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Antimicrobial Peptide Hepcidin TH1-5 induced apoptosis and activates caspase cascades in HepG2 and HeLa cells

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Antimicrobial peptides (AMPs) have shown to exhibit a broad spectrum of cytotoxic activity against cancer cells without harming normal cells. Hepcidin TH1-5 is one of hepcidin synthetic isoform, originally isolated from *Oreochromis mossambicus.* It is a 20-25 amino acid peptide that was reported to exhibit antimicrobial activity and its expression is markedly suppress in cancerous, but not in non-cancerous cells. In order to unearth the anticancer effect of this peptide, the apoptosis effect of synthetically derived Hepcidin TH1-5 against HepG2, HeLa, HL-60, Chang's Liver and NIH/3T3 cell lines was carried out. Proliferation and cytotoxic properties were evaluated using MTT assay. Modes of cell death and cell cycle were analysed using flow cytometric and microscopical analysis. Activation of caspases evaluated using bioluminescence assay. Treatment with HepTH1-5 show potent cytotoxic activity against HepG2 and HeLa, but not on HL-60, Chang and NIH/3T3 and correspondingly induced apoptosis in HepG2 and HeLa. It was also significantly arrest cells in G2/M phase and cell cycle delay in S phase in HepG2 and HeLa cells, respectively. Findings from this study show that HepTH1-5 was significantly induced apoptosis and disturbed cell cycle via activation of intrinsic pathway. These findings will serve as basic data on the development of HepTH1-5 as anticancer agent.

Keywords: Antimicrobial peptide, Hepcidin TH1-5, cytotoxic activity, apoptosis

INTRODUCTION

Hepcidin was identified originally in mammals. This small cation peptide has been shown to possess antimicrobial peptide and also serve as important iron regulatory hormone. (Nicolas et al. 2001; Robertson, 2011). Study also shown that excess iron induced the peptide expression. It can be reduced by anemia and hypoxia. This small peptide is well conserved in fishes and mammals, where three exons separated by two introns and encode a propeptide that consists of a highly conserved signal peptide sequence an acidic propiece and a mature peptide (Chen et al. 2009; Douglas et al. 2003).

Substantial numbers of studies have shown that antimicrobial peptides (AMPs) exhibit a broad spectrum of cytotoxic activity against cancer cells. For instance, Piscidin-1, Chrysophsin-1, Epinecidin-1 and Pardaxin showed antitumor activity on HT1080 cells (Lin et al. 2013; Hsu et al. 2000). Apoptosis has been induced in MDA-MB-468, MCF7, T47-D, MDA-MB-231, SKBR3 cells as well as HepG2, HeLa, MN-11 and U937 cell lines as a result of Pleurocidin, Chrysophsin-1, Pardaxin and Epinecidin-1 treatment (Hilchi et al. 2011; Jaeyong et al. 2012; Lin et al. 2009). Studies have also shown AMPs could modulate the inflammatory response on virus RAW264.7

and JEV by Chrysophsin-1 and Epinecidin-1 (Huang et al. 2011; Jaeyong et al. 2012; Chen et al. 2009).

Hepcidin TH1-5 is one of hepcidin synthetic isoform, originally isolated from *Oreochromis mossambicus.* It is a 20-25 amino acid peptide that was reported to exhibit antimicrobial activity and its expression is markedly suppress in cancerous, but not in non-cancerous cells (Lauth et al. 2005). The amino acid composition (GIKCRFCCGCCTPGICGVCCRF) and secondary structure, hepcidin TH1-5 (HepTH1-5) can be classified into antiparallel ß-sheet with slightly twisted, two-stranded and connected by flexible hairpin loop that curls over the concave surface of the ß-sheet with the presence of disulphide-bonding pattern. Similar to other antimicrobial peptides, hepcidin exhibits positively charged hydrophilic side chains from the hydrophobic ones, where this characteristic will able to disrupt bacterial membranes (Lin et al. 2007). Expression and sequence analyses revealed that iron regulatory function and antimicrobial activity may exist in distinct hepcidin peptides in some fish species (Chen et al. 2009; Douglas et al. 2003).

However, studies on addressing the feasibility of synthetic HepTH1-5 peptide for anticancer therapeutic were limited. Chang et al. (2010) reported that HepTH1-5 was cytotoxic against HT1080, HeLa and HepG2 cell lines. Therefore in present study, we re-evaluated the cytotoxicity effect of HepTH1-5 on several cancer cell lines (HeLa, HepG2, HL-60 and Chang liver) and non-cancer embryonic fibroblast (NIH/3T3). We also examined the apoptosis induction and its pathway activation on these cells as well as cell cycle arrest and compared with standard drug, methotrexate.

MATERIALS AND METHODS

Peptide

Hepcidin TH1–5 was synthesized (GIKCRFCCGCCTPGICGVCCRF) by Mimotopes (Victoria, Australia) at >98% purity. Synthetic peptides were reconstituted in phosphate-buffered saline (PBS; pH 7.4) for the experiments and kept in -80^{o}C prior.

Cell Culture

The HeLa (human cervix adenocarcinoma) cells, HepG2 (human hepatocellular carcinoma) cells, HL-60 (human promyelocytic leukemia) cells, Chang's liver cells and NIH/3T3 embryonic fibroblast cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured using D-MEM and RPMI-1640 media (Gibco, USA) respectively, supplemented with 10% foetal bovine serum (Gibco, USA), antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) (Nacalai-Tesque, Japan), and maintained in an incubator at 37°C with 5% CO_2 in a humidified atmosphere.

Cytotoxicity Assay

Cytotoxicity assay was performed according to Tajudin et al. (2012) with some modification to suit the peptide. Cells were seeded in 96-well plates at a concentration of 1.0 x 10^5 cells/well. The concentration for HepTH1-5, methotrexate (Sigma, USA) and hydrogen peroxide (Sigma, USA) was at 100 µg/mL followed by 2-fold dilutions. The cell viability was measured at 72 hours treatment. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye solution of 5 mg/mL (Nacalai Tesque, Japan) was added at a volume of 20 µL in each well and was incubated for 4 hours. Then the culture medium was removed and 100 µL of DMSO (Sigma, USA) were added to each well to solubilise the formazan crystals. The plates were then read using microtiter plate reader at 570 nm with reference at 630 nm wavelengths (Infinite M200, Tecan, Switzerland). A dose response curve of the percentage of cell viable versus extract concentration was plotted.

Cell Proliferation Assay

Cell proliferation assay were carried out according to Tajudin et al. (2012). Briefly, the cells at concentration of 1.0 x 10^5 cells/mL were incubated with HepTH1-5 and methotrexate at CD_{25}, CD_{50} and CD_{75} values for 24, 48, and 72 hours in 96-wells plates. The MTT assay was performed and the absorbance of each well was read at 570 nm with reference at 630 nm wavelengths as mentioned above. The proliferation graph was established by plotting the percentage of viability versus time.

