INVESTIGATION OF THE EFFECT OF 4'-ALKYLKLAVUZON DERIVATIVES ON NUCLEOTIDE SYNTHESIS AND NUCLEOCYTOPLASMIC TRANSPORT

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ABSTRACT

INVESTIGATION OF THE EFFECT OF 4'-ALKYLKLAVUZON DERIVATIVES ON NUCLEOTIDE SYNTHESIS AND NUCLEOCYTOPLASMIC TRANSPORT

In anti-cancer agent development studies one of the most significant issue is to get an agent that specifically targets cancer cells without any effects on healthy cells. Goniothalamin, that is a styryl lactone isolated from *Goniothalamus* plant species, is an anti-cancer agent that has selective anti-proliferative activity on cancer cell lines. Klavuzon and derivatives, which can be thought as analogs of goniothalamin, are more cytotoxic in cancer cells compared to goniothalamin. Previous structure activity relationship studies implies that α,β-unsaturated δ-lactone moiety is the source of the biological activity. Since it behaves as Michael acceptor, in this thesis possible irreversible inhibitions of two separate intracellular targets are investigated.

In the first part, thymineless death caused by possible thymidylate synthase inhibition has been studied. Anti-proliferative effect of 4'-methylklavuzon in HuH-7 cancer cell line was tested by using MTT. Viability of klavuzon treated cells did not changed significantly in the absence and presence of varying concentration of additional thymidine supplement, and it is concluded that thymineless death is not a crucial mechanism for klavuzon derivatives. In the second part, 4'-methylklavuzon and its derivatives were tested on HeLa cell line to investigate inhibitory effect on the nucleocytoplasmic transport. Immunocytochemistry was used to demonstrate nucleocytoplasmic localization of Riok2 protein which is transferred from nuclei to cytoplasm by CRM1 nuclear export protein. Successfully, all tested klavuzon derivatives inhibit CRM1 nuclear export protein. Potency of the inhibition depends on the size of the alkyl substituent at 4'- position of klavuzon.

ÖZET

4’-ALKİLKLAVUZON TÜREVLERİNİN NÜKLEOTİD SENTEZİ VE NÜKLEOSİTOPLAZMİK TRANSPORT ÜZERİNDEKİ ETKİSİNİN İNCELENMESİ

Antikanser ajan geliştirme çalışmalarda en önemli konulardan birisi spesifik olarak kanserli hücreleri hedef alıp sağlıklı hücrelere zarar vermeyen bir ajanın keşfidir. Goniothalamin, *Goniothalamus* bitki türlerinden izole edilmiş bir stiril lakton olup, kanserli hücrelerin çoğalmalarına karşı seçici aktivitesi bulunan bir anti-kanser ajandır. Klavuzon ve türevleri bir tür goniothalamin türevi olarak düşünülebilirler ve goniothalamine oranla kanser hücrelerine karşı daha sitotoksiklerdir. Daha önceki yapı-aktivite ilişkisi çalışmalarında α,β-doymamış δ-lakton yapısının biyolojik aktivite için gerekli olduğu bilinmektedir. Bu grup Michael akseptörü olarak görev aldığından bu tez kapsamında iki farklı hücre içi mekanizmanın geridönüşümsüz inhibisyonu araştırılmıştır.

Çalışmanın ilk bölümünde, olası bir timidilat sentaz inhibisyonuna bağlı olarak gerçekleşebilecek timidinsiz ölümün varlığı araştırılmıştır. Farklı konsantrasyonlarda timidin varlığında veya yokluğunda klavuzonla muamele edilmiş hücrelerin canlılıklarında herhangi bir değişiklik olmamıştır, bu nedenle timinsiz ölüm olayının klavuzon türevleri için önemli bir mekanizma olmadığı sonucuna varılmıştır. İkinci bölümde ise, 4'-metilklavuzon ve türevlerinin nükleositoplazmik transport üzerindeki etkilerini araştırmak için HeLa hücreleri üzerinde test edilmiştir. CRM1 proteini tarafından taşınan Riok2 proteininin nükleositoplazmik yerini göstermek için immünositokimya kullanılmıştır. Başarılı bir şekilde test edilen tüm klavuzon türevleri CRM1 proteini inhibe etmişlerdir. Maddelerin inhibisyon potansiyeli klavuzonun 4’-konumundaki alkil grubunun büyüklüğüne bağlı olarak değişmektedir.

.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

CH2FH4	N-5,10-methylene-tetrahydrofolate
CRM1	Chromosome region maintenance 1
CRM1/exportin1	Chromosome maintenance protein 1
DHFR	Dihydrofolate reductase
DHFU	Dihydrofluorouracil
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
dTDP	Deoxythymidine diphosphate
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphoshate
FdUMP	Fluorodeoxyuridine monophosphate
FdUTP	Fluorodeoxyuridine triphosphate
FG	Phenylalanine-glycine
FUDP	Fluorouridine diphosphate
FUDR	Fluorodeoxyuridine
FUMP	Fluorouridine monophosphate
FUR	Fluorouridine
FUTP	Fluorouridine triphosphate
GEF	Guanine nucleotide exchange factor
ICC	Immunocytochemistry
Imp α	Importin α
Imp β	Importin β
MTA	Multiple targeted antifolate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NE	Nuclear envelope
NESs	Nuclear export sequences
NLSs	Nuclear localization sequence
NPCs	Nuclear pore complexes
NTF2	Nuclear transport factor 2
NTRs	Nuclear transport receptors
Nups	Nucleoporins
OPRT	Orotate phosphoribosyltransferase
PBS	Phosphate-buffered saline
PRPP	Phosphoribosyl pyrophosphate
Ran	Ras-related Nuclear protein
RanBP1	RAN binding protein 1
RanBP2	RAN binding protein 2
RanGAP	Ran GTPase-activating protein
RanGDP	Guanosine-5'-diphosphate
RanGTP	Guanosine-5'-triphosphate
RCC1	Regulator of chromosome condensation 1
Riok2	Rio Kinase 2

RNA	Ribonucleic acid
RR	Ribonucleotide reductase
TK	Thymidine kinase
TP	Thymidine phosphorylase
TS	Thymidylate synthase
UK	Uridine kinase
UP	Uridine phosphorylase
5-FU	5-Fluorouracil

CHAPTER 1

INTRODUCTION

1.1. Moleculer Trafficking and Cancer

The physical seperation of the genome from the cytoplasm by the nuclear envelope (NE) is a trademark of the eukaryotic cell making a necessity to transport macromolecules over the nuclear layer to contemplate their typical function. This traspport procedure is profoundly composed and is an essential controller of cell signalling. The seperation of proteins from their site of capacity is a repetitive theme guaranteeing the constancy of signal transduction and averting distorted activation. Numerous transcription factors reside in the cytoplasm in an inactive structure until activation that outcome in their translocation into the nucleus. Upon nuclear passage they coordinate with particular transcriptional programs deciding cell fate (Zanella, Dos Santos, & Link, 2013). Cancer cells use the unusual confinement of tumor supressor proteins as a method for their inactivation and to successfully evade anti neoplastic treatments (J. G. Turner, Dawson, & Sullivan, 2012). The unusual confinement of oncoproteins can lead to their deficient activation (Hung & Link, 2011). Our developing comprehension of the components that control the nucleocytoplasmic transport has coordinated the advancement new therapeutic avenues to target growths that show variant protein subcellular localization (Hill, Cautain, de Pedro, & Link, 2014).

