Cancer is one of the common causes of mortality worldwide. The often terminal, aggressive and devious nature of cancer makes it a difficult target to eradicate. Throughout the years, mankind’s knowledge of cancer has progressed leaps and bounds but we are no closer to finding a cure. Cancer incidence worldwide was as high as 14.1 million cancer cases in 2012 alone, with 8.2 million cancer-related deaths reported [1]. The top three forms of cancer affecting humans worldwide are cancers of the lung, breast and colorectal. Globally, leukemia, a form of blood cancer, is placed eleventh [2]. Four major forms of leukemias have been described, acute lymphoid leukemia (ALL), acute myeloid leukemia (AML), chronic lymphoid leukemia (CLL) and chronic myeloid leukemia (CML) [3]. Targeted therapy using Imatinib (Gleevec) can be useful to a certain extent for CML and certain ALL cases [4]. Treatment with chemotherapy can cause some undesirable side-effects that could severely impact a patient’s quality of life. For instance, treatment with Imatinib mesylate can cause several side-effects such as fever, liver dysfunction, fluid retention, skin rashes, diarrhea [5], cytopenia [6], anaemia [7] and thrombocytopenia [8] in some patients. However, survival of CML patients has greatly improved through the use the tyrosine kinase inhibitor (TKI) [9]. Effective treatment with fewer side-effects is highly desirable in cancer therapy. Phytochemicals, such as vitamin E can be explored for such purposes.

Tocotrienols (T3) are members of the vitamin E family, which also includes the tocopherols. Four main isoforms of T3 exist naturally and they are known as alpha- (α), beta- (β), delta- (δ) and gamma- (γ) T3 (Figure 1) [10]. Tocotrienols are structurally similar to the tocopherols, with the exception of an unsaturated side-chain [11]. However, the T3are reported to possess distinct biological properties such as antioxidant, neuroprotective, cardioprotective [12], anti-diabetic [13], anti-inflammatory [14], radioprotective [15] and anti-cancer [16] activities. These properties of T3 make them to be potentially useful in the management of various diseases.

The highly regulated process of apoptosis becomes disrupted in many cancers, leading to cancer cell survival. Gamma-T3 is reported to exhibit potent cytotoxicity towards cancer cells as well as inhibit cell proliferation [17], induced apoptotic cell death [18], as well as suppresses tumour invasion [19]. However, data on the effects of exposing K562 human CML cells to γT3 is not yet available. Induction of apoptosis in cancer cells is desired as apoptotic cell death does not usually result in inflammation and tissue damage commonly seen during necrotic cell death [20]. Therefore, the aim of this study was to investigate the effects of exposing the human K562 CML cells to γT3 as well as to identify differentially regulated genes related to apoptosis in the leukemic cells.

**Methods and Materials**

**Cell culture**

RPMI (Roswell Park Memorial Institute) Medium 1640 (GIBCO, USA); L-glutamine (GIBCO, USA); HEPES (1M) (GIBCO, USA); Penicillin-Streptomycin (10,000 U/mL) (GIBCO, USA); Fetal Bovine Serum (GIBCO, USA) were used.
Test compounds

The gamma-tocotrienol (γT3) was a kind gift from Davos Life Science Pvt. Ltd. (Singapore). The γT3 was weighed and dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, US) to make a 10 mg/ml stock solution. The γT3 used in this study is a natural product extracted from palm oil. The stock solution of γT3 was aliquoted and stored at -20°C prior to use. Doxorubicin (Sigma, USA), used as the positive control, was also prepared in DMSO.

Cells

The K562 human CML cell line was purchased from the American Tissue Culture Collection (ATCC, USA). These cells were maintained in complete medium [RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum [HI-FBS] and 1% Penicillin-Streptomycin (10,000 U/mL)] at 37°C in a humidified 5% CO2 incubator (RS Biotech, UK).