Acridine Orange and Propidium Iodide Staining (AO/PI)

HepG2 and HeLa cells were treated in triplicates with HepTH1-5 for 24, 48 and 72 hours at CD_{50} concentration and methotrexate was used as a positive control. Cells without treatment were used as negative control. After incubations, cells were harvested by trypsinized and centrifuged at 300 g using refrigerated centrifuge model 3-16K;

rotor 11,180 (Sigma, USA) for 10 minutes. The resulting pellets were rinsed with PBS twice. Then the pellets were suspended in 50 µL of acridine orange (10 µg/mL) and 50 µL of propidium iodide (10 µg/mL) for 5 minutes. About 10 µL of stained cells was dropped onto glass slide and covered with a cover slip. A population of 200 cells were examined and the viable, apoptotic and necrotic cells were scored using fluorescent microscope (Nikon TE2000-U, Nikon, Japan) as described in Tajudin et al. (2012).

Flow Cytometric Analysis of Cell Cycle Distribution

The cells were seeded in 6-well plate at concentration of 2.0 x 10^5 cells/well. HepG2 and HeLa cells were treated with HepTH1-5 and methotrexate at CD_{50} and CD_{20} concentrations for 24, 48 and 72 hours. After the incubation, cells were trypsinized and centrifuged at 300 g for 10 minutes. Cell pellets were fixed in 80% (v/v) cold-ethanol and kept at -20°C. After a week, the fixed cells were analysed using cycle test plus DNA reagent kit (Becton-Dickson, USA) according to the manufacturer's instructions.

Flow Cytometry Analysis –Annexin V-FITC/PI

HepG2 and HeLa cells were treated with HepTH1-5 and methotrexate at CD_{50} concentration for 24, 48 and 72 hours. The cells were then harvested and centrifuged at 300 g for 10 minutes and subsequently washed with cold PBS three times. A volume of 100 µL binding buffer (annexin V-FITC Apoptosis Detection Kit I, Becton Dickson, USA), 5 µL of Annexin V-FITC and 5 µL PI solutions were added and incubated in the dark for 15 minutes. Following the incubation, 400 µL 1X binding buffer was added to each tube and gently mixed before analysed by flow cytometer (FACS Calibur, Becton Dickson, USA). Approximately 10,000 events were sorted accordingly into viable, early apoptotic, late apoptotic and necrotic cells.

Bioluminescent assay

A time-dependent study of caspase-3/7, -8 and -9 activities was performed in triplicates using assay kits Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 (Promega, Madison, WI) on a white 96-well microplate (Perkin Elmer, USA) . Both cells at a density of 2 x 10^5 were seeded per well and incubated with HepTH1-5 for 12, 24, 48 and 72 hours. Caspase activities were investigated according to the manufacturer protocol. Briefly, 25 µL of caspase-Glo reagent were added in the dark prior to incubation at 37°C supplemented by 5% CO_2 within 4 hours. The presence of active caspases from apoptotic cells cleaved the aminoluciferin-labeled synthetic tetrapeptide thus release substrate in luciferase reaction and production of light. The caspase activities were measured using a Tecan Infinite H200 Pro (Tecan, Männedorf, Switzerland) microplate reader. The intensity of luminescence signals measured in relative light units (RLU) is directly proportional to the caspase activity.

Statistical Analysis

Data were expressed as mean ± standard deviation (S.D.) of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. This was accomplished via the GraphPad Prism, V5.0 (GraphPad, Software, San Diego, CA, USA). * *p*-value < 0.05 was considered statistically significant.

RESULTS

HepTH1–5 inhibits proliferation of cancer cells in dose and time dependent manner

The cytotoxicity of hepcidin TH1-5 was evaluated against cancer cell lines (HepG2, HeLa, HL-60, Chang and NIH/3T3) using tetrazolium dye 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays whereas methotrexate, a commercial anticancer drug was used as positive control. The effects of HepTH1-5 on the cells were shown in Table 1. It was found that, HepTH1-5 consistently displayed strong cytotoxicity effects on HepG2 and HeLa cells at IC_{50} value of 0.86 µM (2 µg/mL) and 11 µM (11.5 µg/mL), respectively. In contrast, no significant cytotoxicity effects of HepTH1-5 on HL-60 cells (IC_{50} of 85.8 µM or 200 µg/mL). Whereas, weaker cytotoxic effect observed against Chang and NIH/3T3 cell lines. As comparison, methotrexate showed notable cytotoxicity effects on HepG2, HeLa and HL-60 cells while showing less effects against Chang's liver and NIH/3T3 cell lines with IC_{50} values of 50.6 µM (23 µg/mL) and 32.1 µM (14.6 µg/mL), respectively.

Table 1: Effects of HepTH1-5 and methotrexate with IC_{50} values on viability of cancer cells; HepG2, HeLa, HL-60, Chang liver and normal cells; NIH/3T3 cell lines at 72 hours treatment.

Cell Lines	IC_{50}			
	HepTH1-5		Methotrexate	
	µM	µg/mL	µM	µg/mL
HepG2	0.86 ± 0.010	2	4.4 ± 0.075	2
Chang	15.02 ± 0.066	35	50.6 ± 0.010	23
HeLa	11 ± 0.118	11.5	10.3 ± 0.005	4.7
NIH/3T3	16.3 ± 0.09	38	32.1 ± 0.029	14.6
HL-60	85.8 ± 0.003	200	1.54 ± 0.006	< 0.7

All data were expressed as mean ± SD (n = 3)

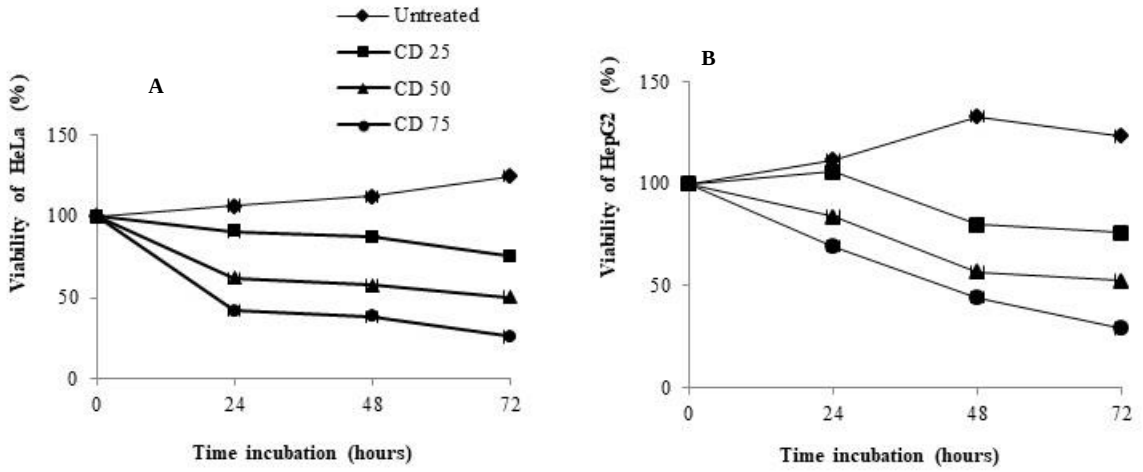


Figure 1: The cell proliferation of HeLa (A) and HepG2 (B) cells after treatment with HepTH1-5 at CD_{25}, CD_{50} and CD_{75} for 24, 48 and 72 hours. Every point represents the mean of triplicate samples and error bars represent the standard deviation.

