INVESTIGATION OF THE ANTI-CANCER PROPERTIES OF NOVEL 4'-ALKYL SUBSTITUATED KLAVUZON DERIVATIVES ON PANCREATIC CANCER CELL LINE

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

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December 2015
İZMİR

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ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my supervisor Assoc. Prof. Dr. Ali ÇAĞIR for the continuous support of my MSc. study and research, for his patience, motivation and enthusiasm. His guidance helped me in all the time of research and writing of this thesis.

Besides my advisor, I would like to thank to the rest of my thesis committee; Assist. Prof. Dr. Özden Yalçın ÖZUYSAL and Assoc. Prof. Dr. Mehmet ATEŞ for their encouragement and insightful comments and Assist. Prof. Dr. Mustafa KÖKSAL who gave access to the laboratory and research facilities.

I would like to thank to Çağır's Lab members; Murat DELMAN for his helps and Murat KARA for the synthesis of compounds which were used in this study.

Also I would like to thank to Biotechnology and Bioengineering Research Center specialists Özgür YILMAZER and Dane RUSÇUKLU for their kindly helps.

Finally I would like to thank my friend Zeynep AYTAÇ and my family for their supports, patients and encouragement during my thesis study. I dedicate my thesis to my father Mehmet ŞEN, my mother Kentar ŞEN, my family and my friend Zeynep AYTAÇ.

This study was supported with 113 Z 146 numbered project of TÜBİTAK.

ABSTRACT

INVESTIGATION OF THE ANTI-CANCER PROPERTIES OF NOVEL 4'-ALKYL SUBSTITUATED KLAVUZON DERIVATIVES ON PANCREATIC CANCER CELL LINE

In anti-cancer agent studies one of the important issue is to discover an agent that target specifically cancer cells with no or minimal effects on normal cells. *(R)*-goniothalamin, which is a styryl lactone isolated from plants, is an anti-cancer agent that is cytotoxic to cancer cell lines with no or minimal effect on normal cells. Also klavuzon molecule, the 1-naphthyl substituted 5,6-dihydro-*2H*-pyran-2-one derivative, was tested on cancer cell lines and showed higher cytotoxicity. In the first part of this study anti-proliferative effect of novel 4'-alkyl substituted klavuzon derivatives were tested on MIA PaCa-2 cancer cell line and normal Human Pancreatic Duct Epithelial Cell (HPDEC) line by using MTT, and it is found that cytotoxic activity of the compounds depends on the size of the substituent at position 4 in 1-naphthyl part. While two of them, methyl and ethyl substituted**,** showed high cytotoxicity to MIA PaCa-2 that IC_{50} values in nano-molar levels. Next studies were continued with 4'-methylklavuzon derivative.

In the second part, the apoptotic effect and cell cycle analysis of 4'-methylklavuzon was studied. Especially at the 10 μM concentration there was increment in both early and late apoptosis. Cell cycle analysis showed that increasing the concentration of molecule caused cell cycle arrest in S and G2 phases.

The next part was testing the inhibitory effect of 4'-methylklavuzon on human topoisomerase I enzyme. This enzyme was chosen as one potential target, because 4'-methylklavuzon caused S and G2 phase arrest. Topo I is an actively working enzyme during S phase and inhibition of its activity causes DNA fragmentation and apoptosis. The results showed that there was time dependent inhibition of Topo I enzyme in vitro.

The last part of the study was to show DNA fragmentation at cellular level by COMET assay, also known as Single Cell Gel Electrophoresis. After Topo I assay has showed the Topo I inhibition activity of 4'-methylklavuzon, COMET assay was performed. Especially at the 5 μM concentration comets were formed and tail moments were parallel with positive control.

ÖZET

4’ ALKİL SUBSTİTÜELİ KLAVUZON TÜREVLERİNİN PANKREAS KANSER HÜCRE HATTI ÜZERİNDEKİ ANTİ-KANSER ÖZELLİKLERİNİN ARAŞTIRILMASI

Anti-kanser ilaç çalışmalarında önemli konulardan biri ajanın kanserli hücreleri öldürürken normal hücrelere zarar vermemesi veya zararın minimum düzeyde olması. Daha önce yapılan çalışmalarda (*R)*-goniothalamin molekülünün, bitkiden izole edilen bir stiril lakton, bu özelliklere sahip olduğu gösterilmiştir. Yapılan çalışmalarda klavuzonun, 1-naftil subsitüeli 5,6-dihidro-*2H*-piran-2-on türevi, gonithalaminden daha sitotoksik olduğu görülmüştür. Bu çalışmada MTT kullanılarak literatürde bilinmeyen 4’-alkil subsitüeli klavuzon türevlerinin MIA PaCa-2 kanserli hücre hattı ve normal Human Pancreatic Duct Epithelial Cell (HPDEC) hücre hattı üzerindeki sitotoksik etkilerinin yanı sıra 4’-metilklavuzon türevlerinin MIA PaCa-2 hücre hattı üzerindeki apoptotik etkisi, hücre döngüsü üzerindeki etkisi, insan topoizomeraz I enzimi üzerindeki etkisi ve DNA üzerindeki etkisine bakılmıştır.

Çalışmanın birinci kısımda gerçekleştirilen MTT hücre canlılık testi sonucunda 4’-alkilklavuzon türevlerinin MIA PaCa-2 hücreleri üzerindeki sitotoksik etkilerinin 1-naftil grununun 4- konumundaki substitüentin büyüklüğüyle ilişkili olduğu gözlemlenmiştir. Özellikle metil ve etil substitüeli klavuzon türevleri nano-molar düzeyde sitotoksik aktivite göstermiştir. Daha sonraki çalışmalara 4’-metilklavuzon ile devam etmiştir

İkinci kısımda ise 4’-metilklavuzonun apoptotik ve hücre döngüsü üzerindeki etkileri çalışılmış ve 10 μM dozdaki uygulamada erken ve geç faz apoptotik hücre sayılarında artma görülmüştür. Hücre döngüsü analizlerinde ise 4’-metilklavuzonun S ve G2 fazlarında döngüyü durdurduğu görülmüştür.

Sonraki çalışmada ise 4’-metilklavuzonun insan topoizomeraz I enzimi üzerindeki inhibisyon etkisi incelendi. Yapılan çalışma 4’-metilklavuzonun ön-inkübasyon süresine paralel olarak topoizomeraz I enzimi üzerinde inhibisyon etkisi olduğunu göstermiştir.

Son kısımda ise tek hücre düzeyinde COMET testi ile 4’-metilklavuzonun DNA üzerindeki etkilerine bakılmıştır. Özellikle 5 μM uygulanan örneklerde kuyruk oluşumları görülmüştür.

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LIST OF ABBREVIATIONS

APAF1	Apoptotic protease activating factor 1
ARF	Adp-ribosylation factor
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated x protein
Bcl2	B-cell lymphoma 2
BID	BH3 interacting-domain death agonist
Cdk	Cyclin dependent kinase
Chk	Checkpoint kinase
CMG	Cdc45-MCM-GINS helicase
Cpt	Camptothecin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
FITC	Fluorescein isothiocyanate
Gadd45	Growth arrest and dna damage inducible 45
HPDEC	Human Pancreatic Duct Epithelial Cell
MDM2	Mouse double minute 2 homolog
MPF	Maturation-promoting factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	Phosphate-buffered saline
PI	Propidium iodide
PS	Phosphatidylserine
R-GTN	(R)-goniothalamin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCF	F-box containing complex
TAE	Tris-acetate-EDTA
TNF	Tumour necrosis factor
Topo I	Topoisomerase I
Topo II	Topoisomerase II

CHAPTER 1

INTRODUCTION

1.1. Cell Division and Cancer

Cell division and cell cycle are very important parts of the cell biology. Cell division contributes to cell differentiation during development of organism and during later stage of life. This contribution is based on tissue formation and having specific functions. Also cell division is necessary for renewal of cells to replace dead cells with new one. Because of these vital functions, cell division need to be very tightly controlled and regulated as it is. Any defects during control of cell division and/or cell cycle could cause diseases such as tumors and cancers.

Cancer is a genetic disease and is basically uncontrolled division of cells. In cancers there are certain mutations in genes, generally that controls cell division or cell grow or both. There are several molecular genetic analyses of human cancers indicating that cancer cells contain more than one genetic deficiency; mutation, translocations and mitotic recombinations (nondisjuntions, gene conversions) (Preston-Martin, Pike, Ross, Jones & Henderson, 1990; Ramel, 1988) which can be occurred during cell division. So this means that increased cell division increases the risk of tumour formation and cancer formation (Ames, 1992; Cohen, & Ellwein, 1990). Mutagenesis can be caused by endogenous or exogenous factors. For instance DNA of an individual cell is hit 104 times per day in the human body (Ames, 1992). These combinations of changes cause alterations in expression of proto-oncogenes to oncogenes and also inactivate tumor suppressor genes and lead cells to form cancer cells (Preston-Martin, Pike, Ross, Jones & Henderson, 1990; Stanbridge, 1990; Stanbridge, 1990; Sager, 1989)

1.2. Cell Cycle

1.2.1. The Eukaryotic Cell Cycle

Mainly cell division consists of four processes; first cell growth, second replication of DNA, third chromosomes distribution to daughter and fourth cell division. Before going to cell division it needs to be big enough to get commitment to divide which is G1 phase. Although cell grows in this phase, DNA is not duplicated. After commitment of division cell progress to S phase and begin to duplicate its DNA. After completion of DNA synthesis cell progress into G2 phase, in this phase cell continuous to grow and synthesize proteins to prepare itself to mitosis. During M phase, cell completed all steps of division and finally progress into cytokinesis. After cytokinesis in most cases cell begin new cycle, however some type of cells divide occasionally such as liver cells, kidney cells or neurons. These type of cells exit from G1 phase and go into G0 phase where they continue their metabolism but inactive to proliferate (Figure 1.1) (Cooper, 2000)

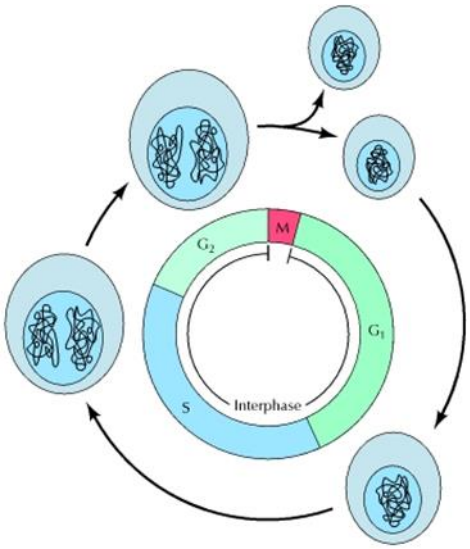


Figure 1.5. The phases of cell cycle
(Source: Cooper, 2000)

1.2.2. Regulation of Cell Cycle

Cell cycle is controlled very rigorously and it is very complex. There are some genes and protein families that have important functions in cell cycle regulation. They

are very important because any defect of function during cell cycle can progress into cancer; cells continue to normal growth but division of cells become uncontrolled. Cyclin dependent kinases (Cdks) are enzyme family of serine/threonine protein kinases that adds phosphate to its protein target either activates or inactivates the target. Cdk's are the major control enzymes during cell cycle causing cells to progress from G1 to S or from G2 to M phase. Maturation Promoting Factors (MPF) trigger cells to progress in cell cycle which includes Cdk and cyclins phase. Another MPF is p53 protein which is a 53 kDa protein, also known as tumor suppressor protein that has very important functions during cell cycle; activates DNA repairing mechanism in case of any mistake during DNA duplication or progress cells in apoptosis if there is an irreparable DNA damage. Although p53 is very important to prevent cells from being cancer, it is most frequent mutations leading cells to form cancer (Surget, Khoury, & Bourdon, 2014)

