ORIGINAL ARTICLE

Cytotoxic Effect of 2,6-bis(4-Hydroxy-3-Methoxybenzylidene) cyclohexanone (BHMC) and Curcumin on Human Liver Cancer Cells, HepG2

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ABSTRACT

Introduction: Curcumin is an active constituent derived from turmeric with a variety of pharmacological activities. It suppressed cell proliferation and induced apoptosis in several cancer cell lines. However, due to its poor bioavailability, derivative analogue of curcumin has been synthesized to enhance the drug-like effects. BHMC was synthesized by removing β-diketone moiety from curcumin structure and modify it into conjugated double bonds. It has been proved to exhibit stronger anticancer effects with improved bioavailability compared to curcumin. Objective: This study aims to investigate the toxicity effect of BHMC and curcumin on human liver cancer, HepG2 and non-cancer mouse fibroblast, 3T3. **Methods:** Both cell lines were purchased from ATCC and cultured in supplemented DMEM. Cell viability was determined via MTT assay and confirmed with trypan blue assay. Morphology hallmarks of apoptosis of both treated cells were analyzed using inverted microscope at 40X magnifications. **Results:** BHMC and curcumin were very potent towards HepG2 and normal 3T3. These data were further confirmed with trypan blue assay which showed that both compounds significantly reduced the percentage of HepG2 and 3T3 cells viability. Both treated cells also displayed all the morphology hallmarks of apoptosis upon treatment. **Conclusion:** BHMC has a greater cytotoxicity effect on HepG2 compared to curcumin despite its non-selective cytotoxicity effect on non-cancer 3T3.

Keywords: BHMC, curcumin, HepG2 cells, 3T3 cells, Apoptosis

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INTRODUCTION

Cancer is defined as a set of diseases characterized by upregulated cell growth, invasion to surrounding tissues and metastasis to other parts of the body. Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and third leading cause of cancer death worldwide (2). It is reported that HCC usually associated with chronic liver infection with 80% of HCC is caused by hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as other important risk factors such as liver cirrhosis from excessive alcohol consumption, genetic liver disease, primary hemochromatosis and also including exposure to dietary carcinogens especially aflatoxin (1). Thus, since HCC is an aggressive tumour that associated with poor prognosis, chemotherapy plays crucial role in this treatment especially in HCC patients with advance stages when other options of treatments like resection and liver transplantation are inapplicable (6). Sorafenib is a standard chemotherapeutic agent for patients with very advanced HCC. However, it only provides limited survival advantage with wide profile of adverse effects and toxic manifestations such as thrombocytopenia, hand foot syndrome and mucositis (3). Therefore, due to the limited therapeutic applications of HCC treatments along with the development of drug resistance, novel strategies were undertaken by great deal of research focusing more on the bioactive compound as an alternative for liver cancer therapy.

Many chemotherapeutic agents are phytochemicals, the secondary metabolites that are naturally found in plants with protective or preventive properties (4). This natural dietary and polyphenolic compounds showed to have various biological activities that target specific pathways and enzymes and effectively fight against carcinogenesis. Curcumin (diferuloyl-methane) is one of the polyphenolic compounds derived from turmeric (*Curcuma longa*). It has been shown to have wide variety of pharmacological activities such as antioxidant, antimicrobial, anti-inflammation and anticancer properties (16). Besides, it has also been shown to regulate a diverse array of cellular signaling pathways,

gene expression, various signaling molecules and can also act as inhibitor of transcription factor nuclear factor-kappa B (NF-κB), downstream gene products, as well as inducible enzyme activity (26). Numerous *in vitro* studies indicated that curcumin capable to suppress cancer cell proliferation and promote apoptosis as well as cell-cycle arrest via modulating two crucial tumour cell survival pathways: NF-κB and protein kinase B (Akt) (3). Although curcumin is remarkably non-toxic and has promising anti-cancer activities, preclinical and clinical studies indicate that curcumin has one major limitation in which it has poor bioavailability and pharmacokinetic profiles (28). Previous studies have also demonstrated lower serum and tissue levels of curcumin irrespective of route of administration, rapid metabolism and elimination as the major factors curtailing curcumin bioavailability. These had been observed occurred in both animals and humans (11, 24) and it is due to its hydrophobic property in nature (14). Moreover, there are two major *in vitro* stability issues that complicate its use as a pharmaceutical that are oxidative degradation and modification and solvolysis (11). Thus, there are attempts to improve the solubility of hydrophobic curcumin and increased its bioavailability (24).