Following the promising cytotoxicity effects of HepTH1-5 against HepG2 and HeLa cells, the effect of HepTH1-5 on proliferation of these cells were examined at CD_{25}, CD_{50} and CD_{75} concentrations. It was found that the proliferation rate significantly reduced in concentration dependent manner (Figure 1). After incubation at 72 hours, viable cells of HepG2 reduced to about 75.9%, 52.6% and 29.3% at CD_{25}, CD_{50} and CD_{75} concentrations respectively. Meanwhile, viability of HeLa cells were also decreased which is 75.5%, 50.5% and 25.1% at CD_{25}, CD_{50} and CD_{75} concentrations.

The proliferation rates of both untreated cells on the other hand were increased (refer to Figure 1A & 1B), as indicated by the cell viability percentage values from to 111.8% to 123.3% and 106.5% to 124.5% from 24 hours to 72 hours on HepG2 and HeLa cells respectively ($p < 0.05$ with respect to the untreated cells of initial cell concentration). To conclude, HepTH1-5 inhibits the proliferation of studied cells in dose- and time-dependent manner.

HepTH1–5-affects membrane integrity and induced apoptosis in HepG2 and HeLa cells.

HepG2 and HeLa cells treated with HepTH1-5 were double stained with AO/PI. A total of 200 cells were counted and scored based on apoptotic, necrotic and viable cells. Figure 2 shows that after 24 hours treatment, approximately 61.2% and 69.2% of HepG2 and HeLa cells, displayed membrane blebbing and nuclear chromatin condensation as a sign of early apoptosis (EA). Whereas 20.7% and 22.2%, respectively were in late apoptosis (LA) representing by intercalated acridine orange amongst the fragmented DNA. As compared to methotrexate treatment, only 18.2% of cells were in early apoptosis, whereas 76.3% were in late apoptosis.

These trends were continued after 48 hours of

treatment but prolonged incubation of 72 hours saw a dramatic change in the apoptotic effect where less than 12% of HepG2 cells and 10.7% of HeLa cells were at early apoptosis while more than 79.7% of HepG2 and 82.8% of HeLa cell lines were at late apoptosis. Most of cells treated by HepTH1-5 shown membrane blebbing and condensed nuclei within two days incubation and performs in shrinkage phases with reddish-orange colour which indicates AO binding towards denatured DNA, thus confirming late stage apoptosis after 72 hours treatment. On the other hand, there was no statistical significant ($p < 0.05$) difference in necrotic counts which undergone stage of necrosis (NC) at different treatment times (24, 48 and 72 hours) as shown in Figure 3. All data were shown as mean ± SD ($n = 3$).

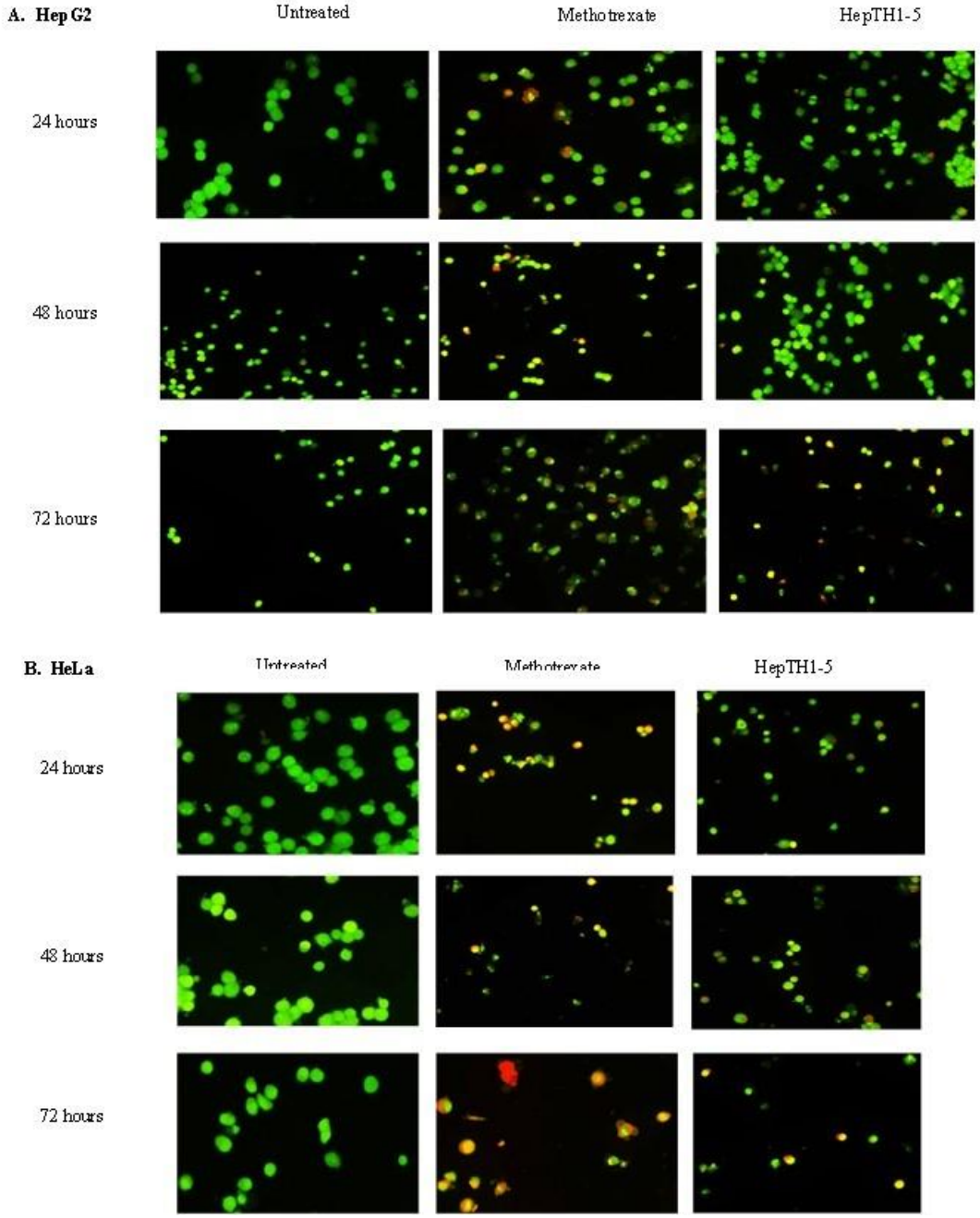


Figure 2: Membrane disruption of (A) HepG2 and (B) HeLa cells after treatment with HepTH1–5 (8.58×10^{-4} µM or 2.0 µg/mL and 0.011 µM or 11.5 µg/mL respectively for 24, 48, and 72 hours followed by incubation without (untreated) or with HepTH1–5 in 200x magnification.

