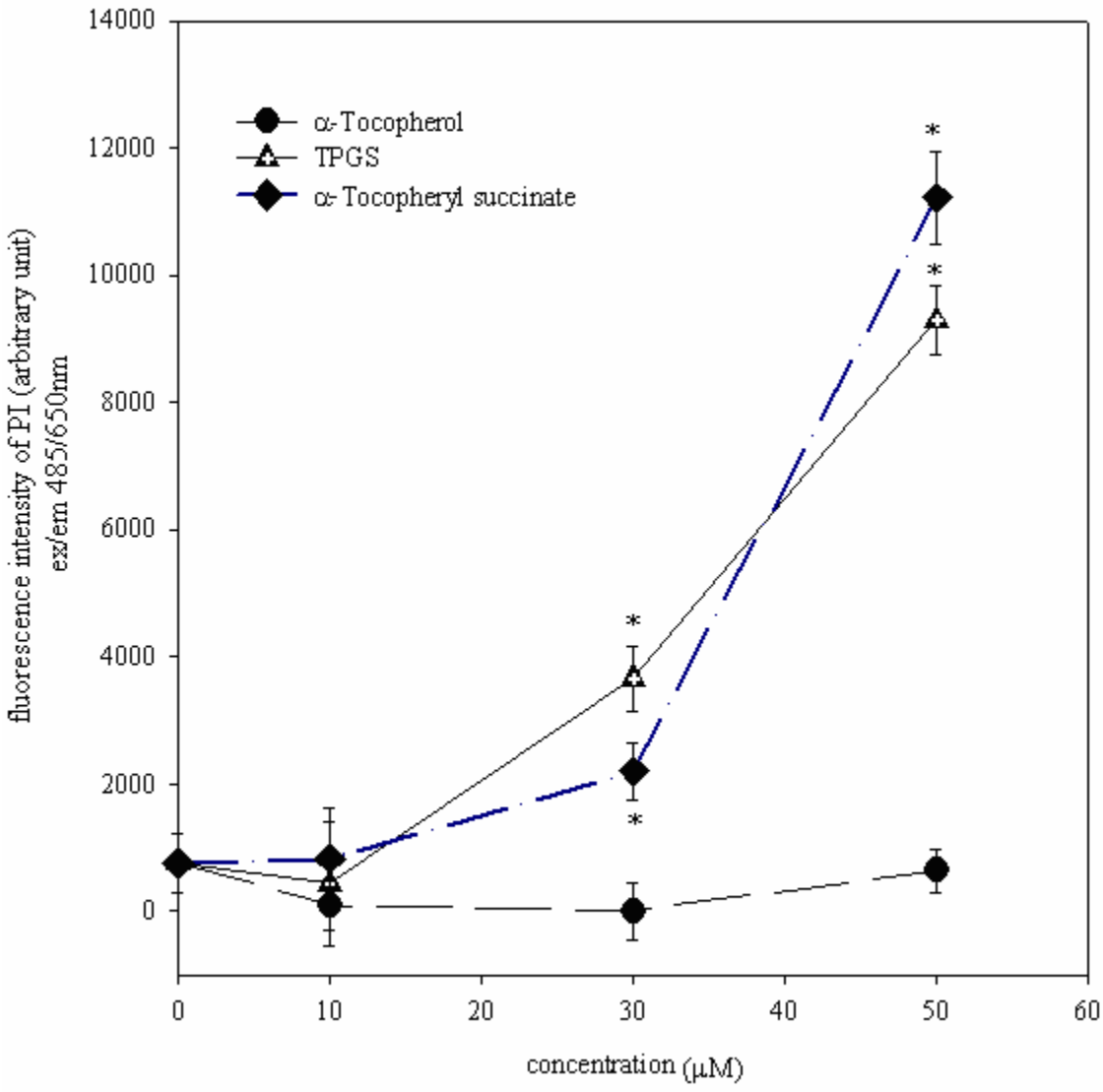


Figure 18. Comparative study of the cytotoxicity of TPGS, alpha-tocopheryl succinate and alpha-tocopherol in RAW264.7 cells. Cells were treated with 10 μM, 30 μM, and 50 μM concentrations of TPGS, alpha-tocopheryl succinate, or alpha-tocopherol for 18 hours and dead cells were assayed using propidium iodide. Values are the mean ± SEM of four wells. * $p<0.05$ compared with untreated cells (0 μM). Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

Comparative Study of the Activation of Caspase 3 by TPGS and Alpha-Tocopheryl Succinate in RAW264.7 Macrophage Cells. Figure 19 shows the activation of caspase 3 by TPGS and alpha-tocopheryl succinate as a function of time. RAW264.7 cells were treated with 50 μM concentration of TPGS or alpha-tocopheryl succinate for the indicated time periods. There was an earlier activation of caspase 3 in TPGS treated cells compared with alpha-tocopheryl succinate.

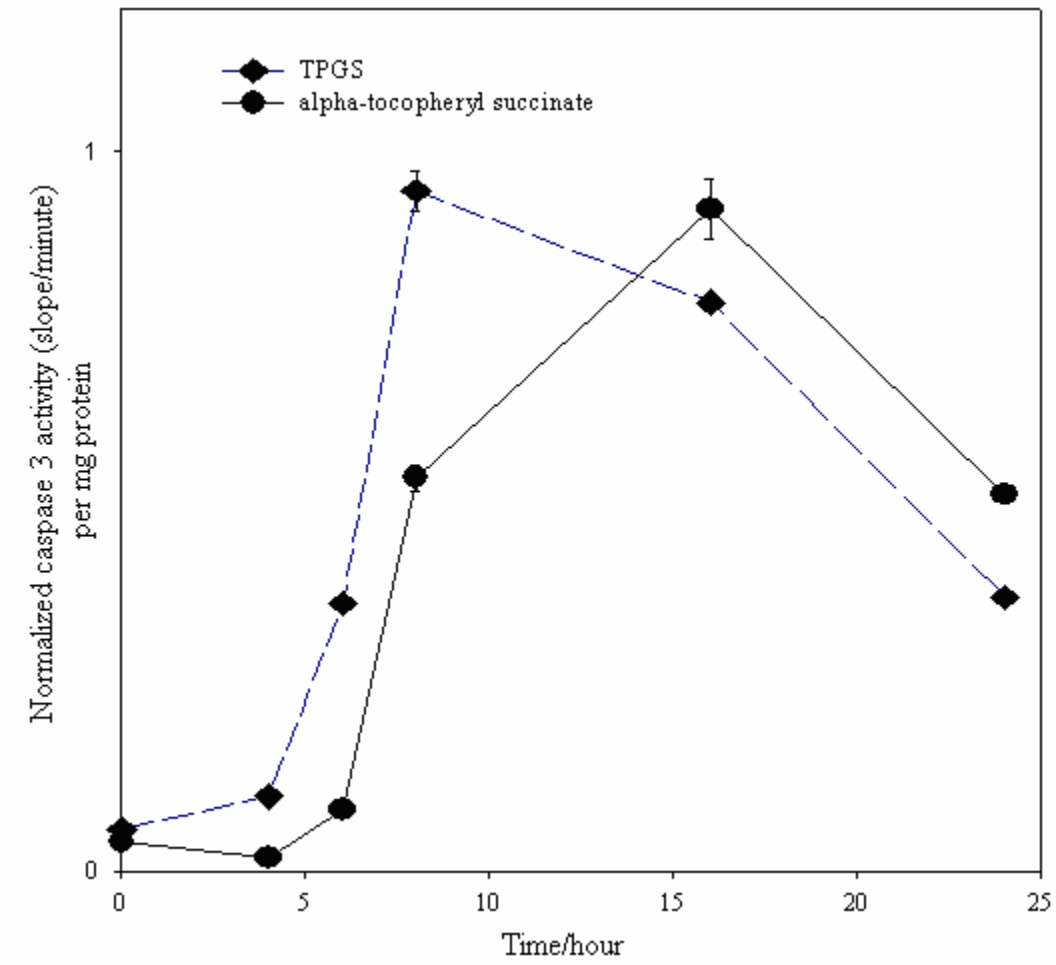


Figure 19. Comparism of the time course for activation of caspase 3 in RAW264.7 macrophage cells by TPGS and alpha-tocopheryl succinate. Cells were treated with 50 μM TPGS or alpha-tocopheryl succinate for the indicate time periods and caspase 3 activity was evaluated fluorimetrically as described in Material and Method Section. Values are the mean ± SEM of six wells.

