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GAMMA-TOCOTRIENOL DOES NOT COMPETE WITH MITOXANTRONE TO BE EFFLUXED FROM ABCG2 OVEREXPRESSING CELLS

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ABSTRACT

The human adenosine triphosphate (ATP)-binding cassette (ABC) subfamily G2 (ABCG2) half transporter of the G-subfamily that is involved in transportation of an extensive range of substrates, including xenobiotics and endogenous compounds. ABCG2 transporter upregulation in many cancerous tissues is often linked to multiple drug resistance (MDR) due to its involvement in efflux of various drugs. Gamma-tocotrienol (γT3) is an isoform of vitamin E that possesses promising anti-cancer effects via various mechanisms, however, transportation of γT3 remains unknown and its therapeutic effects might be limited by MDR. This project, thus, sought to study the anti-cancer effect (i.e. *anti-proliferative effect) of γT3 in an ABCG2-expressing breast cancer cell line [Michigan Cancer Foundation-7-mitoxantrone (MCF7-MX)] and the possibility of γT3 to be transported via ABCG2 transporter. It was demonstrated that after 72 hr treatment with γT3, cell proliferation of MCF7-MX cells was inhibited with* IC_{50}*=43 μM. The co-administration of γT3 with MX, a substrate of ABCG2, has shown that γT3 is not a competitor for MX transport (p>0.05). The data confirms the anti-proliferative role of γT3 in ABCG2 expressing cells and suggests that ABCG2 might have a minimal role in γT3 transport. This result provides an essential basis for the further study of γT3 as an anti-cancer compound.*

Keywords: gamma tocotrienol, ABCG2 transporter, multidrug resistance, mitoxantrone, vitamin E.

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INTRODUCTION

Gamma tocotrienol (γT3) is one of the eight natural forms of vitamin E that can be found abundantly in palm oil and rice bran oil (Aggarwal *et al.,* 2010; Ahsan *et al.,* 2014). The γT3 could be beneficial against various cancers via numerous mechanisms namely anti-proliferative, apoptosis induction, immunomodulatory function aside its well-known antioxidant activity (Ahsan *et al.,* 2014). It is notable that γT3 has multiple molecular targets that are involved in transcription, translation and post-translational protein levels and may interact directly with cellular targets to mediate its anti-cancer effects via various signalling pathways (Aggarwal *et al.,* 2010).

The effectiveness of γT3 as an anti-cancer agent will be highly dependent on its availability for cancer cells. However, cancer cells often overexpress multiple drug resistance (MDR) transporters, such as ABCG2 [adenosine triphosphate (ATP)-binding cassette (ABC) subfamily G2]. ABCG2, also known as breast cancer resistance protein (BRCP), is a 655-amino acid polypeptide chain with a molecular weight of 70 kDa (Doyle *et al.,* 1998). ABCG2 is expressed naturally in various tissues and organs to protect the body from toxicity associated with xenobiotic exposure; however, when expressed on the surface of cancer cells, this protective mechanism often leads to MDR, thus leads to chemotherapy failure due its efflux activity (Nakanishi and Ross, 2012; Mo and Zhang, 2012; Stacy *et al.,* 2013; Horsey

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et al., 2016). For example, ABCG2 has been found to be frequently expressed in hematopoietic malignancies including acute myelogenous leukaemia (AML) and acute lymphoblastic leukaemia (ALL) which lead to poorer disease-free survival rate and lower response rate (Benderra *et al.*, 2004; Uggla *et al.*, 2005; Stacy *et al.*, 2013).

ABCG2 has been shown to transport a broad range of substrates such as chemotherapeutic drugs, hydrophobic or lipophilic substances (Mo and Zhang, 2012). As ABCG2 also transports sulphate and glucuronide conjugates (Suzuki *et al.*, 2003; Imai *et al.*, 2003), it is possible that ABCG2 is involved in excretion of vitamin E since it was proposed that vitamin E metabolite, carboxy-ethyl-hydroxychromanols (CEHC) needs to be sulphonated or glucuronidated to be excreted via transporters (Pope *et al.*, 2000; Kiyose *et al.*, 2001).

