Synergistic effect of hyperforin and paclitaxel on growth inhibition, apoptotic mediator activation in MCF-7 human breast cancer cells

Muttiah Barathan, Ahmad Khusairy Zulpa, See Mee Hoong, Kumutha Malar Vellasamy and Jamuna Vadivelu

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

ABSTRACT

The role of hyperforin in combination anticancer therapy remain unknown. In this study, several doses of combination treatment (paclitaxel and hyperforin) were introduced to the Michigan Cancer Foundation (MCF)-7 cell culture for 24 h. The effectiveness of the drug’s combination effect was determined by the combination index (CI) followed by the anticancer effect through apoptosis, reactive oxygen species (ROS), lactate dehydrogenase (LDH) and caspase 8 assays. It was found that combined doses of paclitaxel (7, 6, 5, 4 µg/mL) and doses of hyperforin (1, 2, 3 and 4 µg/mL) enhanced synergistic growth inhibitory effect. (Hy: 4 µg/mL & Pa: 4 µg/mL) was selected as suitable dose with lowest CI and it significantly induced changes to MCF-7 cell morphology and led to apoptosis with increased in the high level of ROS, LDH and caspase 8 compared to singly treated hyperforin. This study provides the basis for elucidation of combination of hyperforin and paclitaxel on animal models.

1. Introduction

Female breast cancer is now the world’s most commonly diagnosed cancer after lung cancer [1]. According to GLOBOCAN, there were 2.26 million women diagnosed with breast cancer and 6,85,000 deaths globally. The numbers are predicted to increase up to 50% higher in 2040 than in 2020 [2]. However, breast cancer is very treatable if the disease is detected early and an appropriate combination of treatments is given to the patients. Commonly, the combination of localized and non-localized treatments such as radiation therapy, chemotherapy, targeted biological therapy, hormonal therapy and surgery are used to treat microscopic cancer from spreading to another part of the body through the blood and lymphatic vessels [3]. However, there are a few disadvantages in these therapies such as the development of resistant cancer cells towards certain types of treatments; hence this will lead to an increase in toxicity of chemotherapy drugs in the patient’s body and severe side effects [4].

Hence, medication based on natural products derived from microbes, animals and plants has long been considered to be an alternative cancer therapeutic agent purely due to an inexpensive, readily applicable, acceptable and accessible approach [5]. The phytochemicals derived from natural products are also known for their other properties such as antibacterial, antioxidant and anti-diabetic [6]. This is mainly due to the structural and biological variation of natural products [7]. Some of the clinically used natural anti-breast drugs are paclitaxel (taxol) and doxorubicin. Paclitaxel works as an antimicrotubule agent which inhibits breast cancer cells from replicating [8], and doxorubicin is an anthracycline antibiotic that induces severe damage to the DNA of breast cancer cells [9]. In addition, several in vivo and in vitro reports also indicate that natural products tend to work in a synergistic way which can improve overall treatment by doing a multi-target drug approach at low doses [10]. For an instance, a strong synergistic inhibition on the MCF-7 and MDA-MB-231 cells was displayed during the combination treatment of Strobilanthes Crispus leaf extract and tamoxifen [11]. At that same time, combination treatment also had reduced the IC50 dose of tamoxifen and induced signalling apoptosis through mitochondrial cell death pathway along with activation of caspase-8 and caspase-9 which are responsible for both extrinsic and intrinsic pathways [11]. In addition, the naturally occurring alkaloid coralyne potentiated paclitaxel-induced apoptosis and mitigated migration of MCF-7 and MDA-MB-231 cells [12]. Concoction of curcumin and paclitaxel on the other hand triggered apoptosis in MCF-7 cells via modulation of apoptotic mediators simultaneously inhibited EGFR signalling pathway [13]. Meanwhile, several other natural...
products may also inhibit the occurrence of autophagy in cancer cells which will enhance the cytotoxic effects induced by therapeutic agents upon cancer cells. At the same time, activation of autophagy may also induce death in cancer cells as well as reduce tumour size. Curcumin was found to potently inhibit the cell growth of human non-small cell lung cancer culture through autophagy by inhibition of the PI3K/Akt/mTOR pathway. There are few natural products that activate autophagy and resulting in cell death in cancer cells, zerumbone derived from a bitter ginger plant may induce autophagy events in prostate cancer culture and honokiol, lignan isolated from the bark, significantly prevents the growth of melanoma through cell proliferation, viability, clonogenicity, and induces autophagy.

Hyperforin is a phytochemical isolated from St John’s wort plant, and this compound has been used in traditional medicine to treat depression [14]. Recent investigations demonstrated that hyperforin inhibited the proliferation of breast cancer cell lines and induced apoptosis via activation of a mitochondrial pathway [15]. Further, another study has shown that a combination of different phytochemicals from the same plant, hyperforin, and hypericin can synergistically induce cytotoxicity on human malignant cell lines by triggering activation of caspases [16]. However, there is still a need to discover the effectiveness of hyperforin in the combination treatment for breast cancer if using other natural products.

Our preliminary findings showed that hyperforin and paclitaxel inhibited the growth of MCF-7 cell line at certain IC\textsubscript{50} dose hence in this present study, hyperforin and paclitaxel were combined together at various doses and introduced to the MCF-7 cell culture to check whether the combination treatment could elicit better anticancer properties than when used singly.