1.2.2.1. G1 Phase

The fate of a cell is determined in G1 phase; in this phase cell make decision which way to progress; differentiate, proliferate, going apoptosis or become quiescent. The length of G1 phase is an important difference between embryonic and somatic cells. In embryonic cell the length of G1 phase is shorter than somatic cell (Savatier, Huang, Szekely, Wiman, & Samarut, 1994; Becker, Ghule, Therrien, Lian, Stein, Van Wijnen, & Stein, 2006; Fluckiger, Marcy, Marchand, Negre, Cosset, Mitalipov, & Dehay, 2006). Cyclins are the key regulators which interact with specific CDK's and activate them. Cyclin D and cyclin E are two major class cyclin proteins in the G1 phase. Cyclin D interact with CDK4 or CDK6 whereas cyclin E interact with CDK2. After interaction of cyclin D with CDK4 or CDK6, this complex phosphorylates retinoblastoma tumor suppressor Rb protein which is called hypophosphorylated Rb (Sherr, 2000). Hypophosphorylated Rb found in the cell as complex with E2F protein family which is transcription factors. E2F family is very important transcription factor that critical for progress cells into S phase. Although association of E2F with Rb protein is dependent on phosphorylation by cyclin D-CDK4/6 (Ezhevsky, Ho, Becker-Hapak, Davis, & Dowdy, 2001) also this phosphorylation of Rb protein leads to dissociation of histone deacetylases from Rb which increases expression of cyclin E gene through derepression (Harbour, Luo, Dei Santi, Postigo, & Dean, 1999). The protein $p27^{kip1}$

suppresses cyclinE-CDK2 complex, also $p27^{kip1}$ interacts with cyclin D-CDK4/6 which means $p27^{kip1}$ required for cyclin D-CDK4/6 activity (Sherr, & McCormick, 2002). When cyclin D level increase, it needs more $p27^{kip1}$ and sequesters most $p27^{kip1}$ protein in the cell. The depletion of $p27^{kip1}$ causes increasing activity of cyclin E-CDK2 complex. Active cyclin E-CDK2 hyperphosphorylates Rb protein which causing releasing of E2F to initiate expression of critical genes needed for progress into S phase (Sherr, & McCormick, 2002).

1.2.2.2. S Phase

S phase is the phase that DNA replication takes place. After transition from G1 to S phase, cell begins to duplicate chromosomes to segregate its genome to two identical daughter cells. DNA duplication needed to be complete without any mistake otherwise genetically abnormal cells are produced which resulted in cell death or diseased cell. Because of this S phase is tightly controlled. Any problems with DNA replication during S phase trigger a checkpoint. In case of a problem during DNA replication, checkpoint holds replication on until overcome the problem. If problem cannot be resolved, cell is going to apoptosis which is programmed cell death.

During replication H bonds of double strand DNA (dsDNA) are broken and single strand DNA (ssDNA) is formed. Because ssDNA is more prone to breaks, degradation and mutations, replication protein A interact with ssDNA and protect from these dangers, however long RPA coating of ssDNA signals for checkpoint. RPA coated ssDNA is attracted by a specific protein known as ATRIP which recruits ataxia telangiectasia mutated and Rad3 related kinase, ATR to stalled replication fork (Cimprich, & Cortez, 2008; Cortez, Glick, & Elledge, 2004). This association of ATR and RPA activates kinase activity of ATR and arrest S phase for a while. Some researches indicated that ATR also stabilize DNA replication fork that contain ssDNA (Katou, Kanoh, Bando, Noguchi, Tanaka, Ashikari, & Shirahige, 2003). One study suggests that there could be effect of ATR on Mcm2-7 proteins that is core domain of CMG Helicase that unwinds dsDNA to ssDNA.

If DNA replication is completed and all ssDNA is converted to dsDNA, ATR protein is inactivated and cell cycle proceeds. Any ATR signaling pathway defect that caused by mutations, result ssDNA's in dsDNA breaks (Menoyo, Alazzouzi, Espín,

Armengol, Yamamoto, & Schwartz, 2001) **.** These double strand DNA breaks cause activation of ataxia telangiectasia mutated known as ATM. ATM protein is activated to repair dsDNA breaks (Cimprich, & Cortez, 2008) through phosphorylating checkpoint kinase 2, chk2. Chk2 triggers downstream proteins by phosphorylation and ultimately results in repairing of dsDNA breaks. Also when chk2 triggers downstream, phosphorylation of p53 takes place (Caspari, 2000). This activation is a defense mechanism to protect tumor formation.

1.2.2.3. G2 Phase

Activation of ATR and ATM kinases promote DNA repair, cell cycle arrest or apoptosis in case of dsDNA breaks (Abraham, 2001; Harper, & Elledge, 2007)**..** Another important player is p53, which is a tumor suppressor protein, promotes cell cycle arrest and apoptosis. p53 regulates cell cycle in G1 phase as well as in G2 phase. Upregulation of p21 by p53, a Cdk inhibitor, promotes cell cycle arrest in G1 phase in case of DNA damage (Dulić, Kaufmann, Wilson, Tisty, Lees, Harper, & Reed, 1994; Waldman, Kinzler, & Vogelstein,n1995). Also p53 and p21 are required in G2 phase to arrest cell cycle (Bunz, Dutriaux, Lengauer, Waldman, Zhou, Brown, & Vogelstein, 1998), which basically is achieved through pro-mitotic genes repression (Jackson, Agarwal, Yang, Bruss, Uchiumi,nAgarwal, & Taylor, 2005; Taylor, & Stark, G.2001). Amount and type of DNA damage determine the level of p53 protein in cell (Lahav, Rosenfeld, Sigal, Geva-Zatorsky, Levine, Elowitz, & Alon, 2004; Batchelor, Loewer, Mock, & Lahav, 2011) for instance prolonged DNA damage in G2 cause p53 dependent permanent cell cycle arrest (Baus, Gire, Fisher, Piette, & Dulić, 2003).

These functions of p53, DNA repairing, apoptosis and cell cycle arrest, protect from neoplasia. In case of p53-dependent arrest, p53 protein blocks cdc2; the cyclin dependent kinase required to enter mitosis. p53 inhibits Cdc2 through three transcriptional targets simultaneously, Gadd45, p21 and 14-13-3σ. p53 also inhibits cdc2 through repression of *cyclin B1* gene promoter, which Cyclin B1 protein required for activation of cdc2. Also p53 repress *cdc2* gene promoter itself, in case of escaping initial block (Taylor, DePrimo, Agarwal,bAgarwal, Schönthal, Katula, & Stark, 999). Topoisomerase II enzyme, form mitotic condensed chromosomes and required during G2/M transition (Withoff, De Jong, De Vries, & Mulder, 1995), is also downregulated

by p53 protein through repressing its promoter (Sandri, Isaacs, Ongkeko, Harris, Hickson, Broggini, & Vikhanskaya, 1996; Wang, Zhan, Coursen, Khan, Kontny, Yu, & Harris, 1999).

Cdc2 activity is also inhibited by p53-independent pathway during genotoxic stress. ATM and ATR are protein kinases that are members of phosphatidylinositol-3 kinase. These kinases are activated by DNA damage and caused G2 cell cycle arrest (Cliby, Roberts, Cimprich, Stringer, Lamb, Schreiber, & Friend, 1998; Rudolph, & Latt, 1989). ATM and ATR activate serine kinases Chk1 and Chk2 through phosphorylation. Activated Chk1 and Chk2 phosphorylate Cdc25 and sequester it in the cytoplasm. The phosphorylated Cdc25 cannot de-phosphorylate Cdc2/Cyclin B and causes p53-independent G2 cell cycle arrest (Kumagai, & Dunphy, 1999; Lopez-Girona, Furnari, Mondesert, & Russell, 1999).

1.2.2.4. M Phase

In mitosis phase, chromosomes are condensed by Topoisomerase II enzyme, become mitotic chromosomes and sister chromatids attached to mitotic spindle in prometaphase, they are aligned in metaphase, nuclear envelop breaks down and ***(42)*** then chromosomes move to opposite directions. This nuclear division followed by cell division which is called cytokinesis. Basically M phase checkpoints control exact and correct separation of chromosomes during cell division. The spindle checkpoint controls and delay cells to entry anaphase until all chromosomes correctly attached to mitotic spindle. A tension is formed when a chromosome is attached to microtubules at kinetochores from opposite site. The lack of this tension activate spindle checkpoint (May, & Hardwick, 2006). Also lack of attachment of microtubules to kinetochores activates checkpoints and arrest cell cycle at metaphase (Clute, & Pines, 1999; Skoufias, Andreassen, Lacroix, Wilson, & Margolis, 2001)

Anaphase-promoting complex/cyclosome (APC/C) is the main target of the spindle checkpoint. APC/C is a multiprotein E3 ubiquitin ligase adding ubiquitins to a variety of cell cycle regulator to mark them for degradation by 26S proteasomes (Castro, Bernis, Vigneron, Labbé, & Lorca, 2005). Securing is a substrate for APC/C and its destruction by proteasome releases separase and sequentially destroys cohesin. Cohesin holds sister chromatids together and destruction of cohesion allows sister

chromatids to be pulled to opposite poles. Cdc20 and Cdh1 are accessory proteins of APC/C and they present some substrate to APC/C for ubiquitylation (Peters, 2002). Cdc20 is the key target for spindle checkpoint which required for destruction of securing and required for anaphase onset (Hwang, Lau, Smith, Mistrot, Hardwick, Hwang, & Murray, 1998; Kim, Lin, Matsumoto, Kitazono, Matsumoto, 1998).

Generally cytokinesis completes mitosis which gives rise to two daughter cells. In animal cells, actin and myosin II filaments form a contractile ring during cytokinesis. This ring locates at metaphase plate and pulls plasma membrane inward and divide cell in half.

1.3. Apoptosis

Apoptosis is a programmed cell death that allows the cell to die in a controlled way (Hotchkiss, Strasser,McDunn, & Swanson, 2009; Duprez, Wirawan, Berghe, & Vandenabeele, 2009; Wyllie, 2010; Fulda, Gorman, Hori, & Samali, 2010). Apoptosis is an important process for organisms to get rid of infected or damaged cells (Vicencio, Galluzzi, Tajeddine, Ortiz, Criollo, Tasdemir, & Kroemer, 2008; Labbe, & Saleh, 2008). Apoptosis has some morphological properties; exposure of phosphatidylserine to outer of cell membrane surface, cell shrinkage, membrane blebbing and chromatin condensation. There are two well-known pathway, extrinsic and intrinsic pathways (Duprez, Wirawan, Berghe, & Vandenabeele, 2009; Sprick, & Walczak, 2004). Tumor necrosis factor receptors family; TNFR, Fas and TRAIL mediate the extrinsic pathway, which are known as death receptor. After activation of these receptors they recruit and activate cysteine-aspartic proteases known as initiator caspases 8 and 10. Then death inducing signaling complex (DISC) is formed and DISC leads to activate effector caspase 3. Activated caspase 3 is responsible for cleavage of a number of substrates that lead to DNA fragmentation, nuclear fragmentation and membrane blebbing which are hallmarks of apoptosis (Portt, Norman, Clapp, Greenwood, & Greenwood, 2011).