To our knowledge, there is no reported study on tocotrienol (T3) as substrates of ABCG2. As ABCG2 substrates overlap to some extent with ABC subfamily B member 1 (ABCB1) P-glycoprotein 1 (P-gp), studies done on the interaction between ABCB1 and other vitamin E isoforms may well serve as a guideline. Excess γT3 has been shown to upregulate activity and expression of ABCB1 messenger ribonucleic acid (mRNA) and protein (Zhou *et al.*, 2004; Abuznait *et al.*, 2011). Zhou *et al. (2004)* showed that all T3 (α-, β-, γ-, δ-) activate the nuclear receptor, pregnane X receptor (PXR) that mediates expression of various genes, including ABCG2, to regulate xenobiotic detoxification and drug clearance in the liver (Landes *et al.*, 2003; Zhou *et al.*, 2004). Collectively, this suggests that T3 is potentially involved in the upregulation of ABCG2 for efflux purposes to prevent toxicity.

Whilst γT3 has the potential to be utilised as an anti-cancer agent, it is important to study its interaction with ABCG2 to identify any drug-drug interaction and to maximise the therapeutic value of γT3, which often has low bioavailability. This study thus aims to investigate the potential involvement of ABCG2 in the cellular transport of γT3.

MATERIALS AND METHODS

Cell Culture and Reagents

All tissue culture reagents were from Sigma-Aldrich, St Louis, Missouri, USA unless stated otherwise. The γT3 was a gift from Davos Life Science (Singapore) which was a pure compound extracted from palm oil and dissolved in ethanol. The breast cancer cell line MCF7 (ATCC) and MCF7-MX (ATCC) cells that were developed previously (Nakagawa *et al.*, 1992) were grown in Dulbecco's Modified Eagle Medium (DMEM, + 4.5 g litre^{-1} D-glucose, 0.11 g litre^{-1} sodium pyruvate and 0.56 g litre^{-1} L-glutamine) supplemented with 10% (v/v) fetal calf serum (FCS) and 100 units/ml penicillin and 100 μg ml^{-1} streptomycin at 37°C, 5% carbon dioxide (CO_2). Cells were passaged at 80% confluence by trypsinisation. The detached cells were removed from the flask in 5 ml medium and centrifuged at 1300 g for 5 min. The pellets were re-suspended in medium and passaged in 1:5 ratio into new flasks. Where necessary, cell density was determined using a haemocytometer.

Cell Proliferation Assay

The cells were seeded at cell density of 1.2x10^4 cells/well in 100 μl medium and left to attach for 24 hr at 37°C, 5% CO_2. Then, media were changed to fresh media containing 1% FCS in the presence of 0-100 μM γT3 followed by 72 hr incubation in dark, at 37°C, 5% CO_2. Prior to cell treatment, the γT3 stock solution was diluted with equal amount of 100% FCS and incubated in dark for 16 hr, followed by serial dilution with 1:1 mixture of 100% ethanol and 1% FCS media, the final concentration of ethanol in the cell culture was <0.05%. The cell proliferation was then determined with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Fitchburg, Wisconsin, USA) which uses the colorimetric reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulphophenyl)-2*H*-tetrazolium (MTS) and the electron coupling agent phenazine methosulphate (PMS) according to the manufacturer's instruction. Briefly, 20 μl of CellTiter 96® reagent was added to each well and incubated at 37°C in 5% CO_2 for 2 hr. The colour intensity, which is proportional to the number of metabolically active cells, was determined at 450 nm with Multiskan FC microplate photometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Protein Detection Using SDS-PAGE and Western Blot

Cells were harvested as described (Haider *et al.*, 2011). The protein concentrations were determined using a modified Lowry assay method (Lowry *et al.*, 1951) using reagents from detergent compatible (DC) protein assay kit (Bio-Rad, Hercules, California, USA) according to manufacturer's instruction with bovine serum albumin (BSA) as a standard. The cell lysates (10 μg of protein) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%). Following transfer on nitrocellulose membrane, the membrane was blocked using 5% non-fat milk in PBST (PBS with 0.1% (v/v) Tween 20, (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 hr at room temperature. ABCG2 was then bound using primary monoclonal mouse antibody BXP-21 (1:1000 dilution) at 4°C, overnight, followed by secondary antibody, polyclonal rabbit

anti-mouse IgG antibody conjugated with horse radish peroxidase (HRP) (1:2000) for 1 hr at room temperature. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