Human peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers at the medical clinic of the International Medical University (IMU) located in Kuala Lumpur, were used as normal control cells in the cell-based assay. The PBMC were isolated from blood collected in vacutainer tubes containing lithium heparin (BD, USA). The blood was centrifuged (1500 rpm for 10 minutes at 4°C). Theuffy coat layer containing platelets and leucocytes were carefully aspirated into a sterile 15 ml centrifuge tube and subjected to a red cell lysis step using the RBC lysis buffer as recommended by the manufacturer (InTrON, Korea). The cells were then resuspended in complete medium.

The 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay

The 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate the cytotoxicity of γT3 towards K562 CML cells. A two-fold serial dilution of γT3 (0-30 µg/ml) was performed in 96-well plates. Doxorubicin, a chemotherapeutic drug used to treated cancers was used as a positive control. The K562 cells were harvested and seeded into the wells (1 x 104 cells/well) of the 96-well plates. The plate was centrifuged (1500 rpm for 10 minutes at 4°C). Theuffy coat layer containing platelets and leucocytes were carefully aspirated into a sterile 15 ml centrifuge tube and subjected to a red cell lysis step using the RBC lysis buffer as recommended by the manufacturer (InTrON, Korea). The cells were then resuspended in complete medium.

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Bromodeoxyuridine assay

Cell plating and treatment with γT3 was done as described for the MTT assay. The bromodeoxyuridine (BrDU) assay measures the incorporation of BrDU, a thymidine analogue, into the DNA of proliferating cells [21], which is a more accurate tool to establish anti-proliferative activity of any test agent. The BrDU assay used in this study was an ELISA-based assay that was performed as recommended by the manufacturer (Merck-Millipore, USA). The percentage of inhibition of cell proliferation was calculated using the same formula used in the MTT assay.

Cell Death ELISA

The mode and extent of cell death induced in the K562 cells was determined using a commercial cell death ELISA kit as described previously [22]. Briefly, the K562 cells were plated at a cell density of 1 x 104 cells/well and were treated with for 72 hours with three concentrations of γT3 namely IC50 (4.20 µg/ml), IC25 (8.77 µg/ml) or IC12.5 (13.14 µg/ml) values obtained from the MTT assay. The K562 cells treated with doxorubicin (0, 0.05, 0.1 and 0.5 µg/ml) served as positive controls. The cell lysate and supernatant were harvested separately to detect apoptosis (from lysate) and necrosis (from supernatant). Cell death was reported as enrichment factor, which was calculated using the following formula:

\[
\text{Enrichment factor} = \frac{\text{Absorbance of the sample (dying / dead cells)}}{\text{Absorbance of Control (cells without treatment)}}
\]

Gene expression studies

The human apoptosis quantitative polymerase chain reaction (qPCR) array (QIAGEN, Germany) was used to analyze differential gene expression in K562 cells exposed to γT3. This is a commercial qPCR array using a 96-well format where 84 wells are annotated with primers that can detect 84 human genes that have reported to be associated with apoptosis (QIAGEN, Germany). The remaining 12 wells contain primers for house-keeping and other control genes. The K562 CML cells were treated with γT3 for 72 hours. The concentration of γT3 used in this study was the 72 hours, IC50 concentration was determined using the MTT assay. This concentration was chosen as results from the cell death ELISA showed that this concentration induced highest level of apoptosis in the K562 cells. The time point of 72 hours was selected as we observed the highest level of apoptosis with low necrosis in the K562 cells treated with γT3. Untreated K562 cells were used as control. Total RNA was extracted from the K562 cells (γT3 treated and control) using the RNeasy Plus Mini Kits (QIAGEN, Germany) and using a formula [Percentage viability = (Average OD of sample / Average OD of control) x 100]. A graph showing percentage viability versus concentration of γT3 was plotted and the 50% inhibitory concentration (IC50) was determined from the plot.
QIAshredder (QIAGEN, Germany) according to the manufacturer recommended protocols. The absorbance ratios at 260/280 nm and 260/230 nm of the extracted RNA samples were analysed using a nanoquant spectrophotometer (Tecan’s Nanquanot, USA) to determine the RNA quality and concentration. The RNA samples used all had 260/280 nm ratio greater than 1.8 and 260/230 nm ratio greater than 1.7. The RNA samples were also analysed using a bioanalyzer (Agilent Technologies, USA) to ensure that the RNA used in the qPCR array analysis the RNA integrity number (RIN) of all samples were above seven. Three biological replicates were used for the qPCR array assay.

