Can Hinokitiol Kill Cancer Cells? Alternative Therapeutic Anticancer Agent via Autophagy and Apoptosis

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Hinokitiol에 의해 유도된 Autophagy 및 Apoptosis에 의한 대체 항암요법 연구

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ABSTRACT

Cancer is genetically, metabolically and infectiously induced life threatening disorder showing aggressive growing pattern with invasive tendency. In order to prevent this global menace from jeopardizing human life, enormous studies on carcinogenesis and treatment for chemotherapy resistance have been intensively researched. Hinokitiol (β-thujaplicin) extracted from heart wood of cupressaceous is a well-known bioactive compound demonstrating anti-inflammation, anti-bacteria and anti-cancer effects on several cancer types via apoptosis and autophagy. This study proposed that hinokitiol activates transcription factor EB (TFEB) nuclear translocation for autophagy and lysosomal biogenesis regardless of nutrient condition in cancer cells. Mitophagy and β-catenin translocation into the nucleus under treatment of hinokitiol on non-small cell lung cancer (NSCLC) cells and HeLa cells were investigated. Hinokitiol exerted cytotoxicity on HeLa and HCC827 cells: moreover, artificially induced autophagy by overexpression of TFEB granted imperfect sustainability onto HeLa cells. Taken together, hinokitiol is the prominent autophagy inducer and activator of TFEB nuclear translocation. Alternative cancer therapy via autophagy is pros and cons since the autophagy in cancer cells is related to prevention and survival mechanism depending on nutrition. To avoid paradox of autophagy in cancer therapy, fine-tuned regulation and application of hinokitiol in due course for successful suppressing cancer cells are recommended.

INTRODUCTION

Hinokitiol (β-thujaplicin) is a natural bioactive substance found in the wood of cupressaceous used for antimicrobial agent, shows antitumor effects via autophagic signaling pathway [1]. The chemical structure of β-thujaplicin (2-hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one) which contains tropone structure (2,4,6-cycloheptatrien-1-one): seven-membered aromatic ring, is also found in several other phytochemicals with wide spectrum of biological effects and troponoids are naturally synthesized by plants to protect them from bacteria as a defense
mechanism [2]. Autophagy is a physiological self-digestive process that has been evolutionarily conserved for cells to balance energy sources under nutrient deprivation condition or to adapt to the stress conditions in cells [3]. Cells utilize long-lived proteins and destroyed unnecessary cellular organelles for energy sources or materials for cell formation under nutrient deprivation via autophagy [4]. During autophagy in mammalian cells, cytoplasmic constituents sequestered by autophagosome are digested by lysosomal enzymes via lysosome-autophagosome fusion called autolysosome [5]. Initially, the autophagy was regarded as tumor suppression mechanism in that the deleted pattern of autophagy related genes: ATG6 and BECN1, are often observed in 70% of human prostate, breast and ovarian cancers [6]. In the previous studies, the level of autophagy, during the early development stage of cancer, highly increased, whereas far reduced autophagy was shown in malignant stage of pancreatic cancer. This gives an idea that autophagy is in any way actively used during carcinogenesis and the decreased autophagy is essential to keep malignant stage of pancreatic cancer [7]. TFEB is regarded as a key regulator of autophagy due to its prominent capability to upregulate autophagy and lysosome related gene expression [8]. Under starvation condition, dephosphorylated TFEB migrates into the nucleus from cytoplasm in order to activate the target genes [9]. Recently, there has been a report that curcumin: hydrophobic polyphenol extracted from turmeric of the ginger family, directly interacts with TFEB for augmentation of TFEB nuclear translocation which ameliorates transcriptional activity of TFEB [10, 11]. More recent findings suggest that hinokitiol affects cancer cells in various ways depending on the concentration: treatment with non-toxic dose (1 ∼ 5 μM) of hinokitiol does not exert severe cytotoxicity to normal cells but suppression of migration ability of human lung adenocarcinoma cells [12]. Hinokitiol is also related to the suppression of cancer stemness and glioma oncogenicity by downregulation of Nrf2 expression; on the other hand, melanoma is notably inhibited by hinokitiol via increased activity of antioxidant enzymes catalase (AEC) and superoxide dismutase (SOD) as well as downregulation of proteolytic enzymes: matrix metalloproteinase (MMP-2 and −9) [13, 14]. Moreover, the gefitinib-resistant lung adenocarcinoma cells and its proliferation are suppressed and negatively regulated by hinokitiol via three different pathways: inhibition of epidermal growth factor receptor/extracellular signaling regulated kinase (EGFR/ERK) signaling pathway; senescence via s arrest by DNA damage; and autophagy [15]. However, there have been rarely introduced of anticancer effect of hinokitiol against HeLa and HCC827 cells compared to the previous reports on curcumin and other cancer cells [16]. In this study, hinokitiol was used to see its anticancer effects on HeLa and HCC827 cells, and how effectively prevent cancer cell growth compared to normal cells. This study showed the first evidence of nuclear translocation of TFEB under treatment of hinokitiol in HeLa cells. Moreover, anticancer effects with considerable cytotoxicity of hinokitiol were shown in HeLa and HCC827 cells. Prevention of β-catenin nuclear localization was also observed with augmentation of mitochondrial fragmentation in NSCLC cells by treatment of hinokitiol.