2. Materials and methods

2.1. Chemicals

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin/glutamine (100×), phosphate-buffered saline, and trypsin/ ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), hyperforin and paclitaxel were purchased from Sigma-Aldrich (USA). All chemicals used in the experiments were of analytical grade.

2.2. Cell culture and treatment

Luminal type A breast cancer cell line, MCF-7 was purchased from American Type Culture Collection (Rockville, USA). The cells were grown in RPMI-1640 medium supplemented with 10% FBS (Gibco, USA) and 100 units/ml penicillin/streptomycin/glutamine. The cells were maintained at 37°C in a humidified incubator with 5% CO\textsubscript{2}. Before treatment, the culture medium was replaced with a fresh medium. Paclitaxel and hyperforin were dissolved in pure DMSO and stored at −80°C until further use. The compounds were then freshly diluted in the culture medium before adding into the cell culture and the final concentration of DMSO did not exceed 0.01%.

2.3. Determination of cytotoxic effects and combination index

The MCF-7 cells were plated in triplicates on a 96-well plate (Corning, Arizona, USA) at a density of 2 × 10\textsuperscript{3} cells/mL in 100 µL of culture medium and incubated for 18 h. The cytotoxicity synergy of hyperforin and paclitaxel on MCF-7 cells were examined by treating the cells with the various non-constant combination of hyperforin and paclitaxel (µg/mL) such as (1/7), (2/6), (3/5), (4/4), (5/3), (6/2) and (7/1) for 24, respectively. About 10 µL of MTT (5 mg/mL) were added to the cells and incubated for another 4 h in a dark condition. Then, all the media were removed without disturbing the formation of blue formazan, and 100 µL of DMSO was added to the cells to solubilize the formazan crystals. Subsequently, the absorbance was read at a wavelength of 570 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Untreated MCF-7 cells were used as the negative control in this experiment. All experiments were repeated minimally three times. The average values from triplicate plates were calculated after subtracting the average value for the blank. The percentage cell viability was compared with that of non-treated control cells, which were subjectively assigned viability of 100%. The percentage of cell survival was plotted against the concentration of the compounds (µg/mL). To determine whether hyperforin and paclitaxel showed synergistic effects on MCF-7 cells when used in combination, the combination index (CI) values were calculated from the IC\textsubscript{50} values of the dose–response curve. The combined effect (CI) of hyperforin and paclitaxel was then analyzed using the Chou and Talalay method (1984) and CompuSyn software using a non-constant ratio combination design (Biosoft, Cambridge, UK). All tests were performed in triplicates. If the CI value exhibits equal to 1, it is known as additivity, less than 1 is known as synergy, and more than 1, known as antagonism. This experiment was designed based on Fu et al., 2019 [17].

2.4. Assessment of cell morphological changes

The MCF-7 cells were plated in 6-well plate and treated with a respective combination dose for 24 h. The
medium from cell culture was removed, and the cells were fixed with methanol and stained with 4% Giemsa stain for 15 min. The solution was then removed, and the cells were observed under a Zeiss AX10 inverted phase-contrast microscope with epifluorescence and a digital imaging system (Carl Zeiss Microscopy GmbH, Göttingen, Germany).

2.5. Assessment of apoptosis
Annexin V/propidium iodide (PI) double staining assay was performed using the Tali™ Apoptosis Kit-Annexin V Alexa Fluor® 488 & Propidium Iodide according to the manufacturer’s instructions (Life Technologies, San Diego, CA, USA) after the 24 h in vitro combination treatment of hyperforin and paclitaxel on both MCF-7 cells. Briefly, the cells were harvested from 6-well plates via trypsinization, centrifuged and resuspended in Annexin V binding buffer before they were incubated with Annexin V Alexa Fluor 488 in the dark for 20 min. The cells then were subjected to incubation with PI in the dark for 5 min and immediately analyzed using Tali™ Image-Based Cytometer (ThermoFisher). The data analysis was performed via FCS Express Research Edition software (version 4.03; De Novo Software, New Jersey, NJ, USA)