Conversion of RNA to cDNA was performed using the RT2 First Strand Mastermix kit according to manufacturer’s protocol (QIAGEN, Germany). One microgram of total RNA was used per plate. The cDNA obtained was mixed with RT® SYBR Green Fluor qPCR Mastermix (provided with the kit) and water before loading into the wells of a RT® Profiler PCR array human apoptosis plate (QIAGEN, Germany), which were annotated with individual primers of 84 genes from a panel of human apoptosis genes. Real time PCR analysis was done using real-time thermocycler (BIORAD IQ5 Standard edition, version 2.1.95.824, USA). Data analysis was performed using a web-based software (assessed at URL: www.SABiosciences.com/pcarraydataanaylsis.php) provided by the manufacturer (QIAGEN, Germany). Gene expression data is presented as fold change of treated groups compared to control group (untreated cells) calculated based on the ΔΔCT method. The same site also provides web-based software that can be used to show association between the differentially regulated genes. This software was used to analyse the data as well as discuss possible interactions between the putative targets.

Some of the differentially expressed genes identified from the qPCR array were validated using real-time PCR (RT-PCR). The genes selected based on their function in the apoptosis pathway include Casp9 [23], CD40 [24], NOD1 [25] and TNFRSF10B [26]. The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as the house-keeping gene. The primers used to perform the RT-PCR analysis of these genes were purchased from Invitrogen (Invitrogen, USA) according to the manufacturer’s instructions (Actif Motif, USA). The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as the house-keeping gene. The master mix for each reaction contained 0.5 µl of SuperScript® II Platinum SYBR Green one-step qRT-PCR (Invitrogen, USA) was prepared for each gene of interest and the housekeeping gene. The master mix for each reaction contained 0.5 µl of SuperScript® III RT (Invitrogen, USA), 12.5 µl of 2X SYBR® Green Reaction Mix (Invitrogen, USA) and 0.5 µl, each of 10 µM forward and reverse primers of the respective genes. Each master mix was briefly centrifuged and loaded into pre-chilled 0.2 ml RT-PCR strip tubes (14 µl/tube) pre-chilled on ice. The RNA samples were thawed and diluted with RNase-free water (GIBCO, USA) to a final concentration of 100 ng. Then, 11 µl of the diluted RNA was loaded into specific wells containing the master mix for the real time PCR reaction. The strip tubes were loaded into the IQ5 real time PCR detection system (Bio-Rad, USA) set to the following cycling conditions: 50°C for 3 minutes (for the synthesis of cDNA), 95°C for 5 minutes (initial DNA denaturation), 40 cycles of 95°C for 15 seconds and 50°C for 30 seconds and finally, 40°C for 1 minute. All samples were run in triplicates.

Protein expression studies

Total protein was extracted from γT3-treated cells 72 hours post-treatment using the QProteome Mammalian Protein Prep Kit according to the manufacturer recommended protocol (QIAGEN, USA). As the CASP9 protein is a nuclear protein, nuclear protein was extracted from the treated K562 cells using the Nuclear Extract Kit according to the manufacturer’s instructions (Actif Motif, USA). Total protein content of all the extracted protein samples was determined using the Quick-Start Bradford Protein Assay (Biorad, USA). Equal amount of protein was used as starting material for the ELISA. The concentrations of the CASP9, CD40, NOD1 and TNFRSF10B proteins were measured using relevant commercial ELISA kits using the manufacturer recommended protocol (Novatein Biosciences, USA).