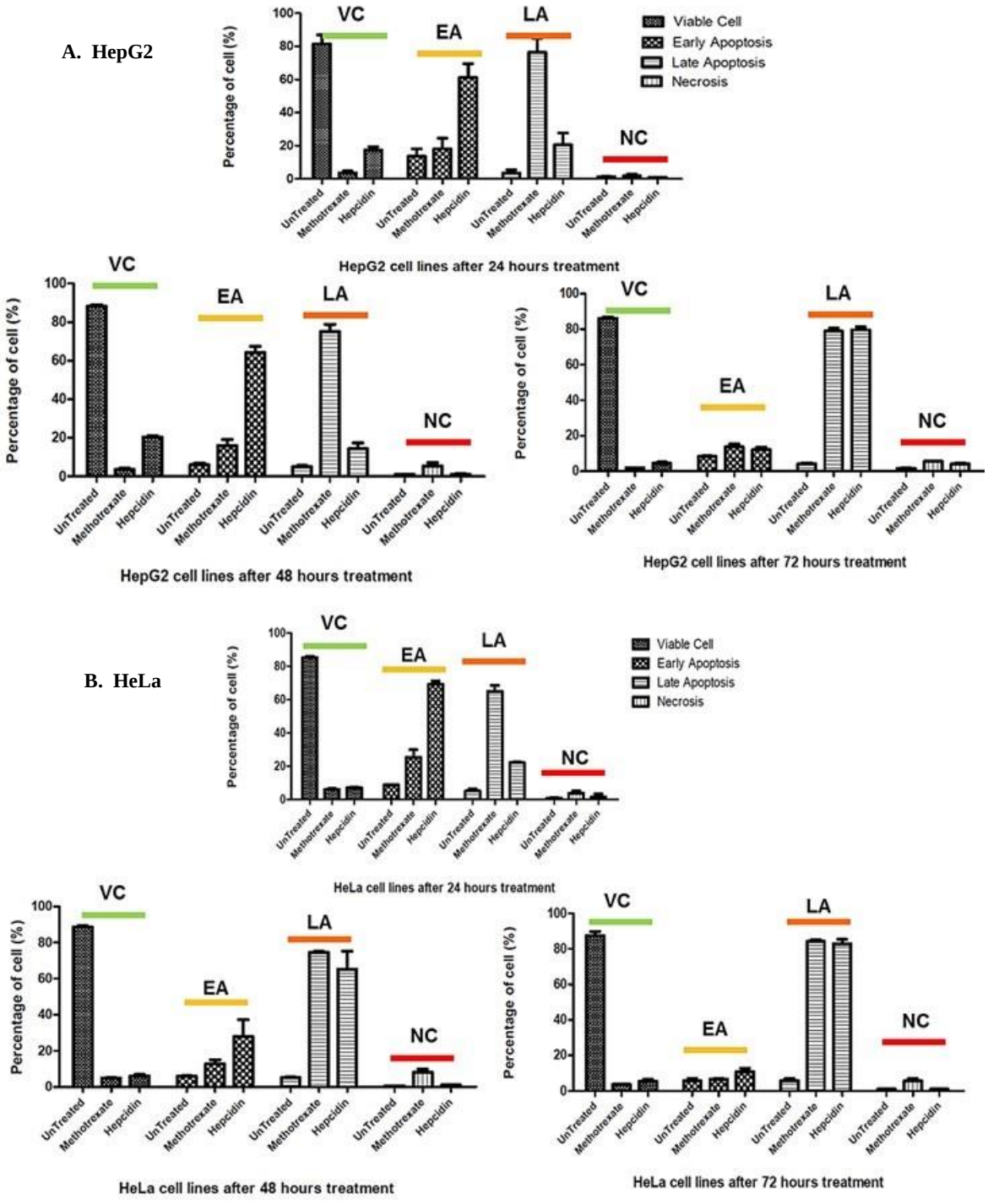
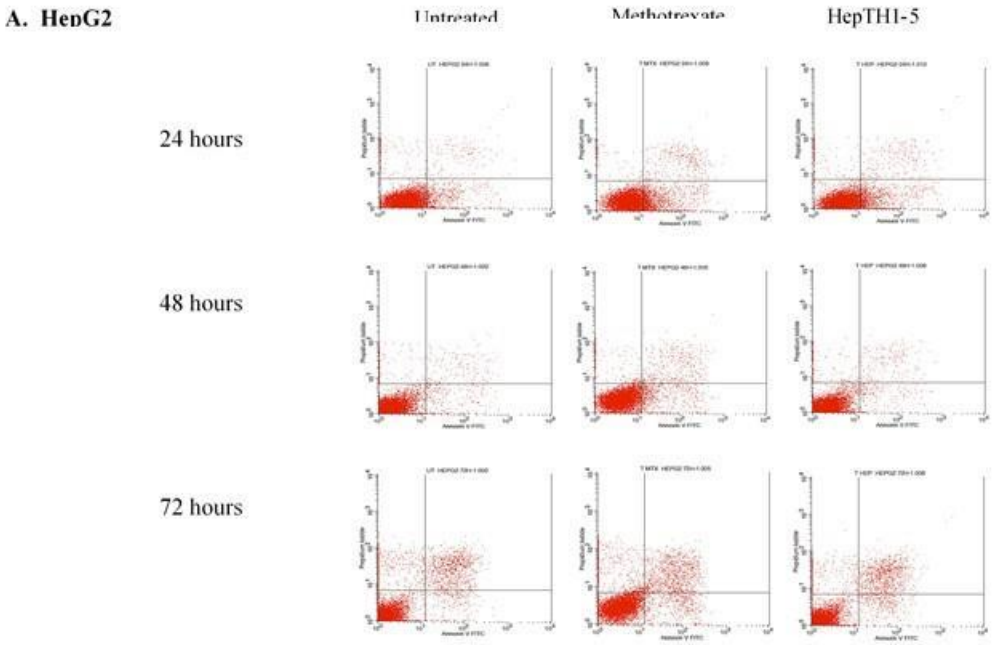


Figure 3: Percentages of viable (VC), apoptotic in early apoptosis (EA) and late apoptosis (LA) and necrotic cells (NC) counted in histogram graph after HepTH1-5 and methotrexate treatments for . HepG2 (A) and HeLa (B) cells.