Intrinsic pathway or cell autonomous pathway is basically regulated by mitochondria (Gupta, Kass, Szegezdi, & Joseph, 2009; Brunelle, & Letai, 2009; Galluzzi, Morselli, Kepp, Vitale, Rigoni, Vacchelli, & Kroemer, 2010). Intrinsic pathway can be activated by various stimuli such as DNA damage or cytotoxicity. Bcl-2 protein families are anti-apoptotic proteins that maintain mitochondrial membrane

integrity by preventing pro-apoptotic proteins, BAX and BAK, from causing mitochondrial damage. In case of cellular stress Bcl-2 homology 3 (BH3) protein is activated and antagonize anti-apoptotic Bcl-2 protein which relieve pro-apoptotic BAK and BAX proteins inhibition. Pro-apoptotic BAK and BAX proteins oligomerize and form channel in mitochondrial membrane, causes releasing of cytochrome c (cyt c) protein into cytosol. Cyt c form complex with Apaf1 and ATP, recruit and activate procaspase 9 called apoptosome (Riedl, & Salvesen, 2007). Activated caspase 9 activates effector caspase -3, -6 and -7 and they execute cell death. Also some other pro-apoptotic proteins are released from mitochondria; such as Smac/Diablo which antagonize inhibitor of apoptosis proteins (IAPs), XIAP, cIAP1 and cIAP2 (Figure 1.2) (Portt, Norman, Clapp, Greenwood, & Greenwood, 2011).

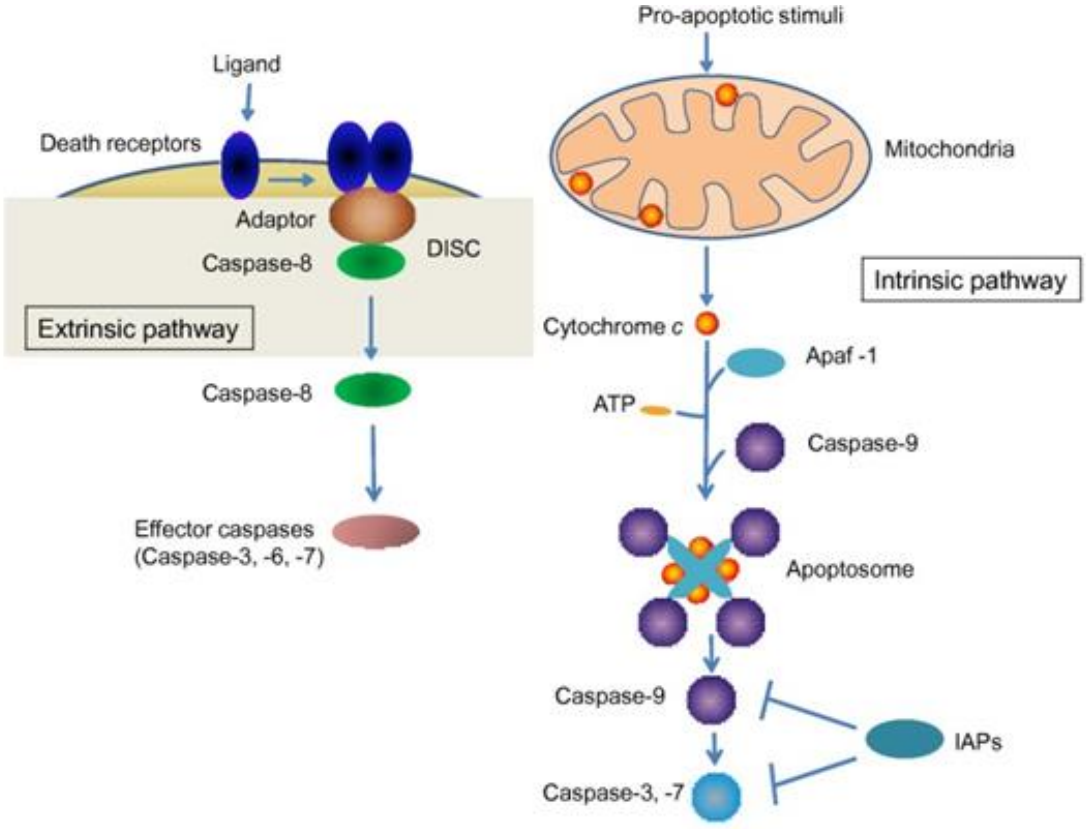


Figure 1.6. Apoptosis; intrinsic and extrinsic pathways
(Source: Li, & Sheng, 2012)

1.4. DNA Topoisomerase I

DNA Topoisomerases are ubiqutious enzymes that remove DNA topological problems during DNA replication, transcription, DNA repair and chromatin condensation by transient single or double strand breaks in the DNA (Cozzarelli, & Wang, 1990; Wang, 1996; Champoux, 2001; Wang, 2002). These enzymes also help to keep steady state of supercoiling of DNA, low enough to let protein-DNA interactions and high enough to prevent high supercoiling deleterious effect (Cozzarelli, & Wang, 1990). Topoisomerases can be divided in two groups based on the number of DNA strand breaks during action, type-I and type-II. Type-I enzymes are monomeric, introduce single strand break and do not require ATP while type-II enzymes are dimeric, introduce double strand break into DNA and require ATP to function (Cozzarelli, & Wang, 1990).

The type I enzymes are further classified in two subtypes; type Ia, including prokaryotic type I topoisomerases, forms covalent bond with 5'-phosphate end of nicked DNA and act only on negative supercoils while type Ib, including eukaryotic type I topoisomerases, attached 3'-phosphate of nicked DNA and act on both negative and positive supercoils.

1.4.1. Topoisomerase I Action Mechanism

Human Topoisomerase I (Topo I) enzyme is 91 kDa weights and can be divided into four domains. First one is N-terminal domain which is highly charged and poorly conserved and dispensable for enzymatic activity because N-terminal truncated enzyme shows same activity with wild type enzyme in vitro (Stewart, Ireton, & Champoux, 1997). Second domain is highly conserved core domain which binds to DNA. Third one is conserved C-terminal domain; this domain contains active nucleophilic tyrosine723 that forms phosphodiester bond with DNA backbone. The last domain is linker domain which connect C-terminal domain with core domain (Figure 1.3) (Carey, Schultz, Sisson, Fazzio, & Champoux, 2003).

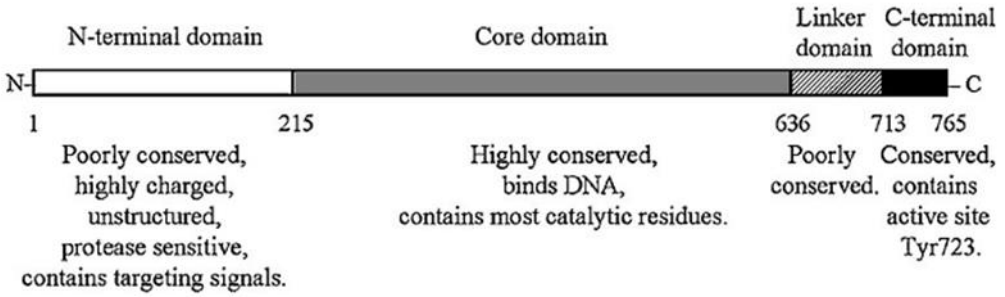


Figure 1.7. Primary structure of Topoisomerase I

Topo I enzyme firstly binds to DNA through core domain, and then O-4 oxygen of Try723 makes nucleophilic attack on the scissile phosphate of DNA backbone forming a phosphodiester bond with 3'-phosphate and releases 5'-hydroxyl which results in single strand break in DNA. After introducing ssDNA break, enzyme linked 3'-DNA rotate around axis of DNA and relax supercoils. Lastly tyr723-3'-bond released and linked with 5'-hydroxyl as reversal action of introducing DNA breaks and enzyme fall off from DNA (Champoux, 2001).

1.4.2. Inhibitor of Topoisomerase I Enzyme

In recent years, topoisomerase enzymes become very popular targets during the search of anti-cancer agents. Inhibitors can bind and block the activity of topoisomerases before binding to DNA and topoisomerase poisons inhibit the ligation step of DNA's break. So without functional topoisomerase enzyme cell cannot replicate its DNA, transcription of genes inhibited or DNA is fragmented and cell is going to apoptosis in case of ligation inhibition. Topoisomerases inhibitors are classified in two groups; class I is topoisomerase poisons including camptothecin (Cpt) and its derivatives irinotecan, topotecan. Class I group inhibitors trap and stabilize the covalent DNA-Topoisomerase complex. Class II is catalytic inhibitors that interfere with topoisomerase-DNA complex without trapping (Topcu, 2001).

1.4.2.1. Camptothecin (CPT)

Camptothecin is an alkaloid extracted from a Chinese tree *Camptotheca acuminate* by Monroe Wall and coworkers (Wall, Wani, Cook, Palmer, McPhail, &

Sim, 1966; Wall, & Wani, 1995; Pommier, 2009). Then it was identified that having antitumor activity on L1210 and P388 murine leukaemia models in 1966 (Wall, Wani, Cook, Palmer, McPhail, & Sim, 1966). In early 1980s it was find out that its antitumor activity comes from effective Topo I inhibition activity (Eng, Faucette, Johnson, & Sternglanz, 1988) and Cpt was the first identified specific Topo I inhibitor.

There are three evident showed that Cpt selectively inhibits Topo I; 1) only naturel Cpt isomers are active on Topo I (Jaxel, Kohn, Wani, Wall, & Pommier, 1989; Hsiang, Liu, Wall, Wani, Nicholas, Manikumar, & Potmesil, 1989), 2) Topo I deleted yeast mutant was immune to Cpt ((Eng, Faucette, Johnson, & Sternglanz, 1988; Nitiss, & Wang, 1988; Bjornsti, Benedetti, Viglianti, & Wang, 1989) and 3) Topo I gene of Cpt resist cells have point mutations (Pommier, 2009).

Cpt binds to DNA-Topo I complex and stabilizes. The first DNA cleavage step is not affected, Topo I properly binds to DNA and attach 3'-phosphate to free 5'-hydroxyl of DNA strand. However at the ligation step Cpt binds to complex and inhibits ligation causes irreversible dsDNA breaks and results in cell death (Figure 4). This inhibition occurs during S phase. Although Cpt considered as cell cycle inhibitor, it showed cytotoxicity while cell do not replicate DNA actively (Chen, & Liu, 1994; Rothenberg, 1997).