2.6. Assessment of ROS
The formation of cellular reactive oxygen species (ROS) after in vitro combinational treatment of hyperforin and paclitaxel on MCF-7 cell culture was measured using the dichloro-dihydro-fluorescein diacetate (DCFH-DA)–based assay using a commercially available kit (Abcam, Cambridge, UK). In brief, after treatment with both compounds, the cells were subjected to incubation with DCFH-DA in the dark for 45 min. Later, the DCFH-DA was removed, and the cells were washed with 1× buffer solution. The outcome was measured and recorded at 485 nm (excitation) and 535 nm (emission) using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The non-treated MCF-7 was investigated as well. The concentration of the R110 released from the MCF-7 cell culture is calculated from the absorbance values at excitation: 470 nm and emission: 520 nm. The comparison of the absorbance of R110 from combined treated MCF-7 cells with untreated cells allows determination of the fold increase in caspase-8 activity. Briefly, MCF-7 cells were treated with hyperforin and paclitaxel together for 24 h, and the cells were collected and lysed using cold 1X cell lysis buffer for 10 min. The cell lysates were incubated with a caspase-3 colorimetric substrate, (Ac-IETD)2-R110 (1 mM). The reaction mixtures were incubated at 37°C for 3 h, and the absorbance was measured at respective absorbance using an ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Assessment of caspase-8
Caspase-8 IETD-R110 Fluorometric and Colorimetric Assay Kit are based on the hydrolysis of the two IETD tetrapeptide substrate, (Ac-IETD)2-R110 by caspase-8 resulting in two successive cleavages which finally lead to the release of fluorescent R110 (Biotium, Fremont, CA, USA). The concentration of the R110 released from the MCF-7 cell culture is calculated from the absorbance values at excitation: 470 nm and emission: 520 nm. The comparison of the absorbance of R110 from combined treated MCF-7 cells with untreated cells allows determination of the fold increase in caspase-8 activity. Briefly, MCF-7 cells were treated with hyperforin and paclitaxel together for 24 h, and the cells were collected and lysed using cold 1X cell lysis buffer for 10 min. The cell lysates were incubated with a caspase-3 colorimetric substrate, (Ac-IETD)2-R110 (1 mM). The reaction mixtures were incubated at 37°C for 3 h, and the absorbance was measured at respective absorbance using an ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.9. Statistical analyses
Statistical analysis was carried out using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA). The levels of significance for comparisons between two samples are compared by T-test and more than two independent samples were determined using ANOVA. Data were presented as mean ± standard deviation, where n refers to the number of independent experiments. The level of significance was considered at *P < 0.05, **P < 0.01, and ***P < 0.001. Groups were compared by one-way or two-way analysis of variance with Bonferroni’s post hoc test applied to explore significance. Meanwhile, the CI values were calculated with help of CompuSyn software (ComboSyn Inc., Paramus, NJ, USA) to examine the interactions between natural products. The synergistic effects of the hyperforin and paclitaxel on MCF-7 was evaluated via the isobologram method. Briefly, the IC_{50} value of hyperforin and paclitaxel was
plotted on the x- and y-axes to form a straight line. The data points in the isobologram correspond to the actual IC\(_{50}\) value of the combination of the natural products. Data points on or near the line indicate an additive effect (\(= 1\)), those below the line indicate synergism (\(< 1\)) and those above the line represent antagonism (\(> 1\)).

3. Results

3.1. Synergistic cytotoxicity of hyperforin and paclitaxel

In the investigation of whether hyperforin (Hy) and paclitaxel (Pa) affected the cell viability of the MCF-7 cell line when used in combination, the CI values were calculated from the IC\(_{50}\) values of the dose–response (Figure 1(A)). The MCF-7 cells were treated with combination of Hy and Pa at different serial concentrations such as (dose 1: Hy: 1 µg/mL and Pa: 7 µg/mL), (dose 2: Hy: 2 µg/mL and Pa: 6 µg/mL), (dose 3: Hy: 3 µg/mL and Pa: 5 µg/mL), (dose 4: Hy: 4 µg/mL and Pa: 4 µg/mL), (dose 5: Hy: 5 µg/mL and Pa: 3 µg/mL), (dose 6: Hy: 6 µg/mL and Pa: 2 µg/mL) and (dose 7: Hy: 7 µg/mL and Pa: 1 µg/mL) for 24 h. At 24 h, about 55%, 56%, 56%, 55%, 50%, 49% and 52% cell survival were recorded with dose 1, dose 2, dose 3, dose 4, dose 5, dose 6 and dose 7, respectively, compared with untreated MCF-7 cells (82%). Meanwhile, synergistic effects on MCF-7 cells were analyzed by CompuSyn software and Chou and Talalay method (Figure 1(B)) [18]. Based on that, it was found only certain tested doses exhibited synergistic effects (CI value below 1), dose 1, dose 2, dose 3 and dose 4 exhibited the CI values of 0.88, 0.87, 0.88 and 0.86, respectively (Figure 2(A)). The combination of Hy: 4 µg/mL and Pa: 4 µg/mL (dose 4) was selected for further experiments since it has recorded the lowest CI among others. The efficacy of treatment was significantly increased with the combination of hyperforin and paclitaxel when compared with paclitaxel-alone (\(P < 0.001\)). When paclitaxel is given alone, about 61% of cell survival was recorded; however, the percentage of cell survival was found to decrease in combination treatment (55%). However, the similar treatment was not significant when compared with hyperforin alone although the percentage of MCF-7 cell survival was reduced from 57% (hyperforin alone) to 55% in combination treatment (Figure 2(B)).
Figure 2. (A): Combined effect of Hy and Pa on MCF-7 cell viability. The viability of MCF-7 after various combination treatments of Hy and Pa was measured using MTT assay. The combination of Hy 4 µg/mL and Pa 4 µg/mL was recorded as the lowest CI. (B): The viability of untreated MCF-7, Hy (4 µg/mL) treated, Pa (4 µg/mL) treated and combination of Hy (4 µg/mL) and Pa (4 µg/mL). The cell viability was measured using the MTT assay. Data are presented as the mean ± SEM. The levels of significance between samples according to one-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant compared with untreated cells.

