ANTIPROLIFERATIVE PROPERTIES OF 2'-ALKOXYMETHYL SUBSTITUTED KLAVUZON DERIVATIVES

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by
Mehmet Salih YILDIZ

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We approve the thesis of **Mehmet Salih Yıldız**

Examining Committee Members:

Assoc. Prof. Dr. Ali Çağır
Department of Chemistry, İzmir Institute of Technology

Prof Dr. Safiye Neşe Atabey
Faculty of Medicine Basic Medical Sciences Department Of Medical Biology, Dokuz Eylül University

Assist. Prof. Gülistan Meşe Özçivici
Department of Molecular Biology and Genetics, İzmir Institute of Technology

27 January 2017

Assoc. Prof. Dr. Ali Çağır
Supervisor, Department of Chemistry,
İzmir Institute of Technology

Assoc. Prof. Devrim Pesen Okvur
Co-Supervisor, Department of Molecular Biology and Genetics,
İzmir Institute of Technology

Assoc. Prof. Dr. Engin ÖZÇİVİCİ
Head of the Department of Biotechnology Bioengineering

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of and Engineering and Sciences

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ABSTRACT

ANTIPROLIFERATIVE PROPERTIES OF 2'-ALKOXYMETHYL SUBSTITUTED KLAVUZON DERIVATIVES

One of the main objectives of studies on anticancer agents is that the agent is expected to show a high cytotoxic activity on cancer cells and show a less cytotoxic effect on the contrary in healthy cells or never show cytotoxic activity. (R)-goniothalamin, isolated from the *Goniothalamus* plant, is a styryl lactone and has been found to have a selective antiproliferative property on cancer cells in studies conducted. The Michael acceptor feature in the structure of goniothalamin is thought to be covalently bonded to the nucleophilic side chains of the enzymes and show activity in this way. In previous studies, it has been shown that 1-naphthyl substituted 5,6-dihydro-2H-pyran-2-one derivatives and 4'-methyl klavuzon derivatives exhibit higher cytotoxic activity on cancer cells than goniothalamin. In this study, antiproliferative properties of newly synthesized 2'-alkoxymethyl substituted klavuzon derivatives have been examined and MIA PaCa-2 pancreatic cancer cell lines and HPDEC pancreatic healthy cell lines were used.

MTT cell viability tests were performed at the first step of this study. As a result of this study, it has been observed that the 2'-isobutoxymethylklavuzon derivative has selective cytotoxic activity on the MIA PaCa-2 cell line. It showed activity at lower concentrations than goniothalamin. Cytotoxic activities of the compounds are associated with the size of the R group at position 2'-. Methoxymethyl substituted the worst selective activity among these compounds whereas isobutoxy derivative the best selective one.

In the second stage of the study, the inhibition on topoisomerase I enzyme was studied. The 2'-alkoxymethyl klavuzon derivatives were found to have Topo I enzyme inhibition properties depending on concentration and time manner.

The study continued with choices methoxy and isobutoxy derivatives and these two compounds caused an arrest at G1 phase and DNA damage. Also, isobutoxy derivative induced apoptosis in the MIA PaCa-2 pancreatic cancer cell lines.

ÖZET

2’-ALKOKSİMETİL SÜBSTİTÜELİ KLAVUZON TÜREVLERİNİN ANTİPROLİFERASYON ÖZELLİKLERİ

Anti kanser ajanları üzerine yapılan çalışmaların ana amaçlarından biri ajanın, kanserli hücreler üzerinde yüksek bir sitotoksik aktivite göstermesi ve sağlıklı hücrelerde tam tersi olarak daha az bir sitotoksik etki göstermesi yada mümkünse hiç göstermemesi beklenilmektedir. *Goniothalamus* bitkisinden izole edilen (R)-goniothalamin, bir stiril lakton olup, yapılan çalışmalarda kanser hücreleri üzerinde seçici antiproliferatif özelliği olduğu bulunmuştur. Goniothalaminin yapısında bulunan Micheal akseptör özelliği, enzimlerin nücleofilik yapıları ile kovalent bağlar kurduğu ve bu şekilde aktivite gösterdiği düşünülmektedir. Daha once yapılan çalışmalarda 1-naftil subsitüeli 5,6-dihidro-*2H*-piran-2-on türevi ve 4’-metil klavuzon türevleri, kanser hücreleri üzerinde goniothalaminden daha yüksek bir sitotoksik aktivite gösterdiği görülmüştür. Bu çalışmada yeni sentezlenen 2'-alkoksimetil sübstitueli klavuzon türevlerinin antiproliferatif özelliklerine bakılmıştır ve MIA PaCa-2 pankreas kanserli hücre hattı ile HPDEC pancreas sağlıklı hücre hatları kullanılmıştır.

Çalışmanın ilk basamağında MTT hücre canlılık testleri yapılmıştır. Bu çalışmanın sonucunda 2’-isobütoksimetilklavuzon türevinin MIA PaCa-2 hücre hattı üzerinde seçici bir sitotoksik aktiviteye sahip olduğu görülmüştür. Goniothalaminden daha düşük konsantrasyonlarda bu aktiviteyi gösterdiği gözlemlenmiştir. Klavuzon türevlerinin sitotoksik aktiviteleri, 2’-konumundaki R grubunun büyüklüğü ile ilişkilidir. Metoksimetil substitueli klavuzon, bileşikler içinde en kötü seçici aktiviteye sahip olan bileşik iken, isobütoksimetil substitueli klavuzon en iyi seçici aktiviteye sahip olan bileşik olduğu görüldü.

Çalışmanın ikinci aşamasında topoisomeraz I enzimi üzerindeki inhibisyonuna bakıldı. Bu çalışmada kullanılan 2’-alkoksimetil klavuzon türevlerinin konsantrasyona ve zamana bağlı olarak enzimi inhibe etme özellikleri olduğu görülmüştür.

Metil ve isobütoksi türevlendirilmiş klavuzon türevleri ile çalışmalara devam edildi. Ve bu iki bileşik hücrenin G1 fazını bloke etti ve DNA hasarına neden oldu. Ayrıca isobütoksi türevi MIA PaCa-2 pankreas kanser hücre hatlarında apoptozu indükledi.

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ABBREVIATIONS

AMP	Adenosine monophosphate
APAF1	Apoptotic protease activating factor 1
ARF	Adp-ribosylation factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated x protein
Bcl-2	B-cell lymphoma 2
CDK	Cyclin dependent kinase
Chk	Checkpoint kinase
CKIs	Cyclin dependent kinase inhibitors
Cpt	Camptothecin
DISC	Death-inducing signal complex
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E2	Ubiquitin-conjugating enzyme
E2F	E2 factor family of transcription factors
FADD	Fas-associated death domain
FITC	Fluorescein isothiocyanate
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
pRb	Retinoblastoma protein
PS	Phosphatidylserine
R-GTN	(R)-goniothalamin
RNA	Ribonucleic acid

ROS	Reactive oxygen species
TAE	Tris-acetate-EDTA
TNF	Tumour necrosis factor
Topo I	Topoisomerase I
Topo II	Topoisomerase II
TRADD	TNFR1-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand

CHAPTER 1

INTRODUCTION

1.1.Cancer and Pancreatic Cancer

Cancer is a genetic illness that can be defined as uncontrolled cell division caused by certain mutations that occur at several control points of DNA (such as p53, p21) that control cell division or cell proliferation lead to cancer. Human or the other organism cancers have some molecular genetic analyzes; This shows that cancer cells have more than one genetic defect; Mutations, translocations and mitotic recombination's that can happen during cell division (no disjunctions, gene transformations) (Ramel, 1988; Preston-Martin et al., 1990). This means that enhanced cell division improves tumor generation and cancer risk (Cohen and Ellwein, 1990; Ames, 1992;). Tumors have more complexity than cancer. Tumors create a microenvironment of their own. So that the tumor cells occur their own characteristics and they look like normal cells (Legarza and Yang, 2006).

Cancer as an evolutionary sickness involves the characteristics of a single-celled organism that develops asexually and connected to historic paradigm model. Pancreatic ductal adenocarcinoma is an especially potent example of this event. (Makohon-Moore and Iacobuzio-Donahue, 2016). Pancreatic cancer begins when pancreatic abnormal cells grow up uncontrollably. Pancreatic cancer constitutes about 2% of whole cancers and is the fourth most common reason for cancer death (Directory, 2003). Also, a pancreatic cancer malignancy has a troubled clinical prognosis. For this reason, improved treatments are urgently needed. Despite significant advances in understanding of its biology, there remains no effective clinical treatment for late stage pancreatic cancer, and current therapy ensure only a few months of survival (Directory, 2003). Survival of pancreatic cancer is average six months, and even though fifty years of research and medicinal development, five year survival keeps less than 5% (Directory, 2003). Alvin and Christine A. foresight for 2016, approximately 53,070 patients can be diagnosed with pancreatic cancer and most can die five years later. This is because, for pancreatic cancer, there is not current clinical screening method at the time of treatment,

and appropriate survival treatments will be the only treatment of for only 10-15% of recently diagnosed cases.(Makohon-Moore and Iacobuzio-Donahue, 2016). Also, pancreatic cancer is divided into two groups. Approximately 95% of pancreatic cancers start within channels carrying pancreatic juices. This region is called the exocrine pancreas. In uncommon cases (5%), pancreatic cancer occurs in the endocrine region, which is responsible for hormone production. The endocrine pancreas is composed of islet cells that produce both insulin and other hormones (Stain, 2003)

1.2.Cell Cycle

Eukaryotic and prokaryotic cells grow and divide regularly according to the cell cycle (Schneider, 2001). Basically, cell cycle take place of four stages. These are cell growth (G1 or Gap1) phase, DNA replication (S) phase, chromosomes distribution (G2 or Gap1) phase and mitosis or cell division (M) phase (Şen, 2015). If we need to clarify some of the events in these phases, cell must reach a sufficient size before the cell divides, which is called the G1 phase. The S phase comes after this process and DNA copies itself at this stage. This stage is known as the DNA synthesis stage. After that, the cell enters to the G2 phase. In this phase, cell growth and protein synthesis continue in order to get ready for cytokinesis (Schneider, 2001). M phase divides the chromosomes of a single nucleus of eukaryotic cell into two identical nuclei. Generally, cytokinesis is begun immediately; it divides nuclei, cytoplasm, organelles and cell membrane into two cells containing roughly equal parts of these cellular components in. Both mitosis and cytokine describe the M phase of the cell cycle. At the end of this phase, the mother cell is divided into two identical cells that are genetically similar to each other (Mitchison and Salmon, 2001). In most cases, cells start a new cell cycle after cytokinesis, whereas some kind of cells cannot divide, such as liver cells, kidney cells. These kinds of cells come out of the G1 phase and enter the G0 phase where they maintain metabolism, deactivated to proliferate.(Şen, 2015) (Figure 1.1)

