THE APOPTOTIC ANALYSIS OF 7α-HYDROXY-β-SITOSTEROL EXTRACTED FROM *CHISOCHETON TOMENTOSUS* (MELIACEAE) IN CANCER CELL LINES

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ABSTRACT

The main objective of the present study is to investigate the cytotoxicity potential and anti-cancer mechanism of 7α-hydroxy-β-sitosterol (CT1), a known stigmastane sterol extracted from bark of *Chisocheton tomentosus* (Meliaceae). *In vitro* exposures of this compound was conducted on five cancer cell lines; breast adenocarcinoma cells (MCF-7), hepatocyte liver carcinoma cell (HepG2), oral squamous carcinoma cell (HSC-4) and (HSC-2) and epidermoid cervical carcinoma (Ca Ski) and in comparison with normal human mammary epithelia cell line (HMEC). Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and Live/Dead cytotoxic/viability assay. The flow cytometric analysis and DNA fragmentation assays were used to determine mode of cell death mediated by CT1. Wound healing assay was performed to investigate the potential of migration inhibitory effect of CT1. Protein levels were examined by Western blot analysis. The results demonstrated that CT1 exposure markedly cytotoxic toward MCF-7, HepG2 and HSC-4 cells in time- and dose-dependent manner. Conversely CT1 did not significantly affect the viability of HSC-2, Ca Ski and HMEC cells within a similar dosage range. *In vitro* scratch assay showed the potential of CT1 to inhibit migration of HSC-4 cells without significant effect observed for MCF-7 and HepG2 cells. Flow cytometric analysis for annexin V/PI dual staining demonstrated that death was achieved via apoptosis followed by secondary necrosis after 24 h post-treatment period at IC_{50} concentrations. Apoptotic effects of CT1 were confirmed by DNA fragmentation which showed laddering of DNA for three tumor cell lines without forming significant laddering in HMEC cells. Cell cycle analysis also demonstrated that CT1 caused an accumulation in the G_0/G_1-phase of cell cycle in MCF-7 cells. Western blotting analysis on apoptotic proteins lysed from MCF-7 cells treated with CT1 suggested that induction of MCF-7 cell death via apoptosis was modulated through both intrinsic and extrinsic pathway. A time-dependent up regulation of Bax/Bcl protein ratio, Fas Ligand and procaspase 8 proteins and down regulation of procaspase 9, procaspase 3, procaspase 6, Bim and ERK 1/2 proteins were detected in MCF-7 cells confirmed the pathway. In conclusion, CT1, a natural compound from the Malaysian plant exhibited its potential use as a cancer chemopreventive agent.

ABSTRAK

Tujuan utama kajian terkini ini adalah untuk mengenal-pasti keupayaan sitotoksik dan mekanisma anti-kanser oleh 7α-hydroxy-β-sitosterol (CT1), sejenis sterol yang dikenali sebagai stigmastane yang diestrak daripada kulit pokok *Chisocheton tomentosus* yang berasal dari keluarga tumbuhan Meliaceae. Sebatian ini didedahkan kepada lima jenis sel kanser iaitu sel payudara (MCF-7), sel hati (HepG2), sel mulut (HSC-4 dan HSC-2) dan sel servik (Ca Ski) dan juga sel normal dari epithelia (HMEC) secara luar dari organisma. Keupayaan sel untuk meneruskan kelangsungan hidup dinilai dengan menggunakan eksperimen MTT dan Live/Dead. Analisis aliran sitometer dan pemecahan DNA digunakan untuk menentukan jenis kematian sel yang disebabkan oleh CT1. Eksperimen pemulihan luka dijalankan untuk menyiasat keupayaan kesan perencatan CT1 terhadap activiti pergerakan sel. Aras protein ditentukan dengan kajian western blot. Keputusan menunjukan bahawa pendedahan CT1 mengakibatkan kesan sitotoksik terhadap sel MCF-7, HepG2 dan HSC-4 dalam keadaan bergantung terhadap dos dan tempoh rawatan. Sebaliknya CT1 tidak memberi kesan yang penting terhadap kelangsungan hidup sel HSC-2, Ca Ski dan HMEC di dalam julat dos yang sama. Eksperimen pemulihan luka memperlihatkan keupayaan CT1 untuk merencatkan pergerakan sel HSC-4 tanpa memberi kesan yang secukupnya di dalam sel MCF-7 dan HepG2. Analisis aliran sitometer dengan menggunakan gabungan annexin V dan PI telah menunjukkan kematian sel disebabkan oleh apoptosis, kemudian diikuti dengan nekrosis sekunder setelah 24 jam sel dirawat dengan IC_{50} masing-masing. Kesan apoptotik yang berada dalam CT1 disahkan dengan pemecahan DNA yang mana mempamerkan pecahan DNA seperti corak tangga untuk ketiga-tiga sel kanser dan tidak bagi sel HMEC. Aliran sitometer juga menunjukan yang CT1 telah mengakibatkan pengumpulan sel di fasa G_0/G_1 di dalam kitaran sel MCF-7. Analisis western blot terhadap protein-protein apoptotik yang diperolehi dari sel MCF-7 yang telah dirawat dengan CT1 menyokong bahawa rangsangan kematian sel-sel MCF-7 melalui apoptosis telah dikawal oleh mekanisma laluan dalam dan luar. Peningkatan terhadap nisbah protein Bax/Bcl-2, Fas Ligand dan procaspase 8 dan penurunan terhadap protein procaspase 9, procaspase 3, procaspase 6, Bim dan ERK1/2 di dalam sel MCF-7 secara bergantung terhadap tempoh telah mengesahkan laluan ini. Kesimpulannya, kompoun semulajadi CT1, yang diperolehi dari tumbuhan Malaysia telah mempamerkan kebolehanya untuk digunakan sebagai agent kimia mencegah barah.