3.2. Cell morphological changes

Giemsa staining was used to evaluate the occurrence of cell morphological changes such as cytoplasmic condensation, cell shrinkage and membrane blebbing in MCF-7 cell culture through an inverted phase-contrast microscope [19]. As mentioned previously, dose 4 (Hy: c and Pa: 4 µg/mL) was selected for further experiment since it has exhibited a lower CI along with hyperforin alone (4 µg/mL), and paclitaxel alone (4 µg/mL) were used to treat MCF-7 cells. The morphological changes in the treated MCF-7 cells were later compared with untreated cells. There was no major morphological change found in the untreated control cells. The structure of MCF-7 cells was seen to be intact and large spherical cells that eventually assumed clumped and/or aggregate forms were also observed in the untreated cells (Figure 3(A)). At the same time, cells treated with hyperforin alone were started to show distinctive and prominent morphological signs of apoptosis-like cells were found to become small, isolated, or detached forms, showing characteristic signs of blebbing and shrinking of the cell membrane (Figure 3(B)). A similar result was observed in paclitaxel-alone treated MCF-7 cells (Figure 3(C)). Meanwhile, more cells were seen to detach and the remaining cells were appeared to be apoptotic bodies in MCF-7 cells subjected to combination treatment (Figure 3(D)). Similarly, reduced cell volume and chromatin condensation were seen in all the treated cells compared with untreated cells.

Figure 3. Apoptotic effects of Hy and Pa on cellular morphology of MCF-7. (A) Cellular morphology of untreated MCF-7 cells. The cells were aggregated and clustered as monolayer forms. (B) Early morphological changes during apoptosis such as cell shrinkage and membrane blebbing in Hy alone treated MCF-7 cells. (C) Cells treated with Pa alone exhibited early apoptosis signs like membrane blebbing and cell shrinkage. (D) MCF-7 cells were elongated and reduction of cell growth was observed with late apoptotic morphological changes in cells. Similar cellular morphology was observed in three independent experiments (magnification × 100).

3.3. Confirmation of apoptosis

Consistently, the anti-proliferative effects of combination treatment with Hy (4 µg/mL) and Pa (4 µg/mL) were further confirmed by using a Tali® apoptosis detection kit, which allows finding the difference between viable cells and apoptotic cells. The results of the Annexin V-FITC assay based on the quantification of apoptotic and viable cells were shown in Figure 4. Following treatment with Hy alone, it was observed a vast increase in the percentage of apoptotic cells (39%) compared with the untreated cells (11%). Similarly, treatment with Pa alone resulted in a significant increase in the percentage of apoptotic cells (41%). On that note, a significant decrease in the number of viable cells, as well as, an increase in the percentage of apoptotic cells (39%) compared with the untreated cells (11%). Similarly, treatment with Pa alone resulted in a significant increase in the percentage of apoptotic cells (41%). On that note, a significant decrease in the number of viable cells, as well as, an increase in the percentage of apoptotic cells, were apparent in the cells undergone combination Hy and Pa treatment (43%) compared with untreated cells. In addition, the increase in the percentage of apoptotic cells in combination treatment and Hy alone was significant, and this is suggesting that combination treatment was found to enhance the apoptotic effects of Hy and Pa in the MCF-7 cell culture. Although, there was a slight increase in the percentage of apoptotic cells from combination treatment compared with the cells from Pa alone treatment, it was not significant.
3.4. Secretion of apoptosis mediator

Apoptosis mediator such as ROS is essential for activation of cell death [20]. Hence, combination treatment on MCF-7 cell culture was found to induce a significant secretion of ROS (61.3cps, count per second) compared with untreated cells (18.5cps). Meanwhile, cells that were treated with Hy alone (62cps) and Pa alone (61.5cps) exhibited a significant release of ROS in the supernatant culture compared with the untreated MCF-7 cells. However, the release of ROS in the culture supernatant of combination treatment was not significant compared with the other two treated groups; treatment of Hy alone and Pa alone was found to induce the slightly higher release of ROS in the culture supernatant compared with the combination treatment (Figure 5).

3.5. Release of LDH

The anti-inhibitory effects of combination treatment of Hy (4 µg/mL) and Pa (4 µg/mL) on the MCF-7 cells were further confirmed with the ability of the treatment to release LDH from damaged cells which indicate the cytotoxicity of the treatment towards cancer cells. Based on the analysis, it was observed that Hy alone, Pa alone, and combination treatment caused the significant release of LDH in the culture supernatant compared with untreated cells ($P < 0.001$) (Figure 6); however, there was no significant release of LDH found among the Hy or Pa alone with combination treatment.

3.6. Activation of caspase 8

Caspase 8 is another well-established apoptosis mediator [21]. Since it was found that Hy and Pa combination treatment induces apoptotic populations and secretion of ROS in the MCF-7 cell culture. Hence, these findings led to examine the effects of combination treatment on caspase activation, especially on caspase-8, an important apoptotic mediator of the extrinsic pathway. Based on the analysis, caspase 8 activity was interpreted by investigating the ability of treatment to cleave a specific substrate (Ac-IETD)2-R110 and generate a fluorogenic product R110. Hence, the release of R110...
and WallerDuncantest.∗

significance between samples according to one way ANOVA

tamoxifen alone (15% of apoptotic cells) [28]. Another
cancer cells (60% of apoptotic cells), compared with

tive was found to effectively induce apoptosis of breast
treatment with lauryl gallate, a gallic acid deriva-
cancer [27], tamoxifen is well-known for its anti-breast
enhance the cell cycle arrest. In the setting of breast
MAPK (p-p38 MAPK, p-ERK1/2) markers which further
the level of endoplasmic reticulum (ATF-6, XBP-1) and

strawberry tree honey has been shown to synergisti-
treatment compared with Hy alone (P