We further confirmed the apoptosis induction by using Annexin V-FITC and Propidium Iodide (PI) double staining method. In mammalian and yeast cells particularly, phospholipid phosphatidylserines exist in the inner leaflet of the plasma membrane and since phosphatidylserine (PS) translocation also occurs during necrosis, Annexin V-FITC and Propidium Iodide (PI) double staining method is the best alternative to discriminate between apoptotic and necrotic cells whereby an early apoptotic cell is indicated by positive fluorescein isothiocyanate (FITC) that conjugated with Annexin V; protein with high affinity binding for PS and negative PI whilst late apoptotic cells are indicated by both positive stainers (Jaeyong et al. 2012; Tajudin et al. 2012). As shown in Figure 4, early apoptotic population significantly increased to 13.6% and 18.1% in HepG2 and HeLa cells where early stage apoptosis were in 5.01% and 9.1% after 24 hours treatments with HepTH1-5 and methotrexate. These findings might due to PS externalization occur in early apoptosis before the cells undergoes nuclear changes, regardless of the initiator of apoptosis catalyst (Looi et al. 2013). From our observation, early apoptotic population were significantly higher than untreated of HepG2 and HeLa cells at 2.09% and 6.7% with reduction of viable cells at 94.9% and 86.9% in HepTH1-5 and methotrexate after 48 hours incubations. However, late stage apoptosis of methotrexate treatments has been detected in both cells. The occurrence of apoptosis continued in time dependent manner. However, after 72 hours of HepTH1-5 treatment on both cells, the population of late apoptotic cells rose significantly at 19.08% and 15.02%. Similar observation was seen when HepTH1-5 was treated with MCF7 cell lines (Hassan et al. 2016; Mohd et al. 2015). It is worth noted that the number of necrosis positive cells in control and treated cells were insignificant during treatment period. These results prove that treatment of HepTH1-5 on HepG2 and HeLa cells induced apoptosis, with possible translocation of PS from cytoplasm to the transmembrane of both cell lines.



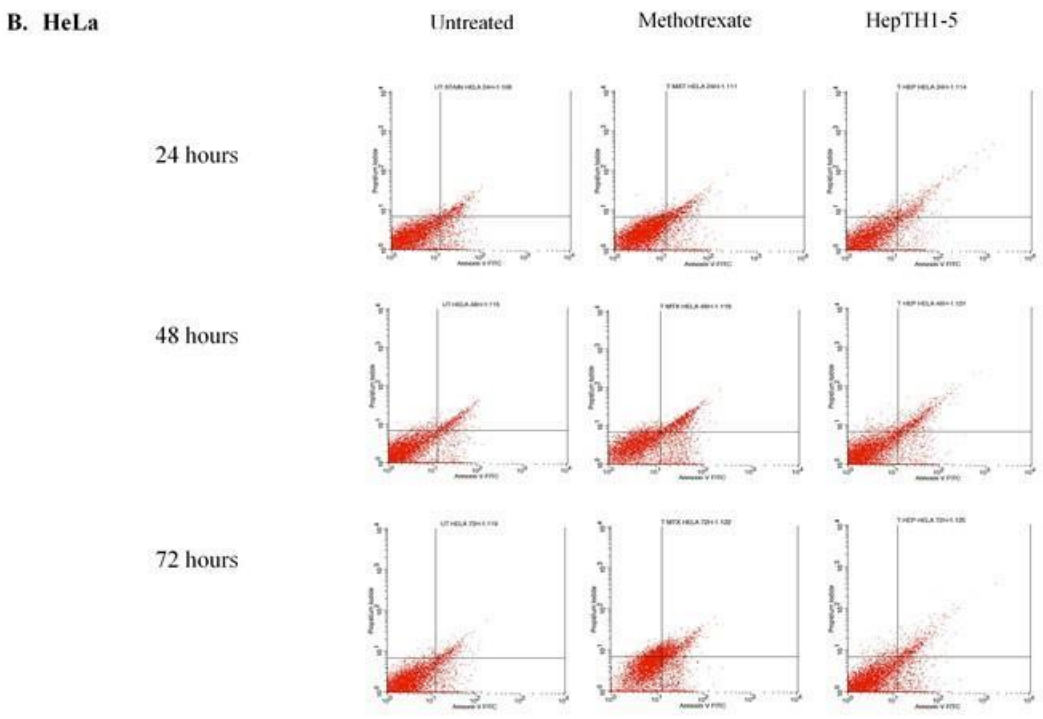


Figure 4: Flow cytometry analysis of HepG2 (A) and HeLa (B) cells depict untreated and treated (HepTH1-5 and methotrexate) stained with annexin V-FITC/propidium iodide (PI). Viable cells were in lower left quadrant, early apoptotic cells were in the lower right quadrant, late apoptotic cells were in the upper right quadrant, and the necrotic cells were in the upper left quadrant. Dot plots were representative of 10,000 cells from a single replicate. All data were expressed as mean ± SD (n = 3).

Effects of HepTH1–5 on G2/M and G0/G1 phase cell cycle arrest in HepG2 and HeLa cells.

To further elucidate the mechanisms of apoptosis induced by HepTH1-5 in HepG2 and HeLa cells, cell cycle analysis were investigated. Cell cycle arrest is a good marker for anticancer activity of drugs or isolated bioactive compounds, which also served as hallmark of apoptosis detection (Jaeyong et al. 2012). Untreated cells showed expected pattern of continuing growth with the highest peak in sub-G1 phase and small peak in G2/M phases. However, after the exposure to 0.86 µM (2 µg/mL) and 11 µM (11.5 µg/mL) for three days, an increase of hypodiploid sub-G0/G1 and sub-G1 DNA accumulation was observed. It was found that HepTH1-5 was able to induce apoptosis in HepG2 and HeLa cells respectively in a time-dependent manner as well as simultaneously induce cell cycle arrest at G2/M and S phases. It has been reported that the G2/M arrest triggers possible phosphorylation of apoptosis-associated proteins in the mitotic phase of cell cycle, which further explains the involvement of G2/M arrest to be associated with apoptosis (Hirt et al. 2000). As demonstrated in Figure 5 and 6, methotrexate induced a marked apoptosis and cause both cells arrest mainly at sub-G1 phases accounted for 56.7% of HepG2 and 72.3% of HeLa cells after 72 hours treatments. This finding (cell cycle delay in HepG2 cells) was not reported in previous study (Chang et al. 2010) where only the event of cell cycle delay on HeLa cells was reported.

HepTH1-5 activates caspase cascades in HepG2 and HeLa cells

The complex of apoptosis activity involves a number of molecules and is classified into by caspase-dependent or caspase independent mechanisms (Looi et al. 2013). Nonapoptotic or necrotic modes of death occurs independent of caspase activation (Hirt et al. 2000). In contrast, caspase-dependent mechanism describes an important role of caspase family in the mediation

of apoptotic progress which can be further divided into extrinsic or intrinsic pathway, as determined by involvement of caspase-8 or caspase-9, respectively.