Cytotoxicity of Polyethylene Glycol (PEG) 1000

Figure 20 shows the PI assay after incubating RAW264.7 cells with various concentrations of PEG 1000 for 24 hours. No significant cell death was observed at any concentration.

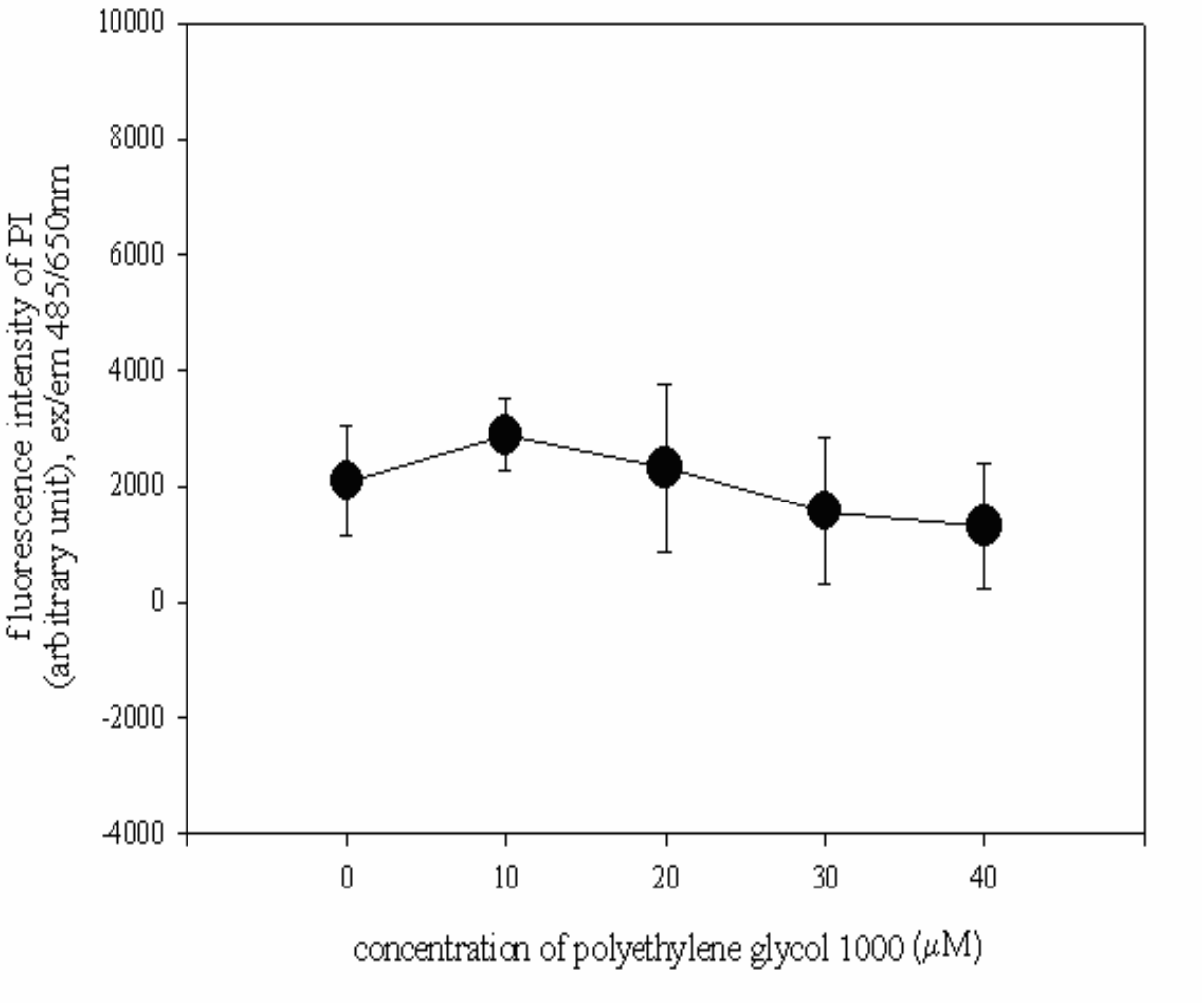


Figure 20. Measurement of dead cell population after exposing RAW264.7 cells to PEG 1000. Cells were incubated with 10 μM, 20 μM, 30 μM, and 40 μM polyethylene glycol (PEG) 1000 enriched cell culture medium for 24 hours and dead cells were evaluated using propidium iodide assay. Data are the mean of eight wells ± SEM.

Figure 21 shows the MTT assay after treating RAW264.7 macrophage cells for 24 hours with various concentrations of PEG 1000. There was no significant change in the viability at any concentration of PEG 1000.

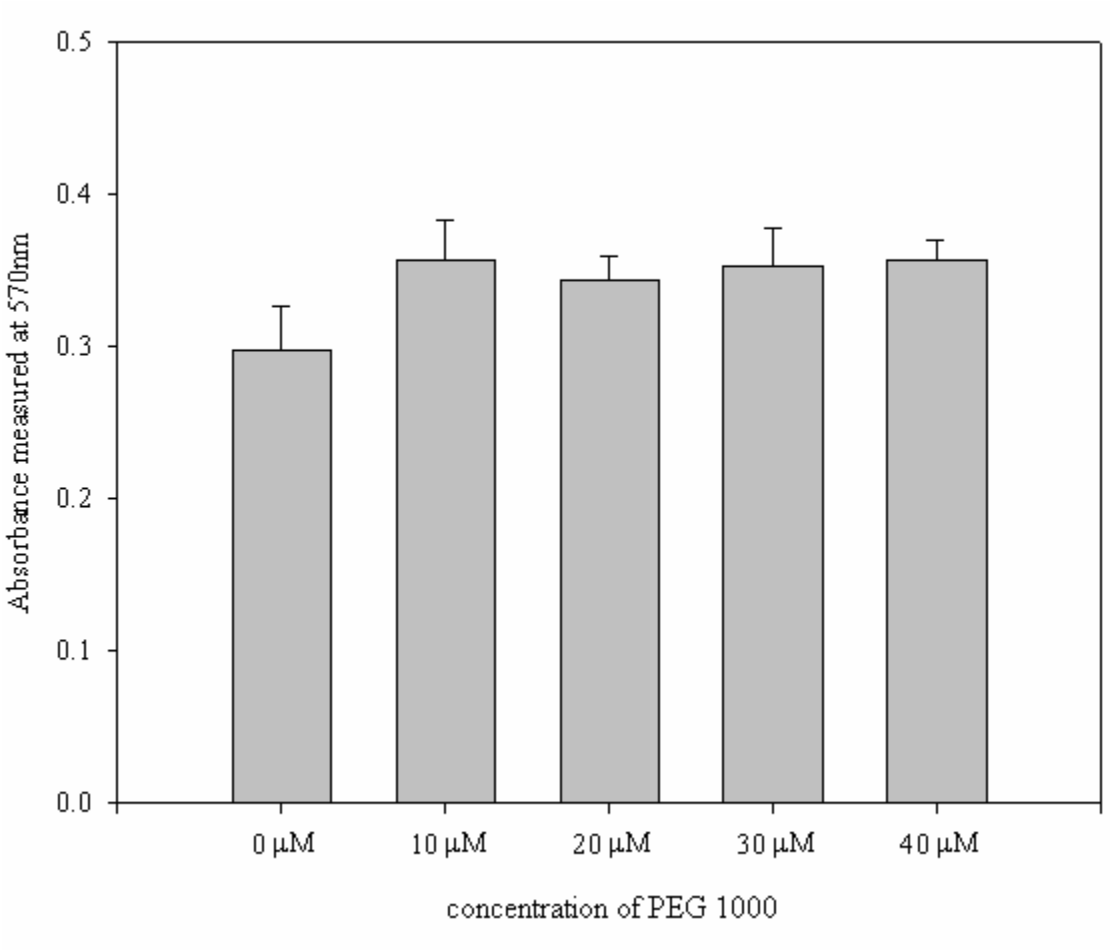


Figure 21. Effect of polyethylene glycol 1000 on RAW264.7 cell viability. Cells were treated with 10 μM, 20 μM, 30 μM, and 40 μM polyethylene glycol (PEG) 1000 for 24 hours. Cell viability was evaluated by the MTT assay as described in Material and Method Section. Values are the mean ± SEM of eight wells.