P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant compared with untreated cells.

indirectly measured the caspase 8 activity in the cell cul-
ture. As predicted, Hy and Pa combination treatment
on the MCF-7 cells induced higher caspase 8 activity
(3.8-fold) compared with untreated cells (1-fold); mean-
while, Hy alone and Pa alone treatments demonstrated
3.2-fold and 3.3-fold, respectively. The presence of cas-
pase 8 activity was significant in Hy and Pa combination

treatment compared with Hy alone (P < 0.05); how-
ever, no significant caspase 8 activity was found when
compared with Pa alone treatment on the MCF-7 cells
(Figure 7).

4. Discussion

It is essential to evaluate the impacts of monotherapy
(single drug) beyond what it can achieve alone; hence
many recent studies are exploring combination anti-
cancer therapy (using multiple drugs) as an option to
treat breast cancer [22, 23, 24]. This may further elicit
the synergistic effects in terms of potency and attain
efficacy with lower doses, as well as reduce the develop-
ment of drug-resistant cancer cells [25, 26]. For instance,
strawberry tree honey has been shown to synergisti-

cantly enhance the chemo-sensitization of 5-fluorouracil
on human colon adenocarcinoma cells by increasing
the level of endoplasmic reticulum (ATF-6, XBP-1) and
MAPK (p-p38 MAPK, p-ERK1/2) markers which further
enhance the cell cycle arrest. In the setting of breast
cancer [27], tamoxifen is well-known for its anti-breast
properties against MCF-7 cells, interestingly combina-
treatment with lauryl gallate, a gallic acid deriva-
tive was found to effectively induce apoptosis of breast
cancer cells (60% of apoptotic cells), compared with
tamoxifen alone (15% of apoptotic cells) [28]. Another

study has demonstrated that gemcitabine and pacli-
taxel were combined together and when given to
anthracycline-treated metastatic breast cancer patients,
it showed an improvement in overall survival at least
22% compared with paclitaxel-alone treatment [29].
In addition, combination treatment using pristimerin
and paclitaxel can also induce autophagy in MDA-MB-
231 cells through ERK1/2 regulation which eventually
leads to cell death. At the same time, under a differ-
ent condition, paclitaxel tends to inhibit autophagy by
blocking formation of autophagosome in breast cancer
cells [27].

In a previous study, it was observed that hyperforin
inhibited the growth of MCF-7 cells with a certain IC50
dose (unpublished data), and in addition, other stud-
ies have similarly proven the ability of hyperforin to
suppress different types of breast cancer cells [30, 31].
Hence in this present study, hyperforin was combined
with paclitaxel, a clinically used drug, and introduced to
the MCF-7 cell culture to check on their potency against
MCF-7 breast cancer cell line and whether if the combi-
nation further enhances chemopreventive effects than
singly used. The doses of hyperforin and paclitaxel used
in this study were found to be non-cytotoxic to the
non-cancerous breast cell line, MCF-10A cells (unpub-
lished data). To our knowledge, this is the first report
regarding the effect of combination treatment involv-
ing hyperforin and paclitaxel on MCF-7 cells. The effi-
ciency of combination treatment was measured using
the CI which is a widely accepted qualitative measure of
the extent of a drug interaction [32]. Based on the find-
ings, paclitaxel potentiated hyperforin-imposed cyto-

toxicity as revealed by the CI values and dose1: Hy:
1 μg/mL and Pa: 7 μg/mL, dose 2: Hy: 2 μg/mL and Pa:
6 μg/mL, dose 3: Hy: 3 μg/mL and Pa: 5 μg/mL and dose
4: Hy: 4 μg/mL and Pa: 4 μg/mL exhibited synergistic
effect towards the growth of MCF-7 cells (CI < 1) that
may lead to reduce the toxicity of a drug along with
inhibiting the development of drug-resistant cells [33],
whereas dose 5: Hy: 5 μg/mL and Pa: 3 μg/mL, dose 6:
Hy: 6 μg/mL and Pa: 2 μg/mL and dose 7: Hy: 7 μg/mL
and Pa: 1 μg/mL displayed antagonistic effects on the
proliferation of MCF-7 cells whereby combination of
hyperforin and paclitaxel at certain doses could lead
to drug inhibition (Figure 1(A,B)) [34]. All these doses
exhibited almost similar potent (induction of cell death)
against MCF-7 cells and almost dose-dependent, with
near maximum cell death achieved when combined
with dose 6. Although, doses 5, 6 and 7 exhibited antag-
onistic effect (CI > 1) towards the growth of MCF-7
cells interestingly lesser number of viable MCF-7 cells
were found in the cell culture treated with those doses.
Nonetheless, the reduction in the number of viable cells
in cell culture treated with doses 1, 2, 3 and 4 was almost
similar to doses 5, 6 and 7. This is additionally indicat-
ing the potential of using a lower hyperforin dose to
achieve the same level of effectiveness as demonstrated
by a high hyperforin dose alone. Of the seven doses, dose 4 (4 µg/mL and Pa: 4 µg/mL) was selected for further analysis since it has recorded the lowest CI value. This dose was also found to induce more cell death in MCF-7 (45%) culture compared with doses 1, 2 and 3. In another set of experiments, potency to induce cell death in MCF-7 cell culture as a single drug was investigated as well. Response of MCF-7 cells towards paclitaxel alone (4 µg/mL) yielded 39% of cell death, whereas hyperforin alone (4 µg/mL) were seen to exhibit 43%. Overall, MCF-7 cells were much more susceptible to combination treatment compared with a single drug.