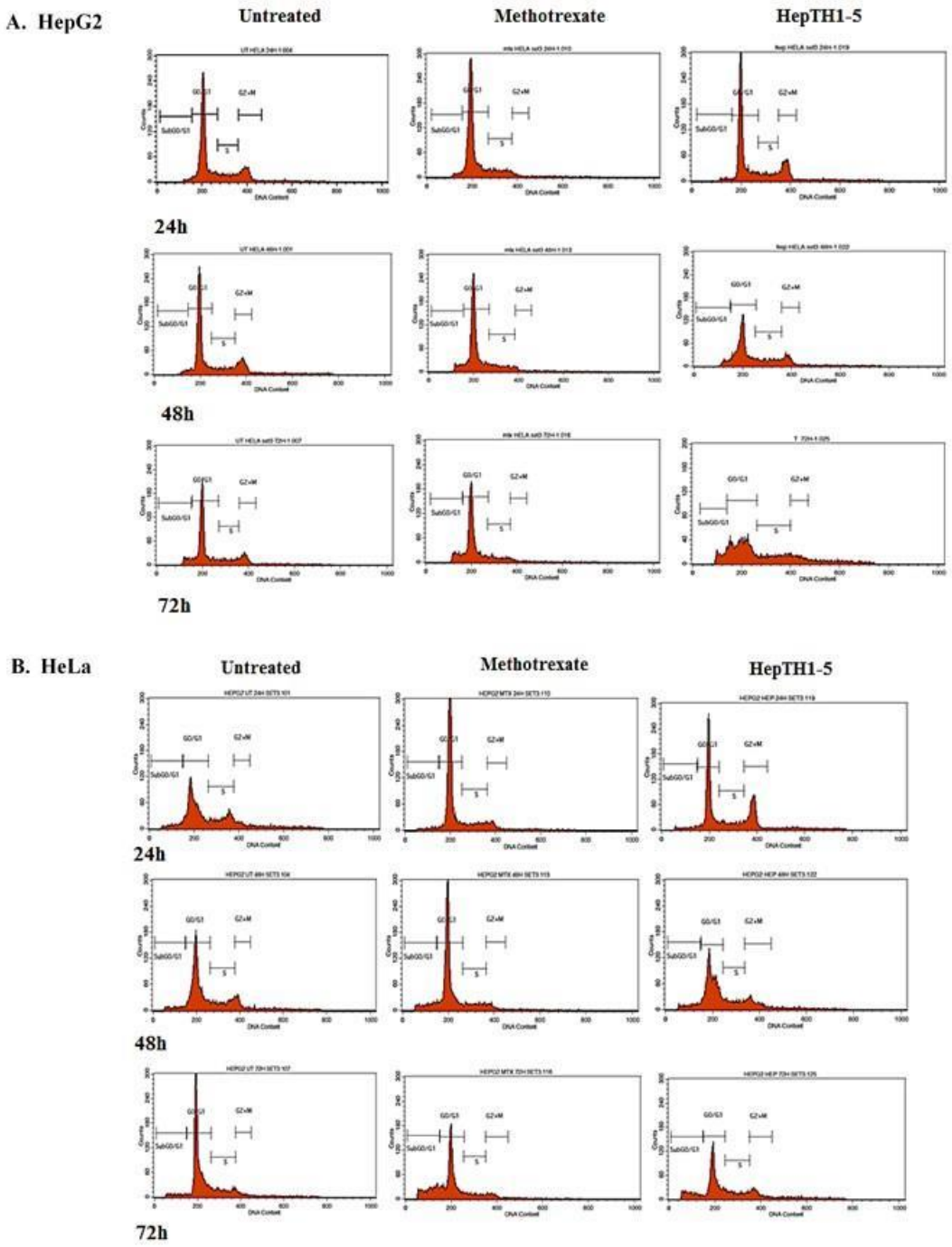


Figure 5: Cell cycle distribution of HepG2 (A) and HeLa (B) cells at 24-72 hours treatment when treated with methotrexate and HepTH1-5.