Numerous synthetic derivative analogue of curcumin has been synthesized in order to improve the bioavailability of curcumin as well as enhancing the anti-tumour activities that have a safety profile similar to curcumin. 2,6-bis-4-(hydroxyl-3-methoxy-benzylidine)-cyclohexanone (BHMC) is one of mono-carbonyl curcumin analogue synthesized based on the chemical structure of curcumin by removing the unstable β-diketone moiety and modifying into conjugated double bonds while preserving the hydroxyl (OH) group (23). The presence of β-diketone moiety causes curcumin to have low bioavailability since it can be rapidly metabolized by aldo-ketoreductase in liver which limited the potential therapeutic effect of curcumin on many types of diseases (13). The difference in the structure of BHMC allows it to be more selective in suppressing various inflammatory mediators as well as demonstrated greater effects of anti-ulcerogenic and anticancer activities compared to curcumin (23, 25). It has also been reported that BHMC was more potent towards breast cancer cell lines MDA-MB-231, MCF-7 and SKBr-3 with low toxicity profile in normal cells (4). In mouse 4T1 triple negative breast cancer (TNBC) cell model, BHMC demonstrated better cytotoxicity effect *in vivo* compared to curcumin via suppression of inflammation, proliferation and metastasis (23). Thus, this project aims to investigate the cytotoxicity effect of BHMC and curcumin on human hepatocellular carcinoma, HepG2.

MATERIALS AND METHODS

Chemical and reagents

Dulbecco's Modified Eagle Medium (DMEM) (4.5G/L Glucose) with L-Gln and sodium pyruvate (Nacalai), penicillin and streptomycin, trypsin EDTA were purchased from PAA Laboratories GmBH (Pasching, Austria) and phosphate buffered saline (PBS) (Oxoid) was purchased from ATOZ Scientific (Japan). Dimethylsulfoxide (DMSO), MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethanol absolute denatured, foetal bovine serum (FBS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). BHMC was 99.9% pure as determined by HPLC meanwhile curcumin was purchased from Sigma Chemical Co. (St. Louis, MO). Curcumin and BHMC were first dissolved in filtered 100% DMSO as stock at 50μM and 100μM, respectively and diluted to appropriate concentration for assays. The final concentration of DMSO in all assays was kept constant at 0.1%.

Cell lines

Human hepatocellular carcinoma cell, HepG2 and non-cancer mouse fibroblast cell, 3T3 were purchased from American Type Culture Collection (ATCC), USA. The cells were cultured in complete DMEM medium and incubated at 37°C in a 5% CO_2 incubator.

Growth inhibition assay

Cytotoxicity activity of BHMC and curcumin was determined using MTT assay in accordance to Danihelova et al. (7) with some modifications. MTT assay involved measuring the metabolism of (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to form an insoluble formazan precipitate by mitochondria dehydrogenase which is specifically present in viable cells. Cells at 70-80% of confluency were harvested with trypsin-EDTA. Initially, 100μL of HepG2 and 3T3 cell suspension of 1 x 10^5 (cells/mL) was seeded in triplicate of 96-well plate. The cells were incubated overnight at 37°C with 5% CO_2 and treated with various concentrations of BHMC and curcumin (0, 0.78, 1.563, 3.125, 6.25, 12.5, 25 and 50 μM). The treated cells were then incubated again at 37°C in a 5% CO_2 humidified incubator for 24, 48 and 72 hours. After 24 hours, 20μl of MTT solution (5mg/mL) in 1x PBS was added to the medium of each well and the plate was left for incubation at 37°C for 4 hours. The absorbance at 570 nm and the reference wavelength of 630 nm were measured with a microplate reader (Opsys MR, USA).