**MATERIALS AND METHODS**

1. **Cell culture and transient transfection**

HeLa and fibroblast were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Welgene, Korea) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA) and 1 × Penicillin-Streptomycin antibiotics (Welgene). On the other hand, HCC827 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI, Welgene) with same supplements. For transfection, HeLa and HEK293 cells were cultured at a confluence of 80% in a 12 well plate (Eppendorf, Germany). One microgram of DNA construct containing the TFEB sequence was incubated with 100 μL of Opti-MEM and followed by mixing with 2 μL of TurboFect (Thermo, USA). The mixture was incubated at the room temperature for 20 minutes and carefully dropped into each well for the transfection. After the transfection, the well plate was incubated overnight at
37°C, 5% CO₂ in a humidified chamber and following day, cells were washed with DMEM or RPMI 1640 for 3 times and added with completed media.

2. HeLa hTFEB-EGFP stable cell lines

For the establishment of stable cell lines which continuously expresses TFEB-EGFP, TFEB coding sequence was amplified by PCR using the primers 5'-CCGAAGCTTATGCGTCACGCATAGGGTTG-3' and 5'-GTCGGAATTCTCACAGCACCAGGCCCTC-3' followed by subcloning into the EcoRI site of the pEGFP-C1 vector. HeLa cells were underwent of transfection with pEGFP-C1 (Cat #: 6084-1, GenBank, USA) which contains hTFEB using TurboFect (Thermo). On the following day, the transfected HeLa cells were cultured with selection media containing 400 μg/mL of G418 (Geneticin, Thermo) for 4 days, consecutively. The cell culture media were replaced every 4 days with higher concentration of G418 up to 1,000 μg/mL for 17 days. After which, each stably green fluorescence expressing colonies were isolated into FACS tube. The stably green fluorescence expressing cells were sorted using FACS ARIA II (Becton, Dickinson and Company, USA) and data were analyzed by FACSDiva version 6.1.3 for better selection.

3. Cell viability test

For live/dead and viability/cytotoxicity test, calcein acetoxymethyl (AM) and ethidium homodimer kit (R37601, ThermoFisher, USA) were used. Live cells were labeled in green and excited at 475 nm; on the other hand, dead cells were labeled in red and excited at 550 nm. Emission filter for green was 500 ∼ 550 nm and 570 ∼ 650 nm filter set was used for red. Objective lenses were 5 × Air NA 0.16 for realistic plate images. Percent cytotoxicity was calculated based on the following equation:

\[
\text{Cytotoxicity (\%)} = \frac{\text{No. of dead cells (red)}}{\text{No. of live cells (green)+No. of dead cells (red)}} \times 100
\]

4. Preparation of hinokitiol and curcumin supplemented Media

In order to prepare hinokitiol and curcumin supplemented media, β-thujaplicin (2-Hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one, hinokitiol) and curcumin (E,E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, Diferuloylmethane) were purchased from Sigma-Aldrich (USA). Each compound was dissolved in DMSO (Sigma-Aldrich) and diluted using RPMI1640 media at 1 mM of final concentration for ready to use.