Results

Gamma-tocotrienol selectively inhibits proliferation of K562 cells

Gamma-tocotrienol exhibited dose and time-dependent effects on the viability of K562 cells (Figure 2). The IC50 value, which was the dose required to kill 50% of cells for γT3 treated K562 was found to be 12.71 µg/ml for 24 hours, before reducing to 9.95 µg/ml for 48 hours and 8.77 µg/ml for 72 hours (Table 1). As human PBMCs were normally used as normal cells in studies assaying cytotoxicity against leukemic cells [27], we also tested the effects of exposing these cells to the same concentrations of γT3. A dose- and time-dependent cytotoxicity was observed (Figure 2), however, the cytotoxicity was markedly lower compared to K562 (Table 1).

As the MTT assay does not provide anti-proliferative information, we also tested the effects of exposing K562 cells to γT3 using the BrdU assay. Gamma-tocotrienol exhibited a dose-dependent reduction in cell proliferation (Figure 3). We also observed a time-dependent reduction in the IC50 values from 24 to 72 hours (Table 2). The percentage of BrdU incorporation into the K562 cells appear to decrease with increasing doses of γT3, indicating that γT3 has anti-proliferative effects on these cells.

Gamma-tocotrienol induces cell death by activating the apoptotic mechanism

The cell death ELISA used can be used to differentiate between two forms of cell death, namely apoptosis and necrosis as well as to quantify the level of cell death [22]. Apoptosis was found to be lowest when K562 cells were treated with γT3 at their IC50 dose and highest at when IC50 concentration was used (Figure 4). The K562

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC50 (µg/ml)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-T3</td>
<td>12.71 ± 0.39</td>
<td>9.95 ± 0.60</td>
<td>8.77 ± 0.43</td>
<td></td>
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<tr>
<td>Doxorubicin*</td>
<td>6.52 ± 0.61</td>
<td>0.22 ± 0.04</td>
<td>0.12 ± 0.03</td>
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</tr>
<tr>
<td>Human PBMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-T3</td>
<td></td>
<td>27.62 ± 1.02</td>
<td>26.07 ± 1.35</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin*</td>
<td></td>
<td>2.59 ± 0.24</td>
<td>2.94 ± 0.70</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: IC50 values obtained from MTT assay for K562 cells and human PBMCs treated individually with γ-T3 and doxorubicin (*Doxorubicin was used as positive control).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC50 (µg/ml)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-T3</td>
<td>10.73 ± 0.47</td>
<td>10.18 ± 0.42</td>
<td>8.34 ± 0.48</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin*</td>
<td>0.37 ± 0.04</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: IC50 values obtained from BrdU assay for K562 cells treated individually with γ-T3 and doxorubicin (*Doxorubicin was used as positive control).
cells were also treated similarly with doxorubicin at their IC<sub>25</sub> (0.06 µg/ml), IC<sub>50</sub> (0.12 µg/ml) and IC<sub>75</sub> (0.27 µg/ml) concentrations, which served as controls. The necrotic fraction appeared to increase in a similar pattern, albeit at a lower rate. The results strongly suggest that gT3 induces apoptosis in the K562 cells.

**Gene expression studies**

The averaged gene expression changes in the γT3 -treated K562 cells was initially visualised in the form of scatter plots (Figure 5). The results show that exposure to γT3 significantly (p < 0.05) regulated 24 genes out of the 84 genes that were annotated in the human apoptosis array by at least two fold or more (Table 3) in the K562 cells. The differentially regulated genes could be categorized into several families such as the BCL2 family, caspase recruitment domain (CARD) family, the caspases, growth arrest and DNA damage (GADD) family, apoptosis inhibitor family, P53
Figure 4: Mode of cell death induced in K562 cells treated with various doses (IC_{25}, IC_{50}, or IC_{75}) of γT3 and doxorubicin (Dox). Cell death ELISA was performed using a commercial Cell Death ELISA kit. Data is represented as mean enrichment factor of cells ± standard deviation (SD). (*p < 0.05 versus control).

