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Enhancement of apoptotic activities on brain cancer cells *via* the combination of γ-tocotrienol and jerantinine A

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ABSTRACT

Background: γ-Tocotrienol, a vitamin E isomer possesses pronounced *in vitro* anticancer activities. However, the *in vivo* potency has been limited by hardly achievable therapeutic levels owing to inefficient high-dose oral delivery which leads to subsequent metabolic degradation. Jerantinine A, an *Aspidosperma* alkaloid, originally isolated from *Tabernaemontana corymbosa*, has proved to possess interesting anticancer activities. However, jerantinine A also induces toxicity to non-cancerous cells.

Purpose: We adopted a combinatorial approach with the joint application of γ-tocotrienol and jerantinine A at lower concentrations in order to minimize toxicity towards non-cancerous cells while improving the potency on brain cancer cells.

Methods: The antiproliferative potency of individual γ-tocotrienol and jerantinine A as well as combined in low-concentration was firstly evaluated on U87MG cancer and MRC5 normal cells. Morphological changes, DNA damage patterns, cell cycle arrests and the effects of individual and combined low-concentration compounds on microtubules were then investigated. Finally, the potential roles of caspase enzymes and apoptosis-related proteins in mediating the apoptotic mechanisms were investigated using apoptosis antibody array, ELISA and Western blotting analysis.

Results: Combinatorial study between γ-tocotrienol at a concentration range (0–24 μg/ml) and fixed IC_{20} concentration of jerantinine A (0.16 μg/ml) induced a potent antiproliferative effect on U87MG cells and led to a reduction on the new half maximal inhibitory concentration of γ-tocotrienol (*i.e.* ${}_1IC_{50} = 1.29$ μg/ml) as compared to that of individual γ-tocotrienol (*i.e.* $IC_{50} = 3.17$ μg/ml). A reduction on undesirable toxicity to MRC5 normal cells was also observed. G0/G1 cell cycle arrest was evident on U87MG cells receiving IC_{50} of individual γ-tocotrienol and combined low-concentration compounds (1.29 μg/ml γ-tocotrienol + 0.16 μg/ml jerantinine A), whereas, a profound G2/M arrest was evident on cells treated with IC_{50} of individual jerantinine A. Additionally, individual jerantinine A and combined compounds (except individual γ-tocotrienol) caused a disruption of microtubule networks triggering Fas- and p53-induced apoptosis mediated *via* the death receptor and mitochondrial pathways.

Conclusions: These findings demonstrated that the combined use of lower concentrations of γ-tocotrienol and jerantinine A induced potent cytotoxic effects on U87MG cancer cells resulting in a reduction on the required individual concentrations and thereby minimizing toxicity of jerantinine A towards non-cancerous MRC5 cells as well as probably overcoming the high-dose limiting application of γ-tocotrienol. The multi-targeted mechanisms of action of the combination approach have shown a therapeutic potential against brain cancer *in vitro* and therefore, further *in vivo* investigations using a suitable animal model should be the way forward.

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Introduction

γ-Tocotrienol is a dietary phytochemical and member of a group of the vitamin E isomers that has demonstrated anticancer effects against cancer cell lines (Park et al., 2010; Sakai et al., 2006; Samant and Sylvester, 2006). However, low-level therapeutic doses have limited the potential of tocotrienols. This has been attributed to metabolic degradation that results from high doses of orally administrated tocotrienols. Consequently, studies have shown that tocotrienols should be used as an adjuvant treatment at low concentrations (Shirode and Sylvester, 2010). On the other hand, jerantinine A is an *Aspidosperma* alkaloid, isolated from the leaves of *Tabernaemontana corymbosa* Roxb ex Wall (Apocy-

Abbreviations: γ-T3, γ-tocotrienol; JA, jerantinine A; U87MG, human brain glioblastoma; MRC5, normal human lung fibroblast; IC_{50}, half maximal inhibitory concentration that induces 50% cell growth inhibition; DMSO, dimethyl sulfoxide; H&E, haematoxylin and eosin; PBS, phosphate-buffered saline; DRI, dose reduction index; CI, combination index; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; DSBs, DNA double strand breaks; SSBs, single strand breaks; Bcl-2, B-cell lymphoma 2; Bid, Bcl-2 homology (BH) 3 interacting domain; Bax, Bcl-2 associated X protein; TRAIL, tumor necrosis factor (TNF)-related apoptosis inducing ligand; XIAP, X-linked inhibitor of apoptosis protein.

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naceae) (Lim et al., 2008). Jerantinine A has been shown to inhibit vincristine-resistant human oral epidermoid (KB) cells (Abubakar and Loh, 2016; Lim et al., 2008). A recent study revealed the inhibition of tubulin polymerization and microtubule disruption as a mode of action for jerantinine A (Raja et al., 2014). However, additional studies are required to further establish the mechanism of action for jerantinine A. Anti-microtubule and tubulin polymerization drugs such as vinblastine and paclitaxel are clinically approved drugs used for the treatment of neoplastic diseases. However, drug resistance, toxicity and limitation of intravenous delivery which result in hypersensitivity continue to limit the potentials of this class of drugs (Tsai et al., 2014).

The use of combinations is an alternative for improving potency as well as overcoming drug resistance and non-selective toxicity of individual compounds. In fact, previous studies have shown that the therapeutic benefits of plants, fruits or vegetables result from the potentiation of their components' activities (Liu, 2004). In this study, we combined two phytochemicals, *i.e.* γ-tocotrienol and jerantinine A at their sub-inhibitory concentrations with the aim of improving the potency of both compounds and reducing the toxicity of jerantinine A to non-cancerous cells.