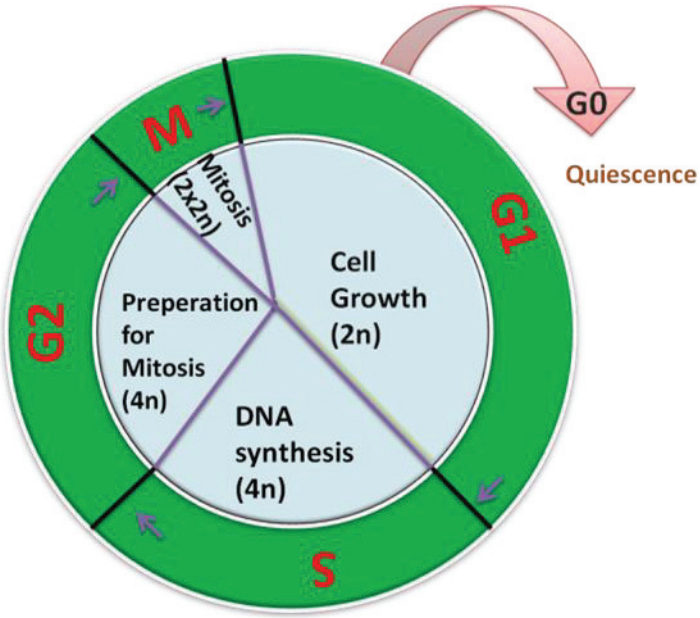


Figure 1.1.The phases of cell cycle.

1.2.1.The Module of Cell Cycle Control

A cell cycle is a phenomenon organized by various enzymes and specific proteins. These basic regulatory components are cyclins and CDKs. Depending on these proteins found in a cell, the cell cycle is able to happen rapid or slow or it may be stopped completely (Mitchison and Salmon, 2001; Paweletz, 2001).

Common results of different studies on eukaryotes have shown that cyclin-dependent kinases (CDKs) play an important role in the progression of cell cycle. CDKs are the family of proteins responsible both activation and inactivation of the cell cycle. CDKs (as the name implies) need the presence of cyclins to become active (van den Heuvel, 2005). Cyclins do not have an enzymatic character, they need to be attached to CDKs to show activity in the cell cycle. CDKs act in unison with various cyclins to increase progression along G1\S and S and G2/ M. CDKs are the main control enzymes that regulate the progress from one phase to another phase throughout the cell cycle (Morgan, 1995). In particular, CDKs phosphorylate their substrates by transferring phosphate groups from ATP to a specific amino acid site chain so that the target protein can be activated or inactivated (When threonine and tyrosine are dephosphorylated, CDK becomes active) (Şen, 2016; van den Heuvel, 2005). Also, the amount and type of CDK in eukaryotic cells vary from organism to organism. For example, there is only

one CDK in yeast, but there are four dissimilar CDKs in vertebrates(Kaldis et al., 2013; Morgan, 1995).

In all eukaryotes, there are several CDKs, and each of that is active at a specific phase of cell cycle. In organisms containing more than one CDK, every CDK is matched with a specific cyclin. For example, while CDK1 and CDK2 bind more than one cyclin (cyclin-A, B, D, and E), CDK4 and CDK6 only match cyclin-D, simply to explain this by an example; Cyclin E and CDK2 complex provide the start of S phase, cyclin A-CDK2 or cyclin A-CDK1 complete the finalization of S phase, cyclin B-CDK1 are responsible for the mitosis. All the cyclins are termed in accordance with the stage they were assembled together with the CDKs such as G1-phase cyclins (Pucci et al., 2000; Cooper, 2000). Especially, any cyclin production or degradation is very important for the cell cycle. Synthesis of new cyclin proteins provides the progression of the cell cycle. When the level of the cyclins falls, the CDKs that are active with them also become passive so cyclin breakdown is as important as its synthesis (Alberts et al., 2002). The cell cycle phenomenon of CDKs is valid for all eukaryotes, but there are minor differences. For instance, mammals and yeast have the little different CDK inhibitors (CKIs) that play a role in cell cycle control. Also, there are not some regulators such as pRb and E2F (E2 factor family of transcription factors) families in single cell eukaryotes, but regulatory genes in mammals have almost more than one subunit (Alberts et al., 2002; van den Heuvel, 2005; Dimova and Dyson, 2005).

Consequently, the CDK controls the production and destruction of the cyclins in the cell cycle. At the same time, CDKs play a critical role in three different cellular activities; transcription, DNA repair and regulation of cell cycle. P21, which is one of the inhibitor proteins that binds CDK and cyclin to stop the cell cycle (Pucci et al., 2000)

1.2.2.Cell cycle regulators

1.2.2.1.Cyclins and CDKs

As discussed above, the cyclins and the CDK protein family play critical roles in every step of the cell cycle. In the G1 phase of the cell cycle, the task of the cyclin D and CDK4 and 6 complexes is to allow the cell to respond to external signals such as

growth factors or mitogens. The transition from G1 to S phase, cyclins E and A, and CDK2 complexes play role and regulates the duplication of centrosomes, and it is important in terms of the end of the G1 phase and the beginning of the S phase. In phase S, the cell cycle is continued by cyclin E and A and CDK2 complexes. (Duronio and Xiong, 2013; Pucci et al., 2000; Branzei and Foiani, 2008) The target of this complex is helicases and polymerases, and in this way, they provide to begin to DNA replication. In the stage of M, Cyclins A and B and CDK1 complexes are a regulator of G2 / M checkpoint, and triggers the entry of the cells into the mitosis (Cooper, 2000). (Figure 1.2)

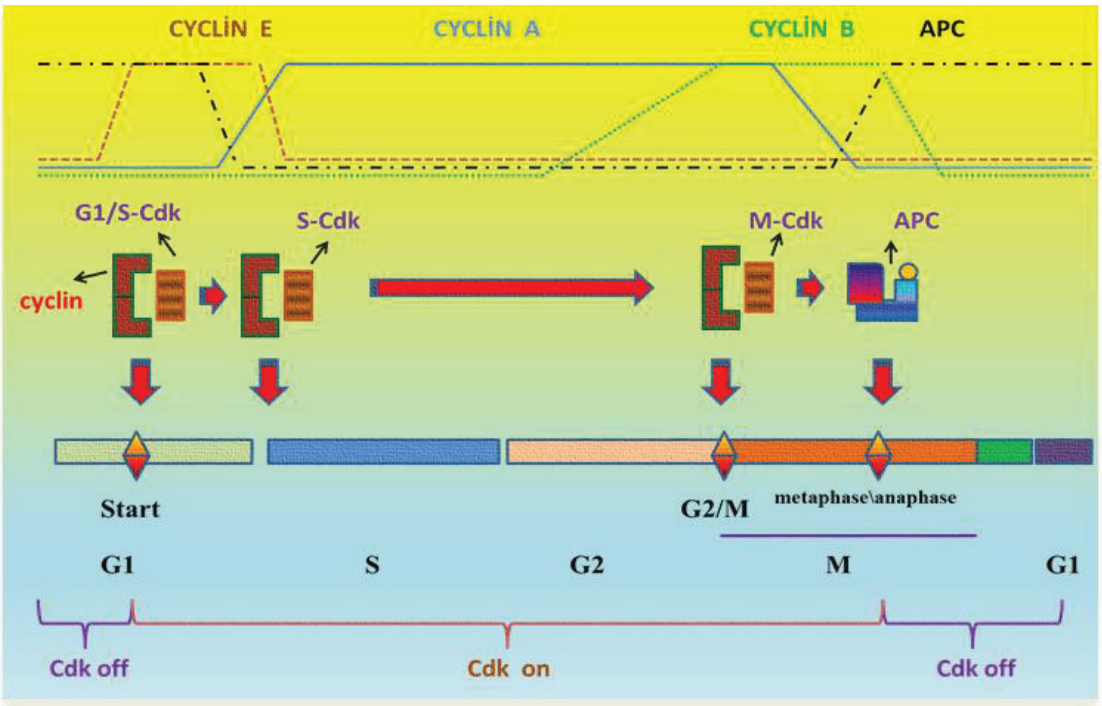


Figure 1.2.Mechanism of cyclin and CDK in cell cycle

1.2.2.2.CDK Inhibitory Proteins (Cip-Cyclin Inhibitory Proteins or Kip- Kinases İnhibitory Proteins)

The role of small inhibitory proteins in the cell cycle together with CDKs is a universal mechanism. The CKI proteins found in mammals are p21, p27 and p57. One of the members of this family, the p27 protein, checks the cyclin-E and CDK52 complexes that play a role in G1. In addition, p27 is interacting with the cyclin D and CDK4/6 complex (Blagosklonny and Pardee, 2013). The increase in the level of cyclin

D present in the cell leads to the depletion of p27. In parallel with p27 depletion, cyclin E activity begin to increase. The cell cycle shifts from the G1 phase to the S phase in the direction of the formation of this active high phosphorylated complex (cyclin E and CDK 4) (Abukhdeir and Park, 2008; Duronio and Xiong, 2013; Blagosklonny and Pardee, 2002). The Retinoblastoma protein helps E2F release and initiates the expression of the required genes (Bertoli et al., 2013)

1.2.2.3.The Rb/E2F Path

Retinoblastoma protein (pRb) is one of the first identified tumor suppressor proteins. and serves as the first control point in the mammalian restriction site (van den Heuvel, 2005) pRb interacts with E2F, thus affecting the expression of the target genes of E2F negatively. The main tasks of these genes; S phase entry and S phase suppression of progression and production of histone modifying enzymes. Phosphorylation of CDKs throughout the G1 phase leads to separation of pRb and E2F from each other and induction genes that involved in the transcription in S phase. The pRb-E2F path is very complex in terms of receiving different signals from the mechanism of G1 control point. A damage that can occur in this mechanism is a level that will affect human life; It results in cancer in particular, or it causes developmental disorders and diseases (in a wide range) (Duronio and Xiong, 2013). pRb does not directly affect CDKs, they use cyclins for this. Depending on cyclins, CDKs can be activated or inactivated form in the cell cycle (Casimiro et al. 2012)

1.2.2.4.Protein Degradation

In eukaryotes, ubiquitin pathway plays a undertaking role in the degradation of selective proteins. The task of ubiquitin, it acts as a marker that enables rapid proteolysis (degradation of polypeptide chains) by marking nuclear and cytosolic proteins. Ubiquitin is a polypeptide composed of 76 amino acids found in all eukaryotes. Proteins are labeled with ubiquitin to allow recognition by the side chain of the lysine amino acid, and this event continues to create the structure of multi ubiquitin chain by continued addition of ubiquitin. This composed polyubiquitinated structure is recognized and degraded by protease complexes with more than one subunit, that is

called a proteosome (Cooper, 2000). During this event, ubiquitin is not degraded and can be used again in the cycle of the cell. During the realization of these events, there is an energy requirement in the form of ATP (Duronio and Xiong, 2013; Lecker et al., 2006). During the ubiquitin binding process, ubiquitin first covalently binds to E1 (ubiquitin activating enzyme) to become active. At this stage, ATP is used and converted to AMP. Then ubiquitin will be transferred to another enzyme, called E2 (ubiquitin-conjugating enzyme). In the last step of the ubiquitin transfer process, ubiquitin is transferred to the E3 (ubiquitin ligase) enzyme to selectively identify the substrates (Callis, 2014).