5. Hanging drop cell culture for 3D spheroids

HCC827 cells were cultured at a 90% confluence and harvested with 0.05% trypsin-EDTA in a 15 mL conical tube for dilution and adjustment of cell number to 100 cells/μL. Around 1,000 cells per well with 70 μL of growth media were seeded into the GravityTRAPTM ULA Plate (inSphero, Switzerland) for 3D formation. To remove the air bubbles, the plate were centrifuged at 250 relative centrifugal force (RCF) for 2 minutes. The plate was incubated at 37°C, 5% CO₂ for seven days consecutively. Media were gently exchanged every two days using a 20 μL pipette for three times remaining 10 μL of residue volume.

6. Immunofluorescent staining

HCC827 cells were seeded into the 35 mm confocal dish (Cat #: 100350, SPL) and after 24 hours incubation, cells were stimulated by conditioned media: 10 μM of hinokitiol for 16 hours. For the immunofluorescent staining, Cells were rinsed twice with 1× Phosphate buffered saline (PBS) and fixed with 4% Paraformaldehyde for 30 minutes at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 for 10 minutes and washed twice with 1× PBS. After which, cells were treated with the blocking buffer; 3% goat serum with 0.1% Triton X-100 in 1× PBS, for 30 minutes and rinsed 3 times with 1× PBS. Primary antibodies against β-catenin (rabbit anti-β-catenin, Cat #: 712700, Invitrogen, USA) were labelled on the shaker in the refrigerator at 4°C overnight. Secondary antibodies; Alexa 488 anti-rabbit IgG, were added into each well at...
room temperature for an hour followed by rinsing with 1× PBS. Nucleus was tagged with DAPI and DraQ5 (Cat # 4084, CST, USA) and each sample was subjected to mounting for confocal microscopy.

7. Immunocytochemistry for active caspase-3

Cells were seeded on the slide glass and cultured for 24 hours. Four hours after exposure to curcumin or hinokitiol, cells were fixed using 4% paraformaldehyde and followed by rinsing with 1× PBS. Cell Conditioning 1 (CC1) standard (pH 8.4 buffer containing Tris/Borate/EDTA) was used for antigen retrieval. For automated immunohistochemistry staining, Discovery XT immunohistochemistry autostainer and Discovery ChromoMap DAB (3,3′-Diaminobenzidine) kit (Ventana Medical Systems, Inc., Tucson, AZ, USA) were used. Slides were incubated with primary antibodies diluted as 1:500; cleaved caspase-3 (C8487, Sigma-Aldrich) for 32 min at 37°C, and secondary antibody; Ultramap anti-rabbit HRP (Ventana, Roche, Switzerland) for 20 min at 37°C. Slides were incubated in DAB with H2O2 substrate for 8 min at 37°C followed by hematoxylin and bluing reagent for counterstain at 37°C. Reaction buffer (pH 7.6 Tris buffer) was used as washing solution in each step.

8. Terminal deoxynucleotidyl transferase dUTP nick end labeling: TUNEL assay

Slide glasses with 80% of cell confluency were subjected to exposure to given curcumin or hinokitiol agents for 4 hours. Cells were rinsed with PBS and endogenous peroxidase was quenched by 3.0% hydrogen peroxide in PBS for 5 min at room temperature. Equilibration buffer was treated on the specimen followed by working strength terminal-deoxynucleotidyl-transferase (TdT) enzyme for 1 hour in the humid chamber 37°C. After which, the specimen was subjected to working strength stop/wash buffer and anti-digoxignenin conjugate. Samples were washed thoroughly and gently tapped off to remove excess liquid and treated with peroxidase substrate for 5 min at room temperature. Counterstain was made using 0.5% (w:v) methyl green and washed with dH2O. All these TUNEL stain kit (S7100) was purchased from Millipore (Germany).

9. Live confocal microscopy and drug treatments

All the confocal microscope images were acquired using a Leica TCS SP8 STED CW System and Leica DMI 6000 inverted fluorescent microscope. The molecules labelled in green fluorescence: EGFP and Alexa 488 dye, were respectively excited using Argon 488 nm laser with 10% of output and 20% of laser power. On the other hand, red fluorescence with Alexa 568, was excited using DSPP 561 nm laser with 5% of laser power. Photomultiplier (PMT) detector and Hybrid Detector (HyD, Hamamatsu) were used for detection of emission and scan speed was 400 Hz using 8× line average with 1 Airy unit pinhole setting. Emission spectral detection ranges were 500~550 nm for green fluorescence and 580~650 nm for red fluorescence. EGFP-hTFEB transfected live stable cells were imaged in real time on the top-stage incubation system (Chamlide TCTM, LCI) which continuously keeps 37°C warm environment and provides 5% CO2 gas. Pre-warmed conditioned media at 37°C were added into the confocal dish using the fluid injector on the stage of confocal microscope.