1.2. Moleculer Trafficking of Protein in Cell

Cellular responses are exceedingly particular procedures in which temporal control is critical for keeping up absolute growth and proliferation. Definitely controlled compartmentalization inside the cell is significant to keeping up complex procedures and supporting life at each level. If the sensitive parity is disturbed in this biological community, it can cause cancer celldevelopment (Ohno, Fornerod, & Mattaj, 1998). Eukaryotic cells are isolated into two same compartments. They are the cytoplasm and the nucleus which are isolated by a physical nuclear membrane. The genome is

protected in the nucleus and nucleus confined on all side by a nucleus membrane which is called nuclear envelope (NE) (Cyert, 2001). Nuclear membrane controls intracellular trafficking. DNA synthesis, protein translation, RNA transcription, RNA transport and cell division are just a couple of the basic and significant fuction of cell that rely on nuclear transport (Bednenko, Cingolani, & Gerace, 2003).

Substrates or cargo proteins must enter or exit the nucleus incorporate a lot of proteins and nucleic acids. Subsequently, in every single eukaryotic cell, NPCs have vital fuction (Katharina Ribbeck & Görlich, 2001). NPCs structure and fuction is widely protected. Their sizes are ~66 MDa in yeast and ~125 MDa in mamalian cells (Rout & Blobel, 1993).

The extensive nucleus mass of NPCs and their ability of mediating gigantic transport fluxes are reflected in an extensive pore. NPCs are able to do permitting the entry of cargo up to ~40 nm (~25 MDa) (Pante & Kann, 2002). The convanient pore size relies on upon the size and surface qualities of molecule or complex. The conventional rule is that particles bigger than ~20–40 kDa must be particularly recognized for transportation. This pathway name is signal dependent transport pathway. It is a active transportation which is required energy to carry larger molecules. Cell always spends energy. Larger molecules can be carried with special proteins which is called import and export proteins. On the other hand, if molecule size smaller than this mass abundantly travel the NPC without particular recognition. This pathway name is called signal independent pathway or passive transportation which is not required energy to carry small molecules. Cell never spends any energy. Small molecules can be carried by diffusion which is basicly equilibrating the cargo molecule between nucleus and cytoplasm.

For instance, small metabolites and proteins abundantly equilibrate between the cytoplasmic and nucleoplasmic compartments. In any case, molecular size is an essential requirement deciding NPC permeability, molecular surface properties are additionally vital since transport receptors of ~100 kDa pass throught the NPC and a minimum effect to the surface of a protein able to significantly influence its NPC permeability (Naim, Zbaida, Dagan, Kapon, & Reich, 2009). Besides, it has lately been proposed that electrostatic connections contribute importantly to NPC penetrability. How the factors representing cargo selectivity and how the proper pore size can quickly

change to permit the transport of a wide range of cargos in both direction are critical basic issues that stay uncertain.

Fragile control of nuclear import and export is given by the karyopherin beta group of proteins and the NPC (Fornerod, Ohno, Yoshida, & Mattaj, 1997). The NPC is comprised of numerous nucleoporins which permits particles under 40 kDa to pasesivelly travel through the nuclear envelope. Active transport for bigger macromolecules requires kanryopherins. These transporters conbine with a chaperone protein (Ran) and the NPC located on nuclear membrane effectively carry >40 kDa proteins into and out of the cell nucleus (Jamali, Jamali, Mehrbod, & Mofrad, 2011). Karyopherins can be sized by infections to promote contaminations and viral replication (Predicala & Zhou, 2013). DNA enhancement and mutation of karyopherins can cause localization of cell parts and can cause cancer.

1.2.1. Nuclear Pore Complex

The cell nucleus is insulated environment by NE.The fairly selective transport of proteins which is bigger than 40 kDa need essentical transport proteins. This transportation can be both into and out of the nucleus through the NPC (Reichelt et al., 1990). The NPC is one of the biggest protein complex structures which are aproximately 125 MDa in the cell. It contain more than 30 unique proteins called nucleoporins (Lim, Aebi, & Fahrenkrog, 2008). The NPC has an origin which is included transporter area containing a nuclear matrix. This matrix helps transportation of macromolecules. NPC has eight fibril proteins that stretch out into the cytoplasm and a nuclear basket structure. Transportation in or out of the cell nuclues is subject to the type of receptor protein and the molecules connected with it (Cook, Bono, Jinek, & Conti, 2007).

Structural components of the NPC broaden ~200 nm throughout the transport hub. It is along ~120 nm horizontally. The origin of pore has interior measurements of ~60–90 nm long and ~45–50 nm at its tightest width. Elastic fibrils extend ~50 nm along the cytoplasm. On the other side of NPC fibrils stretch out aproximately 50–100 nm along the nucleoplasm. Fibrils are appended to the distal ring shape like the nuclear basket (Monecke et al., 2009). The NPC is consisted of more than 450 proteins on the whole called nucleoporins (Nups) (Stewart, 2007). The nuclear pore complex shape is

such as octagonal rotational symmetry. Because it is expected that each Nup is every NPC in a whole number numerous of eight copies. There are about 30 different type o nucleuoporin in yeast and metazoan cells.

Large protein (>40 kDa) has to be bound up with a nuclear export receptor molecule to take shape for nuclear export or import. The greater part of these NPC transport molecules are members of the karyopherin family of proteins. There are almost 19 members of karyopherin receptor family proteins, with each perceiving a particular group of cargo protein or ribonucleic acid (RNA). CRM1 which is known chromosome region maintenance 1 is a pervasive transport receptor protein. CRM1 binds a hydrophobic nuclear export signal peptide sequence of cargo. Three constituents of exported complexes which are formed in the nucleus of the cell are RanGTP, the cargo and CRM1. CRM1 binds the cargo protein weakly. When RanGTP binds to CRM1 collectively, the closeness of CRM1 to both RanGTP and the cargo substrate is enlarged between 500 and 1000-fold (Stoffler et al., 2003). RanGTP is neccessary for the transport of cargo protein. RCC1 is the guanin nucleotide exchange factor and if RCC1 is available, high concentrations of RanGTP can be observed in the nucleus. Concentration difference between the nucleus and the cytoplasm supplies the required energy to catalyze the nuclear export. The CRM1/RanGTP/cargo trimer goes to cytoplasm, then RanGAP hydrolizes RanGTP to RanGDP. This leads to the break up of the trimer and cargoprotein is released into the cytoplasm. For recycling CRM1 and RanGDP goes to the nucleus via the nuclear pore complex (Pante & Kann, 2002).

1.2.2. Nucleocytoplasmic Transport Mechanism

Cargo is traveled through NPCs. It can be choise one of two pathways which are called the signal independent or the signal dependent pathways (Fried & Kutay, 2003). The signal independent pathway is used a passive transportation pathway which is not required energy. The Signal independent pathway carries cargo to equilibrate concentration gradient of cargo through NPC. In contrast, the signal dependent patways carries cargo which must be particularly recognized by receptors, to use energy for transportation cargo through NP (Yang & Musser, 2006).