In line with this notion, the Giemsa staining on treated MCF-7 cells has also confirmed the occurrence of apoptosis by observing the changes in cellular morphology in treated and untreated MCF-7 cells [35]. Cells treated with hyperforin alone and paclitaxel alone have undergone cell shrinkage, plasma membrane blebbing and the formation of apoptotic bodies (small and rounded cells). These apoptotic features were already proven in other studies [36, 37]. Similar observations were found in MCF-7 cells treated with both hyperforin and paclitaxel; however, the magnitude of apoptosis was greater compared with the single drug-treated groups. It is noteworthy that hyperforin and paclitaxel did not induce cells to undergo necrosis since an increase in cell volume (cell swelling) and leakage of cellular contents were not visible on these cells [38]. In addition, changes in cellular morphology after the treatment had promoted the investigation of the presence of apoptotic cells in the cell culture. The apoptotic cells were detected by observing the presence of cells that were positive for Annexin FITC (cells contained green fluorescent) [39]. In addition, combination treatment of hyperforin and paclitaxel enhanced the rate of apoptosis in MCF-7 cells with higher percentages of apoptotic cells compared with the drug alone. In addition, a significant apoptotic effect was found in between hyperforin alone and combined treated cells. This is suggesting partially improved the apoptotic effect of combined hyperforin and paclitaxel treatment against MCF-7 cells. However, there was no significant apoptotic effect when compared with paclitaxel alone and combination treatment.

Elevated levels of ROS during any cancer treatment can eventually induce apoptotic or/and necrotic cell death through mitochondrial cell death [40, 41]. In the present study, hyperforin and paclitaxel were seen to elevate the level of ROS in the MCF-7 cell culture. Meanwhile, synergistic combinations of hyperforin and paclitaxel-induced apoptosis were also supplemented by the elevated level of ROS which may activate the mitochondrial cell death pathway. However, the level of ROS was almost similar in all the treated groups. Formation of ROS in the treated MCF-7 cell culture also will activate initiator caspases that lead to mitochondrial cell death pathway. In addition, ROS was known as an essential entity for the activation of autophagy, an important type of cellular demise is which highly linked with immunogenic cell death and cell survival mechanic. For example, paclitaxel-induced ROS could lead to the formation of autophagy in endometrial carcinoma cells hence inhibition of the ROS by antioxidant N-acetyl-cysteine (NAC) is needed to achieve successful cancer treatment [42]. Combination treatment of hyperforin and paclitaxel triggers apoptosis through activation of caspase 8 with a slightly high level compared with a single drug. This has further confirmed the occurrence of apoptosis via the intrinsic pathway and cleaved many crucial cellular proteins responsible for apoptosis [43, 44]. Additional studies are required to precisely determine whether activation of caspase 8 leads to caspase cascade activation which directly induction of apoptosis [45]. Along with this, LDH, a cytoplasmic enzyme released from damaged plasma membrane that can be measured in cell culture supernatants since several studies have mentioned that the release of LDH is a reliable and fast cytotoxicity assay [46]. Based on the analysis, a low level of LDH was found in the untreated MCF-7 cell culture. This is probably due to the natural occurrence of cell development and aging meanwhile higher release of LDH among the treated groups directly indicates the ability of hyperforin and paclitaxel to induce cytotoxicity in MCF-7 cells. The higher release of LDH due to the damaged plasma membrane of treated cells, which already proven previously in the Giemsa staining. Several studies have shown that the upregulation of LDH in cell culture may subsequently trigger the induction of apoptosis in cancer cells [46].

In summary, herein this is the first study to provide evidence that combined effect of hyperforin and paclitaxel in inhibition of MCF-7 cell growth. An increase in ROS, caspase 8, and LDH levels in combination with treatment of hyperforin and paclitaxel are enough to trigger apoptosis in the MCF-7 cell culture. Additional experiments are needed to confirm signalling mechanisms that lead to apoptosis in these breast cancer cells. Future studies on autophagic and immunomodulatory activity of these combinations are also warranted to fully understand their mechanism of action on breast cancer cells.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work is supported by FRGS Grant from Ministry of Higher Education, FRGS/1/2019/5K15/UM/01/2 (FP109-2019A).

ORCID

Kumutha Malar Vellasamy
http://orcid.org/0000-0001-7661-3928
Jamuna Vadivelu
http://orcid.org/0000-0001-7322-3676
References

[1] Alkabban FM, Ferguson T. Breast cancer. Treasure Island: StatPearls Publishing; 2021. [cited 2021 Aug 7]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482286/

[2] Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Cancer J Clin. 2021;71(3):209–249. DOI:10.3322/caac.21660.