Trypan blue exclusion assay

Trypan blue exclusion assays were perform according to Gevrenova et al. (8) with some modifications. HepG2 and 3T3 (1.5x 10^5cells/mL) were treated with various concentration of curcumin (25 and 50 μM) and BHMC (5, 10 and 15 μM) in 6-well plates with untreated as a control. The cells were incubated at 37°C and 5% CO_2 for 24, 48 and 72 hours. After each incubation time, the existing medium was collected into centrifuged tube and trypsin-EDTA (1 mL) was added into each well. The plate was incubated at 37°C for 10 minutes. Following that, the collected medium was poured into the respective well. Subsequently, 10 μL of trypan blue

dye solution was mixed with equal volume of the cell suspension. The viable and dead cells were counted under an inverted microscope and tabulated. The test was performed in a laminar flow hood. The percentage of the cell viability was calculated with the following formula:

$$\text{Cell viability (\%)}: \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained n unstained)}} \times 100$$

Cell morphology

Based on previous method by Ng *et al*. (22) with some modifications, morphological changes of treated cells were observed under inverted microscope. Both HepG2 and 3T3 were treated with various concentrations of curcumin (25 and 50 µM) and BHMC (5, 10 and 15 µM) in 6-well plate with untreated as a control. Cells were incubated at 37°C and 5% CO_2 for 24 hours. Morphological changes of treated cells were viewed under inverted microscope.

Data analysis

All data was analyzed using the software package Prism GraphPad Programme (GraphPad Software). Error bars represent ± the standard error of the mean (S.E.M.) for the data set. Comparisons within groups of data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett post hoc test using Statistical Package for Social Science (SPSS) version 21.0. The probability of $p < 0.05$ was considered statistically significant.

RESULTS

Cytotoxicity effect of BHMC and curcumin on HepG2 and 3T3 via MTT assay

Both HepG2 and 3T3 were treated with different concentration of BHMC and curcumin (0, 0.78, 1.563, 3.125, 6.25, 12.5, 25 and 50 µM). Data obtained for both compounds following three incubation periods (24, 48 and 72 hours) was used to calculate IC_{50} values (Table I). Each value is the mean ± S.E.M of three independent experiments. Results from this study showed that both compounds exhibited a concentration- and time-dependent anti-proliferative profile in both cell lines. Table I shows the IC_{50} values of BHMC and curcumin as tested in HepG2 and 3T3 at different time incubation. The result indicated that both compounds exhibited cytotoxicity effect on both cancer and non-cancerous cells. Although curcumin appeared to be most toxic towards HepG2 after 72 hours of incubation compared to 24 and 48 hours, BHMC was found to be more toxic towards HepG2 compared to curcumin. BHMC was also appeared to be most potent towards 3T3 with lower IC_{50} value at all incubation time (IC_{50}~ 3µM).

Table I: Cytotoxic effects of BHMC and curcumin on HepG2 and 3T3 reflected by the IC_{50} values as determined by MTT assay.

Incubation time (hours)	IC_{50} (µM)			
	HepG2		3T3	
	BHMC	Curcumin	BHMC	Curcumin
24	16.85 ± 2.49	46.13 ± 0.254	5.50 ± 0.211	35.32 ± 6.27
48	4.97 ± 1.47	26.30 ± 2.76	3.03 ± 0.413	17.67 ± 1.88
72	2.73 ± 0.759	17.93 ± 1.97	3.05 ± 0.446	18.37 ± 1.87

Cytotoxicity effect of BHMC and curcumin on HepG2 and 3T3 via trypan blue assay.