Signal dependent nuclear transport depends on two main properties. The cargo obligate to have the suitable signal which is a lınear sequence (McLane & Corbett,

2009), and, once perceived, the cargo must be passed throught the phenylalanine glycine (FG) network penetrability obstruction. These capacities are given by nuclear transport receptors (NTRs). NTRs need to connect with the FG in the FG networks, and permit bound cargo to pass throught the pore. The biggest class of NTRs is the importin β (Imp β) which incorporates both importins that defines import, and exportins that defines export. Importins and exportins are additionally referred to karyopherinproteins family. NTRs have essential binding side to bind for nuclear localization sequence (NLSs) which is responsible for nuclear import. Also it is responsible for nuclear export sequences (NESs). In some instances, connectors are utilized to connect the cooperation between a cargo's signal sequence and its related NTR. For instance, members of the importin α (Imp α) family have an NLS restricting side and an Imp β restricting domain. This connectors allow freely organize transport pathways (Gorlich & Kutay, 1999). In higher eukaryotes, approximately seven sorts of Imp α have been recognize (Tejomurtula, Lee, Tripurani, Smith, & Yao, 2009).

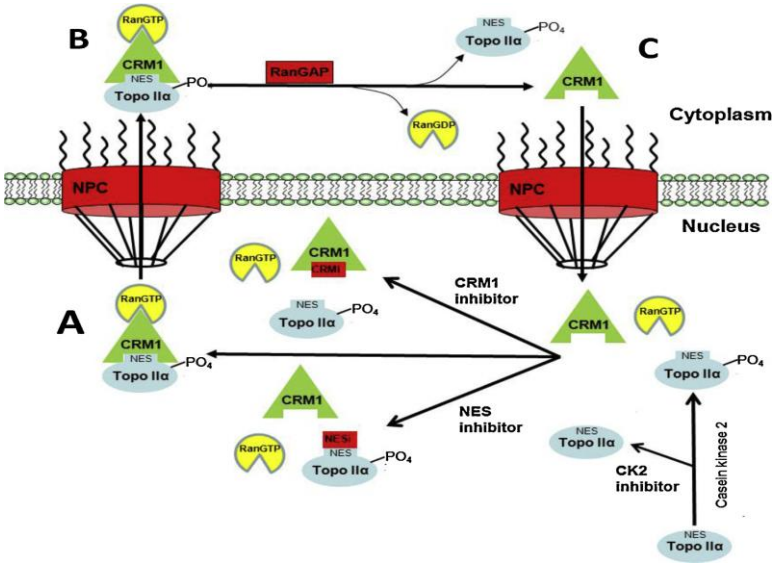

Figure 1.1. Nucleocytoplasmic transport mechanism
(Source: J. G. Turner, Dawson, Cubitt, Baz, & Sullivan, 2014).

Cargo of signal dependent pathway molecules penetrates by using NTRs through the nuclear pore comple

x. On account of import, a fundamental transport complex has a heterodimeric complex including of a cargo particle and a nuclear transport receptor. When Imp α is utilized by a specific import pathway, the transport complex has Imp β, Imp α and cargo complex. Nuclear transport receptor and cargo molecules very similar with each other

and they have very minimal critical differences. Export complexes can be stabilized by GTP-Ran which contains GTP and Ran seperately before being export (Askjae

r, Jensen, Nilsson, Englmeier, & Kjems, 1998). Import and export complexes are reversed the process of effective transport. Both of them dispose of the free cargo molecules. Export and import proteins and RanGTP wıll be used for next cycle of transport (Paraskeva et al., 1999). In import pathway, RanGTP and importins release each other. In contrast, export pathway RanGTP are stable, and initiation of the RanGTPase and complex dismantling require RanGAP and other type of Ran binding protein such as RanBP1 or RanBP2 (Floer & Blobel, 1999). Proficient separation of complex which includes Imp β1 and RanGTP, furthermore requires Imp α . RanGDP is turn back to the nucleus for recyling with nuclear transport factor 2 (NTF2) (K. Ribbeck, Lipowsky, Kent, Stewart, & Gorlich, 1998).

Factors that recognize import complex are exist on the cytoplasmic fibrils of the pore and factors that recognize export complex are exist on the nucleoplasmic side of the pore (Matsuura & Stewart, 2005). For instance, Nup50 is a factor which exist on the nucleoplasmic side of the pore, recognize separation of Imp α with cargo complexes (Matsuura & Stewart, 2005). Nup358 which is called RanBP2,the fundamental segment of the cytoplasmic fibrils, to connect SUMOylated RanGAP and includes RanBP1 homology areas, which connect with Ran (Hutten, Walde, Spillner, Hauber, & Kehlenbach, 2009).

In this manner, transport complex dismantling is planned to happen at the nuclear pore complex toward the end of the transport procedure. The RanGTP is an important component and RanGTP gradient significantly critical for signal dependent nuclear transport. RanGTP is appropriated for import complex dismantling and export complex get together. In the nucleus, concentration of the RanGTP is higher than cytoplasm.

RanGAP exists in the cytoplasm guarantees that the RanGTP gradient is low in this part of cell. In RanGEF is known a guanine nucleotide exchange factor in the nucleus and it is guarantees that RanGTP is recovered from RanGDP in the same place (Gorlich, Seewald, & Ribbeck, 2003). The nuclear transport direction is chanced by RanGTP concentration because concentration of RanGTP gradient manages process of nuclear transport (Nachury & Weis, 1999).

1.2.2.1. Nuclear Export Mechanism

Envelope of the nucleus provide special environment for vital process such as deoxyribonucleic acid (DNA) replication, RNA transcription and generation of ribosomes. The nuclear membrane has a specific physical barriers and complexes which is provided control of the cell division and cycle, especially appoptosis of cell and poliferation of cell.

Nuclear cytoplasmic trafficking controls vital process and components such as RNA and ribosomes which is essential controllers of transcription. Nuclear transport complex which is member of karyopherin proteins family regulate cell cycle (Hodel, Corbett, & Hodel, 2001). In addition, nuclear pore complex which has nuclear transport factor control to transport cell cycle inhibitors and specific drug tragets. For this reason, if there is a misregulation of nuclear export, it leads to pathological problems such as cancer (Kanwal, Li, & Lim, 2002).

Highly specific sequences of amino acids provide transport of protein in the intracellular environment. In the nucleus, proteins have signals which are called nuclear localization signals for import (Bogerd, Fridell, Benson, Hua, & Cullen, 1996). In contras, in the cytoplasm, proteins have signals for export (Ikuta, Eguchi, Tachibana, Yoneda, & Kawajiri, 1998). Thus, molecules and proteins traffiking in the cell is managed by amino acid signalling sequences to the mitochondria (Devi, Prabhu, Galati, Avadhani, & Anandatheerthavarada, 2006), peroxisome (Andres, Dickerson, & Dixon, 1990), golgi apparatus (Zeng, Tran, Tan, & Hong, 2003), lysosome (Bonifacino & Traub, 2003), endoplasmic reticulum (Munro & Pelham, 1987).

All cellular materials must penetrate from nucleus to cytoplasm. It must use specific region of nuclear envelope which is called nuclear pore complex. Transport of small molecules such as small protein and ions penetrate through the NPC to occur diffusion. If proteins bigger than 40-65 kDa can not penetrate through the NPC. Bigger proteins need to be transported through the NPC with the nuclear cytoplasmic transport receptors which is known as karyopherin proteins. The fundamental nuclear cytoplasmic transport receptors are subfamily of the karyopherin β proteins (Cook et al., 2007). Each cargo proteins or RNA is recognized by karyopherin β protein family members. This recognition is occured by a nuclear export or an import signal wich uses specific amino acid sequences of the cargo protein. Lately, just three classes of nuclear

cytoplasmic transport signals have been known. These are corresponding with the essential amino acid nuclear localization signal sequences are occured by importin α and importin β (Lange et al., 2007).