[3] Korde LA, Somerfield MR, Carey LA, et al. Neoadjuvant chemotherapy, endocrine therapy, and targeted therapy for breast cancer: ASCO guideline. J Clin Oncol. 2021;39(13):1485–1505. DOI:10.1200/JCO.20.03399.

[4] Thorn CF, Oshiro C, Marsh S, et al. Doxorubicin pathways: anything new to improve tolerance and reduce sequelae? Front Pharmacol. 2018;9(4):235, DOI:10.3389/fphar.2018.00245.

[5] Dutta S, Mahalanobish S, Saha S, et al. Natural products as reservoirs of novel therapeutic agents. Excli J. 2018;17(4):420–451. DOI:10.17179/excli2018-1174.

[6] Wang W, Li L, Zhang C, et al. Natural products: an upcoming therapeutic approach to cancer. Food Chem Toxicol. 2019;128:240–255. DOI:10.1016/j.fct.2019.04.012.

[7] Attar R, Tabassum S, Fayyaz S, et al. Natural products are the future ofanticancer therapy: preclinical and clinical advancements of Viscum album phytomolecules. Mol Biol Cell. 2015;6(6):62–68. DOI:10.1074/mcb.E114.04-0916.

[8] Wang W, Li L, Zhang C, et al. Natural products as reservoirs of novel therapeutic agents. Excli J. 2018;17(4):420–451. DOI:10.17179/excli2018-1174.

[9] Wang W, Li L, Zhang C, et al. Natural products as reservoirs of novel therapeutic agents. Excli J. 2018;17(4):420–451. DOI:10.17179/excli2018-1174.

[10] Du B, Jiang L, Xia Q, et al. Synergistic inhibitory effects of curcumin and 5-fluorouracil on the growth of the human colon cancer cell line HT-29. Chemotherapy. 2006;52(1):23–28. DOI:10.1159/000090326.

[11] Yaacob NS, Kamal NN, Norazmi MN. Synergistic anticancer effects of curcumin on MCF-7 and MDA-MB-231 human breast cancer cell lines. Molecules. 2014;19:1325–1336. DOI:10.3390/molecules19091325.

[12] Kumari S, Badana A, Mohan GM, et al. Synergistic effects of curcumin and paclitaxel on human breast cancer cell line. Asian Pac J Cancer Prev. 2012;13(8):3283–3285.

[13] Zhan Y, Chen Y, Liu R, et al. Potentiation of paclitaxel activity by curcumin in human breast cancer cell line MCF-7. Anticancer Res. 2013;33(3):1189–1192. DOI:10.21873/ajpcr.2013.05.025.

[14] Schiavone BI P, Verotta L, Rosato A, et al. Anticancer and antibacterial activity of hyperforin and its derivatives. Anticancer Agents Med Chem. 2014;14(10):1397–1401. DOI:10.2174/187152614999998.2808.

[15] Hostanska K, Reichling J, Bommer S, et al. Hyperforin as a constituent of St John’s wort (Hypericum perforatum L.) extract induces apoptosis by triggering activation of caspases and with hypericin synergistically exerts cytotoxicity towards human malignant cell lines. Eur J Pharm Biopharm. 2003;56(1):121–132. DOI:10.1016/s0939-6411(03)00046-8.

[16] Fu Z, Ma K, Dong B, et al. The synergistic antitumor effect of Huiaer combined with 5-fluorouracil in human cholangiocarcinoma cells. BMC Complement Altern Med. 2019;19(1):203. DOI:10.1186/s12906-019-2614-5.

[17] Zhang N, Fu JN, Chou TC. Synergistic combination of curcumin and paclitaxel targeting cancer stem cells through p53-dependent suppression of stemness and anti-invasive pathway. Cancer Res. 2015;75(2):194–204. DOI:10.1158/0008-5472.CAN-14-2179.

[18] Fu Z, Ma K, Dong B, et al. The synergistic antitumor effect of Huiaer combined with 5-fluorouracil in human cholangiocarcinoma cells. BMC Complement Altern Med. 2019;19(1):203. DOI:10.1186/s12906-019-2614-5.

[19] Jaudan A, Sharma S, Malek SNA, et al. Induction of apoptosis by pinoresinol in human cervical cancer cells: possible mechanism of action. Placenta. 2018;13(2):e0191523. DOI:10.1017/journal.pone.0191523.

[20] Pepe L, Ayala E. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med. 2015;86:249–256. DOI:10.1016/j.freeradbiomed.2015.06.09.

[21] Shalini S, Dorstyn L, Dawar S, et al. Old, new and emerging functions of caspases. Cell Death Differ. 2015;22(4):526–539. DOI:10.1038/cdd.2014.216.

[22] Nyaya PN, Kapile D, Srivinas SP, et al. Current trends and challenges in cancer management and therapy using designer nanomaterials. Nano Converg. 2019;6(1):23, DOI:10.1186/s40580-019-0193-2.

[23] Cragg GM, Pezzuto JM. Natural products as a vital source for the discovery of cancer chemotherapeutic and chemopreventive agents. Med Prin Pract. 2016;25(Suppl. 2):41–59. DOI:10.1159/000443404.