Materials and methods

Compounds, cell lines and culture conditions

γ-Tocotrienol isomer with $\geq$ 97% HPLC purity (See Supplementary material) was kindly supplied by Davos Life Sciences Pte Ltd, Singapore. Isolation of jerantinine A from the leaves of *T. corymbosa* has been reported in detail in a previous study (Lim et al., 2008), while the ^{1}H and ^{13}C NMR profiles of jerantinine A are provided as Supplementary material. On the other hand, human brain glioblastoma (U87MG) and normal human lung fibroblast (MRC5) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in cell culture conditions as previously described (Abubakar et al., 2014).

Cell viability study

U87MG and MRC5 cells were seeded at a density of 5×10^3 *per* well in 96-well plates (SPL Life Sciences, Geumgang-ro, South Korea) and incubated overnight to facilitate attachment. The medium was replaced with 200 µl of treatment medium containing either γ-tocotrienol, jerantinine A or these compounds combined at sub-inhibitory concentrations. Meanwhile, vinblastine at a concentration range of 0–8 µg/ml was used as a positive control and U87MG cells treated with plain medium containing an equivalent amount of dimethyl sulfoxide (DMSO) served as untreated control. After 72 h incubation, the cell viability was determined using the neutral red uptake assay protocol as previously described (Lim et al., 2014b) and reflected as 50% cell growth inhibition (IC_{50}) for each individual compound. For combination experiments, a recently described protocol was adopted (Abubakar et al., 2016). The fixed IC_{20} of jerantinine A (0.16 µg/ml) was combined with a concentration range of γ-tocotrienol (0–24 µg/ml) to determine the new potent concentrations of γ-tocotrienol ($_tIC_{50}$) required to induce 50% growth inhibition on U87MG and non-cancerous MRC5 cells. The $_tIC_{50}$ values were determined using the non-linear regression curve fit of the GraphPad PRISM 5 software and results were presented as mean ± SEM of triplicates in three independent experiments.

Determination of pharmacological interaction

The pharmacological interaction and reduction in potent concentrations between γ-tocotrienol and jerantinine A were determined using the combination index (CI) and dose reduction index (DRI) methods, respectively according to the previously described studies (Abubakar et al., 2016; Wali and Sylvester, 2007). Briefly, the CI was determined using the following formula to serve as a preliminary screening for a potential zero interaction, synergism, antagonism or additive effect between different combinations of chemical compounds:

$$CI = \gamma - Tx/\gamma T + JAx/JA$$

where, γ-T and JA are the respective individual IC_{50} concentrations of γ-tocotrienol (3.17 µg/ml) and jerantinine A (0.62 µg/ml). On the other hand, γ-Tx is the $_tIC_{50}$ concentration of γ-tocotrienol (1.29 µg/ml) that induced 50% cell growth inhibition when combined with IC_{20} (0.16 µg/ml) of jerantinine A (JAx). The CI value > 1, < 1 or $= 1$ indicates a potential antagonism, synergism or additive pharmacological interaction, respectively between chemical compounds used in a combinatorial experiment (Bachawal et al., 2010; Chou, 2006; Martinez-Irujo et al., 1996; Wali and Sylvester, 2007). The DRI represents the fold reduction for new concentrations of γ-tocotrienol and jerantinine A used in the combinatorial experiment that induced 50% cell growth inhibition as compared to the IC_{50} of individual γ-tocotrienol and jerantinine A. The dose reduction index values for γ-tocotrienol (DRIt) and jerantinine A (DRIja) were determined according to a previously described method (Wali and Sylvester, 2007):

$$DRIt = \gamma - T/\gamma - Tx$$

$$DRIja = JA / JAx$$

where, γ-T and JA represent the IC_{50} values of individual γ-tocotrienol (3.17 µg/ml) and jerantinine A (0.62 µg/ml), respectively. Whereas, γ-Tx and JAx represent the new IC_{50} values of γ-tocotrienol ($_tIC_{50} = 1.29$ µg/ml) and jerantinine A (0.16 µg/ml), respectively obtained from the combination experiment.

Histochemical staining

Guided by the data generated from cell viability study, the same individual IC_{50} and combined concentrations of γ-tocotrienol and jerantinine A were used in all subsequent assays. Histochemical staining experiment was performed using haematoxylin and eosin (H&E) (Sigma Aldrich, St. Louis, MO, USA) according to a previously described protocol (Abubakar et al., 2016). Briefly, U87MG cells were firstly seeded at a density of 5×10^3 *per* well in 2-well chamber slides (SPL Life Sciences, Geumgang-ro, South Korea) and then treated with IC_{50} of individual γ-tocotrienol (3.17 µg/ml) and jerantinine A (0.62 µg/ml) as well as combined concentrations of γ-tocotrienol (1.29 µg/ml) and jerantinine A (0.16 µg/ml) for 24 h. Untreated control for U87MG cells was also included. The H&E-stained cells were ready to be observed for the changes in morphology.

Single-cell gel electrophoresis (COMET) assay

U87MG cells were seeded at a density of 1×10^4 *per* well and treated separately with the above-mentioned concentrations of individual and combined compounds as well as DMSO serving as the untreated control for 72 h. Thereafter, the COMET assay kit, Oxiselect STA 351 (Cell Biolabs, San Diego, CA, USA) was used to determine the induction of DNA double strand breaks (DSBs) and single strand breaks (SSBs) under neutral and alkaline conditions, respectively according to an established protocol (Lim et al., 2011). DNA damage was quantified using the OPENCOMET software as previously described (Gyori et al., 2014) and results were presented as fold change in percent tail DNA compared to untreated controls from a population of 200 cells.

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