To further confirm the toxicity of BHMC and curcumin on HepG2, trypan blue exclusion assay was performed to measure the viability and toxicity of cells after treated with various concentrations. The assay is based on the basic principle that viable cells do not take up blue dyes, while dead cells do. Only three concentrations (5, 10 and 15 µM) of BHMC and two concentrations of curcumin (25 and 50 µM) were selected based on the IC_{50} values obtained from MTT assay. 3T3 was included to measure the selective cytotoxicity of both compounds. Total number of viable cells were counted using haemocytometer where the cell suspension is mixed with trypan blue solution depending on the time incubation (24, 48 and 72 hours). Fig.1 showed that BHMC significantly reduced more than 40% of HepG2 viability ($p<0.001$) when treated with selected concentrations for 24 hours. More cell populations decreased gradually with increasing concentration and time points. Similar toxicity effects of BHMC were also observed on 3T3 with over 40% of cell death ($p<0.01$)

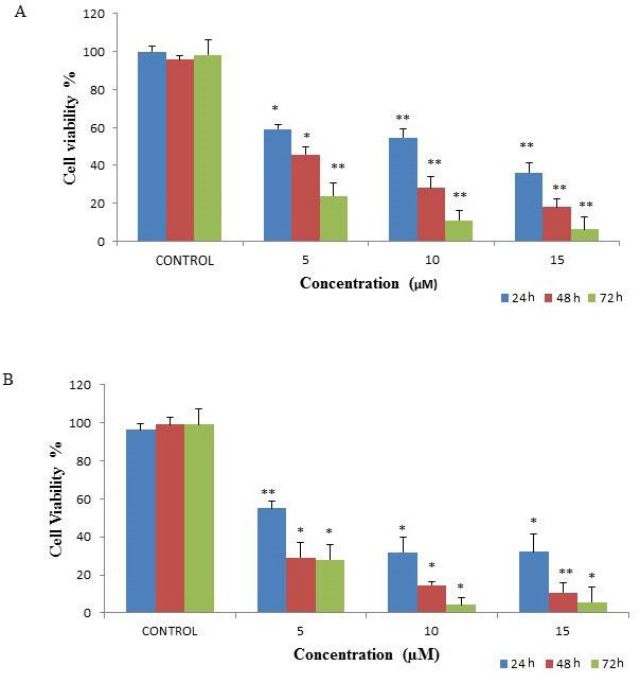

Figure 1: Effect of BHMC on (A) HepG2 and (B) 3T3 cells viability at 24, 48 and 72 hours. Both cells were counted by using a haemocytometer and the percent cell viability was measured using the trypan blue exclusion method. Data are presented as mean ±S.E.M. and represent of two independent experiments. Statistically significant differences are indicated (*$p<0.01$; **$p< 0.001$).

recorded with increasing concentrations and incubation periods. Meanwhile, Fig. 2 showed that both curcumin treated HepG2 and 3T3 cells has reduced cell viability by approximately 20% ($p<0.001$) after 24 hours treatment, and 30%-90% ($p<0.001$) after 48 and 72 hours treatment compared to control. Therefore, this confirms the toxicity effects of both BHMC and curcumin on HepG2 as well as non-cancer 3T3 obtained from MTT assay.