Cytotoxicity of Succinate

The potential cell killing property of succinate was evaluated using the PI assay. RAW 264.7 cells were exposed to 10 μM, 20 μM, 30 μM, and 40 μM succinate for 24 hours and the results shown in Figure 22. No significant cell death was observed in RAW264.7 cells at any concentration of succinate.

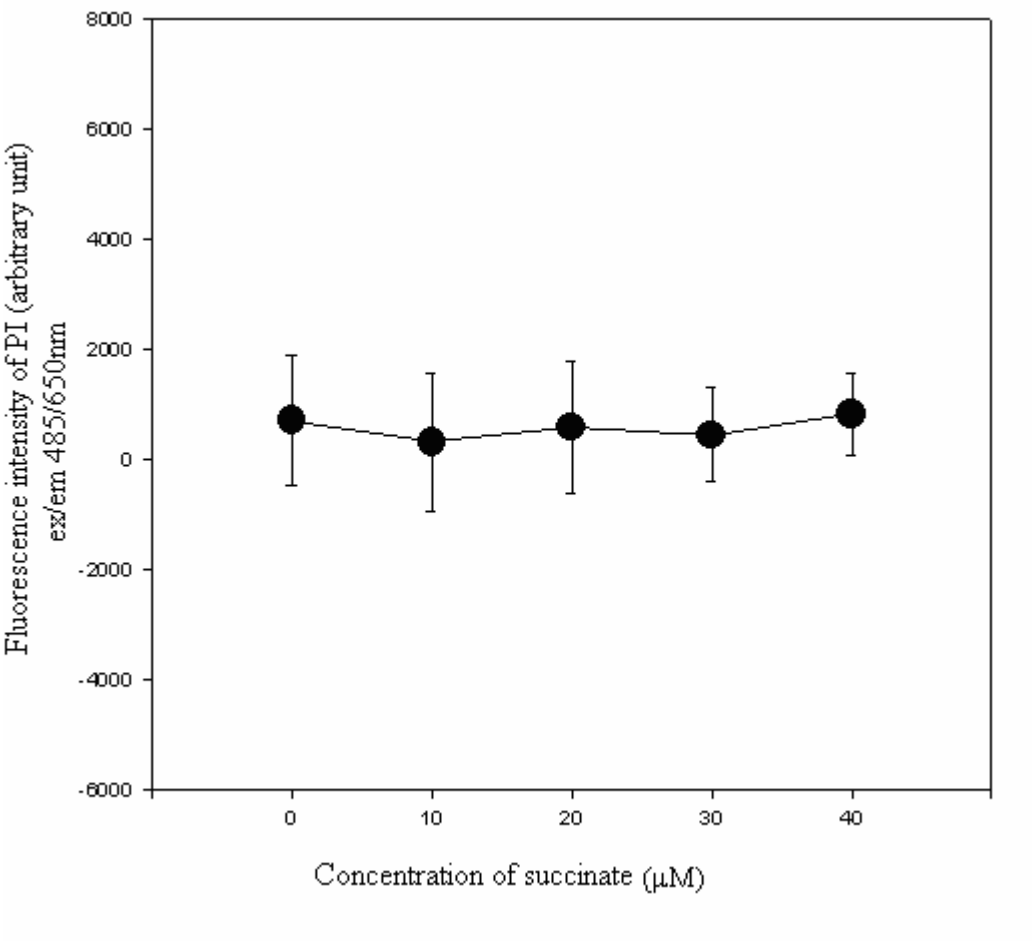


Figure 22. The effect of succinate on cell death in RAW264.7 macrophage cells using the PI assay. Cells were exposure to 10 μM, 20 μM, 30 μM, and 40 μM succinate for 24 hours and the PI assay performed as described in Material and Method Section. The figure shows the mean of eight wells ± SEM.

To confirm this finding, we studied cell viability using the MTT assay and the results are shown in Figure 23. Similarly there was no significant change in the viability of treated cells compared with the untreated cells.

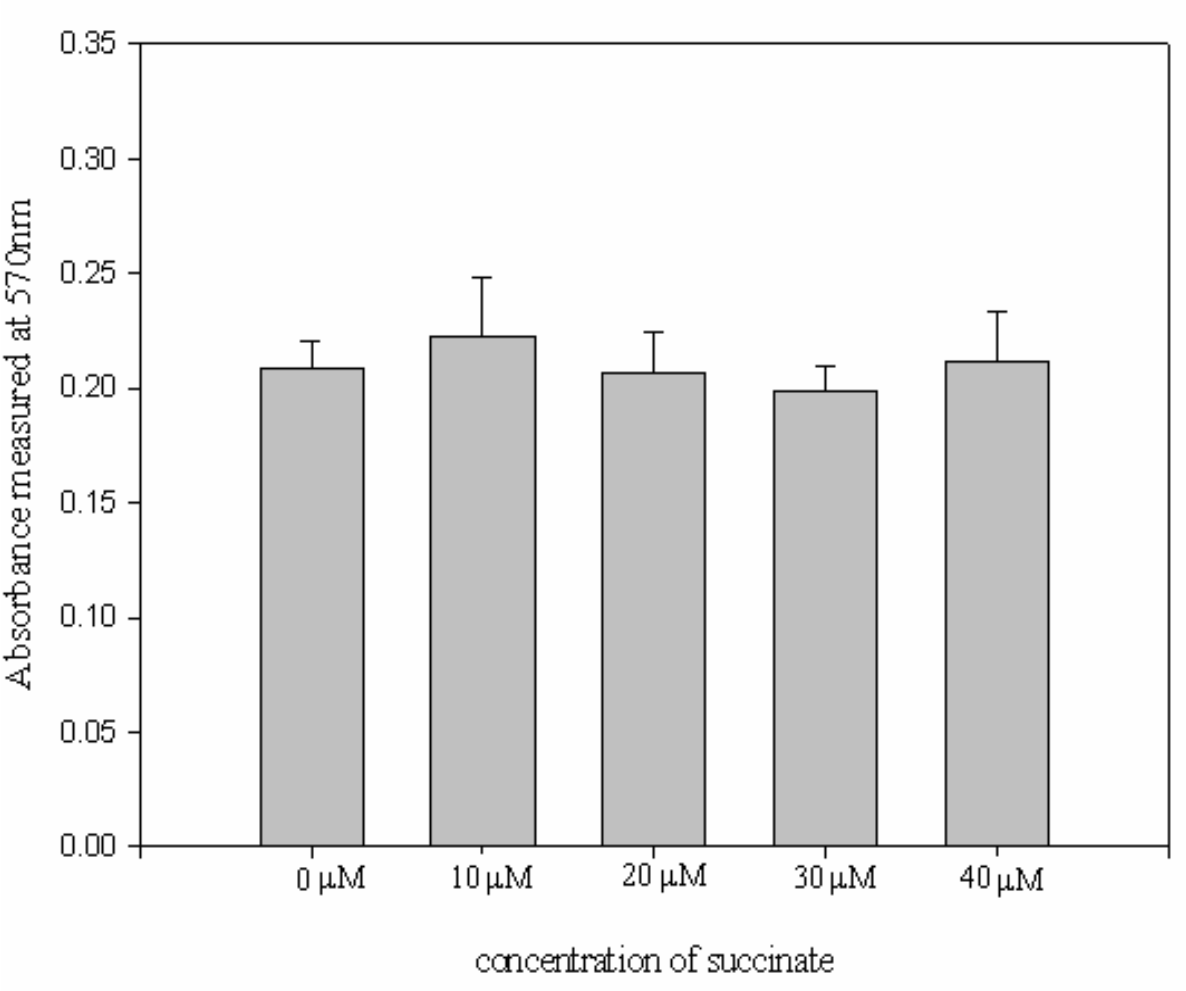


Figure 23. Effect of succinate on RAW264.7 macrophage cell viability. Cells were exposure to 10 μM, 20 μM, 30 μM, and 40 μM succinate for 24 hours and cell viability was evaluated by MTT assay as described in Material and Method Section. Values were the mean ± SEM of eight wells.