10. Confocal image analysis

Nuclear translocation of TFEB labelled in green fluorescence was analyzed using Leica LAS X quantify and analysis function. Intensity based mean value of green fluorescent signal representing hTFEB-EGFP, was automatically calculated with the equation that pixel sum intensity is divided by a region of interest (ROI) area pixel. The ROI size was fixed at 50 μm and same ROI size was applied to measure green signals both in nucleus and cytosol. For molecular counting in β-catenin translocation and lysosomal puncta, confocal images were analyzed by IMARIS image analysis software V. 7.6.2 using add spots and section mode. Estimated diameter of molecules for β-catenin was determined in slice view as follows: the smallest green dot in the images was approximately 100 nm. Lysosomal puncta were measured in the same way.
around 1.2 μm up to 2 μm. Mitochondria tube length and area were measured with angiogenesis function, MetaMorph (Molecular Devices, USA).

11. High content screening (HCS) analysis

Cells were seeded into 96 well plate (CellCarrier 96 Ultra, PerkinElmer, USA) for image acquiring and cell HCS analysis: cytotoxicity, mobility and cell tracking, using Operetta CLS, PerkinElmer. Cells were imaged using z-stack function to find best focus area and well based images were analyzed and showed as heat maps and line graphs combined with merged realistic plate images for cell viability against hinokitiol. Cell count and tracking based on plate images were analyzed using digital phase

![Image of Cell Images and Graphs]

**Figure 1.** Effects of hinokitiol and curcumin on cell viability of normal and cancer cells. Fibroblast, HeLa and HCC827 cells were treated with hinokitiol and curcumin depending on the indicated concentration for 16 hours. (A and B) A realistic plate images of cell viability test on each cell line under treatment of curcumin or hinokitiol. Live cells were labeled with calcein AM colored green and dead cells were colored red with ethidium homodimer-1. Cytotoxicity was calculated based on the ratio analysis of live and dead cells. (C) Cell proliferation analysis after treatment of hinokitiol with indicated concentration. Data are expressed as the mean±SD of hexaplicate determinations. **P<0.01; ***P<0.001. Cell counting was repeated more than three times.
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Figure 2. Immunocytochemistry of cleaved caspase-3 and TUNEL stain in HCC827 cells. Wide-field microscope images of HCC827 cells. Cleaved caspase-3 and TUNEL staining were performed for visualization of apoptosis after 8 hours of treatment with 20 \( \mu \)M of curcumin or hinokitiol. Cleaved caspase-3 and TUNEL positive cells were shown in brown color while cleaved caspase-3 and TUNEL negative cells were shown in blue and green, respectively (upper panel). Quantitative analysis with percentage of cleaved caspase-3 and TUNEL positive cells (lower panel). Data were expressed as the mean±SD of hexaplicate determinations. ***P<0.001. Cell counting was repeated three times. Scale bars, 20 \( \mu \)m.

RESULTS

1. Effects of curcumin and hinokitiol on cell viabilities of cancer cell lines

The cell viability was examined using three different cell lines: Fibroblast, HeLa and HCC827, against curcumin and hinokitiol respectively. As shown in Figure 1A, curcumin gradually exerted cytotoxicity against depicted three cancer cell lines: HeLa (IC\(_{50}=41.52\pm3.96\ \mu\)M) and HCC827 (IC\(_{50}=35.55\pm4.23\ \mu\)M). Similarly, hinokitiol also showed cytotoxicity on those cancer cell lines: HeLa (IC\(_{50}=38.58\pm6.72\ \mu\)M) and HCC827 (IC\(_{50}=37.63\pm5.41\ \mu\)M) (Figure 1B). Interestingly, cancer cell proliferation inhibitory activities were also observed as hinokitiol diluted solution was added into culture media with different concentration: 0, 20 and 40 \( \mu \)M on HeLa and HCC827 cells during sixteen hours live imaging observation using high content screening method (Figure 1C). These data indicates that hinokitiol is alternatively applicable on the mentioned cancer cells in lieu of curcumin for cancer treatment and prevention.