MX Accumulation Assay

Both MCF7 and MCF7-MX cells were seeded as described previously (Haider *et al.*, 2015). The plates were incubated overnight at 37°C, 5% CO_2 before the media was replaced in low light conditions with phenol red free media containing 8 μM mitoxantrone alone or with 8 μM mitoxantrone combined with 500 nM Ko143 or 8 μM mitoxantrone with various concentration of γT3 (3.13-100 μM) for an hour at 37°C, 5% CO_2. Following incubation, the cells were fixed as described previously (Haider *et al.*, 2015). The accumulation of MX in the cells were measured using SpectraMax M2 (Molecular Device, San Jose, California, USA) at excitation wavelength (λ_{Ex}) of 607 nm and emission wavelength (λ_{Em}) of 684 nm. Fluorescence data were corrected for values obtained from incubation with the maximal final solvent concentration used [1% v/v dimethyl sulphoxide (DMSO)].

Cell Fixation and Mounting for Fluorescence Imaging

Prior to cell seeding, the coverslips were sterilised in 70% v/v ethanol and coated with poly-L-lysine as described previously (Haider *et al.*, 2015) in 12-well plates. Both MCF7 and MCF7-MX cells were seeded in a total volume of 1 ml DMEM media and incubated overnight at 37°C, 5% CO_2. The cells were then fixed with 4% paraformaldehyde (PFA) as described above. Cells were incubated with an exogenous amine-containing reagent, 50 mM NH_4Cl in PBS for 15 min followed by incubation in blocking buffer (0.5% (w/v) BSA in PBS) for another 15 min. Coverslips were incubated in primary mouse monoclonal anti-ABCG2 antibody, clone 5D3 (Millipore, Burlington, Massachusetts, USA), diluted at 1:200 in blocking buffer for 30 min. The coverslips were washed four times with blocking buffer and then with secondary Alexa-Fluor 647 goat anti-mouse monoclonal antibody (Life Technologies, Carlsbad, California, USA) diluted at 1:1000 in blocking buffer for another 30 min. The cells were washed several times with blocking buffer and mounted onto microscope slides using FluoroGel (GeneTex, Irvine, California, USA). The slides were viewed using LSM710 confocal laser scanning microscope from Carl Zeiss Microscopy (Zeiss, Oberkochen, Germany). A 1024x1024 image at a 12 bit depth was captured with 647 nm laser at 2% using water-immersion objective C-Apochromat 40x NA 1.20'.

Statistical Analysis

All results presented in the article are expressed as mean and standard deviation (±SD) from three experiments (n=3, each with six internalised repeats). Comparisons between groups were made using one-way analysis of variance (ANOVA) with Dunnett test or t-test as mentioned. The normality of distribution was tested using Shapiro-Wilk normality test. For cytotoxicity assay, the IC_{50} values were calculated using classical dose-response equation: Y=bottom + (top-bottom)/[1+10^(LogIC_{50}-X)*hillslope].

All statistical analyses were performed using GraphPad Prism 7.02, California, USA. A p value of < 0.05 was considered statistically significant.

RESULTS

For this study, a pair of cell lines with negative and positive ABCG2 expression, namely MCF7 and MCF7-MX cells were used. A range of experiments were carried out to analyse the expression of ABCG2 in the target cell lines, the effect of γT3 on cell survival and on the ABCG2-mediated transport of MX.

The expression of ABCG2 at protein level was ascertained by immunoblotting with an ABCG2-specific primary monoclonal antibody, BXP-21 (*Figure 1*). The western blot analysis exhibited a single immuno-reactive band at approximately 72 kDa, corresponding to the expected molecular mass of ABCG2 in all lanes loaded with MCF7-MX cell lysates. However, no band was observed in lanes loaded with MCF7 cell lysates, even when western blots were exposed to photographic film for extended periods of time. This finding indicated that ABCG2 protein was expressed in MCF7-MX cells but was undetectable in MCF7 cells.