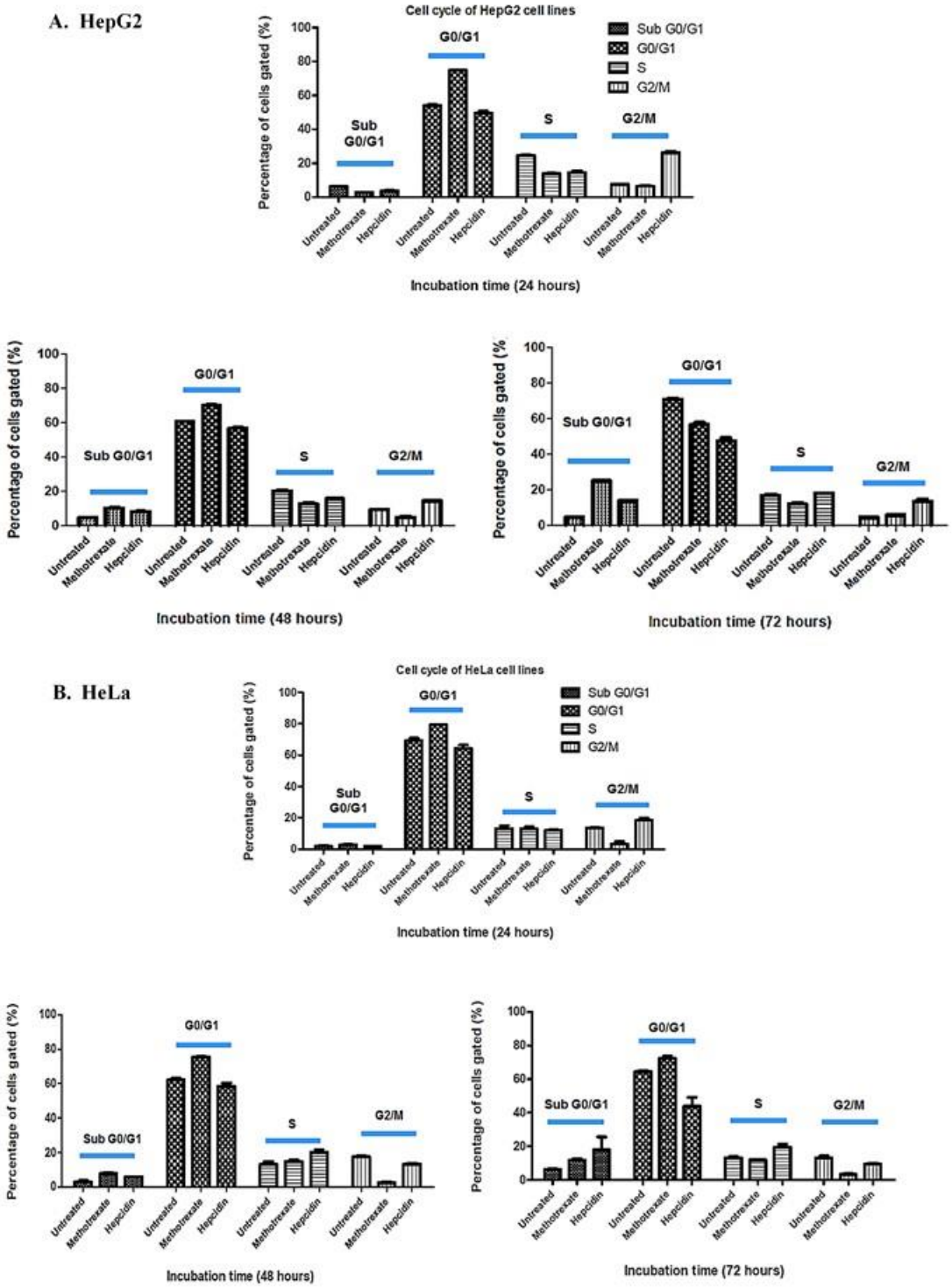
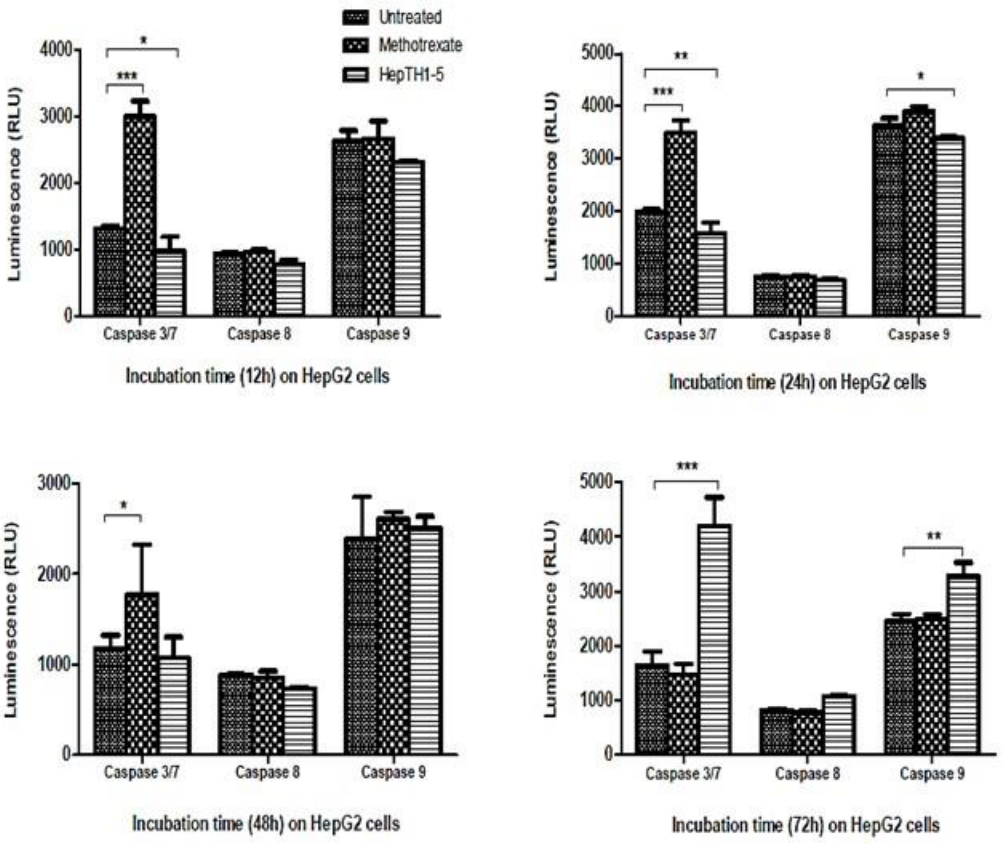


Figure 6: Cell cycle of HepG2 (A) and HeLa (B) cells presented in percentages of cell gated which represents data in mean ± SD. (n = 3).

Both pathway involved activation of caspase-3/7 which is important for inducing downstream DNA cleavage molecules (Looi et al. 2013; Dumont et al. 2000).

We therefore evaluated the effect of HepTH1-5 treatments in caspase 3/7, -8 and -9 activation towards HepG2 and HeLa cells by measuring the luminescence (RLU) intensities every 12 hours and 24 hours. As shown in Figure 7, HepTH1-5 and methotrexate activated caspases-3/7, -8 and -9 in HepG2 and HeLa cells begin at 12 hours. The activity of caspase-9 and -3/7 increase significantly in both cells from 24 to 48 hours but remained high even after 72 hours of HepTH1-5 and methotrexate treatments. These circumstances indicate that both treatments gave high latent effect of apoptosis induction in HepG2 and HeLa cells. However, there was no significant changes in the activity of caspase-8 for the time span of 72 hours in both treatments. Our data suggested that HepTH1-5 and methotrexate induced activation of intrinsic caspase pathway in HepG2 and HeLa cell lines.

A. HepG2



B. HeLa

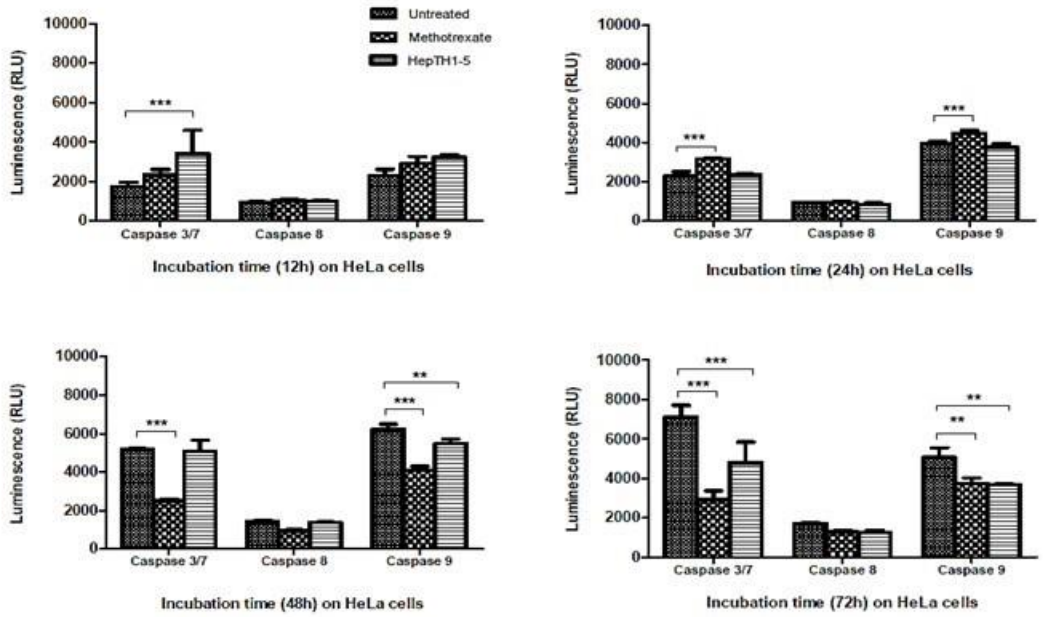


Figure 7: Effects of HepTH1-5 and methotrexate on caspase activation of HepG2 (A) and HeLa (B). Results are expressed as mean ± SD of three replicate experiments. Data were analysed by one-way ANOVA and statistical significance was observed *p <0.05.