Study of the Cytotoxicity of TPGS in LNCaP Cells

LNCaP cells were treated for 22 hours with 10 μM, 20 μM, 30 μM, and 40 μM concentrations of TPGS enriched cell culture medium. We measured dead cells using the PI assay and the results are shown in Figure 24. The results indicate a concentration-dependent increase in dead cells, with significant increase at concentrations of TPGS higher than 20 μM. The total number of cells per well was determine by the digitonin plus PI assay. After measuring the fluorescence intensity of PI corresponding to dead cells, live cells were permeabilized with digitonin and the fluorescence intensity of the PI remeasured which corresponded to total number of cells. The results shown in Figure 25. The percentage of dead cells was calculated as the ratio of the fluorescence intensity of dead cells to that of total cells and the results shown in Figure 26. Concentration-dependent MTT cell viability assays were performed after treating cells with TPGS for 22 hours and the results shown in Figure 27. There was a concentration-dependent decrease in the number of viable cells with a significant decrease beginning at 20 μM concentration of TPGS.

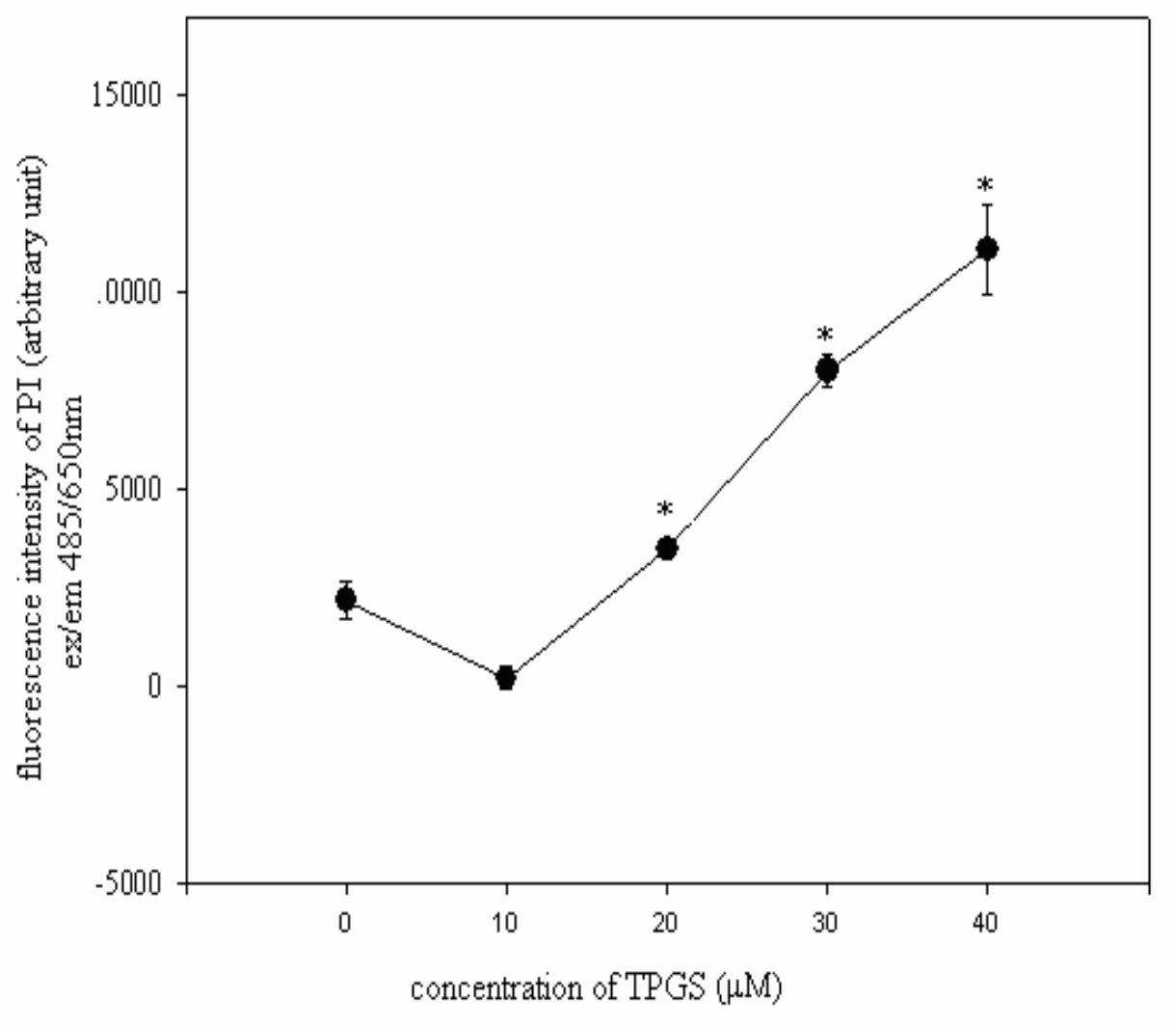


Figure 24. Concentration-dependent cell killing of LNCaP cells by TPGS determined by propidium iodide staining. LNCaP cells were exposed to TPGS (10 μM to 40 μM) for 22 hours. Values are the mean of four wells ± SEM. * $p<0.05$ compared with the untreated cells (0 μM). Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

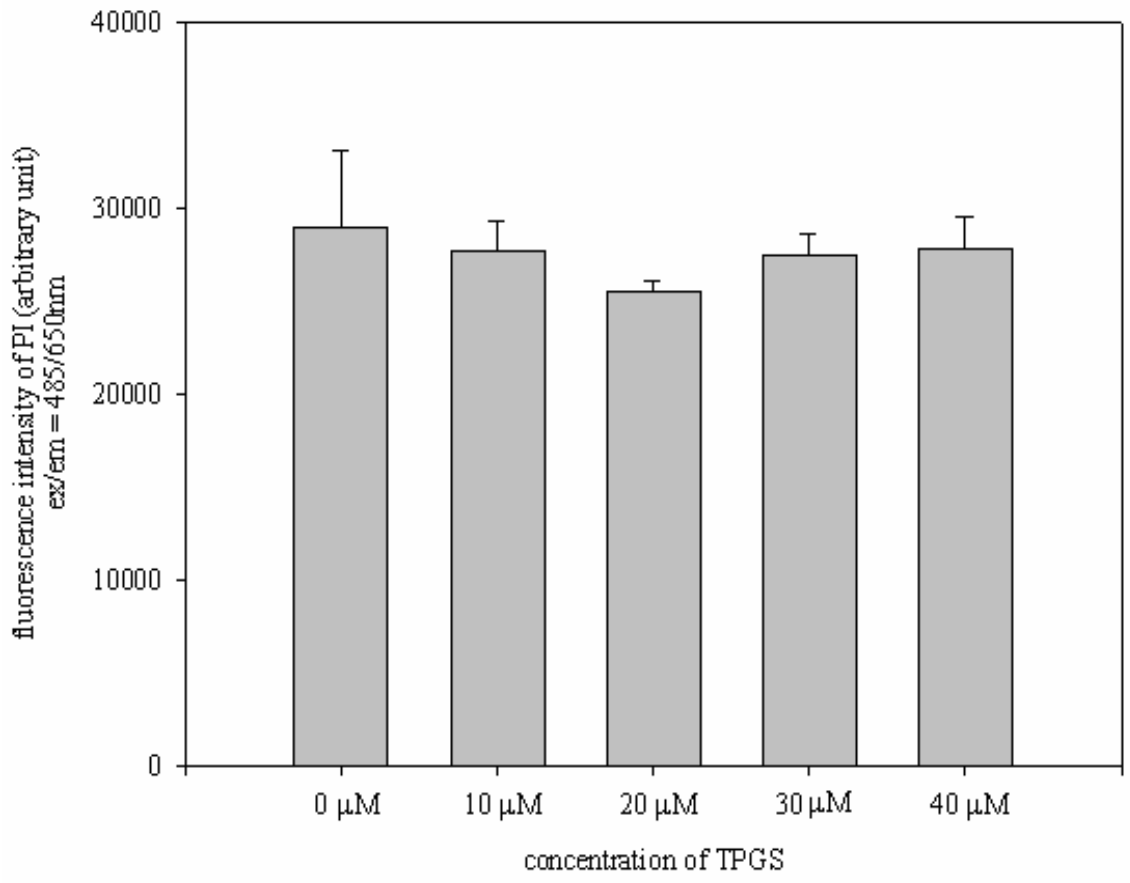


Figure 25. Total number of cells (live plus dead cells) determined by digitonin plus PI assay after exposure of LNCaP cells to TPGS. LNCaP cells were permeabilized with 635 μM digitonin solution. Fluorescence intensity of propidium iodide corresponding to total number of cells was remeasured. Data are expressed as mean of five wells ± SEM.