The entrance of all eukaryotic cells to M (mitosis) phase is partially controlled by cyclin B, a regulatory subunit of Cdc2 protein kinase. Activation of Cdc2 results in chromosome condensation and nuclear envelope destruction. The activation event of Cdc2 is associated with cyclin B. Towards the end of mitosis, Cdc2 allows the ubiquitin proteolysis pathway to switch to the active state for degradation of cyclin B. This breakdown of Cyclin B is caused non-activated Cdc2, and ended the mitosis phase (Vaur, Poulhe et al. , 2004; Lodish et al., 2000)

1.3.Apoptosis

The apoptosis was first proposed by Kerr et al. In 1977 (Elmore, 2007). Apoptosis consists of a controlled cell death event and a complex process (Hengartner, 2000). It is not easy for a cell to make a decision to die, it is the process that is applied in a situation where the life of the cell should come to an irreversible point in order to make this decision (Hengartner, 2000; Alberts et al., 2002). The multi subprograms connected to more than one of these events must be activated and executed in a coordinated fashion to allow the apoptotic process to proceed smoothly after the cell has made its own death decision. (Meier et al., 2000; Hengartner, 2000). Apoptosis is a natural process in multicellular organisms. Apoptosis is a homeostatic mechanism that occurs throughout development and senescent events so that cell populations in tissues are preserved(Elmore, 2007). Apoptosis occurs either as in immune reactions or as defense mechanism when cells are damaged by disease or dangerous agents (Norbury and Hickson, 2001). There are multiple pathological or physiological factors that can trigger the apoptotic pathway. These stimuli need not be the same in all cells. For

example, irradiation or cancer therapy medicines used for the treatment of patients may damage some cells, which may result in triggering of p53-dependent apoptosis pathways (Elmore, 2007)

Caspases are the major molecules that play a role in apoptosis and are found in the cell in an inactive form (proenzyme) under normal conditions. When they pass through the active form they interact with other procaspases (most of the time) and trigger the formation of a protease cascade and proteolytic cascade initiates the apoptotic signaling pathway and the cell is rapidly dragged to death (McIlwain et al., 2013). If the caspases are activated once, it is difficult for the cells to stop the apoptosis pathway. There are ten main caspase types so far defined; Caspases 2, 8, 9 and 10 are initiator caspases, caspase 3, 6 and 7 are effectors or executioners, caspases 1, 4, and 5 are inflammatory caspases (Cohen et al., 1992).

TNF (tumor necrosis factor) receptor plays the main role in the pathway of extrinsic apoptosis and other receptors that help these receptors are Fas and TRAIL. TNF receptors use together extracellular domains rich in similar cysteine, and they own a cytoplasmic field of almost 80 amino acids, which is named the “death domain”. The receptors need to bind with their ligands in order to be able to initiate the signal. When TNF receptor and Fas receptor are activated, they recruit the adaptor proteins FADD and TRADD respectively to generate the death signal pathway (Schneider-Brachert et al., 2013). The adaptor proteins are associated with the induction of procaspases, so that caspase 8 can be induced to the active form (caspase 8) which allow the cell continue apoptosis (McIlwain et al., 2013). In the next step DISC (death-inducing signal complex) is formed and the caspase 3 is transformed to the active form. Caspase 3 causes the division of certain substrates, leading to events such as DNA and nuclear fragmentation, which leads to apoptosis of the cell. (Portt, Norman et al., 2011)

The apoptosis intrinsic pathway is initially organized with mitochondria. This pathway may be activated with various factors such as DNA damage and cytotoxicity. In this pathway the expression level Bak apoptotic protein will be increased and that will cause mitochondrial damage (Wang and Youle, 2009). It has been observed that Bax expression results in the release of cytochrome C, and besides mitochondrial permeability decreases (Liu et al., 2003). The Bcl-2 protein family, a member of antiapoptotic proteins, prevents this damage from occurring and prevents the integrity of mitochondria from deteriorating. Also, the Bcl2 protein family may be anti-apoptotic as well as being pro-apoptotic. It is thought that Bcl2 takes the main role in the release

or not release of cytochrome C. After separation from the mitochondria, cytochrome C interacts with Apaf1 and activates caspase 9 using ATP, thus forming a complex called apoptosome (Adams and Cory, 2007; Wang and Youle, 2009). The active form of caspase 9 that is an initiator caspase activate state caspases-3, 6 and 7 for initiate apoptosis (Shi, 2004). (Figure 1.3)

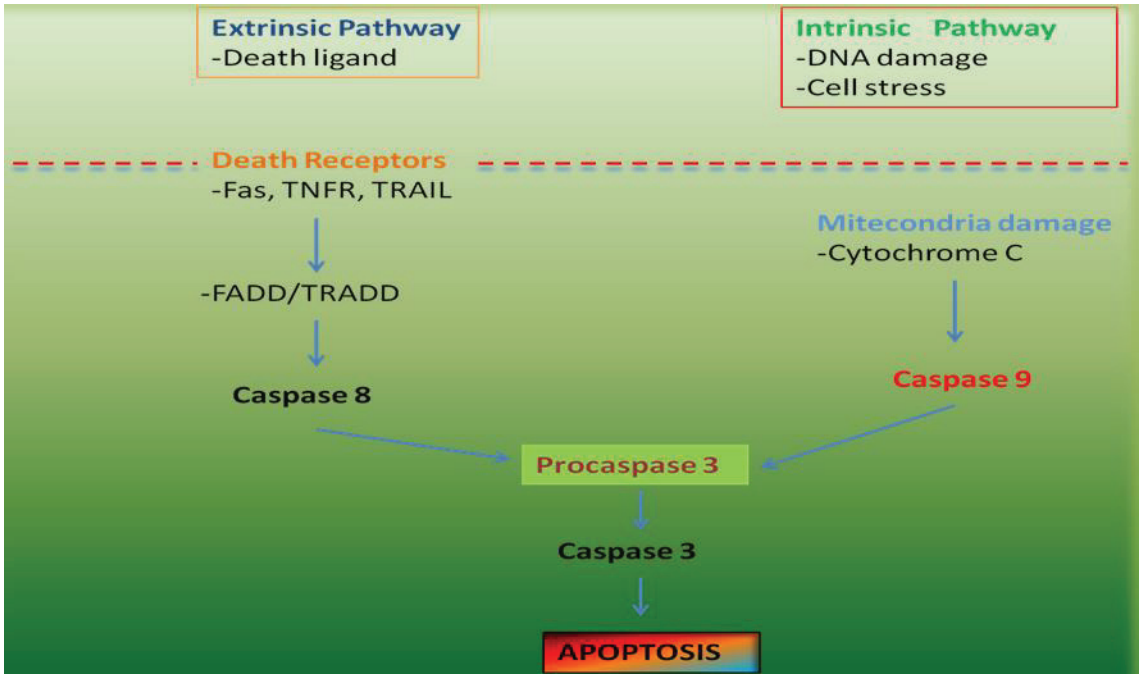


Figure 1.3.Apoptosis; intrinsic and extrinsic pathways

1.4.DNA Topoisomerase I and Camptothecin

The main task of DNA topoisomerases is to play a crucial role in DNA replication, transcription, and recombination. In addition, it is also important for compaction of chromosomes or separation of chromosomes in the nucleus. There are two types of topoisomerase enzyme groups; topoisomerase I and topoisomerase II (Forterre et al., 2014; Ehmann and Lahiri, 2014; Jadaun et al.,2017) Topo I generates a single strand break on DNA but Topo II forms double strand break on DNA (Nagaraja et al., 2017)

As mentioned above, the DNA topoisomerase I enzyme only breaks a single chain of DNA double strand. This broken chain carries over the unbroken chain of DNA and then these breakage points are reconnected.(Nagaraja et al., 2017). As a result, DNA gets loose its supercoiled feature and it becomes ready for replication. Because of

this feature, it is a significant target for anti-cancer drug researches (Barros et al., 2013; Denny, 2013). Topo I is an enzyme with its own catalytic activity and when it breaks DNA, it does not require ATP. C terminal end of Topo I has an active site called tyrosine 723, tyrosine has a nucleophilic character (Denny, 2013) The tyrosine covalently attacks the phosphate groups of DNA's single strand and breaks phosphodiester linkage. Then the DNA helix structure rotates around itself. Tyrosine is bound to the 3'-phosphate group and protects it. At the same time; the phosphodiester bond energy is stored by this complex in order to perform reverse reaction. Finally, the broken ends are united by re-establishing phosphodiester bond and occur the DNA replication fork. (Denny, 2013; Alberts et al., 2002). So, topoisomerase enzymes are considered a good target for cancer drug studies. If topoisomerase enzymes can be inhibited, DNA synthesis is inhibited. Cancer cells cannot replicate itself and cells can trigger the apoptosis (Pommier and Robert, 2001)

Camptothecin is a naturally occurring DNA topoisomerase I poison. It is an alkaloid derivative which is firstly isolated from *Camptotheca acuminate* tree. In the 1980s, it was found that have an antitumor activity. (Eng et al., 1988). Camptothecin has five fused rings and the most reactive of them is ring E. Camptothecin is a powerful anticancer agent and CPT has a very cytotoxic activity. Therefore, efforts are being made to increase the efficacy and to lessen the toxic activity (Zhan et al., 2017).