Traditional nuclear localization signal is occured monopartite or bipartite (Hodel et al., 2001). Karyopherin β2 cargo proteins involve more complex NLS motif. It can contain an N-terminal hydropobic region or another basic region and C-terminal $RX_{2-5}PY$ region (Lee et al., 2006). Chromosome maintenance protein 1 (CRM1/exportin1) which is a nucleocytoplasmic transport protein recognize hydrophobic leucine rich export signals. CRM1 can bind and carry proteins and RNA. To date, approximately 19 karyopherin proteins have been known in human. These are importins, exportins and transportins. To date, how to recognize NLS and NES each other is not clear (Xu, Farmer, & Chook, 2010).

NES includes arrangements of hydrophobic amino acids, including isoleucine, leucine, methionine, phenylalanine, and valine for CRM1 (Kutay & Guttinger, 2005). Despite the fact that there is limited data and a generally high level of sequence variation, an agreement theme for a nuclear export signal is $HX_{2-3}HX_{2-3}$HXH. In this structure, H can be a hydrophobic amino acid such as methionine, valine, isoleucine, leucine, phenylalanine and X is can be any amino acids (Joel G. Turner et al., 2009). The subscripted numbers show the general number of repeats. This accord sequence can be available in more complex protein structures, for example, α-helical proteins (Dong et al., 2009).

In a study utilizing yeast, cells were incubated with inhibitor of nuclear export protein CRM1 which is called leptomycin B. Proteomic investigation of these cells distinguished 285 proteins that were controlled by CRM1. Almost 45% of these proteins included the nuclear export accord sequence. The other proteins can be carried with using mismatching or another alterned nuclear export sequence by CRM1 (Matsuyama et al., 2006).

Every nuclear pore complex can carry cargo into the nucleus and out of the nucleus with utilization of karyopherin and its specific cargo. In the nucleus, concentration of RanGTP is higher than cytoplasm and it is believed that a RanGTP concentration gradient gives energy for nuclear export (Arnaoutov et al., 2005). In the nucleus, guanine nucleotide exchange factor (GEF) preserves the high concentration of RanGTP gradiend to thanks to regulator of chromosome condensation 1 (RCC1).

Chromatin and RCC1 bind each other because of preservation of chromatin (Terry, Shows, & Wente, 2007).

More or less 12–20% of the nucleoporins which are in the nuclear pore complex are included phenylalanine and glycine repeats (FG-repeats) (Rout et al., 2000). CRM1 and importin β family proteins have FG-repeats. For this reason, CRM1 and other importin β proteins can be interacted with nucleoporins. This association cause to change the RanGTP concetration gradient. All these collaborations provide to move the complex in the matrix of nuclear pore complex.

Cargo needs to bind RanGTP dependent factor and CRM1 which is a exportin for movement of the cargo from nucleus to cytoplasm. In this process, RanGTP act to be requlator of transport. Different type of RanGTP dependent export proteins are presented in the cell. Export complex comes together in the nucleus. It consists of three different main parts which are the exportin transport receptor (CRM1), RanGTP and cargo protein. CRM1 binds RanGTP and cargo protein weakly (Monecke et al., 2013). When RanGTP binds to the complex which consists of CRM1 and cargo protein, it increases the binding affinity of members from the complex approximately 500-1000 fold. This changing of affinity is explanied by crystallography of CRM1. CRM1 conformation occurs when cargo protein and RanGTP binds to CRM1 together (Monecke et al., 2009).

In the nucleus, cargo complex which is formed CRM1, RanGTP and cargo protein, pass trought the nuclear pore complex from nucleus to cytoplasm. When it arrives in the cytoplasm, RanGTP which is cargo complex member is hydrolyzed to RanGDP by RanGTPase enzyme. And the cargo complex members disconnect each other. RanGTP and CRM1 turns back to the nucleus to recyle next cycle of nuclear transport (Daelemans, Costes, Lockett, & Pavlakis, 2005).

Cargo proteins need to have a leucine rich nuclear export signal to be bound by CRM1. If cargo protein has this part, it can be recognized and bound by CRM1 (Vogt, Jiang, & Aoki, 2005). After binding, three dimensional conformational of cargo protein can change. Because protein phosphorylation, dephosphorylation, transformation can occur diffirences on the NES to CRM1 (Craig, Zhang, Davies, & Kalpana, 2002).

1.2.2.2. Inhibition of CRM1

CRM1 is a nuclear export molecule which can be found everywhere in the cell. CRM1 binds to a cargo substrate. This cargo substrate which should have hydrophobic NES can be protein or RNA. NESdb is database of NES which is contained CRM1's cargo lastly checked on January 2014 and 241 macromolecules which are exported by and send to by CRM1 are found (Xu, Grishin, & Chook, 2012). Cargo proteins hydrophobic NES binds to the hydrophobic groove of CRM1 which contains an active site Cys528. Normally NES is known by its high leucine content. In any case the characterization of the NES might be better depicted as not particularly leucine rich. However NES has a general hydrophobic pattern. The lenght of NES peptides are 8-15 aminoacid residues. They adjust freely to a consensus sequence of φ1-$X_{1,2}$-φ2-$X_{2,3}$φ3-Xφ4 (φn represents Leu, Val, Ile, Phe, or Met and X can be any amino acid), as inspected in Xu et al. (Xu, Farmer, Collett, Grishin, & Chook, 2012).

Generally CRM1 inhibitors are used direct inhibition of nuclear export. Nuclear export inhibitors sources obtain natural and synthetic molecules (J. G. Turner et al., 2012). The fundamentally of these inhibitors bind to the dynamic site Cys528 contained inside a hydrophobic groove of CRM1 and inconvertible adjust it by a Michael-type covalent addition. Leptomycin B prevent binding side of CRM1 with mutagenesis of Cys528 to Ser528 (N. Kudo et al., 1999). Also ratjadone and other small nuclear CRM1 inhibitors inhibite active side of CRM1 same as leptomycin B inhibition of CRM1 (Hill et al., 2014).

1.2.2.3. Inhibitors of CRM1

1.2.2.3.1. Leptomycin B

Numbers of compounds have been isolated from natural sources and their analogues that inhibit nuclear export by CRM1 have been synthesised. Leptomycin B is the main inhibitor of CRM1. It was isolated from the microorganisms *Streptomyces (Gravina et al., 2015)*. Leptomycin B binds to CRM1 by a Michael type covalent assembly at the reactive site cysteine 528 residue. Alkylation of cysteine 528 side

inhibits CRM1 binding to the leucine rich nuclear export sequence of the cargo protein substrate. This alkylation hindering the structure of the CRM1, cargo and RanGTP complex and successfully blocking nuclear export (Nobuaki Kudo et al., 1999).