Figure 5: Scatter plot comparing normalised expression of all genes in the human apoptosis PCR array following treatment with γT3. The scatter plot represents an average of three biological replicates compared against untreated cells (Control Group). The central line indicates unchanged gene expression. The red dots represent genes up-regulated more than two-fold while black plots indicate unchanged gene expression.
family, tumour necrosis factor receptor superfamily (TNFRSF) and the tumour necrosis factor (TNF) ligand superfamily (Table 3). All the differentially regulated genes were found to be significantly ($p < 0.05$) up-regulated in comparison to the untreated K562 cells. None of the genes annotated in the human apoptosis gene PCR array were found to be under-expressed (i.e. below 2-fold). Many pro-apoptotic genes such as BAD, BAX, BNIP3L, APAF1, NOD1, PYCARD, CASP4, CASP7, CASP8, CASP9, GADD45A, TP53, TP73, CD40, TNFRSF10B, TNFRSF1A, TNFRSF9, CD70, FASLG and DAPK1 found to be up-regulated in K562 cells treated with γT3. We also observed that γT3 treatment also enhanced expression of several pro-survival genes such as BCL2L1, IGF1R, NOL3 and XIAP in the K562 cells. However, as the net effect observed was apoptosis, it is highly likely that the net effect these pro-survival genes were much lower compared to the pro-apoptotic genes that were activated in these cells.

Four genes (CASP9, CD40, NOD1 and TNFRSF10B) of interest were selected to conduct downstream real-time PCR analysis. The CASP9 gene was selected to study the effect of γT3 on the intrinsic pathway of apoptosis [28], whilst the TNFRSF10B gene was chosen to investigate its regulatory effects on the extrinsic pathway of apoptosis [29]. The NOD1 gene was investigated for its apoptotic protease activating factor-1 (APAF-1)-like role in CASP9-dependent apoptosis [23], and the CD40 gene was of interest use due to its anti-tumour activities [30]. The expressions of these genes appear to be time-dependent, as the expression increased from 24 to 72 hours (Figure 6). However, we only observed significant ($p < 0.05$) upregulation of these genes in the γT3-treated K562 cells compared to untreated K562 cells after 72 hours of exposure. Hence, it appears that the apoptosis-inducing property of γT3 was highest at 72 hours, which correlated with the data from the MTT and BrdU assays where γT3 showed strongest cytotoxic and anti-proliferative properties at this time point compared to 24 and 48 hours.

The K562 cells exposed to γT3 induced up-regulation of various genes that encode members of several important apoptotic protein families. We used web-based software (accessed at http://gnpcpro.sabiosciences.com/gncpro/gncpro.php) to map out interactions between some of these genes that we identified from our analysis (Figure 8).

### Protein expression studies

The protein expression of these four genes (CASP9, CD40, NOD1 and TNFRSF10B) were found to be significantly ($p < 0.05$) higher in γT3-treated K562 cells compared to the untreated cells (Figure 7). These findings support the hypothesis that γT3 is an effective bioactive compound that can induce apoptosis in the K562 CML cells.

### Discussion

Several researchers have reported on the anti-cancer effects of tocotrienols in prostate [31] and breast [32] cancers. Although there are numerous publications on the anti-cancer effects in prostate, breast and other human cancers, there are only a few papers regarding the effects of tocotrienols on human leukemic cells [33,34]. However, to date there are no reports on the effects of γT3 on K562 human CML cells. This paper reports on the effects of exposing K562 cells with γT3. The objective of this study was to establish the cytotoxic potential of γT3 on the K562 cells and also to identify genes of the apoptosis network regulated by this form of vitamin E.