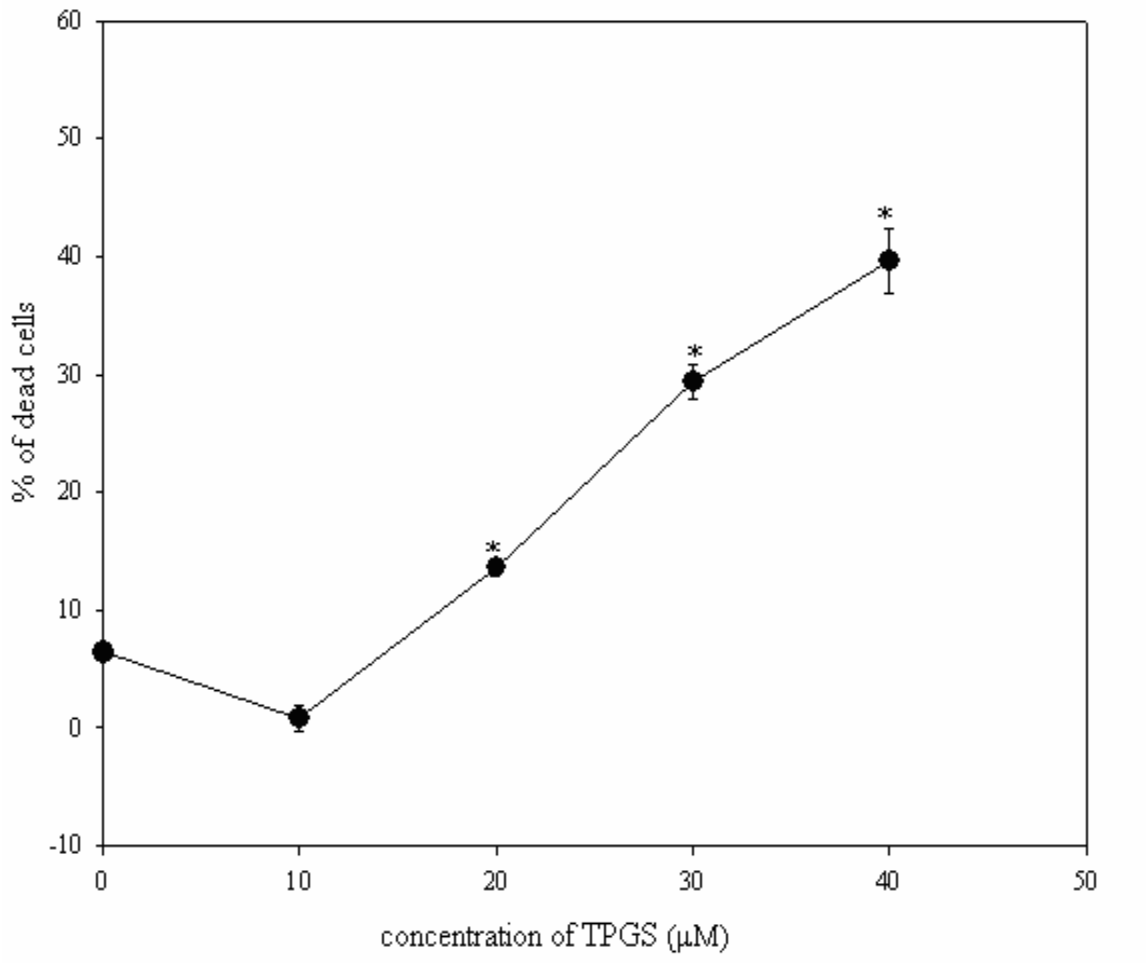


Figure 26. Percentage of dead cells after exposure of LNCaP cells to TPGS. Cells were incubated with10 μM, 20 μM, 30 μM, and 40 μM concentrations of TPGS for 22 hours. '*' ($P < 0.05$), statistically different from untreated cells (0 μM). Death cells increased with the concentration of TPGS. The data show the mean of eight wells ± SEM. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

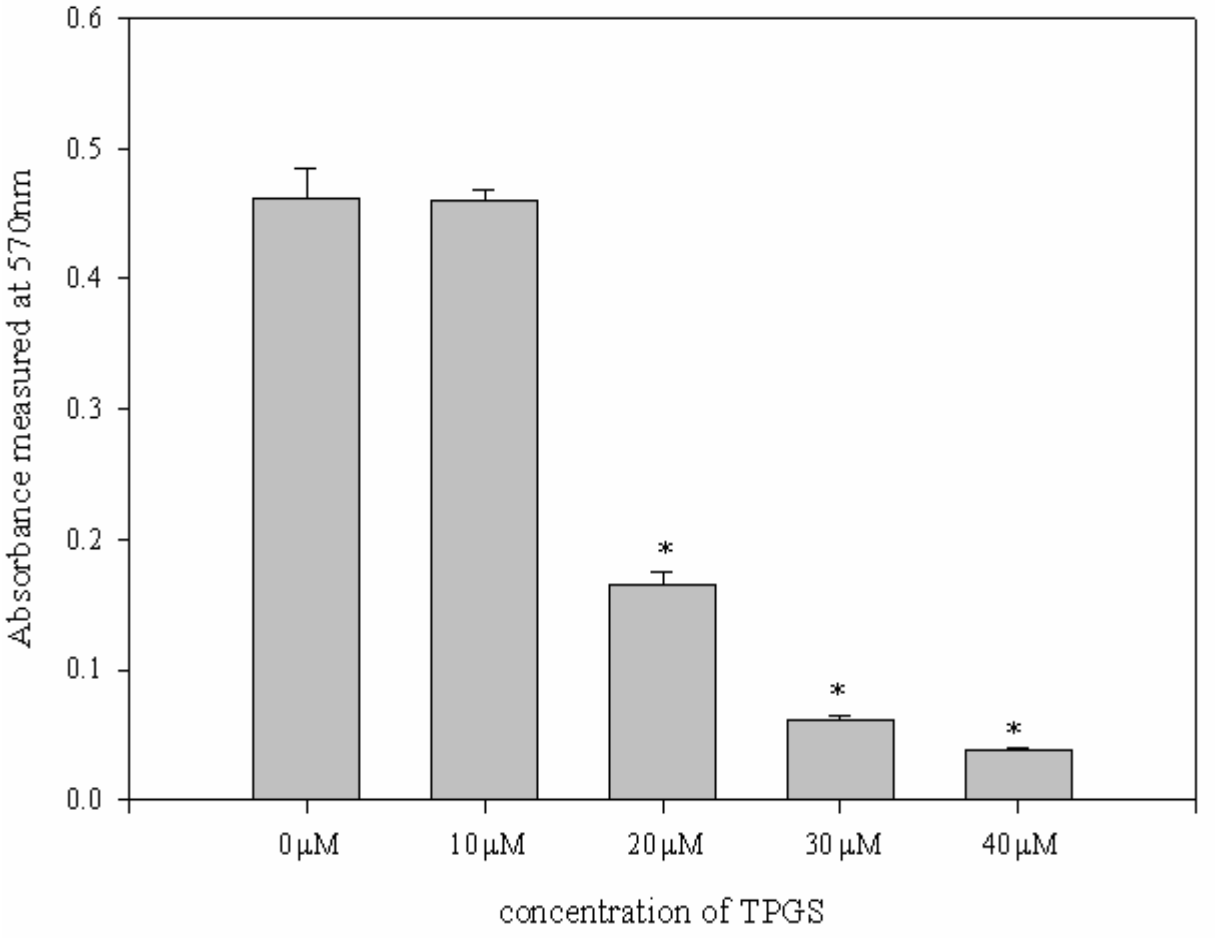


Figure 27. Effect of TPGS on LNCaP cell viability. Cell viability was measured using the MTT assay after treating LNCaP cells for 22 hours with TPGS (10 μM to 40μM). The figure shows the mean of eight wells ± SEM. * $p < 0.05$ compared with the untreated cells (0 μM). Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

To determine the form of cell death induced by TPGS in LNCaP cells, we studied the concentration and time dependent caspase 3 assays. For the concentration-dependent assay, cells (1 x 10^6 cells per well) were treated with 10 μM, 20 μM, 30 μM, and 40 μM TPGS for 22 hours and the results shown in figure 28. These results suggest that caspase 3 was activated in a concentration-dependent manner. For the time dependent assays, we treated LNCaP cells with 40 μM of TPGS for 4 hours, 6 hours, 8 hours, 16 hours, and 24 hours. The results are shown in Figure 29. There was a time-dependent increase in caspase 3 activity reaching a maximum at about 16 hours.