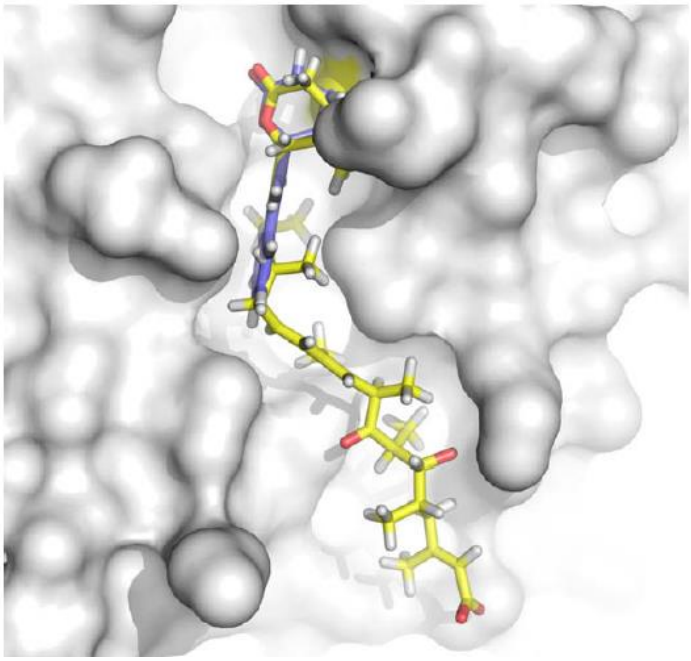

Figure 1.2. Superposition of goniothalamin (blue) and the model of leptomycin B (yellow) binding to CRM1 based on X-ray structures (Source: J.-Y. Wach, Güttinger, Kutay, & Gademann, 2010)

Until this day, most CRM1 inhibitors capacity by modifying of the reactive site cysteine 528 and hinder CRM1 binding to the nuclear export sequence of cargo proteins. Leptomycin B is a powerful inhibitor of CRM1 and is compelling at nanomolar concentration (Nobuaki Kudo et al., 1999; Mutka et al., 2009). In vitro studies have shown that leptomycin B has intense toxicities at concentration <5 nmol/L for 1 hour. Nonetheless, when it was used for phase I clinical trial as an against growth anti cancer compound, leptomycin B was not observed to be clinically helpful because of serious toxicities (Newlands, Rustin, & Brampton, 1996). Right now, leptomycin B inhibition of nuclear export of a protein and leucine rich side of nuclear export signals are the principles to characterize whether a protein is sent out by CRM1.

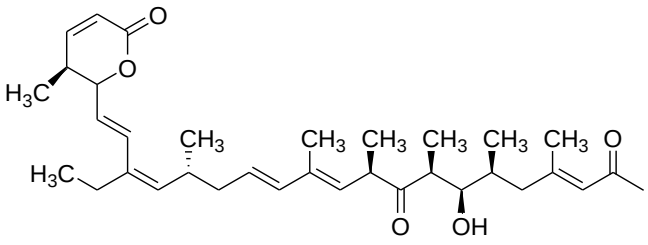

Figure 1.3. Structure of leptomycin B

1.2.2.3.2. Ratjadone

Ratjadones A, B, C and D are anticancer and antifungal CRM1 inhibitors which have been isolated from myxobacterium *Sorangium cellulosum* (Nguyen, Holloway, & Altura, 2012). The chemical structure of ratjadones represents close-range structure with leptomycin B and it act like the same molecular mechanism through modifying CRM1 at its reactive side cysteine 528 (Meissner, Krause, & Vinkemeier, 2004). Thus the interaction between CRM1 and cargo protein was inhibited. Ratjadone compounds' IC_{50} values are in the picomolar range. It was shown that radjadone compounds have the ability to inhibit cell growth and proliferation in bacteria, in yeast, and in the human cancer cell lines Jurkat, HepG2, HeLa, and U87-MG (Burzlaff, Kalesse, Kasper, & Scheper, 2003). Also it is indicated that ratjadone compounds cause cell cycle arrest at G1 phase (Burzlaff et al., 2003).

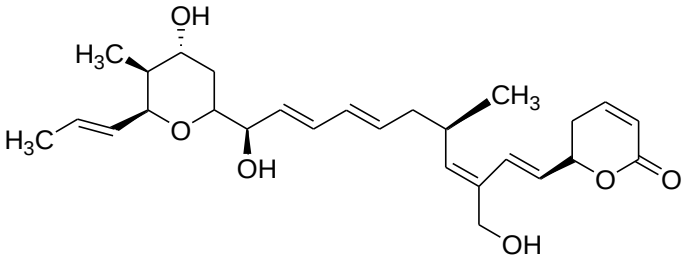

Figure 1.4. Structure of ratjadone

1.2.2.3.3. CBS9106

CBS9106 is a small molecule reversible inhibitor of the nuclear export receptor which is known CRM1 (Nakamura et al., 2000). This CRM1 inhibitor achieved cell cycle arrest and apoptosis. It is a single agent which is used in more than 60 different types of human tumor cell lines, including bladder, colon, breast, endocrine, lung, kidney, pancreatic, prostate, and skin tumor. This molecule induced cell cycle arrest and apoptosis at sub-micromolar concentration. CBS9106 effect is reversible by the removal of the drug. In pull down procedures, it was shown that biotinylated CBS9106 binds specifically to the CRM1 protein. It is competitively inhibited by leptomycin B for the reactive site cysteine 528. Also CBS9106 may react particularly with thiol groups since it is managed by the occurence of N-acetylcysteine. CBS9106 binds and inhibits CRM1. When cysteine 528 residues is replaced by serine, inhibition is not occured. In this

manner, cysteine 528 residue is a key for inhibiton capacity of nuclear export of protein (Sakakibara et al., 2011)**.**

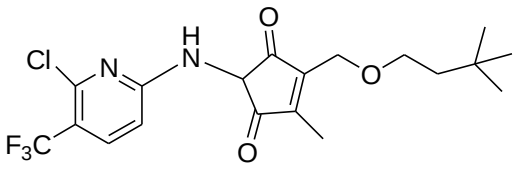

Figure 1.5. Structure of CBS9106

1.2.2.3.4. Goniothalamin

Styryl lactones are auxiliary metabolites isolated from Goniothalamus plant species. To date, there are approximately 100 styryl lactones analogs which are isolated from naturel sources and synthesized. These compounds have been shown to be cytotoxic on cancer cells (de Fatima et al., 2006). Goniothalamin (GTN) a plant styryl lactone isolated from *Goniothalamus andersonii*, achieved cytotoxicity in different type of cancer cell lines including cervical, gastric, ovarian, kidney, breast carcinomas, leukemia (Inayat-Hussain et al., 2010). Goniothalamin and derivatives have α,β-unsaturated δ-lactones in their structures. These α,β-unsaturated δ-lactones have capacity to carry on as a Michael acceptor and these unsaturated carbonyls can bind to the nucleophilic side of target proteins, for example, cysteine, lysine, serine, threonine and glutathione. By Cagir et. al. is demonstrated an expansion of cytotoxic movement of molecules which is replaced of double bound with 1-naphtyl group (Kasaplar, Yilmazer, & Cagir, 2009).

In the context of our synthetic and biological studies on the klavuzon, it has been discovered that derivatives almost the full biological potency of the parent compound which has the structural similarity of this goniothalamin to klavuzon. Therefore, it led to the hypothesis that klavuzon and similar occurring lactones would also constitute inhibitors of nucleocytoplasmic transport leading to potent antiproliferative effects. In this study, we investigated klavuzon and its analogs role in nucleocytoplasmic transport as inhibitor in an in vivo transport assay.

Goniothalamin Klavuzon

Figure 1.6. Structures of goniothalamin and klavuzon

1.2. Thymidylate Synthase

A vital enzyme of nucleotide metabolim is the duplication of pathways. Inhibition of enzyme has many optional pathways. Unlike these enzymes, Thymidylate synthase (TS) is a "bottleneck" catalyst which gives the main method for adding a methyl group to the 5-position of the pyrimidine ring in the thymidine. Thymidylate synthase is the one enzyme in the nucleotide synthesis pathway which rather than metabolizing the 5-FU and its derivatives like natural substrate (Papamichael, 1999).