CPT does not directly inhibit the Topo I enzyme. First, Topo I interact with the DNA to generate a single strand break. When Topo I enzyme recombines the break ends, CPT interacts with Topo I and DNA binary complex and stabilizes it. For this reason, the cell cannot replicate DNA and cell drifts towards death. The main reason why CPT and the other Topo inhibitors are so important that is the presence of elevated Topo I enzyme activity in cancer cells than in normal cells. The fact remains that, camptothecin has a few disadvantages; the absence of easy dissolution of camptothecin in water, and the hydrolyzation of ring E in water (pH 7.4) and to generate the inactive carboxylate form. This form has a high affinity for serum albumin in human. Finally, this inactive form causes a high bladder toxicity. (Pommier and Cushman, 2009; Teicher, 2008) (Figure 1.4)

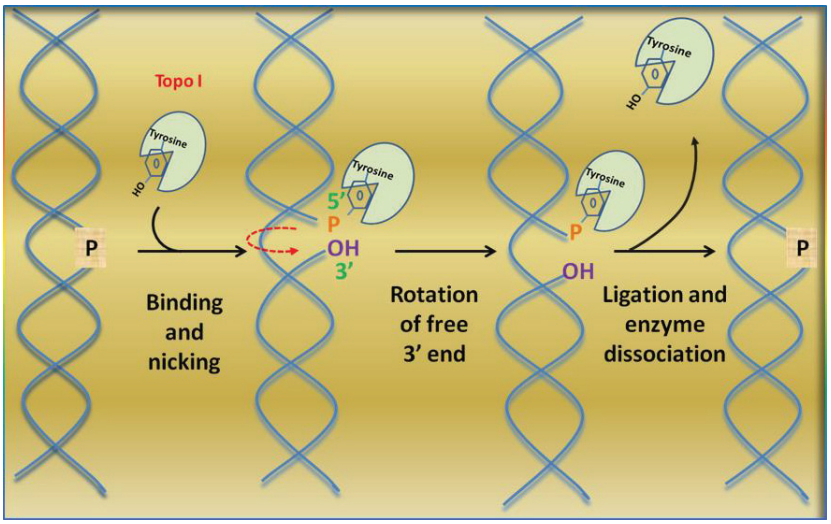


Figure 1.4.The mechanism of Topoisomerase I action

1.5.Goniothalamin

Goniothalamin, like camptothecin, is a plant-derived antiproliferative compound. This styryl lactone derivate is isolated from the *Goniothalamus* plant (Inayat-Hussain et al., 2010) . Goniothalamin has a cytotoxic activity over many cancer cell lines such as MCF-7 (breast carcinoma) and HL-60 (leukemia). Its apoptotic behavior was also shown in the literature(Raitz et al., 2017). Also, it was owned selective cytotoxic feature against cancer cell lines. (de Fatima et al., 2006). Inayat-Hussain et al. observed that goniothalamin trigger apoptosis by activating caspase 3 and caspase 7, which play a role in apoptotic pathways in human Jurkat-T cell lines and in HepG2 cell lines (Inayat-Hussain et al-1999). De Fátima et al. and Inayat-Hussain et al., observed that goniothalamin causes apoptosis via mitochondria path in Jurkat and HL 60 cells. (de Fátima et al.,2005; Inayat-Hussain et al.,2010)

The α,β-unsaturated δ-lactones pharmacophore is found in the structure of goniothalamin, just as it is found in plant-derived compounds, which have many biological activities. These structures have the feature of acting as a Michael acceptor. These unsaturated carbonyl groups can be attached to the nucleophilic moieties found in target proteins such as lysine and cysteine. Kasaplar et al. have replaced this C=C linker and hydrophobic phenyl groups with napthalen-1-yl group, resulting in an increase in the cytotoxic activity of the molecule. This molecule was named as klavuzon by the inventors (Kasaplar et al., 2009)

CHAPTER 2

RESULTS AND DISCUSSION

2.1. MTT Cell Viability Analysis

Klavuzon is a relatively new 5,6-dihydro-*2H*-pyran-2-one derivative and there is a limited structure activity relationship for klavuzon derivatives. Up to now, there are few klavuzon derivatives which possess antiproliferative activity nanomolar concentrations. In one study, it was found that napthalen-1-yl substituted 5,6-dihydro-*2H*-pyran-2-one derivatives are especially more potent compared to other bicycloarly substituted 5,6-dihydro-*2H*-pyran-2-one derivatives (Kasaplar et al., 2009). 4'-methylklavuzon and 2'-methylklavuzon derivatives have remarkable cytotoxicity against cancer cell lines (Akçok et al., 2017).

In another study, heteroatom substituted klavuzon derivatives prepared and tested against cancer cell lines and it was found that one of the dimethoxy substituted klavuzon derivative showed more cytotoxic activity than klavuzon itself. In a recent study, various novel klavuzon derivatives modified at 4'-position by various alkyl groups, were prepared and tested against cancerous and healthy pancreatic cell lines. In some study, it was found that selectivity and potency of the 4'-alkyl substituted klavuzon derivatives were dramatically changed by the size of the alkyl groups. As a continuation to the structure-activity relationship of klavuzon derivative. Biological activities of novel 2'-alkoxymethyl substituted klavuzons will be presented.

Antiproliferative effects of compounds 1-10 were determined by performing MTT viability tests on the HPDEC cell lines (human healthy pancreatic cell line) and of MIA PaCa-2 cell line (human pancreatic cancer cell line). Camptothecin (CPT) and goniothalamin (GTN) were used as positive controls in this study. HPDEC cell lines were tested in collagen coated 96-well microplates, and previously reported that collagen coating has minimal effect on the MTT cell viability results (Sen, 2016). Cytotoxic effect of the compounds was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which basically the reduction of tetrazolium to formazan crystal by mitochondrial reductase enzymes, and absorbance

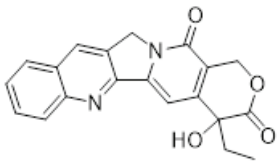
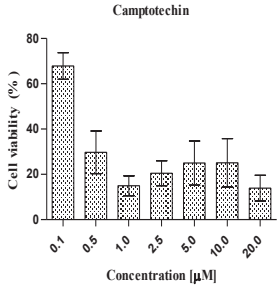
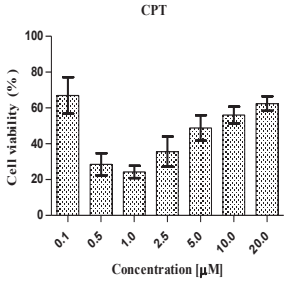
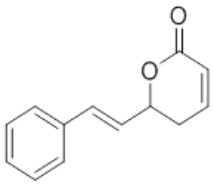
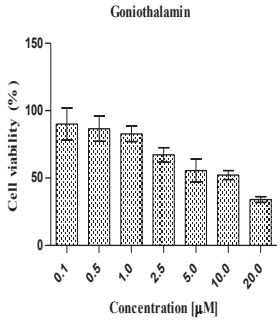
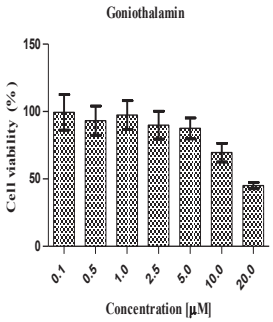
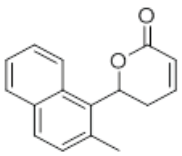
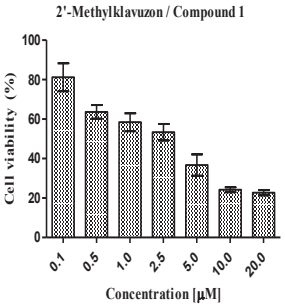
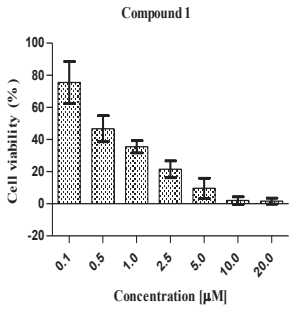
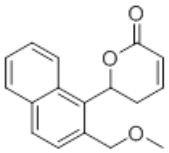
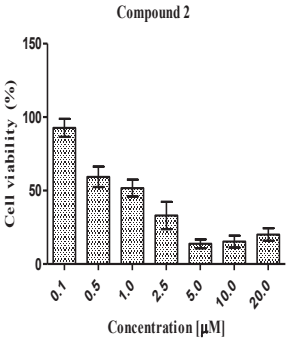
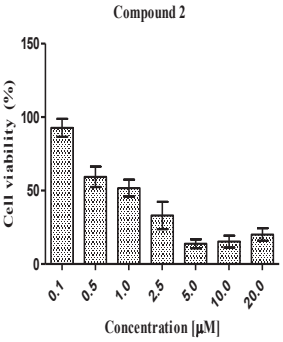
values at 540 nm was correlated with total amount of formazan and living cells (Riss et al., 2004). Finally, IC_{50} (μM) values of the compounds were determined using Graphpad Prism 5 program by using % cell viability rewind. The IC_{50} value is the concentration of a substance to reduce the cell population by 50% relative to the negative control. The results are shown in Table 2.1. Selectivity index is a value that can be calculated by dividing the IC_{50} (μM) value determined for a healthy cell line of a substance by the IC_{50} (μM) value for the cancer cell line.

CPT, which a well-known topoisomerase I poison and was very cytotoxic on two cell lines and without having any selectivity. The probable cause for the lack of selectivity was that the duplication times in the two cell lines used were very close to each other (MIA PaCa-2 duplication time ~ 40 hour, HPDEC ~34 hour) and both cell lines need Topo I enzyme for proliferation. The other positive control and GNT was found to be less effective in both cell lines but it showed two times more selective cytotoxicity in cancer cell line (Table 2.2).

MTT results of compounds 1-10 revealed a clean structure activity for 2'-alkoxymethyl klavuzon derivatives. Although IC_{50} values of compound 2 were higher than those of CPT in both cell lines, compound 2 was found to be the most cytotoxic compound among the newly synthesized klavuzon derivates. Interestingly this compound possessed nonselective cytotoxic activity against MIA PaCa-2 cancer cell line. Compound 10 showed a similar activity compared to compound 1 by having lower IC_{50} values in both cell lines. Similarly compounds 7 and 9 are two other novel derivatives having selective cytotoxicity toward HPDEC cell lines with higher IC_{50} concentrations.

Presence of ethyl or propyl group at 2'-position gave similar potency and selectivity substitution but reduced the cytotoxic potency of klavuzon. Besides, compound 8 showed quite similar selective cytotoxic against cancer cell lines compared to goniothalamin at approximately three times lower doses.