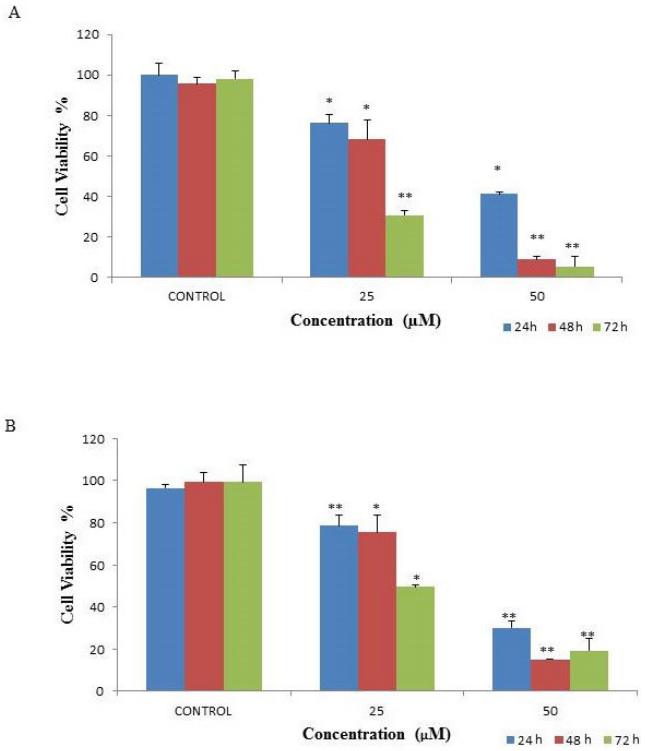

Figure 2: Effect of curcumin on (A) HepG2 and (B) 3T3 cells viability at 24, 48 and 72 hours. Both cells were counted by using a haemocytometer and the percent cell viability was measured using the trypan blue exclusion method. Data are presented as mean ±S.E.M. and represent of two independent experiments. Statistically significant differences are indicated (*$p<0.01$; **$p< 0.001$).

Morphological changes of HepG2 and 3T3 upon treatment with BHMC

Treatment with several concentrations of BHMC showed significant morphological changes in both HepG2 and 3T3 compared to control after 24 hours incubation. At higher concentrations, more cells underwent membrane blebbing followed by apoptotic bodies. Cells population decreased with increasing concentration of BHMC. Similarly, 3T3 also demonstrated morphological changes upon treated with three different concentrations of BHMC (Fig. 3). Cells morphology was observed using an inverted microscope.

Morphological changes of HepG2 and 3T3 upon treatment with curcumin

Treatment with 25µM of curcumin showed significant morphological changes in both HepG2 and 3T3 compared to control after 24 hours incubation (Fig. 4). At higher concentration of 50µM, membrane blebbing started to form with some cells forming apoptotic bodies. Similarly, 3T3 also demonstrated morphological

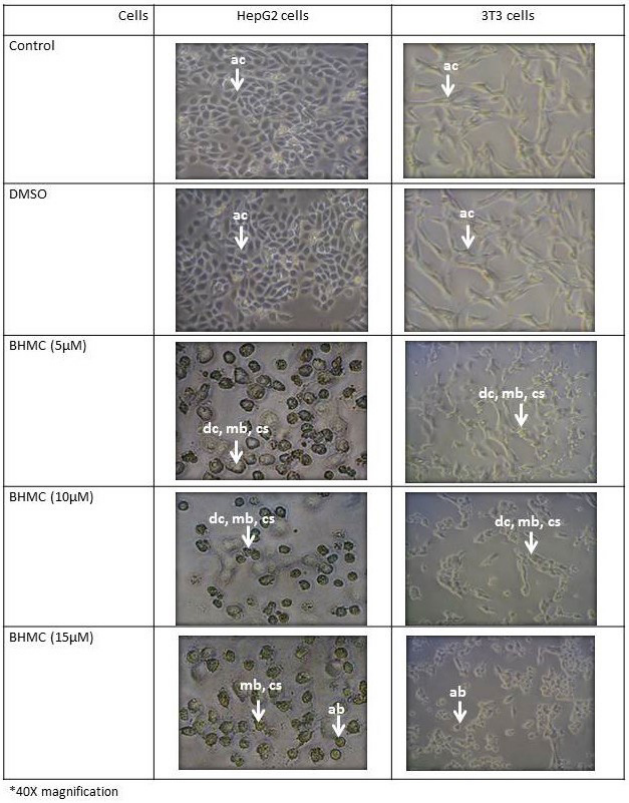

Figure 3: Morphological changes of BHMC-treated HepG2 and 3T3 observed under an inverted light microscope (40X magnifications). Cell population decreased with the increase in the compound concentration. Both treated cells showed the apoptotic features such as cellular detached cell (dc), cell shrinkage (cs) and membrane blebbing (mb) and formation of apoptotic bodies (ab). Healthy cells remained attached (ac) to the surface of the flask.

changes upon treated with both 25 and 50 µM of curcumin. DMSO was included as negative control. Cells morphology was observed using an inverted microscope.