Immunolocalisation of ABCG2 protein in cells was performed on fixed MCF7 and MCF7-MX cells using 5D3 antibody that recognises an extracellular epitope of ABCG2 (Özvegy-Laczka *et al.*, 2005) followed by secondary antibody conjugated to Alexa-Fluor 647. This enables visualisation of protein on the surface of intact cells. The immunofluorescence data as analysed by confocal microscope revealed that ABCG2 was localised on cell surface of MCF7-MX cells (*Figure 2b*) but not on MCF7 cells (*Figure 2a*).

These showed that ABCG2 was expressed and localised on plasma membrane of MCF7-MX cells but was not expressed or expressed below detectable levels in the parental MCF7 cells.

The cytotoxicity of γT3 on both cell lines can be evaluated by determination of the IC_{50} values, which correspond to the concentration of γT3 that are able to reduce 50% of cell viability. After 72 hr treatment with γT3, the log IC_{50} values were -4.66 ±

0.08 and -4.36 ± 0.04 for MCF7 and MCF7-MX cells respectively. This corresponds to Mcf7-MX cells having an approximately two-fold resistance to γT3 (43 μM) compared to parental Mcf7 cells (22 μM). Unpaired t-test analysis indicated that there was a significant difference ($p < 0.01$) in the cytotoxicity of the cell lines to γT3 during 72 hr incubation.

Such differences in cytotoxicity prompted us to consider whether γT3 was a transport substrate of ABCG2 and so we investigated the potency of γT3 to compete with the ABCG2 mediated transport of a well characterised substrate MX. *Figure 3a* confirmed that significant difference ($p<0.001$) in intracellular MX accumulation between the negative control MCF7 cells and MCF7-MX cells with overexpression of ABCG2, in which the latter had lower intracellular MX accumulation. This indicated that ABCG2 was involved in the efflux of MX from the cells. In addition, the administration of the ABCG2 inhibitor, Ko143 resulted in a significant increase in accumulation of MX by seven-fold (7.17 ± 2.3, $p<0.0001$) (*Figure 3c,* second column of data). In contrast, the negative control MCF7 cells showed no significant difference in MX accumulation in the presence and absence of ABCG2 inhibitor, Ko143 (*Figure 3b*). Any compound which inhibits MX transport (either by non-competitive inhibition, *e.g.* by inhibiting the transport mechanism of ABCG2, or by competing with MX for transport) will result in increased accumulation of MX.

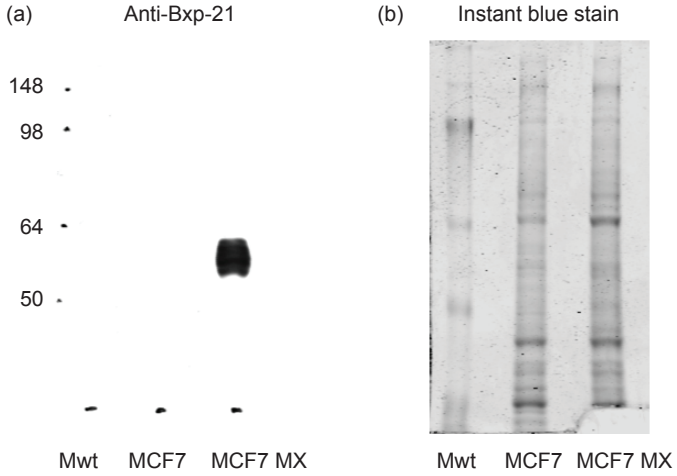
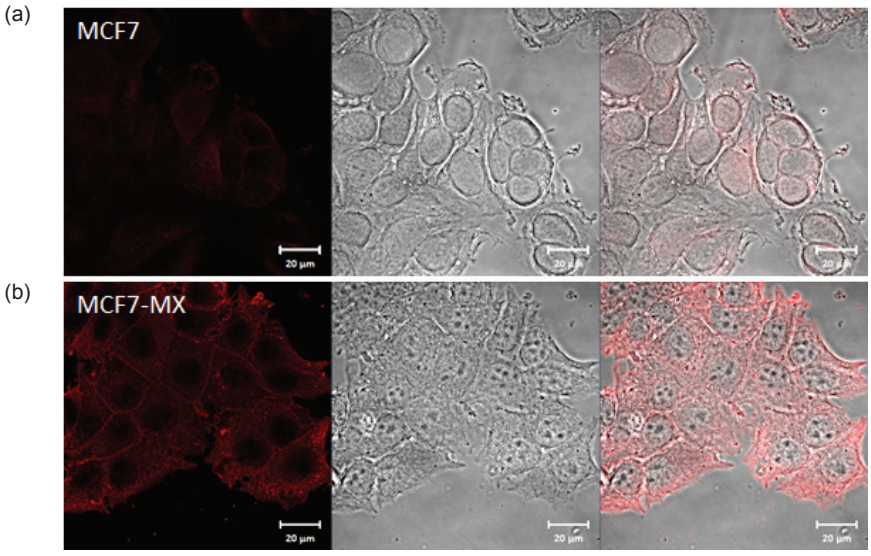