2. Apoptosis induced by curcumin and hinokitiol

The caspase-3 activity was examined to determine how curcumin and hinokitiol increase cytotoxicity in NSCLC cells. Active caspase-3 positive cells were significantly increased in the curcumin or hinokitiol treated groups compared to the control in HCC827 cells. Additional TUNEL stain result was very much consistent with active caspase-3 analysis wherein TUNEL positive cells were slightly increased in curcumin or hinokitiol treated groups compared to the control (Figure 2). These data implied that increased cytotoxicity and apoptotic cell death was caused by curcumin and hinokitiol. The effect of apoptotic death, however, cannot clearly explain the massive cancer cell death which was shown in figure 1 and that casted an inquiry that another mechanism: autophagic death, is possibly related to the cancer cell death by curcumin and hinokitiol.

3. TFEB nuclear translocation and increased lysosomal puncta triggered by hinokitiol treatment in HeLa cells

The hypothesis postulated in this section is hinokitiol activates autophagy under nutrient rich condition via TFEB dephosphorylation and its nuclear translocation possibly leads to the stimulation of lysosome and...
autophagy related genes. To this end, GFP-TFEB construct were transfected into HeLa and HCC827 cells for single cell colony selection under G418 treatment followed by FACS sorting. Unlike the GFP-TFEB HeLa cells, GFP-TFEB HCC827 cells were not successfully established by commercially available transfection methods followed by colony selection and FACS sorting (Data not shown). As introduced in the previous studies, TFEB migrated into nucleus under nutrient deprivation condition in GFP-TFEB HeLa cells. Moreover, TFEB nuclear translocation was clearly observed by treatment of hinokitiol in line with the previously reported study of starvation (Figure 3A). Lysotracker: lysosome labelling dye for live imaging, has been used for detection of autophagy in the previous reports and in this study, the lysotracker was applied for monitoring TFEB and its downstream target gene expression: lysosome. To see whether hinokitiol activates lysosome expression, the TFEB stably expressed HeLa cells were used for evaluation of physical differences of lysosome before and after treatment of hinokitiol. As shown in Figure 3B, the number of lysosomal puncta over 1.2 μm diameter were counted using Imaris V. 7.6.2,

Figure 3. TFEB nuclear translocation and lysosomal puncta enlargement after treatment of hinokitiol and curcumin in HeLa cells. (A) Confocal live images and time dependent GFP N/C ratio analysis bar graphs in HeLa GFP-TFEB transfected stable cells after exposure to serum starvation or hinokitiol, respectively. Hinokitiol triggers GFP-TFEB nuclear translocation under nutrient rich condition in HeLa cells. (B) Enlarged lysosomal puncta after treatment of hinokitiol in HeLa cells. Lysotracker real-time observation in confocal microscope after treatment of hinokitiol and starvation compared to time point 0 hr in HeLa cells. Images were taken at 0, 1, 2 and 4 hours after treatment of hinokitiol and starvation, respectively.
Bitplane. In parallel with the starvation condition, the number of puncta were gradually increased after treatment of hinokitiol in HeLa cells. Importantly, the number of lysosome was increased and giant lysosomes were concomitantly observed under normal nutrient condition. These data suggest that hinokitiol promotes TFEB nuclear translocation with lysosomal activation regardless of the environment naturally controlled by the given nutrient.

4. Impaired cell death caused by artificially induced autophagy in TFEB overexpressed cells and diminished cytotoxic effect of hinokitiol in HCC827 cells introduced by autophagy inhibitor

The hypothesis was established to explore the relationship between autophagy and cancer cell death that increased TFEB in cancer cells and artificially upregulated autophagy by extrinsic stress will negatively react to cancer cell viability. In brief, additionally applied hinokitiol to the cancer cells subjected to autophagy upregulation by TFEB, will synergistically lower the cell viability due to the lost balance of autophagy for its sustainability. To this end, the experiment was designed to see whether TFEB induced autophagy can prolong the viability of cancer cells at the basal level and compare the anticancer effect between wildtype and the autophagy activated group under treatment of hinokitiol. Depending on the concentration of hinokitiol treated, it showed gradually increased cytotoxicity in the wildtype HeLa cells. On the other hand, artificially increased autophagy showed significantly dominant cytotoxicity at higher concentration of hinokitiol and even showed moderately augmented cytotoxicity in the basal level compared to the wild type (Figure 4A). As a result, TFEB transfection into HeLa cells demonstrated a significantly shortened IC50 (19.07±7.14 μM, hinokitiol) with reduced cell viability compared to the previous experiment described in Figure 1. Of note, increased autophagy system in HeLa cells leaded to the lower cell viability compared to the wildtype HeLa cells without exposing to the anticancer agent. When the therapeutic agents were added into the HeLa cells where the additional autophagy was induced by TFEB overexpression, exaggerated cytotoxicity was observed in both experiments with hinokitiol. Another finding was that autophagy inhibition by pretreatment of chloroquine notably reduced the dead cells caused by hinokitiol treatment in HCC827 cells (Figure 4B). Taken together, the massive cell death by hinokitiol is related to the TFEB nuclear translocation and followed by continuous autophagy induction (Figure 4C).