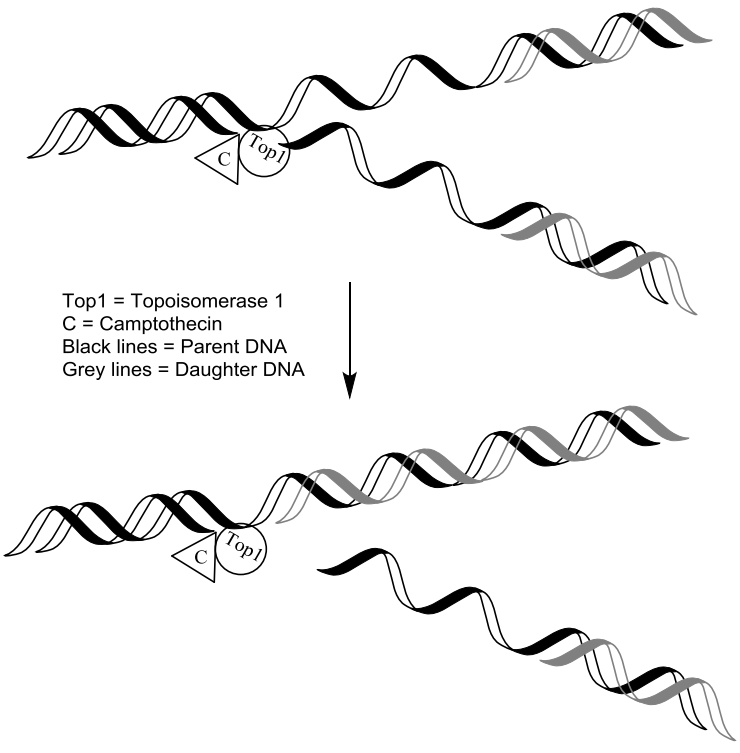


Figure 1.8 The mechanism of topoisomerase I action

Although Cpt has high potency of anti-cancer activity to wide range of cancer type and shows effective activity at low nanomolar concentration, there are some drawbacks because of its physicochemical and pharmaceutical properties. Firstly, Cpt is poorly water-soluble that decreases clinical uses. Secondly, Cpt structure includes an unstable lactone E ring that is highly prone to spontaneous and reversible hydrolysis to an inactive and water-soluble carboxylate form at the physiological pH, 7,4 (Figure 1.5) (Hertzberg, Caranfa, & Hecht, 1989; Mi, & Burke, 1994). This inactive carboxylate form has high affinity to human serum albumin than active form. And thirdly, the inactive carboxylate form cause bladder toxicity (Svenson, Wolfgang, Hwang, Ryan, & Eliasof, 2011; Burke, & Mi, 1994).

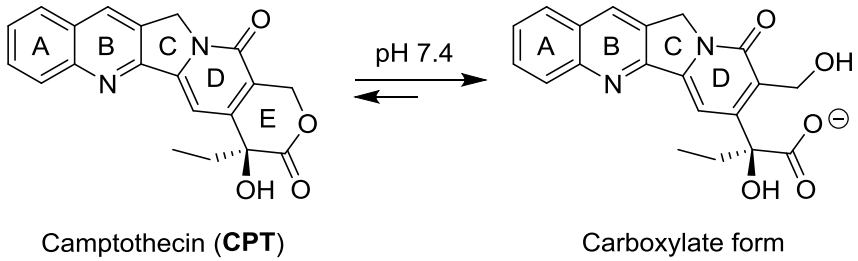


Figure 1.9. Spontaneous hydrolysis of camptothecin lactone E ring

In order to overcome these side effects several Cpt analogues were developed; two of them are FDA approved topotecan (Hycamtin®, GlaxoSmithKline) and irinotecan (Camptosar®, Pfizer) (Kehrer, Soepenberg, Loos, Verweij, & Sparreboom, 2001). Topotecan is a semi-synthetic and water-soluble derivative of camptothecin and it has high anti-cancer activity with Topo I inhibition. Irinotecan is also water-soluble and semisynthetic camptothecin derivative and it is commercially available to treat lung, cervix and ovaries cancers (Svenson, Wolfgang, Hwang, Ryan, & Eliasof, 2011)

Camptothecins are the only Topo I enzyme inhibitors that clinically approved. However there are some limitations of them; first one is their chemical instability, its E ring carbonyl group rapidly converted to carboxylate in blood and become inactivated. Secondly, after removal of camptothecin from Topo I-DNA complex, Topo I ligate DNA breaks and complete its function. Thirdly, there are some side effects cause doses limiting (Pommier, 2009). In order to overcome these drawbacks, several noncamptothecin Topo I inhibitors are produced and these are in clinical development;

the first noncamptothecin Topo I inhibitor that reached to clinical trial is edotecarin. Edotecarin has effects on cellular molecules besides Topo I. Also two family of noncamptotecin Topo I inhibitors are in clinical development indenoisoquinolines and dibenzonaphthyridinones. they are synthetic and chemically stable which overcome the drawback of E ring opening of camptothecin and they also overcome drug efflux-associated resistance of Camptothecin (Pommier, & Cushman, 2009; Teicher, 2008).

1.3. Goniothalamin

Plants are very good source of anti-cancer agent and have long history in treatment of cancer. About 60% of anti-cancer agents isolated from natural sources (Cragg, & Newman, 2005; Bailly, 2009). Some of them are; anthracyclins doxorubicin, daunorubicin and epirubicin, taxanes paclitaxel and docetaxel and camptothecin.

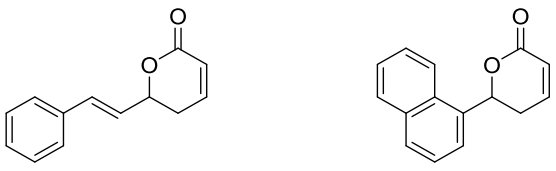
Goniothalamin (GNT) is a styryl-lactone that isolated from *Goniothalamus* which is one of the largest genera of Annonaceae. GNT has wide range of biological activities; anti-cancer (Blazquez, Bermejo, Zafra-Polo, & Cortes, 1999), anti-inflammatory (Vendramini-Costa, de Castro, Ruiz, Marquissolo, Pilli, & de Carvalho,2010), apoptotic effect (De Fátima, Modolo, Conegero, Pilli, Ferreira, Kohn, & De Carvalho, 2006; Inayat-Hussain, Wong, Chan, Rajab, Din, Harun, & Williams, 2009; Inayat-Hussain, Annuar, Din, Ali, & Ross, 2003; Wattanapiromsakul, Wangsintaweekul, Sangprapan, Itharat, & Keawpradub, 2005). GNT has showed cytotoxic effect in variety cancer cell lines including vascular smooth muscle cells (VSMCs), Chinese hamster ovary cells (CHOs), renal cells (Umar-Tsafe, Mohamed-Said, Rosli, Din, & Lai, 2004; de Fátima, Zambuzzi, Modolo, Tarsitano, Gadelha, Hyslop, & Pilli, 2008; CS Souza, de Fatima, da Silveira, & Z Justo, 2012), hepatoblastoma (Al-Qubaisi, Rozita, Yeap, Omar, Ali, & Alitheen, 2011; Al-Qubaisi, Rosli, Subramani, Omar, Yeap, Ali, & Alitheen, 2013), gastric, kidney cells breast carcinoma (Kuo, Chen, Chen, Li, Lan, Chang, & Shiue, 2011), lung cancer cells (Semprebon, de Fátima, Lepri, Sartori, Ribeiro, & Mantovani, 2014), oral cancer cells (Chiu, Liu, Huang, Wang, Chang, Chou, & Wu, 2011; Yen, Chiu, Haung, Yeh, Huang, Chang, & Wu, 2012), and Hela cells (Alabsi, Ali, Ali, Al-Dubai, Harun, Abu Kasim, & Alsalahi, 2012; Alabsi, Ali, Ali, Harun, Al-Dubai, Ganasegeran, & Kasim, 2013). In in vivo models, GNT has showed tumoricidal and tumoristatic activity on Sprague–

Dawley rats with 7,12- dimethylbenzanthracene (DMBA)-induced mammary tumors (de Fátima, Kohn, Antônio, de Carvalho, & Pilli, 2005).

Besides these cytotoxic effects GNT has showed no or low cytotoxicity to normal liver Chang cell lines (Al-Qubaisi, Rozita, Yeap, Omar, Ali, & Alitheen, 2011; Al-Qubaisi, Rosli, Subramani, Omar, Yeap, Ali, & Alitheen, 2013) and normal kidney cell (MDBK); GNT is the only proved agent that cytotoxic to ovarian cancer cell line (Caov-3) and do not caused cell death in normal kidney cell (MDBK) compared with tamoxifen and taxol treated cells (Lin, & Pihie, 2003). Also GNT showed lower cytotoxicity to normal liver cell line (Chang) compared to doxorubicin (Al-Qubaisi, Rozita, Yeap, Omar, Ali, & Alitheen, 2011; Al-Qubaisi, Rosli, Subramani, Omar, Yeap, Ali, & Alitheen, 2013).

GNT induces apoptosis through DNA damage in VSMCs (Chan, Rajab, Ishak, Ali, Yusoff, Din, & Inayat-Hussain, 2006). Apoptosis can be caspase dependent or caspase independent (Zhao, Yang, & Song, 2004; Lee, Abouhamed, & Thévenod, 2006). Although caspase independent apoptosis pathway is not well known, in the caspase dependent pathway caspase has important role through either intrinsic or extrinsic pathways (Chen, Wu, Lan, Chang, Teng, & Wu, 2005). In HepG2 cells GNT induces apoptosis through activation of caspase3, whereas in human Jurkat-T cell caspase 3 and 7 were involved in induction of apoptosis (Inayat-Hussain, Osman, Din, Ali, Snowden, MacFarlane, & Cain, 1999). Also in HL60 and Jurkat cells GNT induces apoptosis through mitochondrial pathway (de Fátima, Kohn, Antônio, de Carvalho, & Pilli, 2005; Inayat-Hussain, Chan, Rajab, Din, Chow, Kizilors, & Williams, 2010).

As many other biologically active natural compounds have α,β-unsaturated δ-lactones in their structures. These α,β-unsaturated δ-lactones have ability to behave as a Michael acceptor and these unsaturated carbonyls can bind to the nucleophilic part of a target enzyme such as cysteine, lysine, serine, threonine or glutathione. Replacing of double bound with 1-naphtyl group by Cagir et. al., showed an increase of cytotoxic activity of molecule (Figure 1.6.) (Kasaplar, Yilmazer, & Cagir, 2009).



Goniothalamin Klavuzon

Figure 1.6. Structures of goniothalamin and klavuzon molecules.