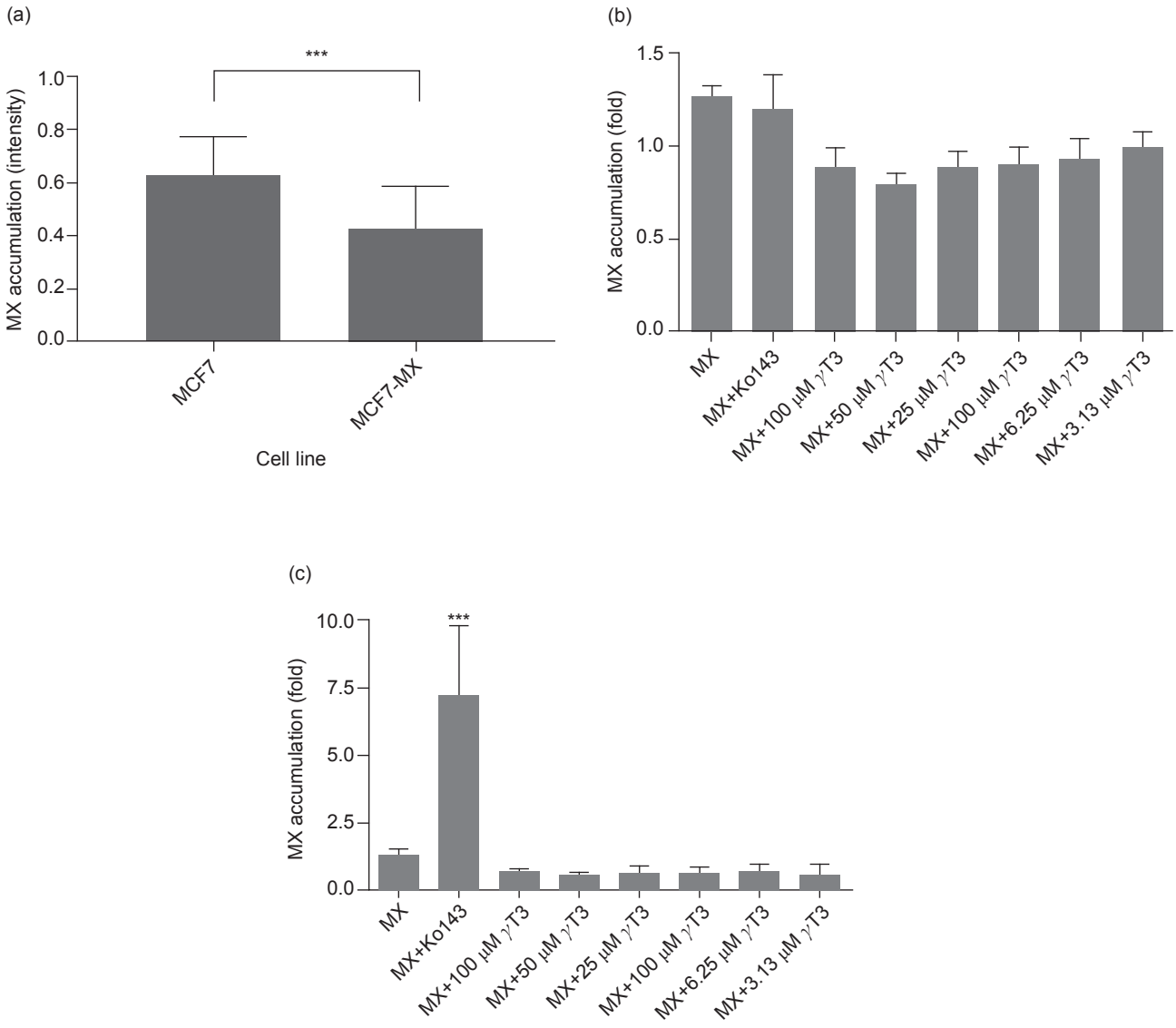
Figure 1. (a) Western blot analysis of ABCG2 [adenosine triphosphate (ATP)-binding cassette (ABC) subfamily G2] expression, and (b) total protein expression in MCF7-MX [Michigan Cancer Foundation-7-mitoxantrone] and MCF7 cells. ABCG2 with molecular weight of approximately 72 kDa was detected only in lanes loaded with MCF7-MX cell lysates but not in lanes loaded with MCF7 cell lysates as shown in (a) band smearing is due to glycosylation of ABCG2 and (b) showed confirmed equal protein loading of cell lysates.