Table 2.1.Concentration dependent cytotoxic activities of CPT, GNT and compounds 1-10 over MIA PaCa-2 and HPDEC cell lines

Structure of compound and name	MIA PaCa-2	HPDEC
 Camptotechin (CPT)		
 Goniothalamin (GNT)		
 2'-methylklavuzon (compound 1)		
 2'-methoxymethyl klavuzon (compound 2)		

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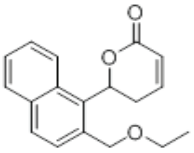
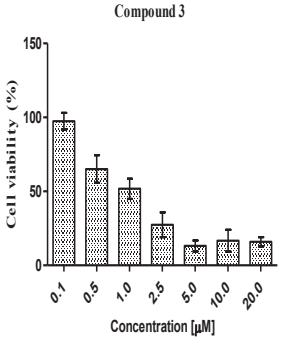
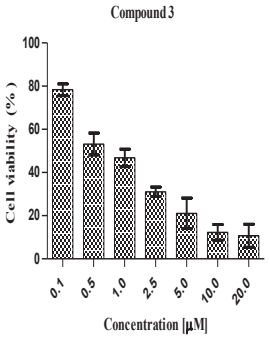
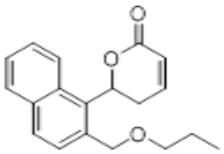
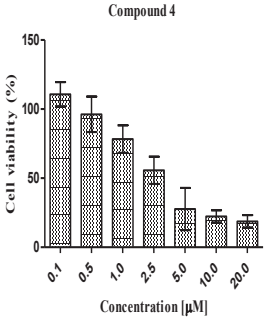
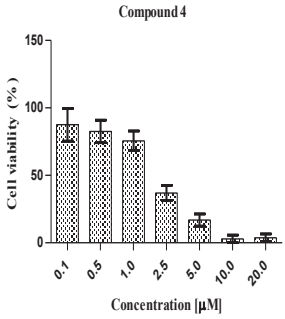
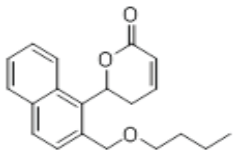
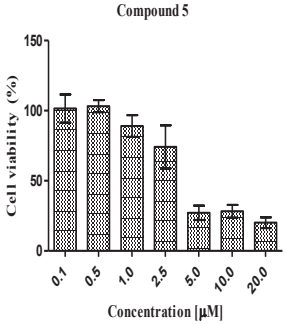
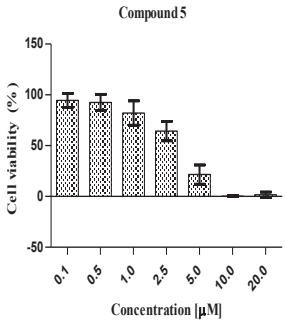
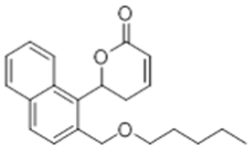
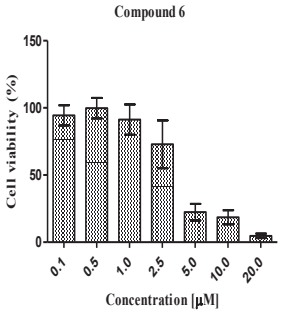
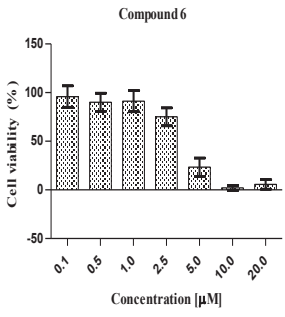
 2'-ethoxymethyl klavuzon (compound 3)		
 2'-propoxymethyl klavuzon (compound 4)		
 2'-butoxymethyl klavuzon (compound 5)		
 2'-pentiloxymethyl klavuzon (compound 6)		

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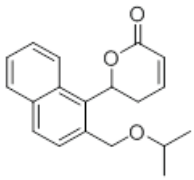
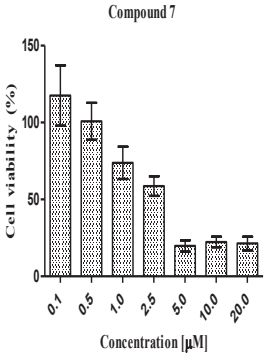
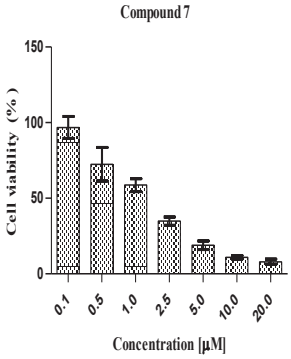
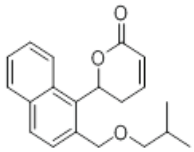
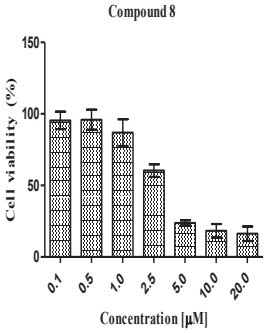
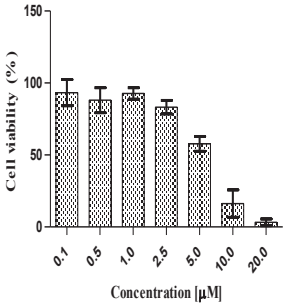
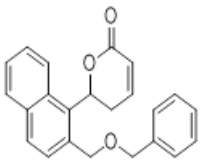
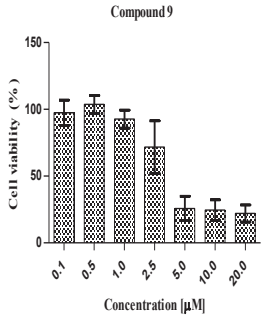
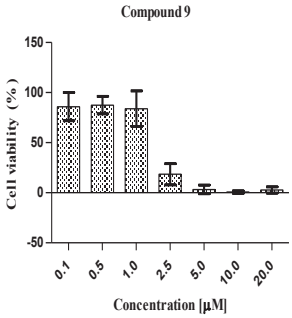
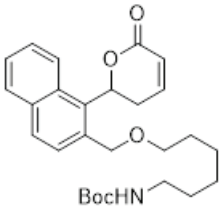
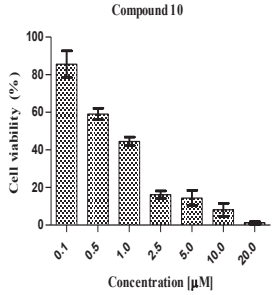
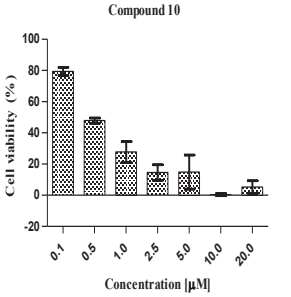
 2'-isopropoxymetyl klavuzon (compound 7)		
 2'-isobutoxymethyl klavuzon (compound 8)		
 2'-benzyloxymetyl klavuzon (compound 9)		
 2'-Boc protected amine derivatized klavuzon (compound 10)		

Table 2.2.Calculated IC50 values and selectivity index for CPT, GNT and compounds 1-10

Compounds	IC_{50} (μM)		Selectıvıty Index
	MIA PaCa-2	HPDEC	(SI)
CPT	0.18 ± 0.01	0.21 ± 0.01	1.17
GNT	8.53 ± 0.88	17.75 ± 0.68	2.08
Compound 1	1.86 ± 0.19	0.53 ± 0.05	0.28
Compound 2	0.61 ± 0.09	0.54 ± 0.10	0.89
Compound 3	0.72 ± 0.11	0.70 ± 0.05	0.97
Compound 4	2.46 ± 0.42	1.98 ± 0.03	0.80
Compound 5	3.63 ± 0.14	3.21 ± 0.23	0.88
Compound 6	3.25 ± 0.39	3.61 ± 0.16	1.11
Compound 7	2.87 ± 0.51	1.23 ± 0.08	0.43
Compound 8	3.02 ± 0.02	5.53 ± 0.22	1.83
Compound 9	3.21 ± 0.19	1.60 ± 0.016	0.50
Compound 10	0.70 ± 0.05	0.42 ± 0.02	0.60

2.2.Results of Topoisomerase I Drug Inhibition Assay

Previously it has been shown that derivates of klavuzons are Topo I inhibitors to evaluate the SAR of MTT assay, Topo I inhibitory properties of newly prepared klavuzon derivates were also studied. Because it has been thought that klavuzons may react with the nucleophilic sites of enzymes and inhibit that, time dependent Topo I inhibition assay was performed to understand the preincubation time that required for full inhibition of Topo I enzyme (Figure 2.1). CPT was used as a positive control in all Topo I mediated supercoiled relaxation assays (Figures 2.1 - 2.1).

To evaluate the effect of preincubation time, Topo I enzyme was preincubated with 50 and 100 μM concentrations of 2'-methyl klavuzon for varying time intervals (0, 1, 5 and 10 minutes). At 100 μM concentration all preincubations time gave strong inhibition of Topo I but at 50 μM concentration of 2'-methylklavuzon there was a weaker inhibition for 5 minutes preincubation time (Figure 2.1.). Hence, 10 minutes preincubation time was used in further studies to evaluate the concentration dependent Topo I inhibition studies of new klavuzon derivates.

As noticed in MTT cell viability results 2'-methoxymethylklavuzon (2) has highest cytotoxic activity and 2'-isobutoxymethylklavuzon (8) is most selective cytotoxic activity against cancer cells. Their concentration dependent Topo I inhibitory properties were shown in figure 2.2. According to this figure, at 50 μM concentration of both compound showed similar potency to inhibit Topo I enzyme but at lower doses (2 and 0.4 μM concentrations) selective isomer, compound 8, showed much better Topo I inhibitory properties, and this could be the source of selectivity for this compound at first glance.

Additionally, to evaluate the length of alkyl group over Topo I inhibition properties, 2'-ethoxymethylklavuzon (3) and Boc protected klavuzon (10) were evaluated for Topo I inhibition. As it can be seen in figure 2.3, 2'-ethoxymethylklavuzon inhibits Topo I at all concentrations strongly but, Boc protected klavuzon failed to inhibit Topo I at 0.4 μM concentration. A similar result was observed from the comparison of Topo I inhibition properties of 2'-propoxymethylklavuzon (4) and 2'-isopropoxymethylklavuzon (7) as shown in figure 2.4. Simply, compound 4 inhibits Topo I at all concentration but, it's isomer compound 7 failed to inhibit Topo T at 0.4 μM concentration. Lastly, long alkyl chain substituted klavuzons (5 and 6) were tested and found that both compounds inhibit Topo I at all tested concentrations.