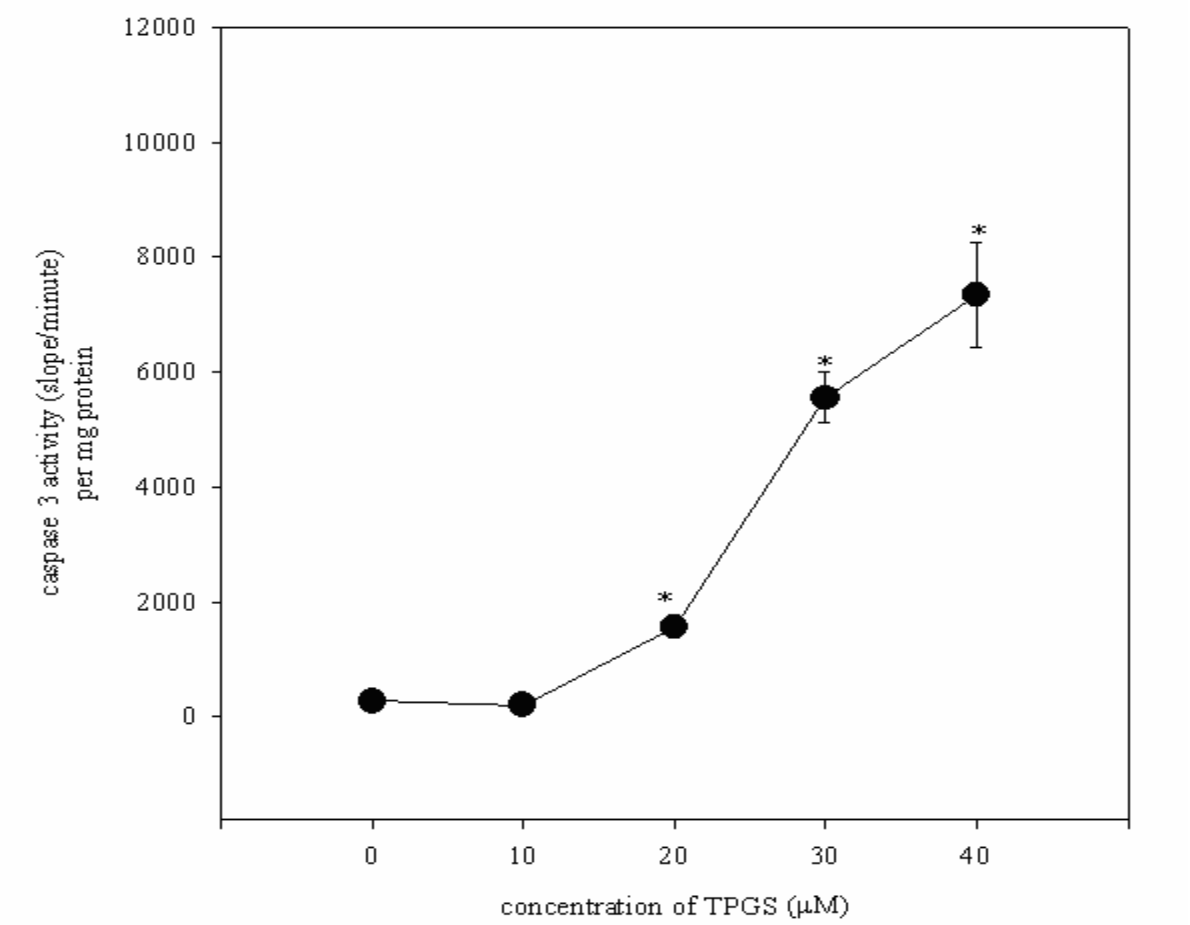


Figure 28. Apoptosis in LNCaP cells induced by TPGS involves activation of caspase 3. The activation was measured as the increase in activity with respect to the control (untreated cell) per mg of protein. '*'$p<0.05$ compared with untreated cells. Values are the mean of five wells ± SEM. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

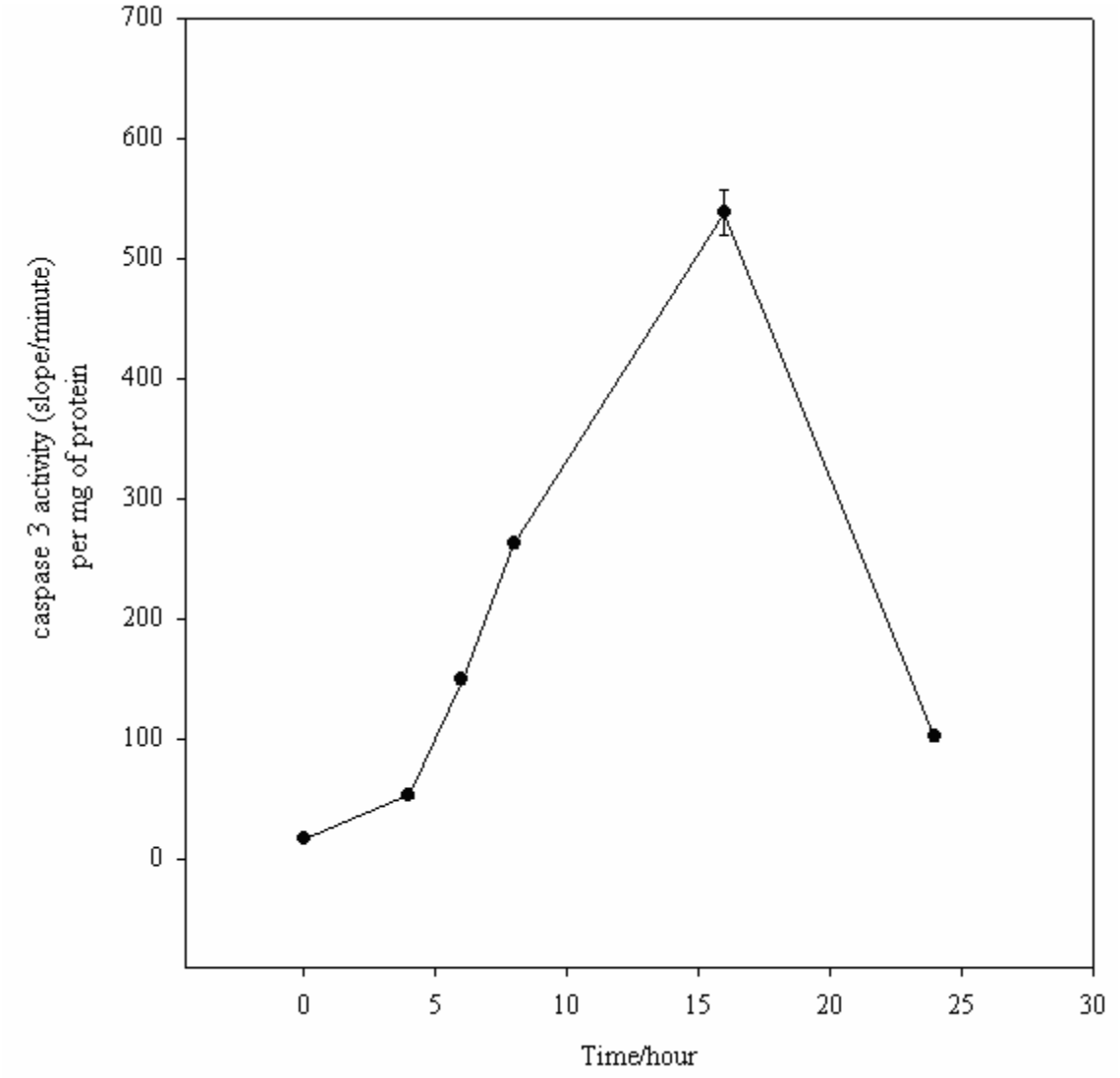


Figure 29. Time-dependent activation of caspase 3 by TPGS in LNCaP cells. Cells were treated with 40 μM TPGS for the indicated time points. The activity was measured as the increase in activity with respect to the 0 hour time point. The data shown are mean of six wells ± SEM.