5. Increased mitophagy and interrupted β-catenin nuclear translocation with lowered cell movement by hinokitiol treatment in cancer cells

Mitophagy is the degradation process of hyper fragmented mitochondria via autophagy in order to remove the damaged and defective mitochondria in cells. Functional damage, impaired mitochondrial dynamics and biogenetics caused by retention of anticancer agent in the mitochondria resulted in significant cell death with increased mitophagy. To see whether hinokitiol induces mitophagy in HCC827 cells, four hours after treatment of hinokitiol, HCC827 cells were labeled with mitotracker for mitochondria and lysotracker for lysosome. Co-localization of mitochondria and lysosome was considerably increased in HCC827 cells compared to mouse embryonic fibroblast (MEF) cells (Figure 5A). This gave the clear evidence that autophagic degradation is intermediated by hinokitiol through the hyper-segmentation of mitochondria and that is maybe related to the massive cell death in HCC827 under treatment of hinokitiol. Cell proliferation was intensively prohibited when hinokitiol was treated on HeLa and HCC827 cells (Figure 1C). Wnt signaling is the key component in cancer biology due to its variety of target genes connected to the cell proliferation. To assess the localization of β-catenin which is the key component in the downstream of Wnt signaling transduction, immuno-fluorescent staining after exposure to DMSO as a control or hinokitiol, was implemented in order to calculate the molecular localization of β-catenin. Indeed, mitigated β-catenin nuclear localization was observed in the confocal microscope and molecular counting image
Figure 4. Impaired cell viability induced by TFEB and alleviated cytotoxic effect by pre-treatment of chloroquine in HCC827 cells. (A) Confocal images of live and dead cells after treatment of hinokitiol in both wild type HeLa cells and TFEB-HeLa cells. Image based quantification data analysis by MetaMorph image analysis software, Molecular Devices. (B) High content screening images of live and dead cells, after treatment of hinokitiol with indicated concentration: 0, 5, 10 and 20 μM in both HCC827 cells and chloroquine, an autophagy inhibitor, pretreated HCC827 cells. Quantification of cytotoxicity with graph was produced by Harmony image analysis software, PerkinElmer. (C) Diagram expatiating the pathway where hinokitiol triggers massive cell death via TFEB activation. Data are expressed as the mean±SD of hexaplicate determinations. *P<0.05; **P<0.01 and ***P<0.001. Cell counting was repeated three times with similar results. Scale bar, 100 μm.

analyzed by Imaris software upon treatment of hinokitiol (Figure 5B). Beta-catenin nuclear localization was reduced in hinokitiol treated group compared to control group. Intriguingly, the 2D migration of HCC827 cells were notably reduced by treatment of hinokitiol (Figure 5C). Cell mobility, measured by cell tracking method, gave the
Figure 5. Co-localization of mitochondria and lysosome and β-catenin translocation with wound scratch migration after treatment of hinokitiol in HCC827 cells. HCC827 cells were treated with hinokitiol for 4 hours before labelling with mitotracker and lysotracker. (A) Mitochondria were shown in green and lysosome is shown in red. Merged images were shown with differential interference contrast (DIC) images. Co-localization between mitochondria (green) and lysosomes (red) were shown in yellow. Co-localization spots are indicated by arrows (blue). Quantification (%) of co-localization by LAS-X image analysis software (Leica, Germany). (B) Confocal images of nucleus (DraQ5) and β-catenin (Alexa 488) under different conditions: DMSO and hinokitiol treatment in HCC827 cells. Image data were analyzed using add spots function in Imaris V. 7.6.2, Bitplane. Nuclear localized β-catenin ratio graph depending on different chemical treatment. (C) Wound scratch and cell migration analysis data. High content screening images were acquired using 10×/0.3 NA, Zeiss for 24 hours continuously, along the indicated condition: control and 10 μM of hinokitiol. Scale bar, 10 μm.
clue that cytotoxicity of anticancer agent not only affects the physiological changes: increased lysosome expression, introduction of giant lysosome, mitochondria segmentation and mitophagy, but also physical depravation like loss of movement. Significant cytotoxic effects shown in Figure 1 were remarkably demonstrated by hinokitiol in microtissues made of HCC827 cells depending on the concentration applied to the test (Figure 5D). Hinokitiol indeed lowered cancer cell proliferation through $\beta$-catenin signaling intervention and arrested cancer cell mobility depending on the concentration.