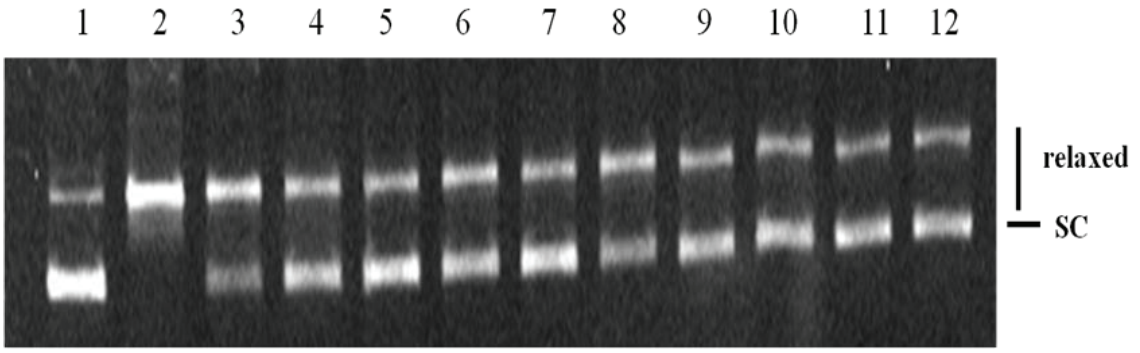


Figure 2.1.Time-dependent Topo I enzyme inhibition by 2'-methylklavuzon (compound 1). Lane 1: 1 μL pUC19 (Supercoiled DNA, pHOT1 0.25 μg/μL); Lane 2: 2 μL relaxed DNA (1 ng / μL); Lane 3: pUC19 + Topo l (2U) + 1 μL DMSO; Lane 4: pUC19 + Topo I + 50 μM CPT; Lanes 5-6: 10 minutes of preincubation for pUC19 + Topo I + 100 and 50 μM compound 1 respectively; Lanes 7-8: pUC19 + Topo I + 5 minutes of preincubation for 100 and 50 μM compound 1 respectively; Lanes 9-10: pUC19 + Topo I + 1 minutes of preincubation for compound 1 respectively; Lanes 11-12: no preincubation pUC19 + Topo I + 100 and 50 μM compound 1, respectively

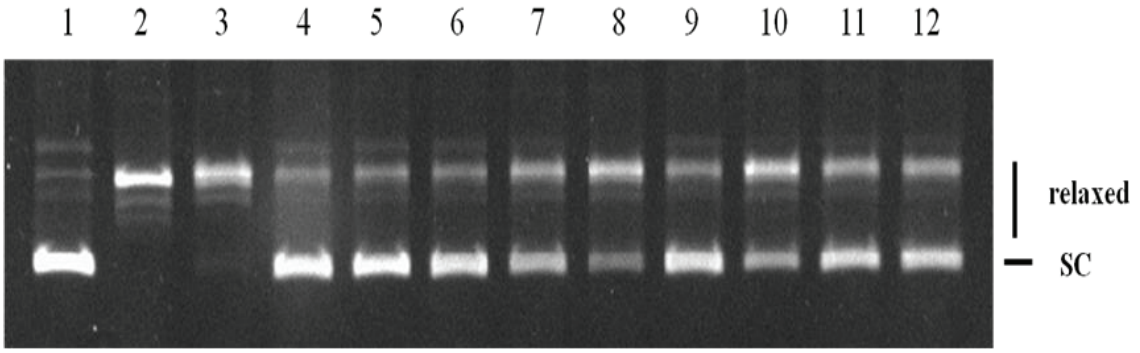


Figure 2.2.Concentratiıon dependent Topo I inhibition after 10 minutes of preincubation with 2'-methoxymethylklavuzon (2) and 2'-isobutoxymethylklavuzon (8). Lane 1: 1 µL pUC19 (Supercoiled DNA, pHOT1 0.25 µg/µL); Lane 2: 2 µL relaxed DNA (1 ng / µL); Lane 3: pUC19 + Topo l (2U) + 1 µL DMSO; Lane 4: pUC19 + Topo I + 50 µM CPT; Lanes 5-8: pUC19 + Topo I + 50, 10, 2 and 0.4 µM 2'-methoxymethylklavuzon (2) respectively; Lanes 9-12: pUC19 + Topo I + 50, 10, 2, and 0.4 µM 2'-isobutoxymethylklavuzon (8), respectively.

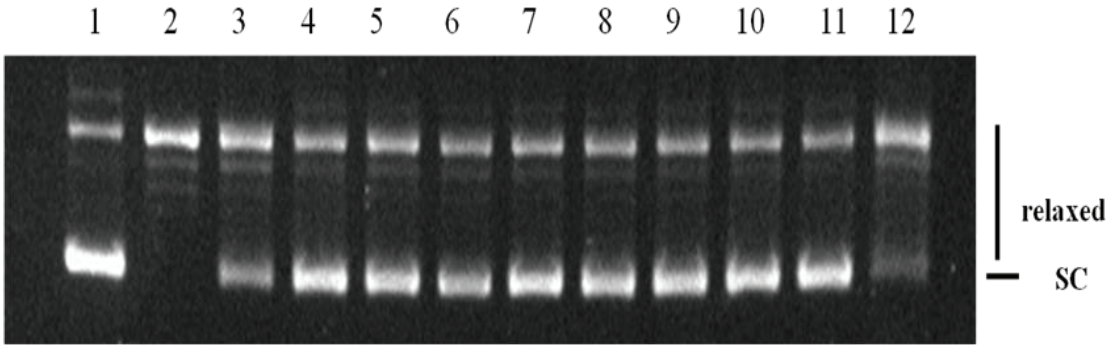


Figure 2.3.Concentratiıon dependent Topo I inhibition after 10 minutes of preincubation with 2'-ethoxymethyklavuzon (3) or 2'-Boc protected amine derivatized klavuzon (10). Lane 1: 1 µL pUC19 (Supercoiled DNA, pHOT1 0.25 µg/µL); Lane 2: 2 µL relaxed DNA (1 ng / µL); Lane 3: pUC19 + Topo l (2U) + 1 µL DMSO; Lane 4: pUC19 + Topo I + 50 µM CPT; Lanes 5-8: pUC19 + Topo I + 50, 10, 2 and 0.4 µM 2'-ethoxymethylklavuzon (3) respectively; Lanes 9-12: pUC19 + Topo I + 50, 10, 2 and 0.4 µM 2'-Boc protected amine derivatized klavuzon (10), respectively.

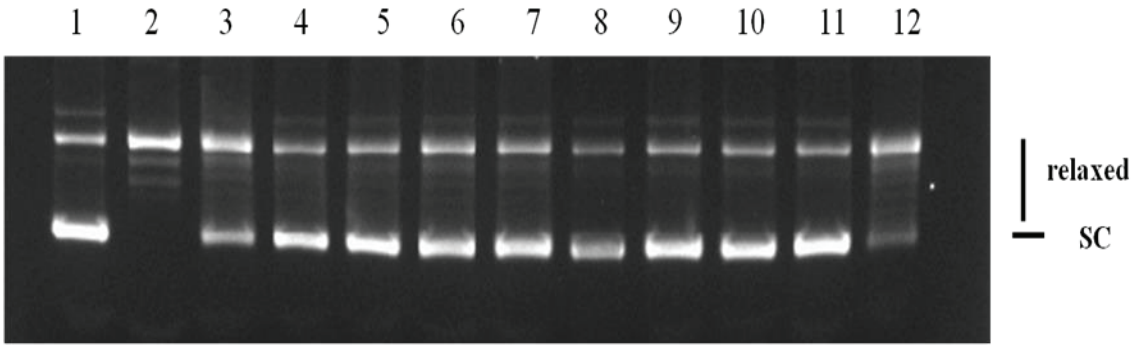


Figure 2.4.Concentratiıon dependent Topo I inhibition after 10 minutes of preincubation with 2'-propoxymethylklavuzon (4) or 2'-isopropoxymethylklavuzon (7). Lane 1: 1 µL pUC19 (Supercoiled DNA, pHOT1 0.25 µg/µL); Lane 2: 2 µL relaxed DNA (1 ng / µL); Lane 3: pUC19 + Topo l (2U) + 1 µL DMSO; Lane 4: pUC19 + Topo I + 50 µM CPT; Lanes 5-8: pUC19 + Topo I + 50, 10, 2 and 0.4 µM 2'-propoxymethylklavuzon (4) respectively; Lanes 9-12: pUC19 + Topo I + 50, 10, 2 and 0.4 µM 2'-isopropoxymethylklavuzon (7), respectively.

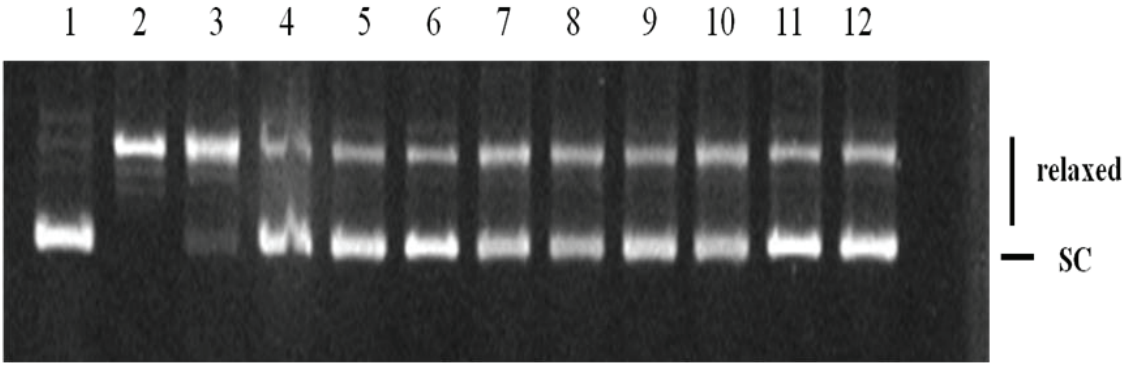


Figure 2.5.Concentratiıon dependent Topo I inhibition after 10 minutes of preincubation with 2'-butoxymethylklavuzon (5) or 2'-pentyloxymethylklavuzon (6). Lane 1: 1 µL pUC19 (Supercoiled DNA, pHOT1 0.25 µg/µL); Lane 2: 2 µL relaxed DNA (1 ng / µL); Lane 3: pUC19 + Topo l (2U) + 1 µL DMSO; Lane 4: pUC19 + Topo I + 50 µM CPT; Lanes 5-8: pUC19 + Topo I + 50, 10, 2 and 0.4 µM 2'-butoxymethylklavuzon (5) respectively; Lanes 9-12: pUC19 + Topo I + 50, 10, 2 and 0.4 µM 2'-pentyloxymethylklavuzon (6), respectively.