The results from MTT assay indicated that γT3 was highly cytotoxic towards the K562 cells. Palau and colleagues [33] investigated the cytotoxicity of γT3 on HL-60 and K562 cells and reported the 72 hours IC$_{50}$ values to be 36.297 μM (14.90 μg/ml) and 34.947 μM (14.35 μg/ml), respectively, which were twice the dose obtained in our study. In a previous study, we found

<table>
<thead>
<tr>
<th>Group</th>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Fold change ($p &lt; 0.05$)</th>
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<tr>
<td>BCL-2 family</td>
<td>BAD</td>
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<td>Nucleolar protein 3 (apoptosis repressor with CARD domain)</td>
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<td></td>
<td>DAPK1</td>
<td>Death-associated protein kinase 1</td>
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Table 3: List of differentially expressed genes from the apoptotic pathway in gamma-T3-treated K562 cells at IC$_{50}$ dose.
Figure 6: Time-course real time PCR for the examination of CASP9, CD40, NOD1 and TNFRSF10B gene expression. The fold change was calculated in comparison with their corresponding untreated controls at the respective time-points (24 hours, 48 hours or 72 hours). Data is represented as mean fold change ± standard deviation (SD). (*p < 0.05 versus control).

Figure 7: Quantification of (a) CASP9, (b) CD40, (c) NOD1 and (d) TNFRSF10B in K562 cells exposed to γT3 for 72 hours using ELISA compared to untreated cells (negative control). The nuclear protein was standardised for each sample to 2.9 mg/ml per well using the results from the Bradford assay. Similarly, total protein concentration for each sample was standardised to 10 mg/ml per well. (*p < 0.05 versus control).
that γT3 induced 50% cell death in CEM-SS cells, which are human acute lymphoblastic leukemic (AML) cells at a much lower dose of 4.4 μg/ml [34]. The potent cytotoxicity of γT3 did not appear to extend towards normal cells as we and others have found that γT3 was visibly less potent in inducing human PBMCs cell death [28]. From the BrdU assay results, we observed that γT3 showed dose-dependent anti-proliferative properties towards the K562 cells. The results from the BrdU assay appear to corroborate the findings from the MTT assay, suggesting that γT3 can kill the K562 cells. The mode of cell death was established to be mainly apoptosis using a commercial ELISA kit that can quantify amount of cell death due to apoptosis or necrosis. We have previously used the same ELISA kit to establish that tocotrienols induced apoptotic cell death in human breast cancer cells [35].

As γT3 treatment induced mainly apoptosis in the K562 cells, we analysed the expression of genes related to human apoptosis using a commercial qPCR array kit that contained primers of 84 genes related to human apoptosis. Apoptosis can mainly occur via two main pathways; the death receptor pathway (extrinsic apoptosis) and the mitochondrial pathway (intrinsic apoptosis). However, other mechanisms including the endoplasmic reticulum (ER)-mediated apoptosis have also been reported. Gamma-tocotrienol has been reported to play a role in inducing apoptosis via all three pathways [28,36].

Gamma-tocotrienol up-regulated several members of the BCL-2 family that are pro-apoptosis such as BAX, BAD, BCL2L1 and BNIP3L genes (Figure 8), which regulate the intrinsic pathway of apoptosis [37]. The BAX gene induced in K562 cells treated with γT3 was also found to be highly expressed in the HT-29 human colon carcinoma cells [18] and Hep3B human hepatoma cells [38]. The high ratio of BAX to BCL-2 proteins plays an important role in the intrinsic apoptosis pathway as an excess of BAX to BCL-2 is reported to favour apoptotic death rather than survival [39].