CHAPTER 2

RESULTS AND DISCUSSION

2.1. Cell Viability Analysis

In cytotoxicity experiments, the anti-proliferative activities of 5 compounds that are derivatives of klavuzon were examined on MIA PaCa-2 and HPDEC cell lines. These compounds are: 4’-methylklavuzon **(1)**, 4’-ethylklavuzon **(2)**, 4’-propylklavuzon **(3)**, 4’-buthylklavuzon **(4)** and 4’-penthylklavuzon **(5)** (Figure 2.1)

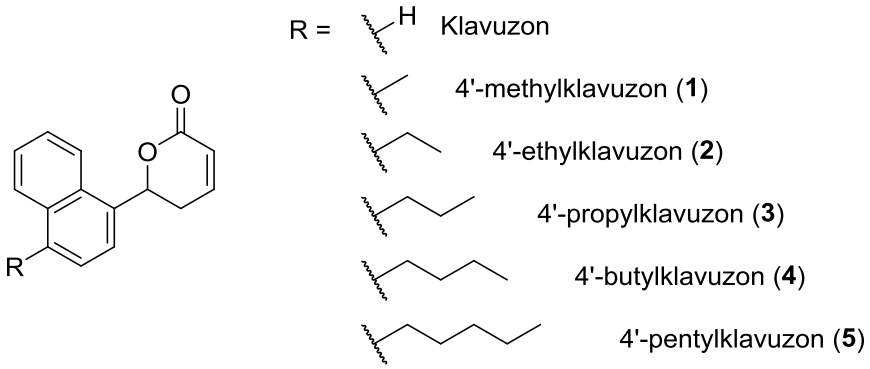


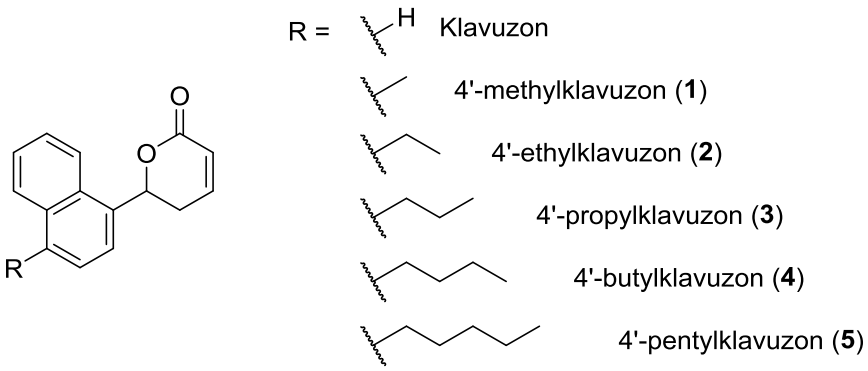
Figure 2.1 Structures of klavuzon derivatives

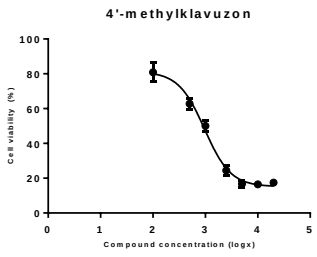
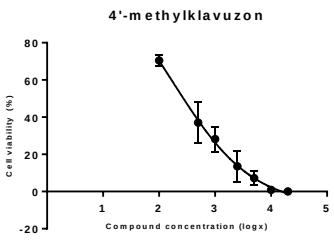
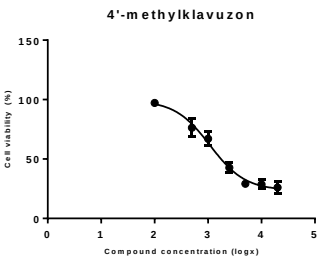
Cytotoxicity of the compounds was determined by the reduction of tetrazolium dye MTT to insoluble formazan. The amount of formazan crystals were determined by the measuring absorbance at 540 nm. The IC_{50} values of cytotoxic compounds were calculated by Graphpad Prism 5 program through nonlinear regression analysis of at least three separate triplicate experiments. The results of MTT assays are listed in table 2.1.

Previous study has showed that klavuzon and 4’-methylklavuzon had showed high cytotoxicity against PC-3 cells and MCF-7 cells when compared to (R)-Goniothalamin. 4’-methyl substituted klavuzon was even more cytotoxic than klavuzon (Kasaplar et al., 2009). Compounds **1**, **2**, **3**, **4** and **5** are the derivatives of klavuzon that have longer 4’-alkyl chain. The cytotoxicity effects of these compounds were examined on MIA PaCa-2 and HPDEC cell lines. The IC_{50} values for MIA PaCa-2 cells were; for 4’-methylklavuzon 0,96 μM, for 4’-ethylklavuzon 0,91 μM, for 4’-propylklavuzon 1,53

μM, for 4'-butylklavuzon 6,32 μM and for 4'-pentylklavuzon 6,71 μM. Although 4'-methylklavuzon was more cytotoxic than klavuzon molecule on PC-3 cells and MCF-7 cells (Kasaplar et al., 2009), increasing the length of alkyl chain did not cause an increment in cytotoxicity on MIA PaCa-2 except 4'-ethylklavuzon. The reason could be increasing the length of side chain decreases the interaction between the molecules and any interacting target proteins. The IC_{50} values for HPDEC cells were very low which is unexpected; one probable explanation could be the interaction of 4'-alkyl substituted klavuzon derivatives with the HPV16 E6 and E7 immortalization pathway of HPDEC cells. Because HPDEC cells have weak attachments, flasks and 96 well plates were coated with collagen before HPDEC cells passage and inoculation to increase attachment of cells to surface. To analyze if there was any effect of collagen on cytotoxicity of molecules; 4'-methylklavuzon, 4'-propylklavuzon and 4'-butylklavuzon molecules were tested on MIA PaCa-2 cells that were inoculated on plates coated with collagen, however the effect of collagen was limited (Table 2.1).

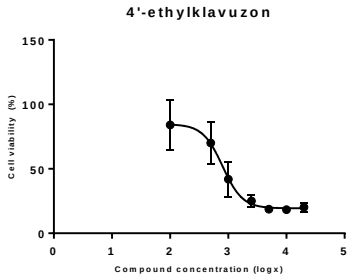
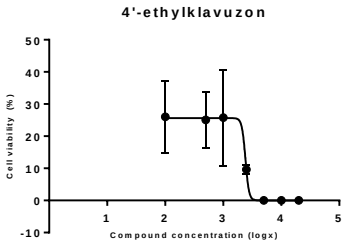
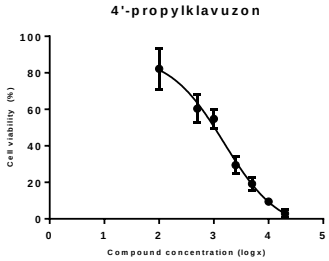
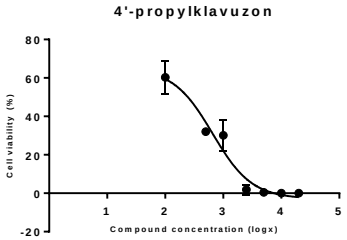
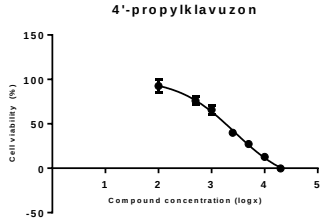
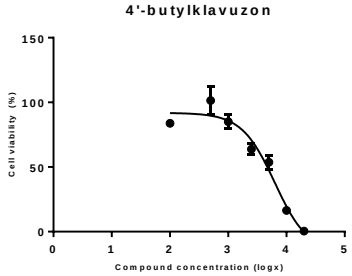
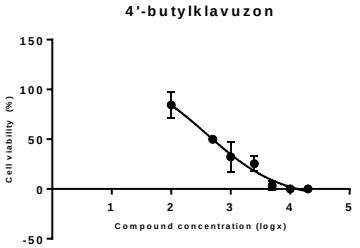
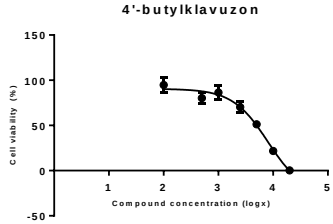
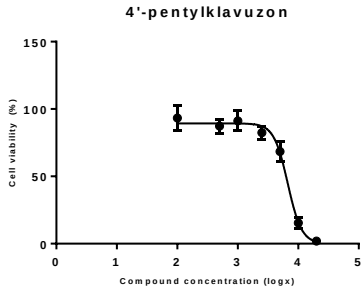
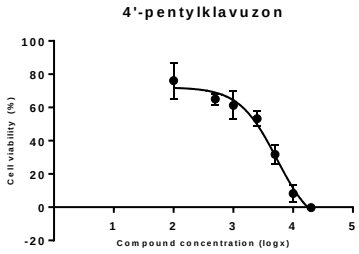
Table 2.1. The cytotoxicity profiles of compounds of **1**, **2**, **3**, **4** and **5** on MIA PaCa-2 and HPDEC cell lines.



MIA PaCa-2 cells	HPDEC cells	MIA PaCa-2 cells (collagen coated plates)
		
IC_{50} = 0,96 ± 0,17 μM	IC_{50} = 0,18 μM	IC_{50} = 1,23 ± 0,34 μM

(Continued on next page)

Table 2.1. Continued

 IC_{50} = 0,83 ± 0,12 μM		
 IC_{50} = 1,5 ± 0,61 μM	 IC_{50} = 0,65 μM	 IC_{50} = 2,62 ± 1,09 μM
 IC_{50} = 6,3 ± 0,73 μM	 IC_{50} = 0,39 μM	 IC_{50} = 7,37 ± 1,21 μM
 IC_{50} = 6,7 ± 0,20 μM	 IC_{50} = 5,3 μM	

2.2. Apoptosis Analysis

One of the most important characteristic of apoptosis is localization of phosphatidylserine (PS) to outer membrane. In normal cell PS is located in inner cell membrane, however during apoptosis PS translocases to outer plasma membrane. In this experiment Annexin V/FITC Screening kit was used to detect apoptosis in 4'-methylklavuzon applied cells. Annexin V is a Ca^{2+} dependent phospholipid binding protein and has high affinity to PS. Annexin V is conjugated to a fluorochrome fluorescein isothiocyanate (FITC) which binds to externalized PS during early apoptosis and can be detected by flow cytometry. To distinguish early apoptosis from late apoptosis or necrosis, propidium iodide agent is used, which is an intercalating agent of DNA and could not pass through normal cell membranes. Live cells are negative for both Annexin V and PI, early apoptotic cells are positive for Annexin V and negative for PI (still has intact plasma membrane), late apoptosis and necrotic cells are positive for both stains.

The cytotoxicity results showed that 4'-methylklavuzon is the one of the most cytotoxic molecule among them and experiments focused on this molecule. The apoptotic effect of 4'-methylklavuzon was examined on MIA PaCa-2 cell line. The experiment was based on concentration dependent, 3 concentrations were applied; 1 μM, 5 μM, 10 μM and results were obtained from three separate experiments (Figure 2.2). 4'-methylklavuzon has showed promising apoptosis activity in concentration dependent manner and necrotic cell percent was very low which is very attractive for an anti-proliferative molecule.