Thymidine is the main nucleotide precursor particular to DNA. TS is very well known target for anti-cancer agents. The catalyst's action is a two phase process. In the first, deoxyuridine monophosphate (dUMP) binds to a receptor site and this instigates a configurational change which opens a contiguous restricting site for N-5,10-methylene-tetrahydrofolate (CH2FH4). The folate's one carbon group is then exchanged to the uridine ring. In this transfer occurs resulting in deoxythymidine monophosphate (dTMP) and dihydrofolate dTMP is in this manner phosphorylated by a kinase to deoxythymidine diphosphate (dTDP) and deoxythymidine triphosphate (dTTP). The product is well known and a necessary base for DNA synthesis (Santi, McHenry, & Sommer, 1974).

1.2.1. Mechanism of Action of 5-FU

5-Fluorouracil (5-FU) is very similar with uracil. Uracil has hydrogen atom at the C-5 position and 5-FU has fluorine atom at the C-5 position. For this reason, cells can use 5-FU for same facilities like uracil. It has been realized that fluorodeoxyuridine monophosphate (FdUMP) which is a 5-FU metabolite, intensely represses TS. This is one of the principle systems fundamental for 5-FU activity (Santi et al., 1974). It binds

surely at the same place and with the same proclivity as dUMP. However, it is not at all like hydrogen, the fluorine atom at the 5′-position can not be dislodged. In this way, FdUMP and the decreased folate turn into covalently bound with TS and its structure form a ternary complex and a cysteine thiol of TS is joined to the 6′-position of FdUMP, with the one carbon group of the folate beside F at the 5′-position.

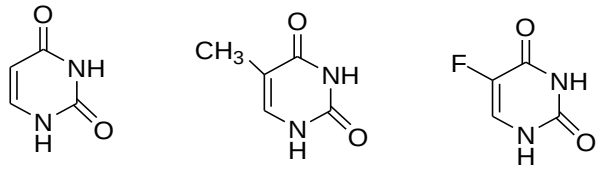

Figure 1.7. Structures of uracil, thymdine, 5-FU

Inside the cell, 5-FU has different several main metabolites because of uracil mechansm: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP).

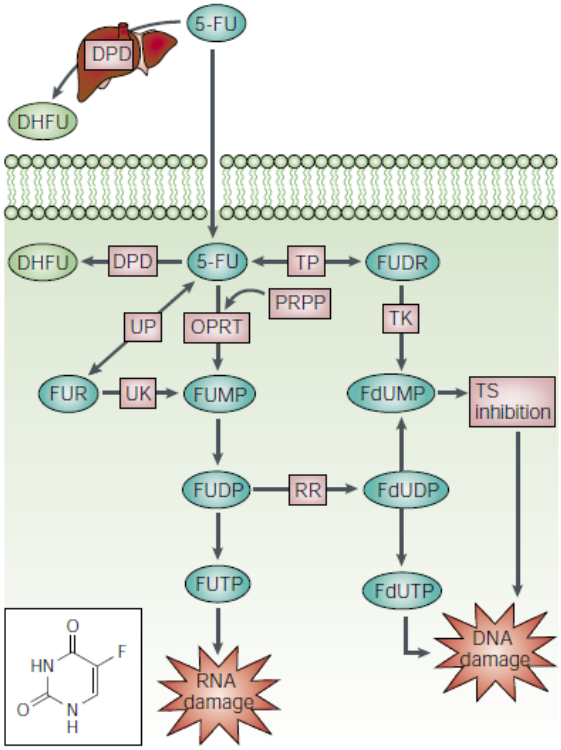

Figure 1.8. 5-Fluorouracil metabolism
(Sorce: Longley, Harkin, & Johnston, 2003)

The principle mechanism of 5-FU activation is transformation to fluorouridine monophosphate (FUMP). This pathway can use enzyme with phosphoribosyl pyrophosphate (PRPP) as the cofactor or the other pathway to produce fluorouridine

(FUR) using with uridine phosphorylase (UP) enzyme. FUMP is at that point phosphorylated to fluorouridine diphosphate (FUDP), which can be phosphorylated to the dynamic metabolite fluorouridine triphosphate (FUTP) or changed over to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR) (Wohlhueter, McIvor, & Plagemann, 1980).

Thusly, FdUDP can be phosphorylated and dephosphorylated to create the active metabolites FdUTP or FdUMP. The other pathway to produce FdUMP is thymidine phosphorylase (TP) enzyme which is catalyzed change of 5-FU to fluorodeoxyuridine (FUDR). Then FUDR phosphorylated by thymidine kinase (TK) to FdUMP.

Dihydropyrimidine dehydrogenase (DPD) is an enzyme in the liver and It can transfer 5-FU to dihydrofluorouracil (DHFU). This pathway is for catabolism of 5-FU in typical and tumor cells. Up to 80% of directed 5-FU is separated by DPD in the liver. (Diasio & Harris, 1989)

1.3.2. Mechanism of Thymidylate Synthase Inhibition by 5-FU

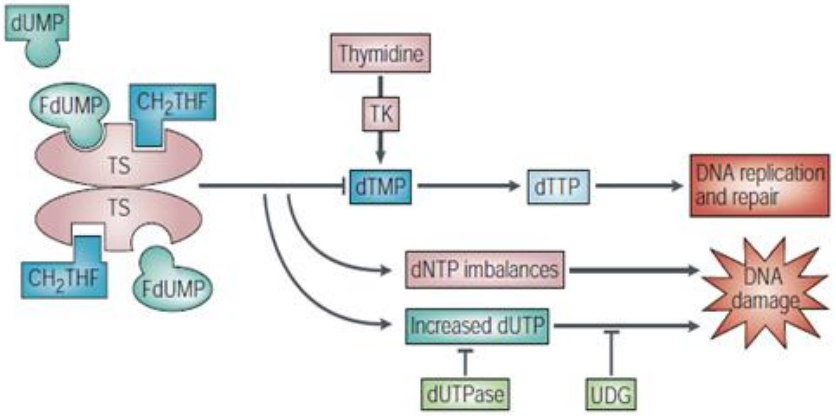

Figure 1.9. Inhibition of thymidylate synthase mechanism by 5-fluorouracil (Source: Longley et al., 2003).

TS catalyze the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) and the reduced folate 5,10-methylenetetrahydrofolate (CH2THF) is used the methyl donor . This reaction is the source of thymidylate, and thymidylate is important for DNA replication and DNA repair. TS protein is 36 kDa and its shape as a dimer (Wohlhueter et al., 1980). All subunits of TS contain a nucleotide restricting site and an official site for CH2THF (Diasio & Harris, 1989). FdUMP which is metabolite of 5-FU, binds to the nucleotide

restricting site of TS. This structure constitute a stable ternary complex with the compound and CH2THF (Ullman, Lee, Martin, & Santi, 1978). In this way blocking binding of the ordinary substrate dUMP, also inhibiting dTMP synthesis (Santi et al., 1974).