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LIST OF ABBREVIATION

^{13}C NMR	13-Carbon NMR
α	Alpha
β	Beta
δ_C	Carbon chemical shift
δ	Chemical shift
^{o}C	Degree Celsius
m/z	Mass per charge
λ	Maximum wave length
±SD	Mean Standard Deviation
µ	Micro
µg/ml	Micrograms per Mililitre
µl	Microlitre
µM	Micromolar
$[M]^+$	Molecular ion
1D-NMR	One Dimension Nuclear Magnetic Resonance
%	Percent
±	Plus-minus
+ve	Positive control
^{1}H NMR	Proton NMR
2D-NMR	Two Dimension Nuclear Magnetic Resonance
(v/v)	Volume per Volume
(w/v)	Weight per Volume
A	Absorbance
AIF	Apoptosis Inducing Factor
ANOVA	Analysis of Variance
Apaf-1	Apoptotic Protease-Activating Factor-1
APS	Ammonium Persulfate
ATCC	American Tissue Culture Collection

ATP	Adenosine Triphosphate
Bax	Bcl-2 Associate X Protein
Bcl-2	B-cell Lymphocyte 2
Bcl-X_L	B-cell Lymphocyte extra large
BD	Becton Dickenson
BH	Bcl-2 Homology Domain
Bim	Bcl-2 Interacting Mediator
bp	Base Pairs
BSA	Bovine Serum Albumin
CA	California
CARD	Caspase Recruitment Domains
CARIF	Cancer Research Initiative Foundation
Caspase	Cystein Aspartate Protease
$CDCl_3$	Deuterated chloroform
CDK	CyclinDependant Kinase
CERI	Cytoplasmic Extraction Reagent I
CER II	Cytoplasmic Extraction Reagent II
cIAP	Cellular Inhibitor of Apoptosis Protein
cm	Centimeter
cm^2	Centimeter Square
CO_2	Carbon dioxide
COSY	^1H-^1H Correlation Spectroscopy
COX-2	Cyclooxygenase-2
d	Doublet
dATP	Deoxy Adenosine Triphosphate (dATP)
DEPT	Distortioness Enhancement by PolarizationTransfer
dH_2O	Distilled Water
DISC	Death Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic Acid
EDTA	Ethylene diamine tetra acetic acid
ER	Estrogen Receptor
ERK	Extracellular-Signal Regulated Kinase
EtBr	Ethidium Bromide
EthD-1	Ethidium Homodimer-1
et al.	and other
FBS	Fetal bovine serum
FADD	Fas Associated Death Domain
Fas	FS9 Associated Surface Antigen
FasL	FS9 Associated Surface Antigen Ligand
FITC	Fluorescence Isothiocyanate
g	Gravity
G	Gram
G_0	Quiescent State
G_1	Gap 1
G_2	Gap 2
GCMS	Gas Chromatography Mass Spectroscopy
GI	Growth inhibition
h	Hour
HCl	Hydrochloride Acid
HEPES	N-2-Hydroxylethyl-Piperazine-N-2-Ethane-Sulfonoc
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HPV	Human papilloma virus
HRP	Horseradish peroxidase
Hz	Hertz
IAP	Inhibitor of Apoptotic Protein
IC_{50}	50% Inhibitory Concentration
IL	Illinois

Inc.	Incorporation
IR	Infrared
kDa	Kilodalton
kg	Kilogram
L	Litre
m	Multiplet
m	Meter
M	Mol
mA	Miliampere
MD	Maryland
max	Maximum
MEGM	Mammary Epithelia Growth Media
mg	Milligram
min	Minimum
mins	Minutes
ml	Milliliter
mM	Milimolar
MMC	Mitomycin-C
MS	Mass Spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular Weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NCI	National Cancer Institute
NCR	National Cancer Registry
ND	Not Determined
NER	Nuclear Extraction Reagent
NIH	National Institute of Health
ng	Nanogram
ng/µl	Nanogram Per Microliter

nm	Nanometer
NMR	Nuclear Magnetic Resonance
NSAID	Nonsteroidal Anti-Inflammatory Drug
NY	New York
OD	oligomerisation domain
OD	Optical Density
p	*p*-value of Data Statistical Significant
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
pH	Potential of Hydrogen
PI	Propidium Iodide
PS	Phosphatidylserine
RNA	Ribonucleic Acid
Rnase H	Ribonuclease H
RPMI	Rosewell Park Memorial Institute
s	Singlet
SD	South Dakota
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
sec	Seconds
S phase	Synthetic Phase
spp.	Species
TBE	Tris-Borate-EDTA
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TGS	Tris-Glycine-SDS
™	Trademark
TNFR	Tumor Necrosis Factor Receptor
TRADD	TNFR Associated Death Domain
U	Units
UMMC	University of Malaya Medical Center

U/ml	Unit PerMililitre
USA	United State of America
US FDA	United State Food and Drug Administration
UV	Ultraviolet
V	Volts
Vol.	Volume
WHO	World Health Organization
WT	Wild Type
X	Times/Multiple
XIAP	X-linked Inhibitor of Apoptosis Protein

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