DISCUSSION

The cytotoxicity activity of HepTH1-5 against cancer and normal cells were evaluated. Different cell lines responded to the treatment differently, which exhibited cytotoxicity against cancer cells in order of HepG2 > HeLa > Chang's liver > HL-60. Chang's liver cells showed less toxicity as compared to HepG2. This could be due to the facts that Chang's liver cells were not derived from malignant tissue (Schuurman et al. 1961). Intriguingly, it showed less toxicity against normal cells NIH/3T3. As comparison to standard drug treatment, methotrexate, it was effectively cytotoxic to HL-60 cells compared to HepTH1-5. This data indicates that HepTH1-5 was more selective in its action. This kind of cytoselectivity may reduce a side effect which commonly occurred during chemotherapy (Jia et al. 2012).

We have also monitored proliferation activity of HeLa and HepG2 cells treated with HepTH1-5. It was found that HepTH1-5 inhibits HeLa and HepG2 cell proliferation in time and dose-dependent manner. Close examination on treated cells showed evidence of apoptosis. Apoptotic cells share a number of common features such as cell shrinkage, nuclear condensation, membrane blebbing, chromatin cleavage and formation of pyknotic bodies of condensed chromatin (Doonan & Cotter, 2007). Although certain integrity of plasma membrane of apoptotic cells is still intact but morphology of the cells had changed with blebbing plasma membrane and condensed nuclear chromatin (Darzynkiewicz et al. 1997). The apoptogenic features of HepTH1-5 treated cells were documented in acridine orange/propidium iodide double staining. The HepTH1-5 treated cells might having disrupted membrane integrity through holes forming and subsequently trigger the apoptosis events in cancer cell death. Membrane-active AMPs are potential cancer therapies due to low risk for induction of resistance (Liu et al. 2011; Hassan et al. 2016; Mohd et al. 2015). The electrostatic interaction between the peptide's positive charges and the membrane's negative charges contribute to the binding of AMPs to the cell membrane, which crucial for the cytotoxicity of this peptide in cancer cells (Hoskin & Ramamoorthy, 2007; Mohd et al. 2015), especially the malignant one. Chen and co-workers reported that TH1-5 that belongs to the tilapia hepcidin family have functions like a lytic peptide as cecropin family (Silvestro et al. 2000) which targets non-polar lipid cell membranes resulting in formation of ion-permeable channels which leads to an outflow of

cell contents and ultimately death of target cells (Chen et al. 2009).

In this study, we also found that substantial number of cells at an early apoptosis in HepTH1-5-treated cells. In contrast to methotrexate-treated cells, whereas the cells at late apoptosis as early as after 24 hours treatment. In the early apoptosis, cells still retain membrane integrity with chromatin super aggregation (highly condensed chromatin). Meanwhile, at the late apoptosis, cell membranes were compromised with condensed and lumping chromatin. The increase of the percentage from early to late apoptotic cells after prolonged incubation with HepTH1-5 might due to synergistic activity of double staining that made PS translocated from the inner leaflet of the plasma membrane to the outer leaflet without damaging plasma membrane permeability (Madeo et al. 1997; Hassan et al. 2015). The differences in apoptotic execution between HepTH1-5 and methotrexate seen in this study indicate that both molecules have a different mechanism of action. Our earlier studies also shown similar findings (Hassan et al. 2016; Hassan et al. 2015)

Cell cycle analysis was carried out to determine the effects of HepTH1-5 treatment on cell cycle. Cell cycle study revealed that G2/M phase is a dominant phase in cells treated with HepTH1-5 in a time-dependent manner with late apoptosis. In contrast, G0/G1 phase is dominant in cells treated with methotrexate. This finding indicates that at range of concentration studied, the cytotoxicity and anti-proliferative effect of HepTH1-5 on HepG2 and HeLa could be attributed primarily to the induction of G2/M arrest, with less contribution of cell division rather that DNA synthesis. This observation corroborated with the studies by the Acadeia Sinica institute (Huang et al. 2011; Lin et al. 2009, Kuo et al. 2018; Wu et al. 2012), where the tested several small cystein-rich marine antimicrobial peptide against several cancer cell lines.

The evidence of apoptosis induction by HepTH1-5 was further shown by caspase activation analysis. Caspases are implicated in the degradation of cytoskeleton causing fragmentation of cells into apoptotic body. Caspase-3 has a vital role in apoptosis. It starts with initiating of a cascade of events involving the release of cytochrome c, which then binds to apoptotic protease activating factor-1 (Apaf-1), which will triggers the activation of caspase-9. Activation of caspase-3 may also follow the extrinsic pathway involving FAS ligand binding to a surface cell receptor, which activates caspase-8. In present study, we observed that HepTH1-5 causes a time-dependent activation of caspase-9, while caspase-8 activities remained at basal level. The increase in caspase-9 activity was associated with the increase in caspase-3/7 activity, indicating that mitochondria membrane permeabilization had occur by releasing cytochrome c which subsequently induced apoptosis. In a study on MSP, an marine antimicrobial peptide against human osteosarcoma where he peptide triggers FAS ligand binding with its receptor and activates caspase-8 protein (Kuo et al. 2018). Data from our work suggest that HepTH1-5 induced apoptosis via mitochondrial- dependent intrinsic pathway.

CONCLUSION

The finding from this study supported earlier report on the notion of cytotoxicity property of HepTH1-5. It had shown that HepTH1-5 exerted a cytotoxic effect on HepG2 and HeLa cells, but not effective against Chang's liver and HL-60 cells. It is not toxic to non-cancer 3T3/NIH cells. Apoptosis induction was shown by morphological observation and flow cytometry analysis on stained cells. Intrinsic pathway might be involved in the apoptosis induced in HepG2 and HeLa, as shown by caspase activity. Taking together, the current findings permit further work on HepTH1-5 as a novel chemotherapeutic agent.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

WA, AMA and KSM contributed to the conception and design of the study. KSM responsible for the designing and approved the apoptosis analysis. WA was responsible for all the experiments. KSM was contributed in revising and approved this paper.

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