The expression of several members of the TNFR superfamily such as CD40, TNFRSF10B, TNFRSF1A and TNFRSF9 genes involved in the extrinsic pathway of apoptosis were also induced in K562 cells following exposure to γT3 (Figure 8). A study investigating the radio-protective effects of γT3 using murine gastrointestinal cells reported down-regulation of several pro-apoptotic genes compared to pro-survival. In these cells, exposure to γT3 down-regulated the expression of the CD40 gene whilst other pro-apoptotic genes that belong to the TNFRSF such as TNFRSF10B and TNFRSF1A were over-expressed [15]. The expression of TNFRSF10B gene, which encodes the death receptor 5 (DR5) was induced in several cells following exposure to γT3 [29]. The upregulation of TNFRSF10B gene by γT3 was reportedly dependent on the expression of extracellular-signal-regulated kinase 1 (ERK 1) as well as TP53 and BAX genes [29].

Gamma-tocotrienol induced the expression of four caspase family members [CASP4, CASP7, CASP8 and CASP9] in the K562 cells (Figure 8). The CASP4 gene encodes Caspase 4, a pro-inflammatory enzyme, which functions in endoplasmic reticulum (ER) stress-induced apoptosis [40]. The ER stress-induced apoptosis is triggered when there is extensive damage to the ER caused by protein aggregation of unfolded protein [41]. Caspase 12 (CASP12) was reportedly the key mediator of ER stress-induced apoptosis in mice; however a functional CASP12 protein has not been reported in humans [41]. In humans, several studies have reported that CASP4 show similar properties to mouse CASP12 and contributes to the
execution phase of ER stress-induced apoptosis in the absence of CASP12 [40, 42]. Caspase 4 can also directly activate the initiator caspase, CASP9 in SH-SY5Y human neuroblastoma cells, by cleaving pro-caspase 9 at the aspartic acid ASP-315 residue [40]. Caspase 9 together with CASP8 is initiator caspases reported to play an important role in γT3 treated cells [28]. Inhibition of these caspases was found to be sufficient to suppress γT3-induced apoptosis in human T-cell lymphoma, JURKAT cells [28]. Similarly, activation of the CASP9 and CASP8 was observed in human hematopoietic Hesp3B cells treated with γT3 [38] and in two human breast cancer cell lines, MDA-MB-231 and MCF-7 [43]. In the MCF-7 cells line, γT3-treatment also induced the expression of the effector caspase, CASP7 as evidenced by the detected cleaved (activated)-form of CASP7 [36].

In the present study, we also found treatment with γT3 induced the expression of the NOD1 gene in the K562 cells. The NOD1 gene [25] along with CASP9 [44], APAF-I [45], NOL3 [46] and PYCARD [47] belong to the CARD family, where each member contains a long amino (NH₂) domain. True to their namesake, CARD members play a role in recruiting caspases to their upstream apoptosis signalling complex [48]. An example of CARD-CARD interaction would be APAF1’s interaction with CASP9 during intrinsic apoptosis [41]. The NOD1 gene is also a CARD member, which is highly similar to APAF1, and participates in CASP9 activation [25].

Treatment of K562 cells with γT3 also up-regulated the expression of the pro-survival genes such as BCL2L1, XIAP and IGFIR. The pro-survival BCL2L1 gene was reported to suppress expression of the BAX and TP53 genes [49]. The X-linked inhibitor of apoptosis protein (XIAP) gene, which belongs to the inhibitor of apoptosis (IAP) family inhibits apoptosis by forming a heterodimer with Caspase 9 monomers [50], thus preventing their activation. This inhibitory effect can be reversed by the second mitochondria-derived activator of caspases (Smac/DIABLO), which is induced by the BAX protein [51].