DISCUSSION

Curcumin is a bioactive compound derived from *Curcuma longa* and has been acknowledged to have potential pharmacological activities. At cellular levels, curcumin exhibited anti-apoptotic activity on a variety of cancer cell lines such as human colon cancer (17), colorectal (12), breast (16) and prostate (27). In certain cell culture systems, curcumin possesses anti-microbial, anti-fungal as well as anti-viral activities. It also demonstrated anti-nociceptive activity in ganglion neurons, anti-parasitic, anti-malarial and anti-oxidant properties in blood plasma and platelets as well as in numerous cell lines (9). Meanwhile, at molecular level, curcumin is able to modulate the expressions of various proteins such as inflammatory cytokines and enzymes, transcription factors and gene-products that linked with cell survivals and proliferation (19). However, due to its poor solubility, instability and interference in several modes of assay *in vitro*, its efficacy has been improved through some chemical modification producing several analogues to maximize its antitumor effect (23). These synthetic ana-

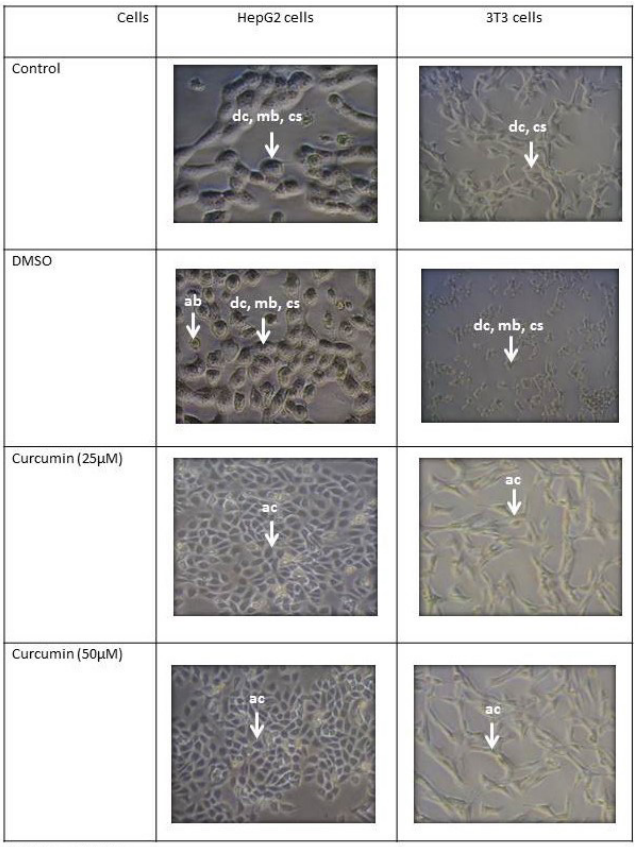

Figure 4: Morphological changes of curcumin-treated HepG2 and 3T3 observed under an inverted light microscope (40X magnifications). Cell population decreased with the increase in compound concentration. Both treated cells showed the apoptotic features such as cellular detached cell (dc), cell shrinkage (cs) and membrane blebbing (mb) and formation of apoptotic bodies (ab). Healthy cells remained attached (ac) to the surface of the flask.

logues of curcumin is claimed to possess similar safety profile as parent compound as well as improved bioavailability (28).