Consequence of TS inhibition is aggregation of dUMP, which could be end with arise level of deoxyuridine triphosphate (dUTP) (Douillard et al., 2000). Both dUTP and the 5-FU metabolite FdUTP perchance misincorporated into DNA. Restore of uracil and 5-FU-containing DNA by the nucleotide excision which also restore enzyme uracil-DNA-glycosylase is worthless when existence of FdUTP/dTTP is high level it is end with extra false nucleotide incorporation (Mitrovski, Pressacco, Mandelbaum, & Erlichman, 1994). Misincorporation, excision and repair making a worthles cycles which finally ends with DNA strand breaks and destruction of the cell (Webley, Hardcastle, Ladner, Jackman, & Aherne, 2000). Because of dUTP misincorporation DNA can be damaged. This is extremly depend on ratio of the pyrophosphatase dUTPase, which restriction to intracellular accumulation of dUTP (Ladner, 2001). Thymidylate can be saved from thymidine via activity of thymidine kinase, So that, effects of TS deficiency is reducing. This saving way is indicate the possible mechanism to strugle against to 5-FU (Greenhalgh & Parish, 1990).

1.3.3. Other Inhibitors of Thymidylate Synthase

An essential component of nucleotide digestion system is the duplication of pathways. Inbition of enzyme can be accomplished by one or more routes. At the present time, there are a lot of TS inhibitors. Some of them have been designed for folate binding side of TS. These folate binding side inhibitors directly interact with special side for folate binding side of TS. Nolatredex, raltitrexed, ZD9331, LY231514 are well know example for folate binding side inhibitors of TS.

In spite of the fact that, other pathway of TS inhibition is 5-FU which is major inhibitor for RNA and DNA synthesis. TS inhibition is increased in amount of dUMP which is intracellular component of TS mechanisms. 5-FU and dUMP compete for binding to TS. As opposed to, folate binding side inhibitors can inhibit TS and they do not influence other TS mechanisms. Also, dUMP increases the binding of folate analogs to TS (Papamichael, 1999).

1.3.3.1. Raltitrexed

Raltitrexed is well known antifolate inhibitor of TS. It is a water soluble antifolate compound. Raltitrexed has a terminal glutamate residue and in the cell, this terminal glutamate residue is changed to a polyglutamate form. Polyglutamates have potential important inhibition property for enzyme inhibition. Additionally it held in the cell for long time. Potential causes of imperviousness to the medication would emerge from the way that it requires a particular transport protein to cross the cell membrane, as well as the way that it capacities as a prodrug for its polyglutamate structure (Jackman et al., 1995) a new thymidylate synthase inhibitor with clinical anti-tumor activity in a range of solid tumours. (Blackledge, 1998)

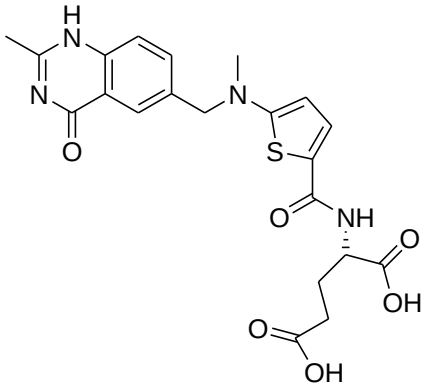

Figure 1.10. Structure of raltitrexed

1.3.3.2. Nolatredex

Nolatredex is another folate side binding TS inhibitor which is water soluble and lipophilic agent. This compound does not include glutamate side chains. It can use passive diffusion to enter into the cell. For this reason, nolatredex is different than other folate inhibitors such as multiple targeted antifolate and raltitrexed (Jodrell et al., 1999). Nolatredex can stay very short time in the plasma. It can not shape polyglutamates and therefore be held in cells, this requires a drawn out infusion (Papamichael, 1999). Its range of toxicities is more like 5-FU than raltitrexed. Restricted movement in colorectal and pancreatic cancer growth has been watched. In recent time, while in stage II assessment, nolatrexed was pulled back from further clinical study (Rafi R, Boddy AV, Cavete JA et al.). Preclinical and phase I clinical studies with the nonclassical antifolate

thymidylate synthase inhibitor nolatrexed dihydrochloride was given by prolonged administration in patients with solid tumors. (Papamichael, 1999)

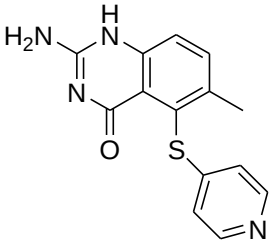

Figure 1.11, Structure of nolatredex

1.3.3.3. LY231514

LY231514 is a multiple targeted antifolate (MTA) agent for TS inhibition. It causes inhibition of a range of catalysts required in folate metabolism, the most essential being TS, dihydrofolate reductase (DHFR), human monofunctional glycinamide ribonucleotide formyltransferase, and aminoimidazole carboxamide ribonucleotide formyltransferase. MTA is transported over the cell membrane utilizing the lessened folate carrier system. It has low proclivity to folate receptors, and in addition being a perfect substrate for the enzyme folylpolyglutamate synthase (FPGS) (Rinaldi DA, Burris HA, Dorr FA et al.). Initial phase-I evaluation of the novel thymidylate synthase inhibitor, LY231514 was used by the modified continual reassessment method for escalation (Rusthoven et al., 1999).

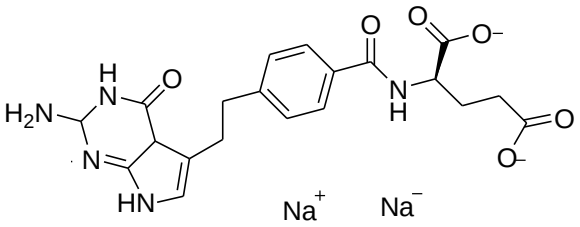

Figure 1.12. Structure of LY231514

1.3.3.4. Eniluracil

Eniluracil is being effective in small quantities as inhibitor for DPD which is the main catalyst in a degradation pathway (Grem et al., 2000). The late acknowledgment of the clinical significance of DPD has led to new potential methodologies for 5-FU

application with safe and effective way (B. E. Harris, Song, Soong, & Diasio, 1990). The significance of DPD on 5-FU pharmacology has been further accentuated by consequences of studies recommending the impact of DPD on pharmacokinetics, efficacy, bioavailability and toxicity of 5-FU (Barry E. Harris, Carpenter, & Diasio, 1991). Relationship of dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels can be an evidence for circadian variation of 5-fluorouracil levels in cancer patients receiving protracted continuous infusion (Baccanari, Davis, Knick, & Spector, 1993).

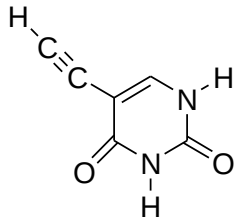

Figure 1.13. Structure of eniluracil

1.3.3.5. 5-FU Analogues

After the discovery of 5-FU, many 5-FU analogue was synthesized as an inhibitor. There are various different fluoropyrimidines. They have been synthesized as prodrugs for 5-FU. For example, uridine phosphorylase is used to remove ribosyl group from 5'-deoxy-5-fluorouridine (doxifluridine) to produce 5-FU

Another 5-FU analog is 1-tetrahydrofuranyl-5-fluorouracil which is called ftorafur (UFT). Hepatic P-450 microsomal enzymes and cytosolic enzyme catalyse from ftorafur to 5-FU. It has benefit of bioavailability. (36 the use of thymidylate) Its action is enhanced by the coadministration of uracil in a 4/1 molar ratio. It stops debasement by DPD. It gives more drawn out centralization of 5-FU in tumor tissues.