2.3.Results of Comet Assay

In this study, comet assay, a sensitive method to study DNA fragmentation at an individual cell level, was performed. The technique is based on micro gel electrophoresis of DNA cells. Basically, cells were embedded in low melting point agarose, fragmented and electroporated. While the damaged DNA creates a comet-like tail as it moves in the electric field, the undamaged DNA remains like a comet head.

Based on the results of the MTT assay, compound 8, the compound with the best selectivity, and compound 1, the compound with the worst selectivity, were selected for Comet analysis. Results of Topo I were also observed that these two compounds inhibit the enzyme of topoisomerase I. A Comet assay was established to determine if a compound caused DNA damage at the cellular level. Three concentrations (1 μM, 5 μM and 10 μM) of each compound were used in the experiment. 50 μM of H_2O_2 as positive control was applied for last 2 hours of incubation. Looking at the results of this experiment observed an increase in tail number and tail size at increasing concentrations of compound 1. Additionally, it was observed that the results 10 μM of compound 1 was higher in the Comet size and Comet quantity than positive control results. The tail length and quantity at 5 μM of compound 8 were observed close value to the positive control. Compound 8 was also found to cause DNA damage in the cell. However, Compound 8 was observed to have a lower than positive control as the count and size of Comets at all three concentrations. In addition, tail sizes at 5 μM of compound 8 were observed to be longer than tail sizes at 10 μM of compound 8. In this study, both compounds were found caused a noteworthy DNA damage.

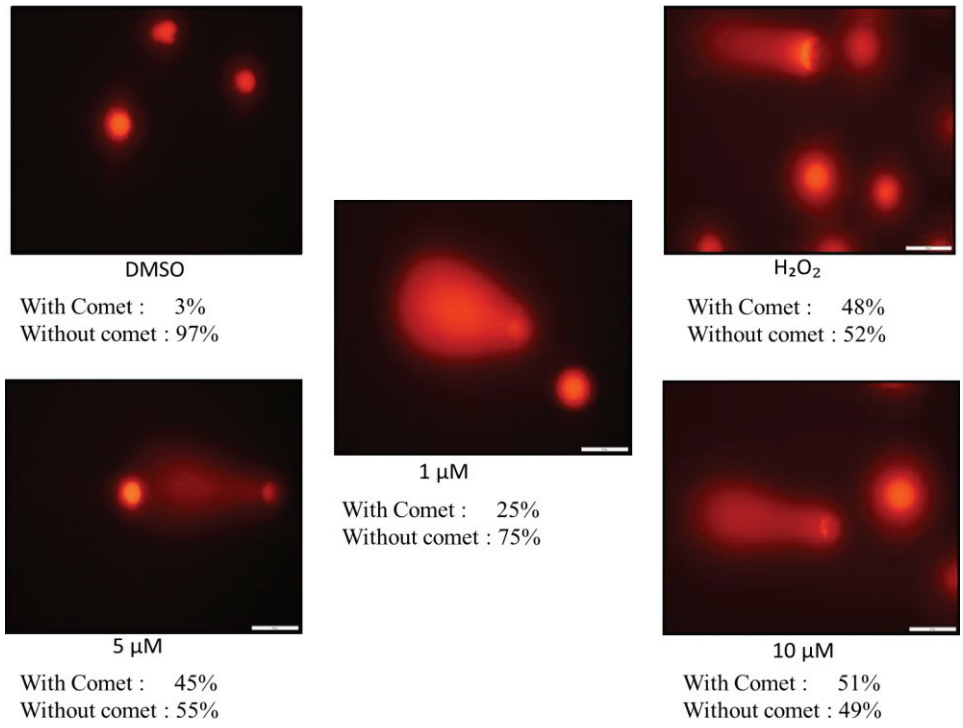


Figure 2.6.Comet assay results of MIA PaCa-2 cells; incubated with 1 µM, 5 µM and 10 µM of compound 1 for 24 hours. H_2O_2 was applied as positive control at 50 µM concentration, DMSO was applied as negative control.

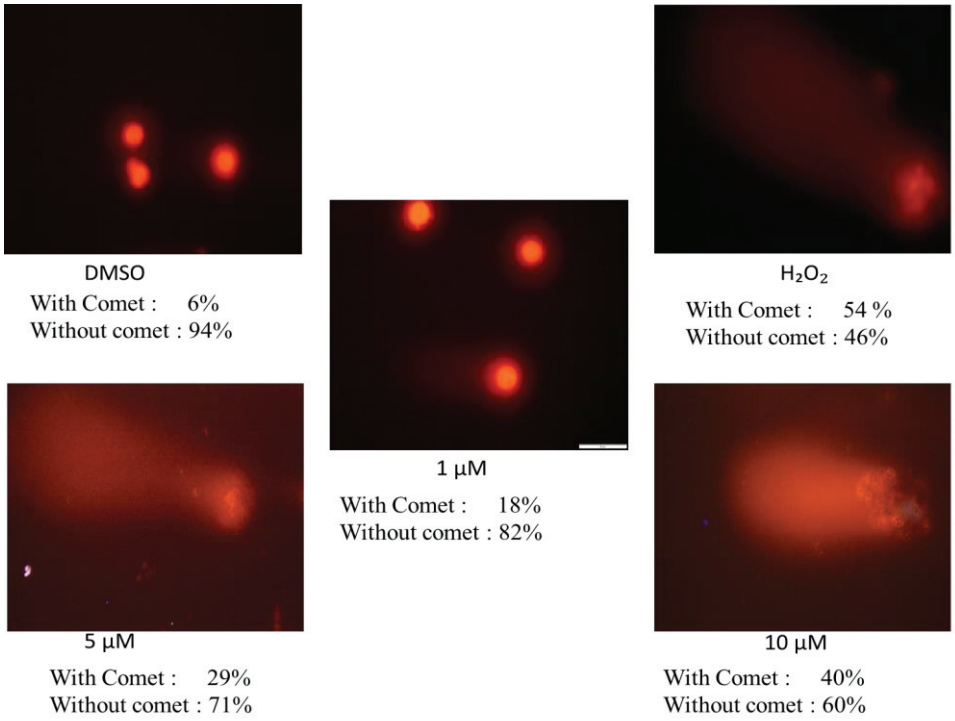


Figure 2.7.Comet assay results of MIA PaCa-2 cells; incubated with 1 µM, 5 µM and 10 µM of compound 8 for 24 hours. H_2O_2 was applied as positive control at 50 µM concentration, DMSO was applied as negative control.

2.4.Results of Cell Cycle Analysis

The cell cycle basically has two main stages; Interphase and mitosis. Proteins are synthesized in the G1 phase of the cell and the cell contents are doubled. DNA is replicated in the S phase and the additional protein synthesis required before mitosis in the G2 phase of the cell. In mitosis, the phase cell become large enough to divide into two cells. The PI dye binds to the DNA as a fluorochrome agent. Depending on the amount of DNA, the intensity of the fluorescence increases or decreases. For example, the amount of DNA is high in the S-phase, so that an amount of fluorescence increases. DMSO was used as negative control. Three different concentrations (1 μM, 5 μM and 10 μM) were used for both compound 1 and compound 8.

In this study, it was observed that cells cycle arrest occurred at the G1 phase for both compounds (compound 1 and compound 8) to MIA PaCa-2 cells were observed. In the literature, CPT arrests the cells in S phase. It was also observed that there was an increase in a number of cells arrested in the G1 phase by an increase in the concentrations of the tested compounds. According to the Topo I results, it was observed that the compounds inhibited the Topo I enzyme before to the DNA binding. This means that DNA synthesis was never started. DNA is synthesized in S phase. Therefore, compounds can be stopped the cell cycle before entering the S phase. Since the compounds inhibit Topo I, the probability of the cells to arrest in G1 phase is higher than the S phase.

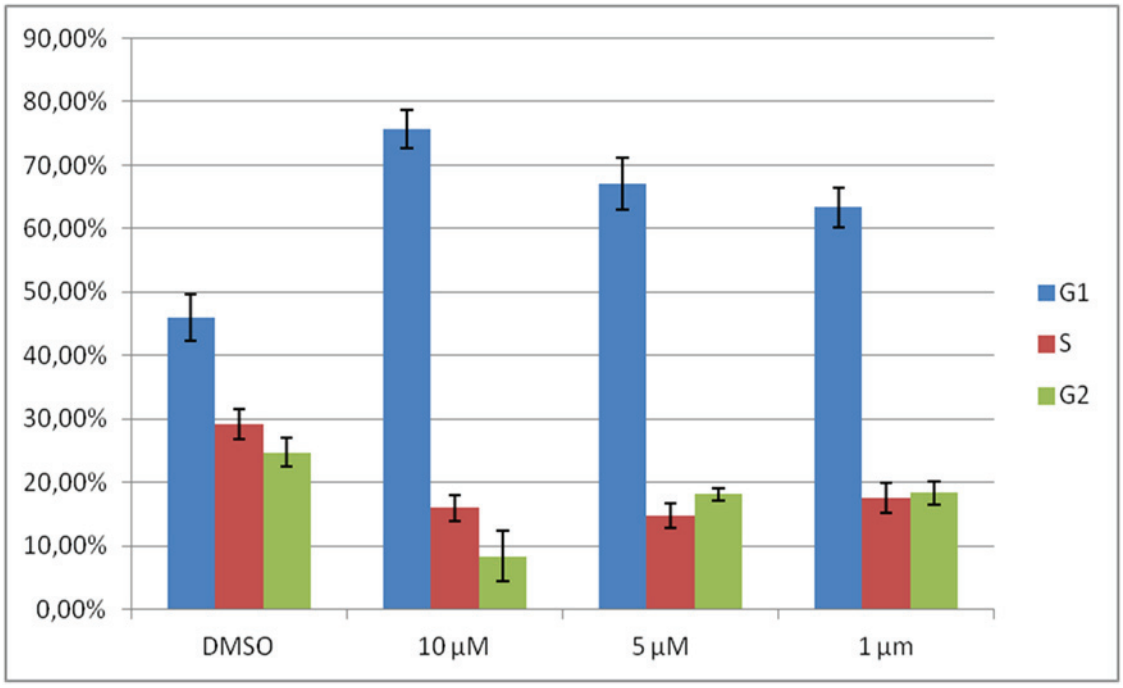


Figure 2.8.Concentration dependent cell cycle arrests on MIA PaCa-2 cells that were incubated with compound 1for 24 hours. DMSO was applied as negative control.