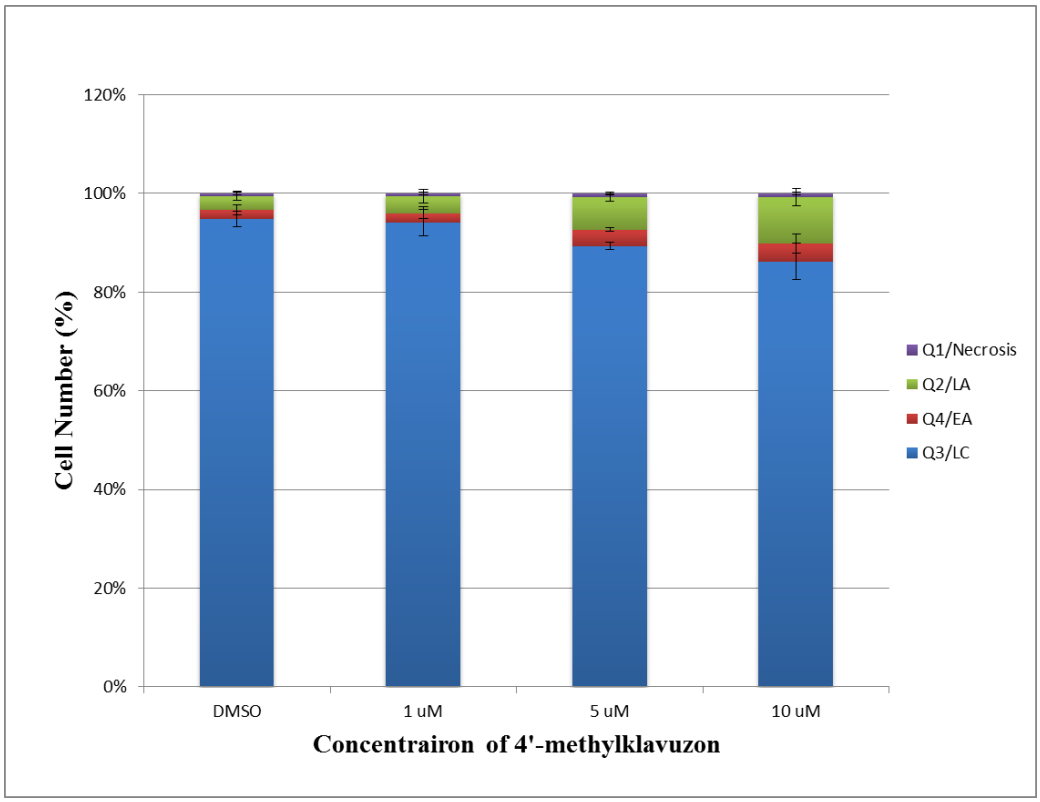


Figure 2.2. Concentration dependent apoptosis on MIA PaCa-2 cells that induced by 4'-methylklavuzon. Incubation time was 24 hours (LA: late apoptosis, EA: early apoptosis, LC: live cell)

2.3. Cell Cycle Analysis

Cell cycle basically has two major phases; interphase and mitosis. As mentioned above during interphase cell prepare itself to division, in G1 phase cell synthesize proteins and doubles cell contents. In S phase DNA is replicated and in G2 phase cell continues to synthesis additional protein that needed during mitosis. In mitosis phase cell begins to divide which completed replication of DNA and big enough to divide in two cells. In order to analyze cell cycle arrest of 4'-methylklavuzon applied MIA PaCa-2 cells PI dye used which is a fluorochrome DNA intercalating agent. In S phase cell has more DNA than G1 phase which means S phase cell takes up more PI dye and fluorescent more brightly. G2 cell completed DNA replication and fluorescent twice as G1 cell.

In the cell cycle analysis of 4'-methylklavuzon applied MIA PaCa-2 cells, especially at 5 μM and 10 μM, were arrested at S and G2 phase. During the S phase cell replicate DNA which means cell needs especially proteins and enzymes that related with DNA replication to progress into G2 phase. One of the important enzyme is

topoisomerase I which removes supercoils during replication. Inhibition of Topo I causes inhibition DNA uncoiling however poisoning of Topo I inhibits DNA religation function of Topo I and causes DNA fragmentation. Cpt is a known Topo I poison and causes S phase arrest and also there are studies indicating inhibition of Topo I by GNT. Because of structural similarities of 4'-methylklavuzon and Cpt and GNT, Topo I could be potential target of 4'-methylklavuzon.

Also there is DNA damage check point in G2 phase that prevent cell to progress into M phase in case of DNA damage or incomplete DNA replication. Arresting of MIA PaCa-2 cells in G2 phase probably caused by Topo I dependent DNA damages or Topo I dependent incomplete DNA replication.

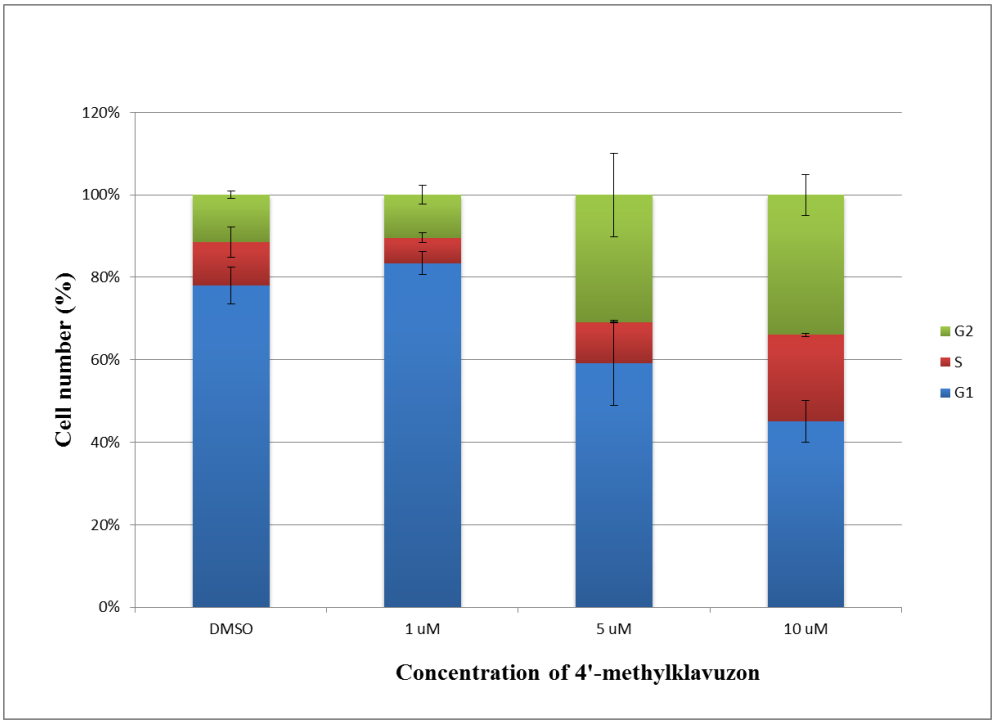


Figure 2.3. Concentration dependent cell cycle arrests on MIA PaCa-2 cells that were induced by 4'-methylklavuzon. Incubation time was 24 hours.

2.4. Topoisomerase I Drug Screening Assay

As mentioned above Topo I is a potential target for 4'-methylklavuzon. To clarify the effect of 4'-methylklavuzon on Topo I enzyme, time and concentration depended Topoisomerase I Drug Screening assays were performed. In time depended experiments, Topo I enzyme was pre-incubated with 4'-methylklavuzon for 0, 1, 5 and

10 minutes before adding of supercoiled DNA. This procedure was applied for 3 different concentrations of 4'-methylklavuzon; 2 μM, 50 μM and 100 μM.

According to the gel results, there was slight reduction of Topo I relaxation activity of 2 μM 4'-methylklavuzon applied tubes, nearly supercoiled DNA was relaxed and there was very weak band on supercoiled DNA line (Figure 2.4). However 50 μM and 100 μM 4'-methylklavuzon applied gels showed that, pre-incubation of 4'-methylklavuzon with Topo I decreased Topo I activity in both pre-incubation time and concentration depended manner (Figure 2.5-2.6). Especially 5 and 10 minutes pre-incubation of Topo I with 100 μM 4'-methylklavuzon showed important reduction of relaxation activity. These results indicate that probably 4'-methylklavuzon is Topo I inhibitor.

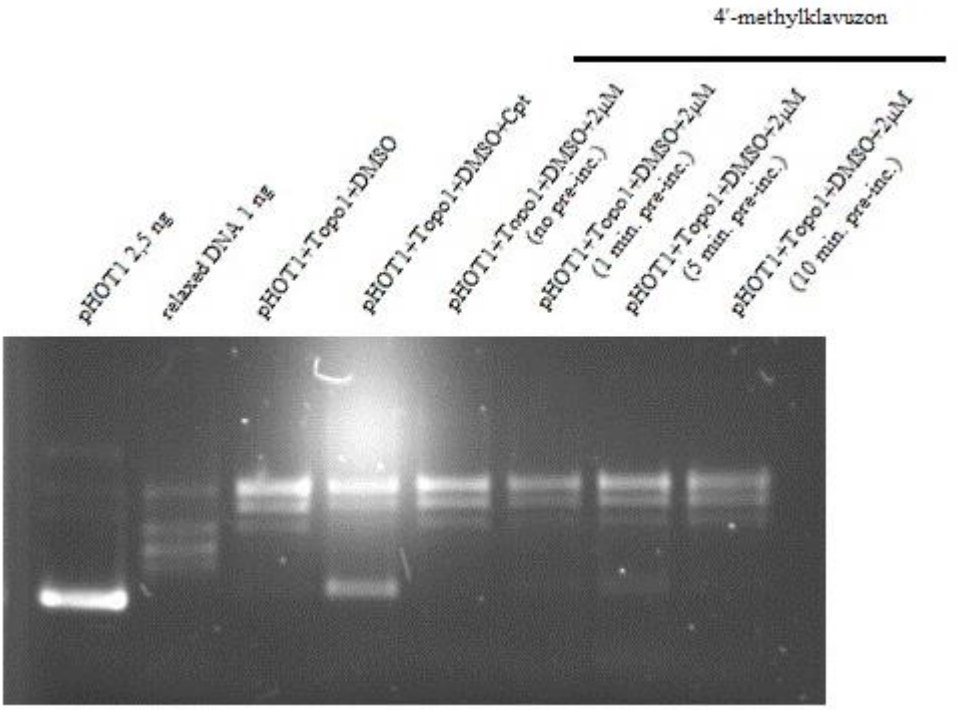


Figure 2.4. Topoisomerase I Drug Screening assay of 4'-methylklavuzon gel result, concentration of 4'-methylklavuzon was 2 μM.

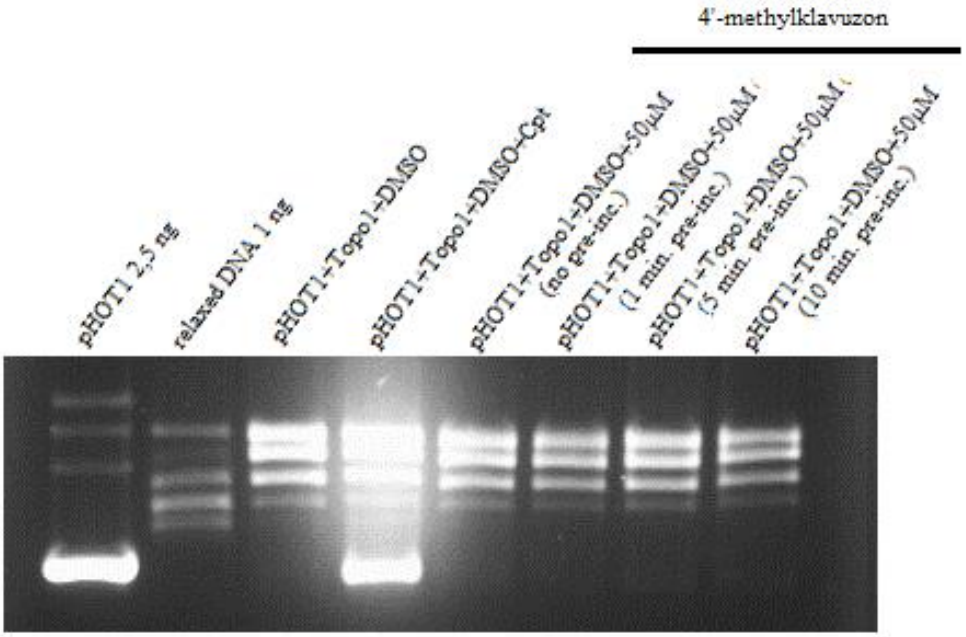


Figure 2.5. Topoisomerase I Drug Screening assay of 4'-methylklavuzon gel result, concentration of 4'-methylklavuzon was 50 μM.