Study of the Cytotoxicity of Alpha-Tocopheryl Succinate in LNCaP Cells

Figure 30 shows the result of the PI assays after incubating LNCaP cells with 10 μM, 30 μM, and 50 μM of alpha-tocopheryl succinate for 24 hours. Significant cell death was noticed at 50 μM concentration of alpha-tocopheryl succinate. Viability of LNCaP cells after treatment with alpha-tocopheryl succinate was also studied using the MTT assay and the results shown in figure 31. There was a concentration-dependent decrease in the population of viable cells, with a significant decrease as from 30 μM.

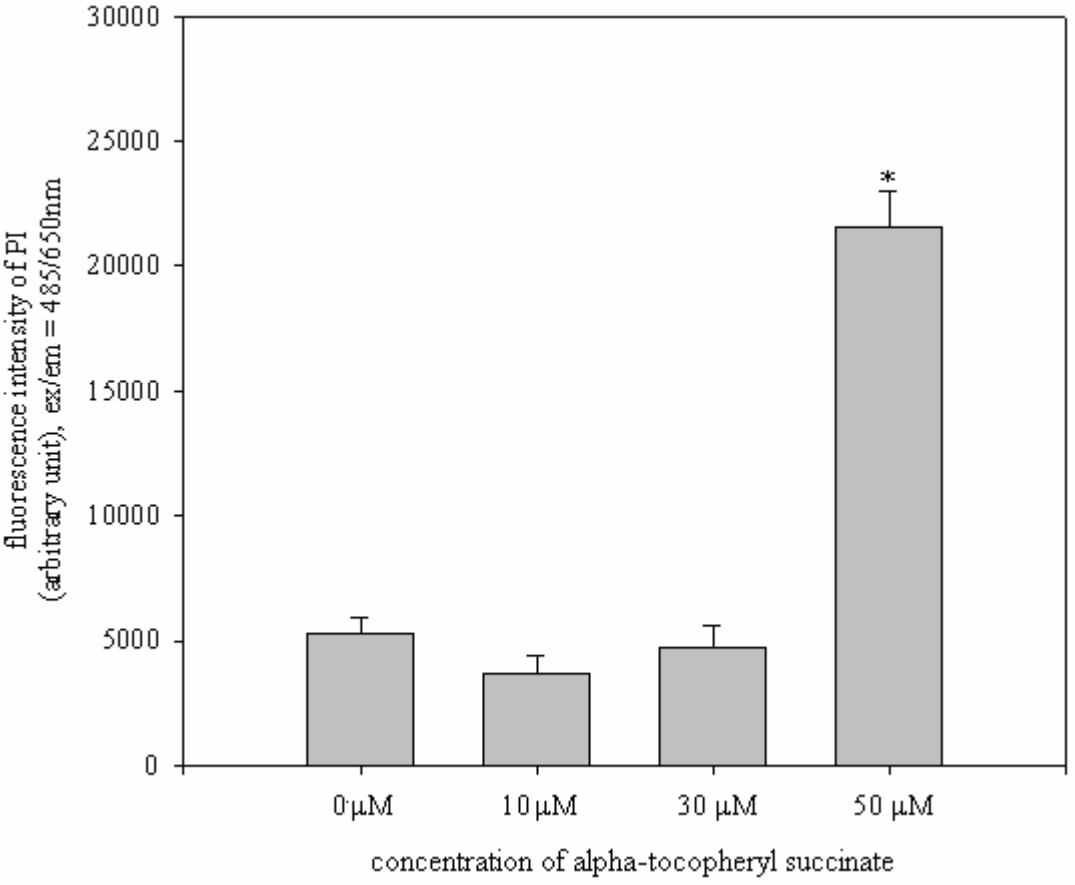


Figure 30. Measurement of cell killing in LNCaP cells after treating cells with alpha-tocopheryl succinate. Cells were incubated with 10 μM, 30 μM, and 50 μM alpha-tocopheryl succinate for 24 hours. Dead cells were evaluated using PI assay as described in Material and Method Section. '*' $P < 0.05$ compared with untreated cells. Values are the mean of eight wells ± SEM. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

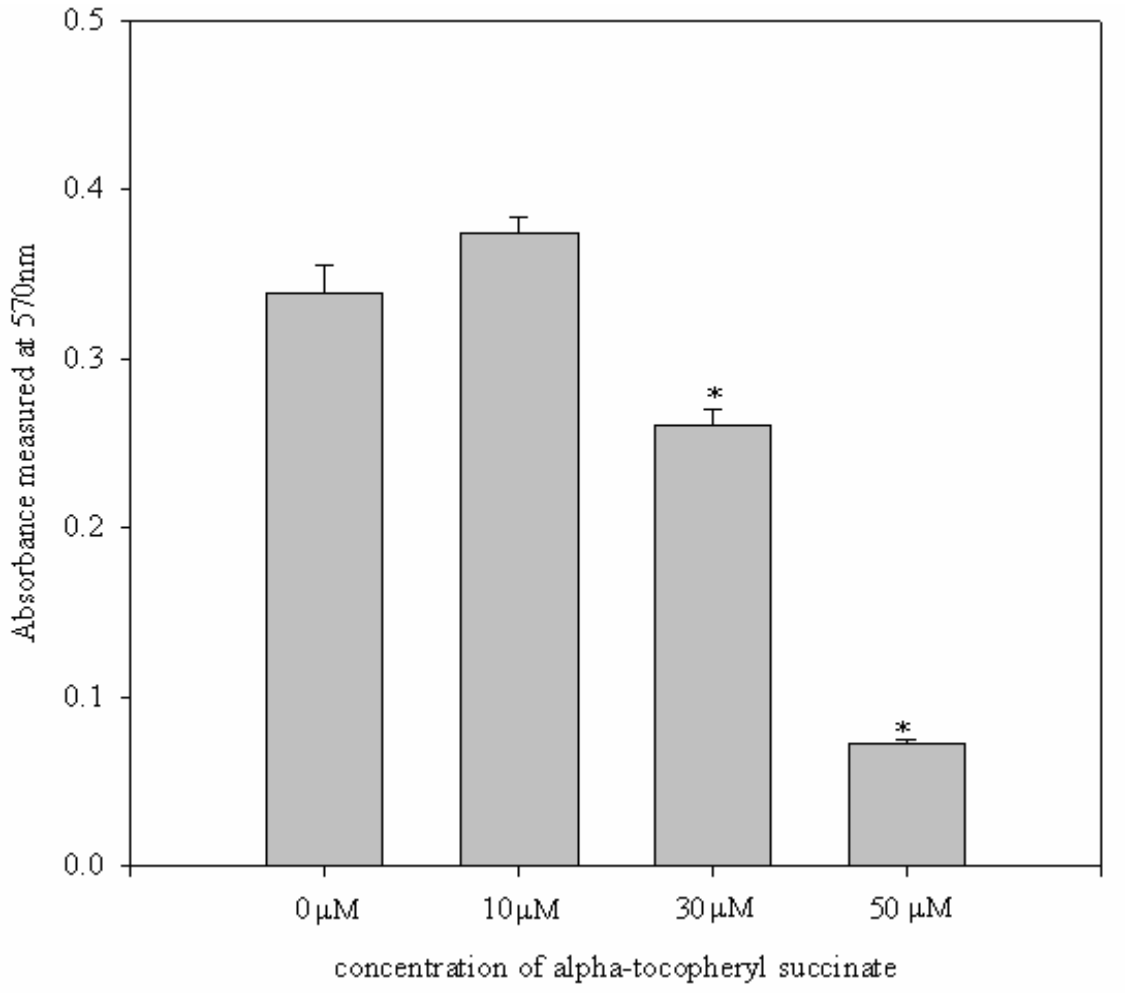


Figure 31. Effect of alpha-tocopheryl succinate on the viability of LNCaP cells. Cells were incubated with 10 μM, 30 μM, and 50 μM alpha-tocopheryl succinate for 24 hours. Cell viability was evaluated by MTT assay as described in Material and Method Section. Values are the mean ± SEM of eight wells. * $p<0.05$ compared with the untreated cell. Statistical analysis was conducted by One-Way ANOVA in SPSS12.0 software package.