Note: Scale bar=20 μm.

Figure 2. Confocal images of ABCG2 [adenosine triphosphate (ATP)-binding cassette (ABC) subfamily G2] expression. (a) MCF7 (Michigan Cancer Foundation-7), and (b) MCF7-MX (mitoxantrone) cells.

To validate the possibility of γT3 as a substrate of ABCG2, γT3 was administered at various concentrations with MX and the accumulation of MX in the cells was measured. The results presented in *Figure 3* as mean ± standard deviation of three independent experiments, each with six internalised repeats. All data were normalised against DMSO only control. In both the parental MCF7 cells (*Figure 3b*) and the ABCG2-expressing MCF7-MX cells (*Figure 3c*), no significant difference in MX accumulation was observed with the addition of γT3 to MX despites at its highest concentration (100 μM) ($p>0.05$). All the data obtained passed the Shapiro-Wilk normality test (alpha=0.05).



Note: *** $p<0.001$, t-test. **** $p<0.0001$ compared to control (MX alone) as indicated by one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test.

Figure 3. Gamma-tocotrienol does not inhibit mitoxantrone (MX) transport in both MCF7 (Michigan Cancer Foundation-7) and MCF7-MX cells. (a) MCF7 cells showed significant difference in the MX accumulation compared to MCF7-(MX) cells in normal condition with MCF7-MX had a lower MX accumulation. (b) MCF7 cells showed no significant difference in MX accumulation with the addition of inhibitor, Ko143 or with combination of γT3. (c) MCF7-MX cells showed a significant increase in MX accumulation by approximately seven-fold (7.17 ± 2.3) with the presence of ABCG2 [adenosine triphosphate (ATP)-binding cassette (ABC) subfamily G2] inhibitor, Ko143. However, no significant difference was shown between cells treated with MX alone and cells treated with combination of MX and γT3 at various concentrations in both cell lines. Data presented as mean ± SD, n=3 (each with six internalised repeats).

DISCUSSION

Overexpression of ABCG2 in various cancer cell lines often results in MDR and leads to the failure of chemotherapy due to the efflux activity of the transporter (Scharenberg *et al.*, 2002; Islam *et al.*, 2005; Mo and Zhang, 2012). As γT3 has been shown to display potent anti-cancer activity via various mechanisms (Shah *et al.*, 2003; Sylvester *et al.*, 2005; Kannappan *et al.*, 2010), it was of interest to study the possibility of γT3 being a transport substrate of ABCG2, which might limit its therapeutic value.