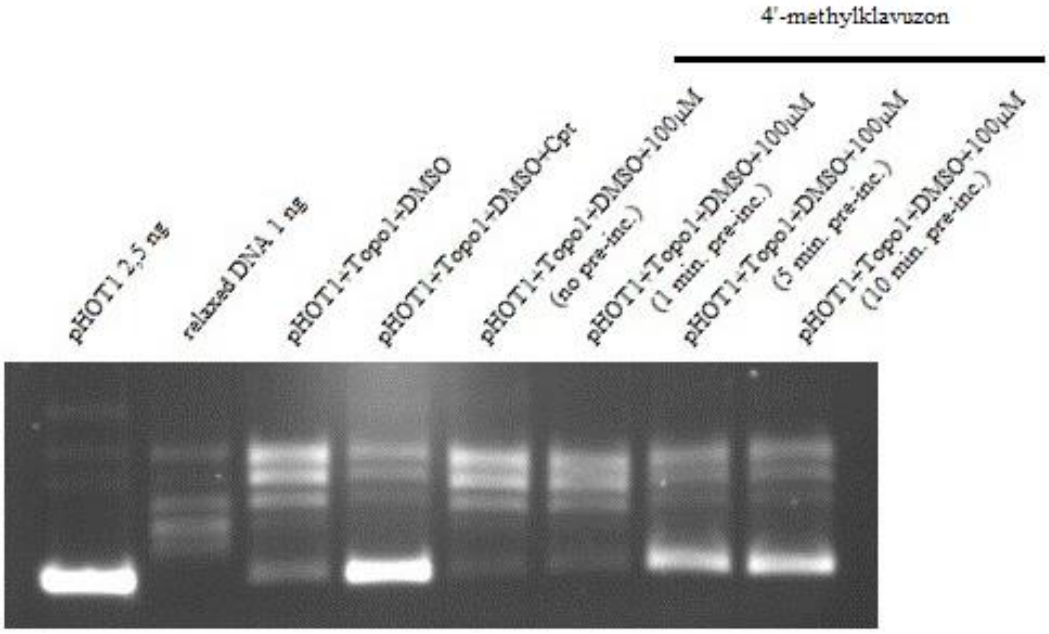


Figure 2.6. Topoisomerase I Drug Screening assay of 4'-methylklavuzon gel result, concentration of 4'-methylklavuzon was 100 μM.

2.5. COMET Assay

DNA fragmentation is one of the important indicator of apoptosis. In this study COMET assay was performed which is a sensitive method to examine DNA fragmentation in level of individual cell. Technique is based on micro-gel

electrophoresis of DNA cell. Basically cells were embedded in low melting agarose, lysed and electroporation was applied. While damaged DNA moves in the electric field and forms a tail like comet, the undamaged DNA stays its place like head of comet.

Apoptosis analysis showed that 4'-methylklavuzon caused apoptosis rather than necrosis. Also Topo I assay results indicated pre-incubation time and concentration depended inhibition of topoisomerase I activity. In order to analyze DNA damage in cellular level COMET assay was performed. Three concentrations of 4'-methylklavuzon were applied; 0,1 μM, 5 μM and 10 μM. For positive control 50 μM and 100 μM H_2O_2 was applied for 2 hours. 0,1 μM applied cell did not show any comets, results were similar with negative control. However 5 μM applied cells showed comet formation. According to the figures the tail moments of comets were shorter than positive control but number of comets was noteworthy. 10 μM applied cell results also showed comets formation however the number of comets was smaller than 5 μM applied cell results, the round shapes of nuclear DNA contents were lost and it was seemed having high damage. This result consistent with apoptosis results, there were an increment in percent of late apoptotic cell.

Table 2.2. The number of comets formed by 0,1 μM, 5 μM and 10 μM 4'methylklavuzon applied Mia PaCa-2 cells for 24 hours. For positive control 50 μM H_2O_2 was applied for 2 hours.

			4'methylklavuzon		
	Negative Control	Positive control (H_2O_2)	0,1 μM	5 μM	10 μM
Number of comets	6	59	0	30	14
Total cell counted	105	109	36	56	33

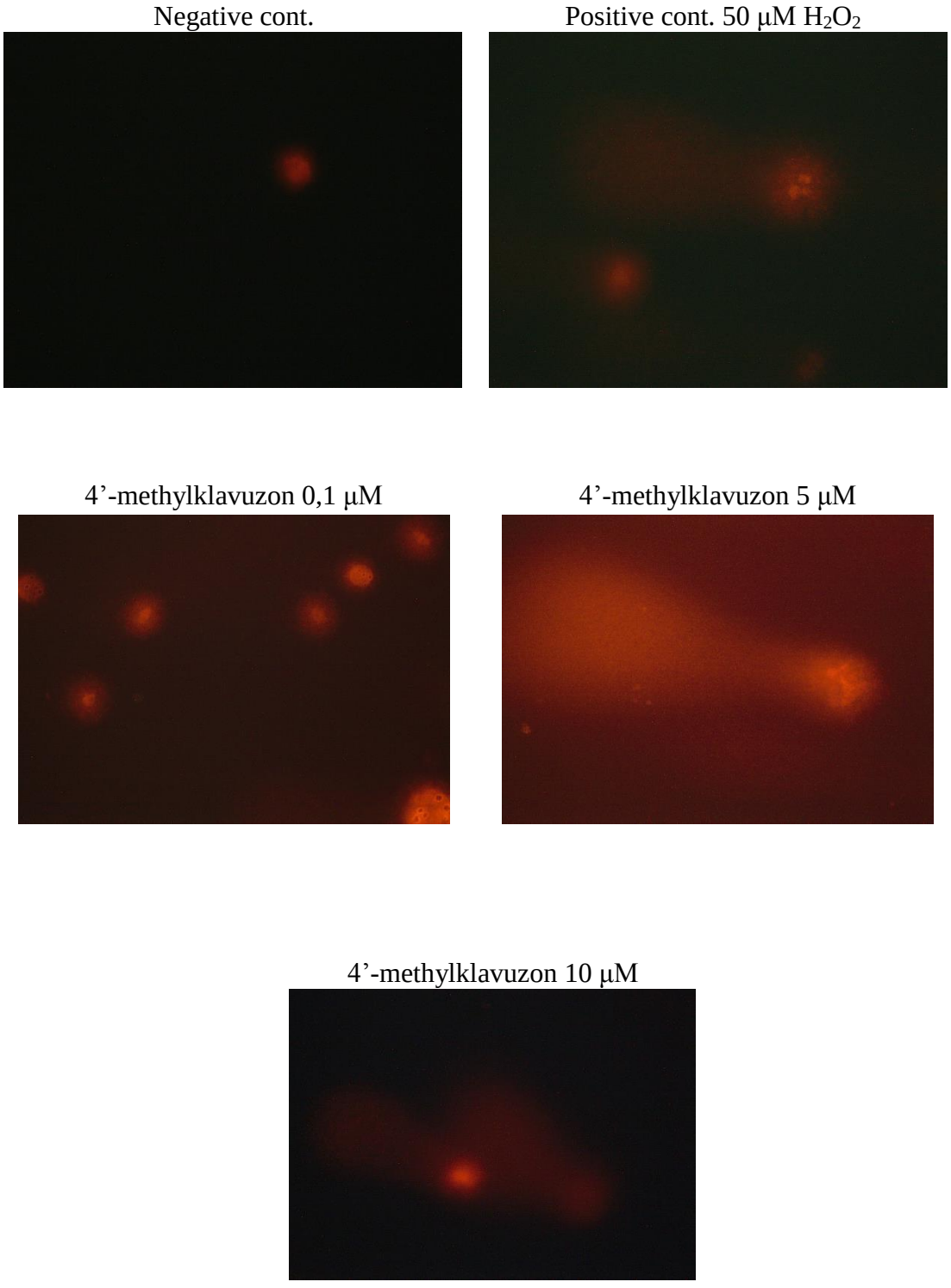


Figure 2.7. Comet assay results of MIA PaCa-2 cells; concentrations of 4'-methylklavuzon were 0,1 μM, 5 μM and 10 μM incubated for 24 hours. H_2O_2 was applied as positive cont. with 50 μM concentration (Images were taken at 40X magnification).

CHAPTER 3

EXPERIMENTAL

3.1. Thawing out Cells

Vial of frozen MIA PaCa-2 (pancreas) cells was taken from -80 ^{0}C and transferred to 37 ^{0}C water bath for thawing 2-3 minutes. After thawing cells were transferred to falcon tube and centrifuged at 800 rpm for 5 minutes. Then the supernatant was removed and pellet was dissolved with new media, Dulbecco's modified Eagle's medium supplemented with 10 % fatal bovine serum, 1 % penicillin-streptomycin and 1 % non-essential amino acids (complete medium). These cells were added to T75 flask and incubated in incubator at 37 ^{0}C 5 % CO_2.

Vial of frozen HPDEC (healthy pancreatic cells) cells was taken from liquid nitrogen and transferred to 37 ^{0}C waterbath for thawing 2-3 minutes. After thawing cells were transferred to falcon tube and centrifuged at 1500 rpm for 5 minutes. Then the supernatant was removed and pellet was dissolved with new media, PriGrow (abm) medium supplemented with 20 % non-heat inactivated fatal bovine serum and 1 % penicillin-streptomycin (complete medium). These cells were added to T75 flask and incubated in incubator at 37 ^{0}C 5 % CO_2.

3.2. Passaging Cells

3.2.1. MIA PaCa-2 Cells

Before passage of MIA PaCa-2, complete DMEM media and trypsin (0,05%) were warmed to 37 ^{0}C in waterbath. The medium on the surface of flask was removed and flask was washed with 3 mL medium to remove any residual. Then 3 mL of trypsin was added to flask and incubated at 37 ^{0}C 5 % CO_2 incubator for 3-5 minutes. After detachment of cells from flask 9 mL of media was added and transferred to falcon tube to centrifuge at 800 rpm for 5 minutes. Supernatant was removed and pellet was

dissolved with fresh media and transferred to new flask and incubated at 37 ^{0}C 5 % CO_2 in incubator.

3.2.2. HPDEC Cells

Before passage of HPDEC cells, flask was coated with type I collagen (ABM's Applied Cell Extracellular Matrix) according to manufacturer protocol. Then complete PriGrow media, collagenase (0,4%) and trypsin (0,05%) were warmed to 37 ^{0}C in waterbath. The medium on the surface of flask was removed and flask was washed with 1 mL medium to remove any residual. Then 1 mL of collagenase was added to flask and incubated at 37 ^{0}C 5 % CO_2 incubator for 15 minute. 3 mL of Prigrow medium was added to flask to inhibit collagenase and cells were collected to falcon tube. Then 1 mL of trypsin (0,05%) added to flask and incubated at 37 ^{0}C 5 % CO_2 incubator for 5 minute. 3 mL of Prigrow medium was added to flask to inhibit trypsin and cells were transferred to same falcon tube. Cells were centrifuge at 800 rpm for 5 minutes. Supernatant was removed and pellet was dissolved with fresh media and transferred to new flask and incubated at 37 ^{0}C 5 % CO_2 incubator.