One of the curcumin's derivative is known as 2,6-bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone or BHMC has been synthesized with mono cyclic ketone replacing the diketone moiety of curcumin (21). It possesses α,β-unsaturated bis-enone system which is important to enhance several biological effects including anticancer as in chalcones and bis-chalcones (20). Numerous studies had reported the anti-nociceptive effect of BHMC on mice is triggered through inhibition of various inflammatory mediators (18, 25). It also demonstrated anti-metastasis effect *in vivo* using mouse model 4T1 with lower tumour burden, less mitotic cells in the tumour and lung metastasis upon treatment with BHMC (23). Meanwhile, anti-proliferative effect of BHMC was demonstrated in *in vitro* models using several cancer cell lines. Comparatively, BHMC has greater cytotoxic effect on human breast cancer cells, MCF-7 and MDA-MB-231 than curcumin. It inhibits the signaling pathway that consists of NF-kB, activator protein (AP-1) and mitogen-activated protein kinase (MAPK). It also reduced the expression of matrix-metalloproteinase-9 (MMP-9) that is related to cell migration and invasion as well as membrane type 1 matrix metalloproteinase (MT1-MMP) that is associated with the degradation of the matrix in invadopodia formation (10). Results from this study demonstrated that curcumin and its analogue, BHMC were able to exhibit cytotoxicity effect against HepG2 cells. However, BHMC exhibited lower cumulative IC_{50} value for HepG2 compared to curcumin (Table I). These inhibitory effects were in time- and concentration- dependent manner. Toxicity effects of curcumin and BHMC has been further confirmed via trypan blue assay which indicated that BHMC was more cytotoxic towards HepG2 compared to curcumin.

Despite being a very potent antitumor compound, comparative toxicology study has been demonstrated that curcumin and its analogues exhibit lowest toxicity in normal human hepatocytes, rat hepatocytes and human fibroblast *in vitro* (3). However, in this study both curcumin and BHMC exerted their toxicity effect towards the non-cancer 3T3 based on the reduced number of cell viability and low IC_{50} values. Although the exact mechanism has not been fully elucidated, it is indeed necessary to evaluate and verify the toxicity effect of BHMC on several other normal cell lines as well as *in vivo* study (28).

Morphologically, BHMC- and curcumin-treated HepG2 at respected concentrations has a rounded up morphology, detached from the surface of the flask and shrunk. Fig. 3 and 4 showed that BHMC had a greater cytotoxic effect compared to curcumin towards HepG2 based on the present of more cells shrinkage as well as a decrease in the appearance of viable cells. The treated cells were deformed and demonstrated morphologic hallmark of apoptosis such as nuclear compaction, cytoplasmic constriction and reduction in cell volume. They also demonstrated membrane blebbing, nuclear condensation and apoptotic bodies which were later being phagocytosed by neighboring cells such as macrophages and parenchymal cells. This process is important to control abnormal growth of cancer cells. However, our qualitative results of curcumin-treated HepG2 demonstrated less cells with apoptotic features compared to BHMC. Although the exact mechanism underlying BHMC's anti-proliferative effect is not been fully elucidated, this compound has the potential to be developed into new chemotherapeutic agent to combat malignancy.

Majority of curcumin's analogues has been reported to be able to exert better anti-proliferative effect on cancer cells compared to curcumin through induction of apoptosis. Some mono-carbonyl analogues not only have enhanced anti-tumour activities *in vitro* but also have better pharmacokinetic profiles *in vivo* (28). Although curcumin analogue of 3,30-hydroxy was able to significantly induced apoptosis in HepG2 via ROS mediated pathway (15), there was no report on the effect of BHMC on this cell line. Mono-carbonyl curcumin analogue of

GL63 also has been reported to have better effect on the activation of caspases-3 and -9, which play major role in regulating apoptosis (28). Incorporation of multiple pairs of methoxy groups at either end of the compound as well as cyclohexanone linker between the two benzene rings in curcumin analogue is essential to increase the toxicity effect against cancer cell lines (5). Although all these derivatives were able to induce anticancer effects in both *in vivo* and *in vitro*, exact molecular mechanism underlying these activities are still unclear (14).

CONCLUSION

This study demonstrated that BHMC was cytotoxic towards HepG2 in concentration- and time-dependent manner compared to curcumin. This has been confirmed with trypan blue assay and morphological studies of treated cells. Although curcumin's analogue, BHMC was relatively toxic towards non-cancer 3T3, several normal cell lines should be used to confirm the toxicity of BHMC.

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