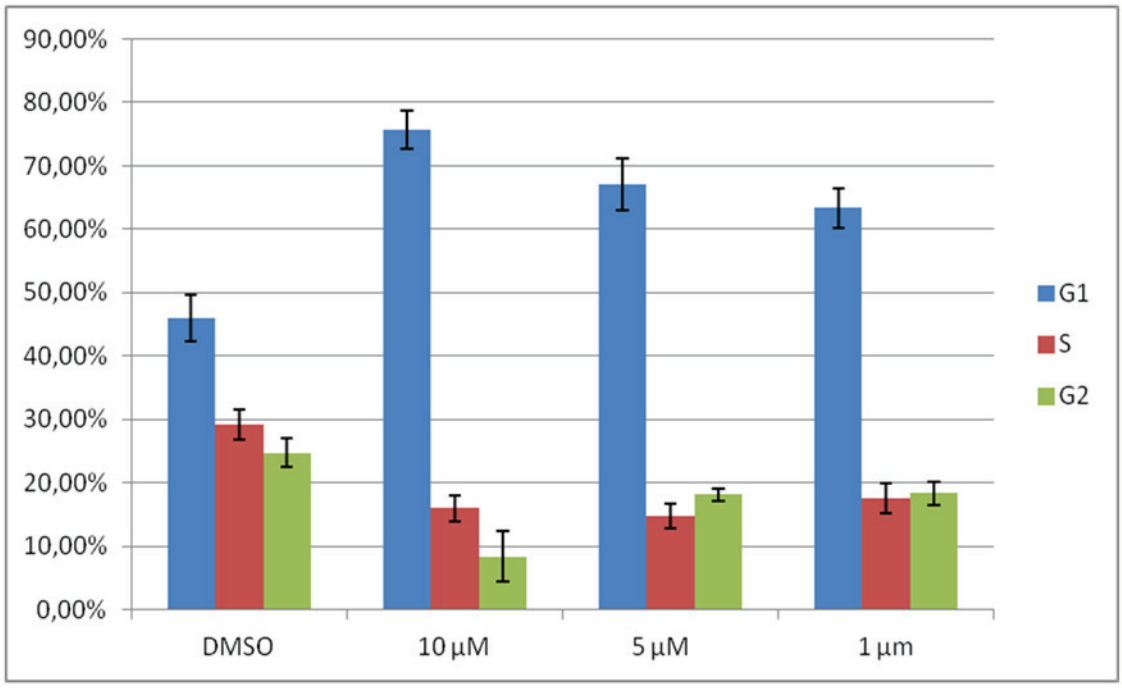


Figure 2.9.Concentration dependent cell cycle arrests on MIA PaCa-2 cells that were incubated with by compound 8 for 24 hours. DMSO was applied as negative control.

2.5.Results of Apoptosis Analysis

Phosphatidylserine (PS) constructs are very important in apoptosis experiments. PS is normally located the inner the cell membrane. But in cells that undergo apoptosis, these structures migrate to the outer of the cell membrane. The Annexin V / FITC Scanning Kit used in this assay is binding to this structure. Annexin V is a Ca^{2+} dependent phospholipid binding protein and has a high affinity for 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. PI is used as an auxiliary dye. The reason for this is to determine whether the cell has early apoptosis, late apoptosis or necrosis. PI dye is a substance that binds to DNA. In addition, PI cannot cross the membrane of healthy cells. The cells that are not bonded to the two dyes are healthy cells. The cells to which both dyes are bonded that are cells in the condition of late apoptosis. Only early apoptotic cells are bonded to the Annexin V dye (still has intact plasma membrane). Only the PI staining is bonded to the cells in the necrosis state, Annexin V does not bind. The assay was conducted for compound 8 having selective cytotoxic effect in this study.

Three different concentrations (1 μM, 5 μM and 10 μM) of the compound 8 were tested on the MIA PaCa-2 cell line. A remarkable increase in the number of cells in late apoptosis has been found, depending on the increase in concentrations. The number of cells undergoing early apoptosis declined to depend on increased concentration. The amount cells that underwent necrosis was observed very low. A low necrosis in antiproliferative studies of a compound is seen as an attractive result for this compound.

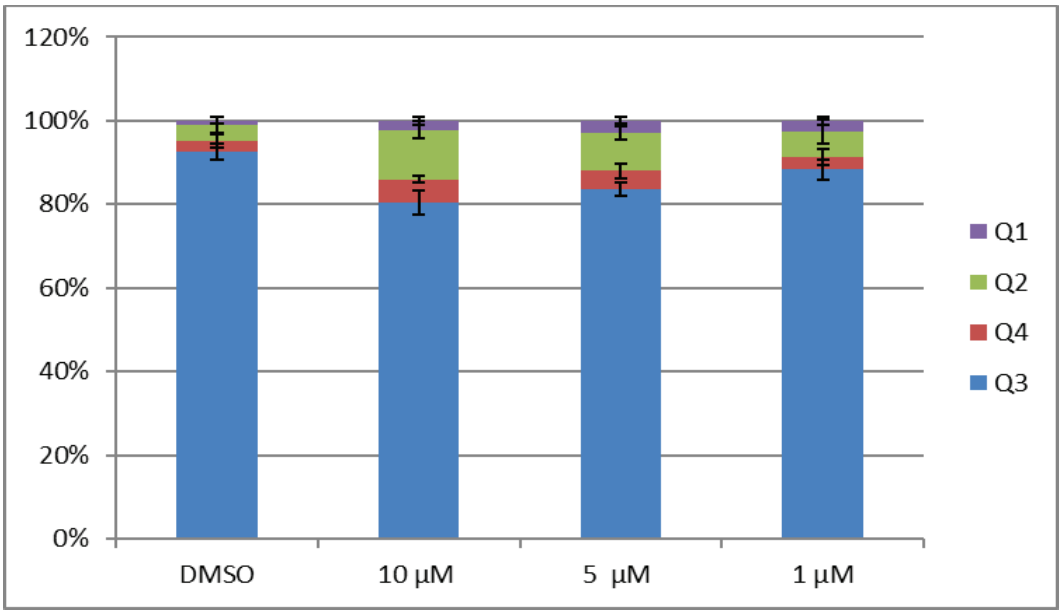


Figure 2.10.Concentration dependent apoptosis on MIA PaCa2 cells that incubated with compound 8 for 24 hours (Q2: late apoptosis, Q4: early apoptosis, Q3: live cell, Q1: necrosis). DMSO was applied as negative control.

CHAPTER 3

EXPERIMENTAL

3.1.Thawing out Cells

Vial of frozen MIA PaCa2 (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 T25 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 water bath for thawing 2-3 minutes. After thawing cells were transferred to falcon tube and centrifuged at 1500 rpm for 3 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 PaCa2 Cells

Before passage of MIA PaCa2, complete DMEM media and trypsin (0,05%) were warmed to 37 ^{0}C in water bath. 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 water bath. 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 minutes. 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 minutes. 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.

3.3.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 squares 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)

3.4.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 PaCa2 cells were stored at -80 ^{0}C, HPDEC cells were stored at liquid nitrogen for long storage.

3.5.MTT Cell Viability Assay

For viability assay of MIA PaCa2 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 was 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]

3.6.Apoptosis Analysis

For 2'-alkoxymethylklavuzon derivates, 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 three different concentrations of 2'-alkoxymethylklavuzon derivates (compound 1 and compound 8) were prepared by dissolved with sterilized 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.

3.7.Cell Cycle Analysis

For 2'-alkoxymethylklavuzon derivates, 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 three different concentrations of 2'-alkoxymethylklavuzon derivates (compound 1 and compound 8) were prepared by dissolved with sterilized 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.

3.8.Topoisomerase I Drug Screening Assay

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 2'-alkoxymethylklavuzon derivates which were dissolved in DMSO. Then Topo I enzymes were pre-incubated with 2'-alkoxymethylklavuzon derivates at 37 ^{0}C for 10 minutes and 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.

3.9.Comet Assay (Single Cell Gel Electrophoresis assay)

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 three different concentrations of the 2'-alkoxymethylklavuzon derivates (compound 1 and compound 8) were prepared by dissolving with sterilized DMSO and filtered. Then 20 µl of each concentration of 2'-alkoxymethylklavuzon derivates (compound 1 and compound 8) were applied to wells and incubated at 37 ^{0}C 5% CO_2 for 24 hours. 50 µM of H_2O_2 (20 µl) 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 cells 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

CONCLUSION

Previous studies have shown that goniothalamin has a cytotoxic activity on cancer cells. As a result of this study, it was observed that the 2'-alkoxymethylklavuzon derivatives have a better cytotoxic activity in MIA PaCa-2 pancreatic cancer cells than (R)-goniothalamin. Also, it was also found that this cytotoxic activity was at the nanomolar level in some 2'-alkoxymethylklavuzon derivatives (compound 2, 3 and 10) by using the MTT cell viability test. 2'-methoxymethylklavuzon is the compound with the highest cytotoxic activity but its selective index is lower than goniothalamin. Whereas the selective index value of 2'-isobutoxymethylklavuzon is very close to goniothalamin. At the same time, this compound showed a similar antiproliferative effect at a concentration of almost three times lower than the goniothalamin on cancer cells. Cytotoxic activity of 2'-methoxymethylklavuzon is reduced in both cell depending on the length of the alkyl groups. When comparing the compounds having the same number of carbons in their alkyl groups, it was observed that the selectivity of the compounds was better for the iso forms, for example, compound 5 and compound 8(Table 2.2.).

In previous researches, klavuzon derivatives were found to be topoisomerase I inhibitors. In this study, it was observed that 2'-methoxymethylklavuzon derivatives also inhibited the Topo I enzyme is interesting is that compound 2 that has best cytotoxic activity than the others, was demonstrated a low inhibition of Topo I at low concentrations whereas compound 8 that is the selective compound, showed a high inhibition Topo I even at low concentrations. It is thought that these compounds may cause DNA damage because Topo I inhibition. Examination results for compound 1 and compound 8 selected for the test for cell cycle and comet showed that the DNA was damaged and the cell cycle was stopped at G1 phase. It has also been observed that compound 8 leads MIA PaCa-2 to apoptosis. The above described results have increased the activity of both compounds on the MIA PaCa-2 cell line depending on the concentration. As a result, we saw that the compounds in this study had a cytotoxic effect on the MIA PaCa-2 cell line and cause Topo I enzyme inhibition. In addition,

compound 8 with selective cytotoxic activity, while causing DNA damage on the cell line, stopped the cells at the G1 phase and drifted the cells into apoptosis.

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