[24] Pritchard JR, Lauffenburger DA, Hemann MT. Understanding resistance to combination chemotherapy. Drug Resist Updat. 2012;15(5–6):249–257. DOI:10.1016/j.drup.2012.10.003.

[25] Palmer AC, Sorger PK. Combination cancer therapy can confer benefit via patient-to-patient variability without drug additivity or synergy. Cell. 2017;171(7):1678–1691.e13. DOI:10.1016/j.cell.2017.11.009.

[26] Afrin S, Giampieri F, Cianciosi D, et al. Strawberry tree honey in combination with 5-fluorouracil enhances chemosensitivity in human colon adenocarcinoma cells. Food Chem Toxicol. 2021 Oct;165:112484. DOI:10.1016/j.fct.2021.112484.

[27] Samani K G, Farrokhi E, Tabatabaee A, et al. Synergistic effects of lauryl gallate and tamoxifen on human breast cancer cell. Iran J Public Health. 2020;49(7):1324–1329. DOI:10.18502/ijph.v49i7.3586.

[28] Colomer R. Gemcitabine in combination with paclitaxel for the treatment of metastatic breast cancer. Womens Health. 2005;1(3):323–329. DOI:10.2217/17455075.1.3.323.

[29] Kwon J, Oh KS, Cho SY, et al. Estrogenic activity of hyperforin in MCF-7 human breast cancer cells transfected with estrogen receptor. Planta Med. 2016;82(16):1425–1430. DOI:10.1055/s-0042-112594.

[30] Chiang IT, Chen WT, Tseng CW, et al. Hyperforin inhibits cell growth by inducing intrinsic and extrinsic apoptotic pathways in hepatocellular carcinoma cells. Anticancer Res. 2017;37(1):161–167. DOI:10.21873/anticanres.11301.

[31] Fouquier J, Guedj M. Analysis of drug combinations: current methodological landscape. Pharmacol Res Perspect. 2019 Dec;7(6):e00549. DOI:10.1002/prp2.149.
[33] Yao M, Yuan B, Wang X, et al. Synergistic cytotoxic effects of arsenite and tetrandrine in human breast cancer cell line MCF-7. Int J Oncol. 2017;51(2):587–598. DOI:10.3892/ijo.2017.4052.

[34] Liu K, Cang S, Ma Y, et al. Synergistic effect of paclitaxel and epigenetic agent phenethyl isothiocyanate on growth inhibition, cell cycle arrest and apoptosis in breast cancer cells. Cancer Cell Int. 2013;13(1):10, DOI:10.1186/1475-2867-13-10.

[35] Kumar S, Sharma VK, Yadav S, et al. Antiproliferative and apoptotic effects of black turtle bean extracts on human breast cancer cell line through extrinsic and intrinsic pathway. Chem Cent J. 2017;11(1):56, DOI:10.1186/s13065-017-0281-5.

[36] Ziegler U, Groscurth P. Morphological features of cell death. News Physiol Sci. 2004;19:124–128. DOI:10.1152/nips.01519.2004.

[37] Zhang Y, Chen X, Gueydan C, et al. Plasma membrane changes during programmed cell deaths. Cell Res. 2018;28(1):9–21. DOI:10.1038/cr.2017.133.

[38] Miller MA, Zachary JF. Mechanisms and morphology of cellular injury, adaptation, and death. Pathol Basis Vet Disease. 2017: 2–43.e19. DOI:10.1016/b978-0-323-35775-3.00001-1.

[39] Crowley LC, Marfell BJ, Scott AP, et al. Quantitation of apoptosis and necrosis by annexin V binding, propidium iodide uptake, and flow cytometry. Cold Spring Harb Protoc. 2016;2016(11): DOI:10.1101/pdb.prot087288.

[40] Liou GY, Storz P. Reactive oxygen species in cancer. Free Radic Res. 2010;44(5):479–496. DOI:10.3109/107157610-03667554.

[41] Aggarwal V, Tuli HS, Varol A, et al. Role of reactive oxygen species in cancer progression: molecular mechanisms and recent advancements. Biomolecules. 2019;9(11):735. DOI:10.3390/biom9110735.

[42] Giampieri F, Afrin S, Forbes-Hernandez TY, et al. Autophagy in human health and disease: novel therapeutic opportunities. Antioxid Redox Signal. 2019 Feb;1;30(4): 577–634. DOI:10.1089/ars.2017.7234.

[43] MacKenzie SH, Clark AC. Targeting cell death in tumors by activating caspases. Curr Cancer Drug Targets. 2008;8(2): 98–109. DOI:10.2174/156809080783769391.

[44] Parrish AB, Freel CD, Kornbluth S. Cellular mechanisms controlling caspase activation and function. Cold Spring Harb Perspect Biol. 2013;5(6):a008672. DOI:10.1101/cshperspect.a008672.

[45] Kumar P, Nagarajan A, Uchil PD. Analysis of cell viability by the lactate dehydrogenase assay. Cold Spring Harb Protoc. 2018;2018(6): DOI:10.1101/pdb.prot095497.

[46] Maes M, Vanhaecke T, Cogliati B, et al. Measurement of apoptotic and necrotic cell death in primary hepatocyte cultures. Methods Mol Biol. 2015;1250:349–361. DOI:10.1007/978-1-4939-2074-7_27.