**DISCUSSION**

This study was started from the hypothesis that autophagy induced by hinokitiol is possibly related to the TFEB nuclear translocation regardless of nutrient manipulation and it is related to the cancer cell death. Autophagy is a well conserved self-degradative mechanism observed in eukaryote for cleaning long lived organelles, malfunctioning proteins and protecting cells from pathogen: besides recycling junk proteins for energy resource under nutrient deprivation [3, 5]. TFEB is a well-known transcription factor up-regulating autophagy and lysosomal biogenesis related genes during malnutrition period [9, 17]. In the previous studies, hinokitol was described as an autophagy inducer and cancer inhibitor via HIF-1 or AKT/mTOR signaling pathway [18, 19]. To confirm whether TFEB nuclear translocation and autophagy activation is connected to the hinokitiol treatment on cancer cells, GFP-TFEB stable cell line was produced and relations between TFEB nuclear migration and lysosomal puncta were analyzed (Figure 3). In this experiments, curcumin was used as a positive control to hinokitiol due to its well-established anticancer effects as a natural derived bioactive compound in the previous report [20]. Indeed, hinokitiol satisfactorily showed anti-cancer effects on cervical and lung cancer cells along the increased concentration and exposure time (Figure 1) in consistent with earlier reports [12, 13]. Interestingly, there have been the reports that autophagy is used for cell survival not only during nutrient deprivation condition of normal cells but also cancer cells as a self-protecting mechanism for sustaining tumor growth against toxicity of cancer therapy [21]. On the other hand, there are many reports that autophagy can be used for cancer cell treatment [22]. TFEB overexpressed HeLa cells showed comparatively exacerbated cell viability with increased lysosomal puncta inferring active lysosome biogenesis and autolysosome formation (Figure 3 and 4). More interestingly, inhibition of autophagy with treatment of chloroquine showed reduced cytotoxicity in HCC827 cells compared to the hinokitiol treated group (Figure 4B). Mitochondria are highly dynamic organelles that can transform their morphology by fission or fusion in order to adjust to the different environments. These dynamics are also regulated by the BCL-2 protein and somehow affect the respiratory, ROS-generating, pro-inflammatory, and lethal signaling functions of mitochondria [23]. Mitochondria elongation which is the protective mechanism of cells when undergoing autophagy is the well-established theory [24, 25]. Hyperfusion and fragmentation of mitochondria is the reaction to the stress. Hyper fragmented mitochondria is tiny small and it can be the target of mitophagy [26]. Mitophagy is the degradation process of hyper fragmented mitochondria via autophagy in order to remove the damaged and defective mitochondria in cells [27]. Functional damage, impaired mitochondrial dynamics and biogenetics caused by retention of anticancer agent in the mitochondria resulted in significant cell death with increased mitophagy [28]. Autophagy is also relatively decreased by aging, and the expression of several key components: ATG5 and ATG7 are shown to be diminished among aging individuals [29]. Furthermore, the previous in vivo study revealed that artificially stimulated autophagy may increase the healthy lifespan in multiple experimental models including mice and primates [30]. In that regard, hinokitiol and its multiple application with other health supplements for senior healthcare as preventive and adjuvant remedies can be positively considered. The key component of Wnt signaling, $\beta$-catenin plays pivotal role in cell proliferation [31]. Failure in regulation of Wnt/
β-catenin signaling is widely linked to physical and physiological disorders: cancers, neurodegenerative diseases, birth defects and other diseases [32]. The level of β-catenin is regulated by destruction complex composed of Axin, GSK3β and adenomatous polyposis coli (APC) via phosphorylation, ubiquitination and proteosomal degradation in the cytoplasm during Wnt signal off state. Conversely, dephosphorylated β-catenin migrates into the nucleus and acts as a transcription factor for cell proliferation [33]. In the latest research, it has been reported that β-catenin and its relevant signaling pathway have important roles in proliferation of bladder cancer cells in three-dimensional organoid culture [34]. In line with the previous studies, nuclear localized