The expression of insulin-like growth factor-1 receptor (IGFIR) gene was induced in the K562 treated with γT3 cells. This gene encodes the IGFIR tyrosine kinase protein, which is responsible for the growth and survival of several types of cancers via the activation of pathways such as the PI3K-Akt pathway and the ras-rasaf-MEK-ERK pathway [52]. The relationship between IGFIR and TP53 may reduce IGFIR’s pro-survival activity as wild-type TP53 was reported to inhibit IGFIR by suppressing the promoter of the IGFIR gene [53]. TP73, which is coincidentally expressed following γT3-treatment in K562 cells, was also reported to inhibit IGFIR promoter activity and decrease IGFIR levels in human colon cancer cells, regardless of the TP53 status [54].

Results from the human apoptosis PCR array suggest that γT3 induced apoptosis in K562 CML cells is through the regulation of genes involved in the intrinsic and extrinsic pathway of apoptosis. This is because exposure to γT3 induced the over-expression of various genes involved in the intrinsic [APAF1, CASP9, BAX, BCL2L1 and BAD] and extrinsic apoptosis pathway [FASLG, CASP8, TNFRSF1A and TNFRSF10B] in the K562 cells [20]. There is also evidence to suggest that γT3 may induce apoptosis by an alternative apoptotic pathway such as the ER stress-induced pathway due to its role in up-regulating the CASP4 gene. However, further assays are warranted before this can be confirmed by investigating the expression of some of the upstream genes involved in ER-stress induced pathway.

The real time PCR carried out for the four selected genes revealed that CASP9, CD40, NOD1 and TNFRSF10B genes were highly expressed following 72 hours treatment with γT3 indicating a time-dependent increase in gene expression. Their corresponding proteins were also highly expressed at 72 hours after treatment with the γT3-treated K562 cells when compared to control, which confirms their participation in γT3-mediated apoptosis in the K562 cells. CASP9 activation signals the beginning of the execution phase of intrinsic apoptosis as it can trigger the activation of CASP3 and the subsequent caspase cascade [23]. The activation of CASP9 involves interaction with the apoptosis complex, which comprises APAF1 and CASP9 in the presence of deoxyadenosine triphosphate (dATP) and cytochrome C. Although NOD1 is able to activate CASP9 by CARD interaction; the expression of CASP9 was essential for NOD1-mediated apoptosis [25].

The upregulation of the CD40 protein in the γT3-treated K562 cells suggest involvement of the extrinsic apoptosis pathway. Activation of CD40 in carcinoma cells by its ligand actively triggered FAS, TNF-related apoptosis-inducing ligand (TRAIL) and induced the expression of FAS [24, 55]. Furthermore, CD40 is capable of activating the initiator caspase, CASP8 [24] and inducing the executioner caspase, CASP7 [52]. The CD40 protein also reportedly induced expression of TNFRSF10B, which was also up-regulated by γT3 [55]. In a study by Kannappan and colleagues [29], γT3 augmented TRAIL-induced apoptosis by causing the overexpression of two death receptors, DR4 and TNFRSF10B (DR5) [36]. The induction of the TNFRSF10B protein was observed in several cell lines such as A293, KBM-5, U266, Panc-1, and MiaPaca-2 cells [29]. TNFRSF10B executes apoptosis via the interaction with CASP8 during extrinsic apoptosis during the formation of the death-inducing signalling complex (DISC) which results in the activation of CASP8 [56].

**Conclusion**

Gamma-tocotrienol is an effective cytotoxic and anti-proliferative agent towards the human CML cell line, K562. It also induced a strong apoptotic response in the K562 cells. The results from this study strongly suggest that γT3 induce apoptosis in the K562 cells through regulating members of the intrinsic and extrinsic apoptosis pathway. This study further supports the potential beneficial properties of tocotrienols, which needs to be further supported with future studies experimental or clinical models.

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**References**


*Corresponding author: Ammu K. Radhakrishnan, Pathology Division, School of Medicine, International Medical University, Bukit Jalil, 57000 Kuala Lumpur, Malaysia, Tel: 6032-7317-205; Fax: 6038-6567-229; E-mail: ammu_radhakrishnan@imu.edu.my

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