Studies confirmed that ABCG2 acts as an efflux pump to reduce intracellular drug accumulation (Ross *et al.*, 1999; Miyake *et al.*, 1999). Thus, it was of interest to determine whether ABCG2 localisation to the plasma membrane in the MX-resistant MCF7-MX cells corresponds to its function as drug efflux pump. Similar to previous studies (Ross *et al.*, 1999; Austin Doyle and Ross, 2003), the ABCG2 is overexpressed in MCF7-MX cells and localised on plasma membrane (*Figures 1* and *2b*). Minimal fluorescent was detected intracellularly in MCF7-MX cells (*Figure 2b*), this might due to the endocytosis of ABCG2 upon 5D3 binding, where the internalised ABCG2 will be partially degraded or restored to cell surface (Studzian *et al.*, 2015).

In this study, γT3 inhibited cell viability of both MCF7 and MCF7-MX cells after 72 hr treatment, in a concentration dependent manner (*Figure 4*). The IC_{50} value of γT3 were 22 μM on MCF7. The IC_{50} value obtained is quite close to the previous study reported that 10 μM of γT3 results in a significant inhibition of cell proliferation of same cell lines (Hsieh *et al.*, 2010). However, another study reported the IC_{50} value of γT3 as 7.2 μM (Ramdas *et al.*, 2011) in MCF7 cells which is three times lower compared to the results obtained. The differences observed could be due to differences in cell density (Riss *et al.*, 2013) or incubation period as the inhibitory effect of γT3 is also time dependent (Patacsil *et al.*, 2012). To the best of our knowledge, to date there are no studies on the cytotoxicity of γT3 on MCF7-MX cells. Here, γT3 has an IC_{50} value of 43 μM in MCF7-MX cells, two-fold greater than in parental MCF7 cells, which raised the possibility that ABCG2 mediated γT3 efflux.

The addition of Ko143, a promising ABCG2 inhibitor completely reversed ABCG2-mediated export of MX thus leads to increase in accumulation of MX by approximately seven-fold (7.17 ± 2.3) in MCF7-MX cells (*Figure 3c*). This result is in agreement to previous findings showing increased ABCG2 substrates accumulation upon Ko143 administration (Özvegy-Laczka *et al.*, 2005; Wu *et al.*, 2016). The results also demonstrated that ABCG2 overexpressed in MCF7-MX cells are functionally active. As ABCG2 is undetectable in MCF7 cells, administration of Ko143 has no effect on MX accumulation level (*Figure 3b*). The possibility of γT3 to be transported via ABCG2 was then tested with co-administration of known ABCG2 substrate, MX. As no significant difference is observed in MX accumulation in MCF7-MX cells, the data (*Figure 3c*) suggested that γT3 does not compete with MX to be transported via ABCG2 and has no effect in ABCG2-mediated γT3 transportation.

ABCG2 has also been proposed to be involved in cell proliferation as various studies suggested that inhibition of ABCG2 decreased cell proliferation and induced G0/G1 phase arrest in ABCG2 expressing cells (Chen *et al.*, 2010; Liu *et al.*, 2014; Zhang *et al.*, 2016), which also would provide a possible explanation for the difference in IC_{50} values during a long-term incubation period.

The γT3 is always co-administered with other compounds, such as statins (Wali and Sylvester, 2007; Wali *et al.*, 2009; Sylvester, 2012) and celecoxib (Shirode and Sylvester, 2010; Shirode and Sylvester, 2011) resulting in synergistic anti-proliferative effects on mammary tumour cell growth. As anti-cancer drugs are often substrates of the ABC transporters that are found to be overexpressed in various cancer cell lines, understanding the interaction between γT3 and ABC transporters is essential. Previous studies reported that γT3 upregulated ABCB1 expression in human intestinal epithelial cell line (LS180) and this has raised the concern on potential effects of food-drug interaction especially during drug treatment (Zhou *et al.*, 2004; Abuznait *et al.*, 2011). Study also showed that γT3 has inhibitory effects on HMG-CoA reductase and reversed the MDR in MCF7/Adr cells. Unlike atorvastatin, the effect of γT3 inhibition was not solely mediated by mevalonate pathway. In fact, γT3 inhibited the ABCB1 expression, increased the accumulation of doxorubicin in cells, led to enhanced G2/M arrest and cell apoptosis (Ding *et al.*, 2017). Collectively, these studies showed that γT3 affects ABCB1 expression and are able to reverse the MDR effect to ABCG2. However, in our study, unlike in ABCB1 transporter, γT3 did not reverse the MDR effect of ABCG2 as the MX level showed no significant difference when co-administered with various concentrations of γT3. This showed that γT3 functions differently in ABCB1 and ABCG2 transporter although both transporters are from the same family.