CHAPTER 4

DISCUSSION

The main objective of this research was to study the cytotoxicity of TPGS in RAW264.7 and LNCaP cells. Both apoptotic cell death and decreased cell viability were observed in RAW264.7 and LNCaP cells exposed to TPGS. Dead cells were stained with PI, and total cells, including live cells, stained with PI after adding digitonin. The percentage of dead cells was obtained as the proportion of the fluorescence intensity of dead cells to that of total cells. The data in Figure 10 revealed that TPGS kills RAW264.7 cells in a concentration-dependent manner, while the data in Figure 11 indicated that the total number of cells did not substantially changed throughout the experiment. The results of the percentage of dead cells (Figure 12) illustrated the concentration-dependent cell killing property of TPGS in RAW264.7 cells; at 20 μM of TPGS about 6% of cells died with respect to the control, at 30 μM TPGS this number is 17%, and at 40 μM TPGS the percentage increased dramatically to about 40%. These results suggest that TPGS is less cytotoxic at lower concentrations ($\leq$ 10 μM) but increasing toxic at higher concentrations.

This finding was supported by the cell viability assay results (Figure 13), which demonstrated a dose dependent suppression of cell viability by TPGS in RAW264.7 cells. Cell viability was significantly suppressed starting at concentration of TPGS greater than 20 μM. Cells lose the integrity of their plasma membrane when exposed to cytotoxic drugs and subsequently become permeable irrespective of whether the form of cell death is necrotic or apoptotic.

In order to distinguish between the forms of cell death triggered by TPGS in RAW264.7 cells, a caspase 3 enzymatic assay was performed and the results (Figure 14) indicated that cell death induced by TPGS was apoptotic and not necrotic. Caspase 3 was activated in a concentration-dependent fashion with significant activation at concentration of TPGS greater than 20 μM.

The biochemical pathways involved in TPGS induced apoptosis were investigated in RAW264.7 cells and the results (see Figure 15) indicated that TPGS induced apoptotic cell death in RAW264.7 cells by the activation of the effector caspase 3, which has been shown to be one of the key executioner caspases in apoptosis.[38] Caspase 3 activation occurred after the fourth hour of treatment and was preceded by the activation of caspase 8, the apical caspase of the extrinsic or death receptor pathway of apoptosis; this occurred before the fourth hour of treatment. On the other hand, the activation of caspase 9, the apical caspase of the intrinsic or mitochondrial pathway, occurred only after the fourth hour of treatment. These findings suggested the importance of the death receptor or the extrinsic apoptotic pathway. The activation of caspase 9 also suggested that activated caspase 8 might have indirectly activated caspase 9 via the cleavage of the proapoptotic Bcl-2 family member Bid.[39] The cleaved fragment of truncated Bcl-2 interacting protein (tBid) facilitates the oligomerisation of the proapoptotic protein Bax, and insertion into the mitochondrial outer membrane, leading to cytochrome *c* release.[40] Released cytochrome *c* induces the formation of an Apaf-1/procaspase 9/dATP/ATP complex known as the apoptosome and a subsequent activation of caspase 9.[41] More work needs to be done in this area to specify the apoptotic pathway involved.

The involvement of the hydrolysis products of TPGS were also investigated in RAW264.7 cells. The results (Figures 16 and 17) revealed the cell killing and cell viability suppression, respectively, of alpha-tocopheryl succinate in RAW264.7 cells. The apoptotic cell killing of alpha-tocopheryl succinate has been reported in many papers.[42,43] The data for the comparative study of the cytotoxicity of TPGS, alpha-tocopheryl succinate and alpha-tocopherol in RAW264.7 cells (Figure 18), confirmed the cell killing potentials of TPGS and alpha-tocopheryl succinate. In contrast, no cytotoxicity was observed for alpha-tocopherol.

The results of the comparative study for the time course for caspase 3 activation in RAW264.7 cells by TPGS and alpha-tocopheryl succinate (Figure 19) indicated an earlier activation of caspase 3 in the TPGS treated cells compared to the activation observed with alpha-

tocopheryl succinate. For the TPGS treated cells caspase 3 activity was significantly visible after the fourth hour of treatment and reached a maximum at the eighth hour, then started to decline as cells die. Whereas for cells treated with alpha-tocopheryl succinate caspase 3 activity was evident only after the sixth hour of treated and attained a maximum at about 16 hours. These results suggest that alpha-tocopheryl succinate and TPGS induced apoptotic cell death via different pathways or that the mechanisms through which they entered the cells were different. Studies on the cytotoxicity of PEG 1000 and succinate did not reveal significant cell death or suppression of cell viability at any concentration (Figures 20, 21, 22, and 23), suggesting that the cytotoxicity observed in TPGS could be due to TPGS itself or to alpha-tocopheryl succinate but not alpha-tocopherol, PEG 1000, or succinate.

The cytotoxicity of TPGS was also studied using LNCaP cells and the results (see Figures 24 and 25) for the percentage of dead cells (Figure 26) indicated a concentration-dependent cell killing of LNCaP cells by TPGS; at 20 μM TPGS about 5% of cells died, at 30 μM about 24% died, and at 40 μM about 33% died with respect to the untreated cells. This was supported by the results of the cell viability assay (Figure 27) that indicated a significant decrease in viable cells with increasing concentration of TPGS. The dose and time–dependent activation of caspase 3 in LNCaP cells by TPGS (Figures 28 and 29 respectively) revealed that cell death was apoptotic and not necrotic. The activation of caspase 3 by TPGS in LNCaP cells occurred in a concentration-dependent manner, with significant activation occurring after 20 μM of TPGS. The time course for the activation of caspase 3 increased steadily reaching a maximum at about 16 hours of treatment then started to decline as cells die. Because we established that alpha-tocopheryl succinate could kill RAW264.7 cells, it was important to study its cytotoxicity in LNCaP cells. The results for cell killing and cell viability assays (Figures 30 and 31 respectively) indicated that alpha-tocopheryl succinate significantly killed LNCaP cells and suppressed their viability in a dose dependent manner. It has been reported that alpha-tocopheryl succinate induced apoptosis in human prostate cancer.[43] Prostate cancer is the second most

deadly form of cancer for men in the United State after lung cancer.[44] The growth of prostate cancer progresses from an androgen-dependent (LNCaP cells) to an androgen-independent (PC-3 cells) stage. The standard treatment for the androgen-sensitive form of prostate cancer is hormonal therapy, but within 2 years of treatment, the cells become androgen-insensitive and do not respond to hormonal treatment.[45] There is currently no effective treatment for androgen-independent prostate cancer.[46] However, several chemotherapeutic drugs including some derivatives of vitamin E which have antiproliferative properties, cause cell cycle arrest, and induce apoptosis have been reported to selectively inhibit the growth of prostate cancer.[47,48] Our studies demonstrated inhibition of cellular proliferation and killing of LNCaP cells by TPGS. The findings suggest that TPGS can serve as a chemotherapeutic drug for the treatment of prostate cancer.

CHAPTER 5

CONCLUSION

TPGS was shown for the first time to induce apoptotic cell death and decrease cell viability in RAW264.7 macrophage cells and LNCaP cells. Apoptotic cell death, as measured by caspase 3 activation, occurred in a concentration and time-dependent manner in RAW264.7 and LNCaP cells treated with TPGS. Based on the results obtained, our working hypothesis is that the interaction of TPGS with death receptors activates the caspase 8 enzyme in RAW264.7 macrophage cells. Activation of caspase 8 also leads to cleavage of Bid causing the release of cytochrome c from the mitochondria and a subsequent activation of caspase 9.

The hydrolysis products of TPGS which are alpha-tocopherol, polyethylene glycol 1000, and succinate were found not to be cytotoxic to RAW264.7 macrophage cells. In marked contrast, alpha-tocopheryl succinate is cytotoxic to both RAW264.7 and LNCaP cells, indicating that the cytotoxicity observed in TPGS may be due to one of its product of hydrolysis, alpha-tocopheryl succinate. The early activation of caspase 3 in RAW264.7 macrophage cells treated with TPGS compared with cells treated with alpha-tocopheryl succinate suggests that TPGS and alpha-tocopheryl succinate might be triggering apoptotic cell death via different pathways or that they entered the cells through different mechanism.

Because TPGS could kill LNCaP cells, it may be useful in treating cancer cells. At present the cytotoxicity of TPGS on androgen-insensitive PC-3 cells and normal prostate epithelial (RWPE-1) cells remain to be addressed. Based on the observation that caspase 3 is activated earlier in TPGS treated cells compared with alpha-tocopheryl succinate, further evaluation of the apoptotic pathways triggered by TPGS and alpha-tocopheryl succinate is needed in order to distinguish between their pathways.

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