β-catenin level were diminished after treatment of hinokitiol, meaning cancer cell proliferation is prohibited by hinokitiol. Moreover, cancer cell mobility was deterred by treatment of hinokitiol (Figure 5B and C). In short, increased autophagy system induced by the external therapeutic agent in HeLa and HCC827 cells seems deteriorate of survival rate and increase cytotoxicity. Directly or indirectly, all these events: upregulation of lysosome, giant lysosome, mitochondria fission, mitophagy, inhibited proliferation, slow-downed cell movement, inhibition of β-catenin translocation and massive cancer cell death, are seemingly connected. Slowing down cell mobility and stagnant proliferation rate under treatment of anti-cancer agent seems lead cancer cells to self-protection state against extreme cytotoxic stress. However, exposure to the high dose of hinokitiol infallibly leads to the deterioration state in cancer cell viability. In the many studies, anticancer therapeutics using natural bioactive compounds are described as novel remedies replaceable to the present chemotherapy [35]. Contrarily, autophagy has also been disputed as both tumor-suppressing mechanism and sustaining tumor growth in many studies [36]. In our previous study, co-treatment of hinokitiol and curcumin showed synergistic anticancer effect via apoptosis in NSCLC cells. However, the massive cell death caused by hinokitiol remained uncertain [37]. This study presented the first evidence that hinokitiol activate TFEB nuclear translocation and it upregulates autophagy and lysosomal biogenesis related gene expression leading to cancer cell death. Reportedly, anticancer effects of phytochemicals is largely divided into three major pathways: necrosis, apoptosis and autophagic death [38]. Artificially induced autophagy granted cancer cells poor viability under treatment of hinokitiol while hinokitiol exerted cytotoxicity with high dose overloading (Figure 4). Indeed, hinokitiol is the prominent autophagy inducer and it works with TFEB nuclear translocation. Downstream of Wnt signaling was prohibited by hinokitiol (Figure 5) and it was known that autophagy inhibits Wnt signaling under starvation condition [39]. Wnt signaling is the prevalent theme in caner biology due to its variety of target genes connected to the cell proliferation [40]. Signaling relations among Hinokitiol, TFEB and Wnt signaling under nutrient rich condition in order to prevent cancer is needed for further study. For successful application of hinokitiol as anticancer agent to suppress tumor cells, other autophagy related signaling pathways and in vivo experiments have to be precisely studied on dose-dependent manner to avoid unexpected side effects. Hinokitiol can be an alternative and preventive anticancer agent for senior healthcare. The results of this study underline the application of hinokitiol for prevention and treatment of NSCLC cells. In particular, massive cancer cell death caused by hinokitiol is mediated by several innate mechanism of programmed cell death and organelles: lysosome and mitochondria. Hinokitiol differently showed cytotoxicity on normal cells with mild toxicity but severe cancer cell death in HeLa and HCC827 (Figure 1). In cancer, apoptotic cell death and autophagy are well balanced for cell survival. When this balance, however, is collapsed by hinokitiol showing autophagy stimulation, cancer cells appear to maneuver to autophagic death (Figure 4C). Hinokitiol, a natural product derived phytochemical, needs to be assessed as alternative adjuvant treatment for NSCLC cells due to its low cytotoxicity on normal cells. Based on this study, hinokitiol is the prominent candidate as a health supplement in alternative medicine and a therapeutic supplement for the preventive...
medicine in senior healthcare. In conclusion, this study presents that a potent anticancer agent: hinokitiol, exerts intensified cytotoxicity against cervical cancer and NSCLC cells. Induced cancer cell death is mediated by caspase-3 in apoptosis and subsequent enlarged lysosomes which are considered as sequestration of anticancer agents and that possibly leads to the lysosomal damage and increased autophagy followed by extreme cell death. The Development of alternative therapeutic strategy using hinokitiol, a natural product derivative, can be duly achieved by continuing efforts with further studies for establishing proper treatment mechanism in NSCLC with minimum cytotoxicity and maximized therapeutic effects.

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