In summary, our results indicate that γT3 is not competing with MX to be transported via ABCG2. However, the assay in this study is limited to determine compounds that are competitive with MX for ABCG2, thus ABCG2 might still have a potential role in the transportation of γT3. As γT3 was also found to reduce cell proliferation in ABCG2 expressing cancer cells, therefore more studies need to be done in the future to investigate the interaction of γT3 with ABCG2 protein to overcome its low bioavailability and to increase the utilisation of this biological compound in cancer therapy.

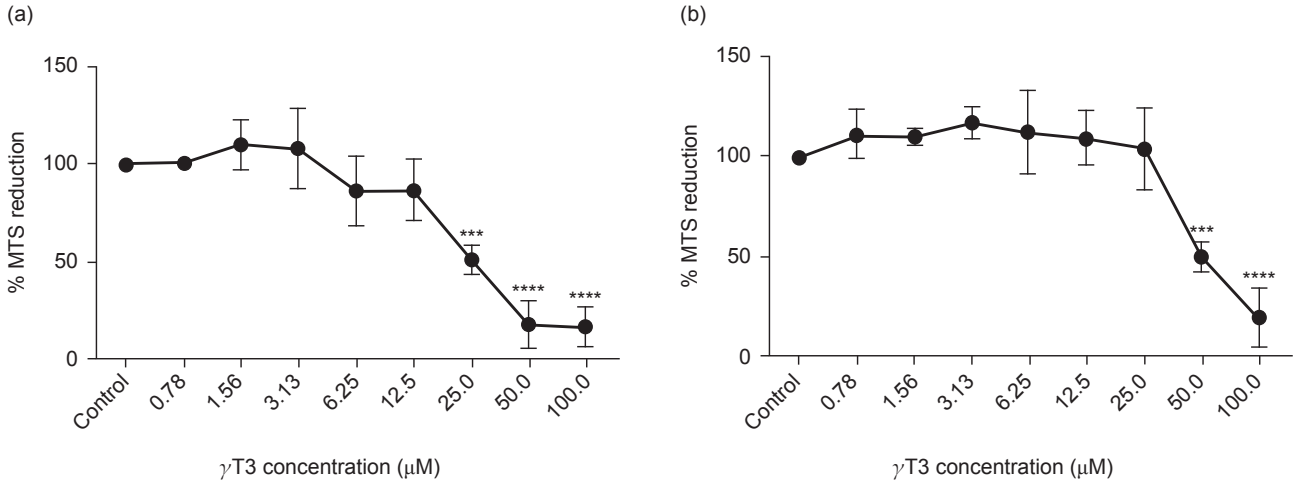


*Figure 4. Cytotoxic effect of γ-tocotrienol (γT3) on (a) MCF7 (Michigan Cancer Foundation-7) cells and (b) MCF7-MX (mitoxantrone) cells as assessed by MTS assay. The γT3 was shown to be toxic to MCF7 and MCF7-MX cells at ≥ 25 μM and ≥ 50 μM, respectively. The cell viability was reduced by 50% at 25 μM (50.73% ± 7) and 50 μM (49.35% ± 6.8), respectively for MCF7 and MCF7-MX cells. Non-linear regression was not shown due to the level of uncertainty of fitting showed. Data presented as mean ± SD, n=3 (each with six internalised repeats *** p<0.001, **** p<0.0001 compared to control as indicated by one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test.*

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