3.2.3. Cell Counting

450 µL trypan blue mixed with 50 µL cell suspension in 1,5 µL centrifuge tube. About 20 µL of mix was transferred to Neubauer counting chamber and covered with coverslip. The chamber divided in two and each side has four counting squares. Totally eight square were counted and divided in eight; calculation was done according to dilution factor and chamber area as written below:

Cells per mL = the average count per square x the dilution factor x10^4(Chamber factor)

3.3. Freezing Cells

Cells detached from flask as described above and centrifuged at 800 rpm for 5 minutes. After removing supernatant, pellet was dissolved freezing medium (50% DMEM, 40%FBS, 10% dimethylsulfoxide (DMSO)) and aliquoted in 1 mL

cryopreservation tubes. Then MIA PaCa-2 cells were stored at -80 ^{0}C, HPDEC cells were stored at liquid nitrogen for long storage.

3.4. Cell Viability Assay (MTT Assay)

For viability assay of MIA PaCa-2 cell 95 µL of cell suspension including 5000 cells was added to each well of 96 well plate (Corning® Costar®) and incubated at 37 ^{0}C 5% CO_2 for 24 hours. Next day 7 concentrations of each 6 compounds were dissolved in sterile DMSO and filtered. 5 µL of each concentration was added to wells for triplicate assay and each experiment was done as triplicate. Compounds applied plates were incubated at 37 ^{0}C 5% CO_2 for 48 hours in incubator. Then cytotoxicity of compounds were determined by MTT (Sigma) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) based colorimetric assay. 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated at 37 ^{0}C 5% CO_2 for 4 hours in incubator. Then plate was centrifuged at 1800 rpm for 10 minutes and supernatant was removed. 100 µL DMSO added to each well to dissolve formazan crystals and kept on shaker at 130 rpm for 15 minutes. Lastly absorbance was determined at 540 nm.

Before viability assay of HPDEC cells, each well of plate was coated with type I collagen according to manufacturer protocol. Then 95 µL of cell suspension including 2000 cells was added to each well of 96 well plate and incubated at 37 ^{0}C 5% CO_2 for 24 hours. Next day 7 concentrations of each 6 compounds were dissolved in sterile DMSO and filtered. 5 µL of each concentration was added to wells for triplicate assay and each experiment was done as triplicate. Compounds applied plates were incubated at 37 ^{0}C 5% CO_2 for 48 hours in incubator. Then cytotoxicities of compounds were determined by MTT as explained above. The viabilities of cells were determined in percentage by the formula below:

% viability=[(ODs-ODB/ODc-ODB)x100]

3.5. Apoptosis Analysis

For 4'-methylklavuzon, apoptosis assay was performed on MIA PaCa-2 cell line. Apoptosis assay was tested by using Annexin V-FITC Detection Kit (BioVision). $5x10^5$ cells in 1980 µL was cultured in 6-well plate (Corning® Costar®) and incubated at

37 ^{0}C 5% CO_2 for 24 hours. Next day 3 concentrations of the 4'-methylklavuzon were prepared by dissolved with sterile DMSO and filtered. Then 20 µL of each concentration was applied to wells and incubated at 37 ^{0}C 5% CO_2 for 24 hours. After incubation supernatant of each well was collected to separate falcon and remaining cells were trypsinized and collected in the same falcon. Then falcons were centrifuged at 1800 rpm for 10 minutes, pellet were washed with 5 mL PBS and centrifuged again. Pellets were dissolved in 200 µL of binding buffer and 2 µL of Annexin V-FITC and 2 µL of propidium iodide (PI) were added to each tube and incubated at room temperature for 15 minutes in dark place. Analysis was done by flow cytometer.

3.6. Cell Cycle Analysis

For 4'-methylklavuzon, cell cycle assay was performed on MIA PaCa-2 cell line. Cell cycle assay was tested by using PI staining (BioVision). $5x10^5$ cells in 1980 µL was cultured in 6-well plate (Corning® Costar®) and incubated at 37 ^{0}C 5% CO_2 for 24 hours. Next day 3 concentrations of the 4'-methylklavuzon were prepared by dissolved with sterile DMSO and filtered. Then 20 µL of each concentration was applied to wells and incubated at 37 ^{0}C 5% CO_2 for 24 hours. After incubation supernatant of each well was collected to separate falcon and remaining cells were trypsinized and collected in the same falcon. Then falcons were centrifuged at 1800 rpm for 10 minutes, pellet were washed with 5 mL PBS and centrifuged again. The pellets were dissolved in 1 mL cold PBS and on the vortex 4 mL of -20 ^{0}C absolute ethanol (Merck) was added dropwise and incubated at -20 ^{0}C at least 24 hours. After incubation cells were centrifuged at 1800 rpm at +4 for 10 minutes and pellets were dissolved in 5 mL of PBS and centrifuged again. Pellets were dissolved in 200 µL phosphate buffer including 0,1% Triton X-100 and 20 µL RNase A (200 µg/mL) was added to each tube and incubated at 37 ^{0}C for 30 minutes. After incubation 20 µL PI (1 mg/mL) was added and incubated at room temperature for 15 minutes in dark. Cell cycle distributions were determined by flow cytometer.

3.7. Topoisomerase I Drug Screening Assay

In order to determine the effects of compounds on Topo I enzyme, Topoisomerase I Drug Screening kit (Topogen) assay was performed. The experiment was designed to indicate concentration dependent Topo I inhibition. In a centrifuge tube reaction mixture was prepared as followed; final volumes were completed to 20 μL with dH_2O, 2 μL TGS buffer (10X TGS; TGS Buffer (1X) is 10 mM Tris-HCl pH 7.9, 1 mM EDTA, 0.15 M NaCI, 0.1% BSA, 0.1 mM Spermidine, 5% glycerol), 2u of Topo I enzyme (Topogen) and 2 μL test 4'-methylklavuzon which was dissolved in DMSO. Then Topo I enzymes were pre-incubated with 4'-methylklavuzon at 37 ^{0}C for different times (0, 1, 5 and 10 minutes). 1 μL supercoiled DNA (pHOT1 concentration of 0.25μg/mL; 25 μg pHOT1 DNA in 100 μL TE buffer, 10 mM Tris-HCl, pH7.5,1 mM EDTA) added to tubes and then tubes were incubated at 37 ^{0}C for 30 minutes. After incubation reactions were terminated by adding 10% sodium dodecyl sulfate (SDS), final concentrations of SDS were 1%, 2 μL of loading dye was added. Samples were loaded to 1% agarose gel and run 1-2,5 V/cm until dye reached end of gel. Gel was stained with ethidium bromide 0.5 μg/mL (EtBr) hold on shaker for 30 minutes and destained with dH_2O for 15 minutes on shaker. Gels were monitored with UV transluminator.

3.8. COMET Assay (Single Cell Gel Electrophoresis assay)

The COMET assay is a sensitive assay to examine DNA fragmentation in level of individual cell. In this assay there are two kinds of lysis solution; alkaline lysis solution to detect double and single strand breaks or neutral lysis solution to detect double strand breaks (Nature Protocol). In this experiment alkaline lysis solution was applied to examine any effect of 4'-methylklavuzon that causes DNA damage.

Samples were prepared as followed: $5x10^5$ cells in 1980 μL was cultured in 6-well plate (Corning® Costar®) and incubated at 37 ^{0}C 5% CO_2 for 24 hours. Next day 3 concentrations of the 4'-methylklavuzon were prepared by dissolving with sterile DMSO and filtered. Then 20 μL of each concentration of 4'-methylklavuzon were applied to wells and incubated at 37 ^{0}C 5% CO_2 for 24 hours. 20 μL 50 μM of H_2O_2 as positive control was applied for last 2 hours of incubation (Benhusein, Mutch, Aburawi,

& Williams, 2010). After incubation mediums of each well were collected to falcons, cells were trypsinized and collected in the same falcon. Then falcons were centrifuged at 1800 rpm for 10 minutes then pellet were washed with 5 mL PBS and centrifuged again. Pellets were dissolved in 500 μL of PBS and each sample was counted as explained above.

After sample preparation, slides were prepared as following: 1% normal melting agarose (NMA) was prepared in 50 mL of PBS, microwaved until near boiling and dissolve agarose. 1% low melting agarose (LMA) was prepared in 10 mL of PBS, microwaved until near boiling and dissolve agarose. LMA was aliquoted in 5 mL samples and refrigerated until needed. When needed, melt in microwave and keep in water bath and stabilize temperature as 40 ^{0}C. Slides were dipped in methanol and burned with flame to remove dust. Then slides were dipped in hot NMA, underside of slides were wiped to remove agarose and laid on a flat surface to dry (Slides can be prepared before the day of experiment). 75 μL of LMA was mixed with about 10.000 in 5-10 μL of cells, pipetted on NMA covered slides and covered with coverslip. Slides were placed on ice pack until second agarose layer hardened (5-7 minutes). Then coverslips were removed gently and samples were placed in freshly prepared cold lysis solution (1,2 M NaCl, 100 mM Na_2EDTA, %0,1 Sodium Lauryl Sarcosinate (SLS), 0,26 M NaOH, pH adjusted 13 or higher) and incubated at +4 ^{0}C for overnight. After incubation slides were removed, rinsed with fresh made cold alkaline/electrophoresis solution dropwise onto slides kept for 20 minutes and repeated 2 more times (0,03 M NaOH, 2 mM Na_2EDTA, pH adjusted to 12,3). Then slides were placed in fresh electrophoresis solution in electrophoresis tank run for 20 minutes at 0,6 V/cm and current was 40 mA. After electrophoresis slides were gently removed from electrophoresis tank, neutralized with neutralization buffer by dropwise coat slides for 5 minutes 3 times and let them dry (0,4 M Tris-Cl, ph adjusted to 7,4). 100 μL of 10 μg/mL PI solution pipetted onto slides, incubated for 20 minutes and rinsed with dH_2O. Comets were analyzed with microscope under UV light.

CHAPTER 4

CONCLUSSION

Previosuly it was shown that *(R)*-goniothalamin is a naturally occurring anti-cancer agent. It is the lead compound of 4'-methylklavuzon showing enhanced cytotoxic activity over cancer cell lines compared to both *(R)*-goniothalamin and klavuzon. In this thesis cytotoxic activity of 4'-methylklavuzon and four new 4'-alkylklavuzon derivatives on MIA PaCa-2 was studied by using MTT and it is observed that presence of a carbon chain longer than two carbons causes a decrease in the activity. Both 4'-methylklavuzon and 4'-ethylklavuzon have IC_{50} values at nanomolar concentrations.

A selected compound, 4'-methylklavuzon caused a dose dependent increment of apoptosis in MIA PaCa-2 cells and it also causes S and G2 cell cycle arrest at high concentrations. Previously it was shown that klavuzon derivatives may inhibit the calf thymus TOPO I-DNA adduct. In here, time dependent Topo I inhibitory property of 4'-methylklavuzon was studied and it is shown that activity of the human topoisomerase I pre-incubated with 4'-methylklavuzon loses its activity. Because of the presence of an increment in S phase of cell cycle and topoisomerase I inhibitory properties it is expected that there should be a DNA damage in the cells. To show such DNA damage the effect of 4'-methylklavuzon on DNA was examined on single cell with COMET assay and there were comets which was indicator of DNA fragmentation. According to results 4'-methylklavuzon and 4'-ethylklavuzon are promising molecules and studies should proceed to determine the potential target or targets of the molecule.

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