Capecitabine (Xeloda), is another orally controlled and tumor specific fluoropyrimidine carbamate. When Capecitabine is taken, it is directly passed through gastrointestinal mucosal barrier without losing its intact structure and its efficiency. It can be absorbed at least 70% percent without variability. It is changed over to 5-deoxy-5-fluorouridine by a successive triple enzyme pathway. The last tumor specific compound response is interceded by the tumor associated angiogenic component thymidine phosphorylase, when it is metabolited to 5-FU. It has two noteworthy points of interest, which may interpret into a therapeutic index. Firstly, it improved medication

fixation at the cancer site and consequently more prominent anti-tumor activity. Secondly, it reduced drug level in nontumor tissues, with a resulting reduction in systemic toxicity. Preclinical information have recommended an enhanced adequacy profile more than 5-FU and oral UFT (Di Costanzo, Sdrobolini, & Gasperoni).

CHAPTER 2

RESULTS AND DISCUSSION

2.1. Cell Viability Analysis

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In this study, anti-poliferative activity of the 4'-methylklavuzon at 20, 10, 5, 2.5, 1.25, 0.625 and 0.1 μM concentrationes and different concentration of thymidine were evaluated against HuH-7 cell line by the reduction of tetrazolium dye MTT to insoluble formazan. The amount of formazan crystals were determined by measuring absorbance at 540 nm. The IC_{50} values of cytotoxic compound were calculated by Graphpad Prism 6 software through non-linear regression analysis of at least two separate triplicate experiments. The results of MTT assays are listed in table 2.1.1

Inhibition of thymidylate synthase (TS) enzyme is one of the most popular mechanisms in anti-cancer agent discovery studies. Thymidylate synthase can catalyze the transformation of uracil to thymine in the cell. Inhibition of TS by an anticancer agent can halt the DNA synthesis in the cells. Among TS inhibitors, 5-fluorouracil is the best well-known example. Michael addition of the nucleophilic thiol residue of TS protein to the 6-C position of 5-fluorouracil is the source of irreversible inhibition of TS. A similar Michael acceptor site also exist in the structures of klavuzon and it can also react with the same thiol side chain and inhibits TS irreversibly.

One of the simple ways to account TS inhibition is to determine percent cell viability in the lack and presence of additional thymine supplement. For this purpose, the effect of the amount of thymine supplements in HuH-7 cells was studied by MTT cell viability analysis. As it can be seen in figure 2.1.A addition of thymine at 50 μM concentration or higher did not have any significant effect on the cell growth for 48 hours.

Then two separate set of cell viability test were performed. In the first one HuH-7 cells were incubated with varying concentrations of 4'-methylklavuzon alone (Figure 2.1.B) or in the presence of additional 4 μM and 40 μM concentrations of thymidine (Figure 2.1.D and F respectively). In this data set 4'-methylklavuzon and thymidine were appled on cell with the same time to measure competion of 4'methylklavuzon and

thymidine. Addition of low dose of thymidine supplements further increases the anti-proliferative effect of the 4'-methylklavuzon while addition of 40 μM concentration of thymidine did not cause any significant effect on IC_{50} values.

In the second set of experiments, HuH-7 cells were first incubated with varying concentrations of 4'-methylklavuzon for 24 hours then growth medium is aspirated. Then new growth medium containing 0, 50, and 100 μM concentrations of thymidine were added over HuH-7 cells and incubated for further 48 hours to recover from possible thymidineless death (Figure 2.1.C, E, and G respectively). After 4'-methylklavuzon were aspirated, the main aim to added thymidine to constıtute thymidine pool to recover from possible thymidineless death. Although presence of additional thymidine supplements increases the IC50 values, their effect were not strong enough to fully recover the cells from death (Cox & Harmenberg, 1992).

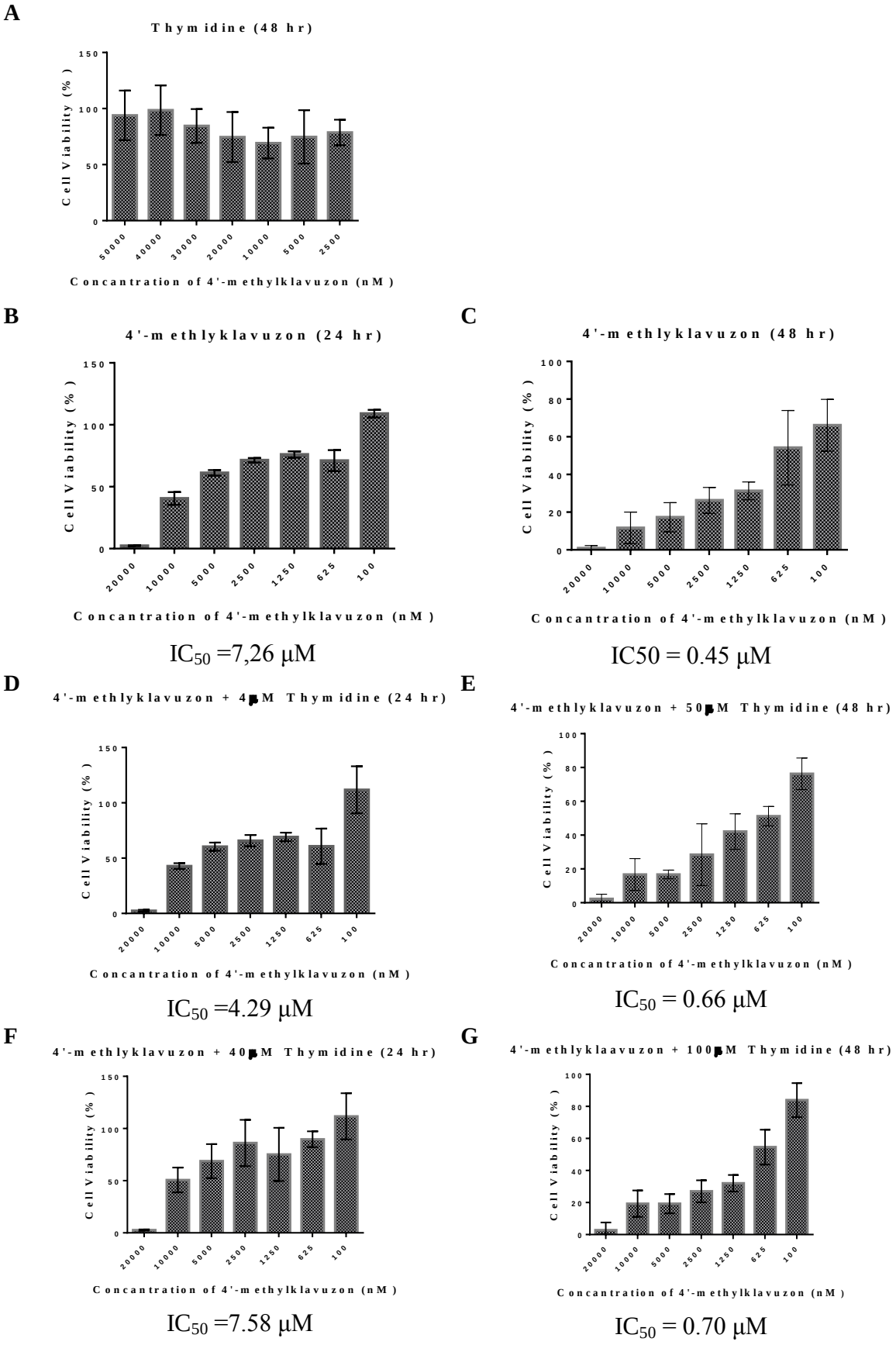


Figure 2.1. The anti-poliferative actitivity of compounds of 4’-methylklavuzon and thymidine on HuH-7 cell line

2.2. Immunocytochemistry Analysis

The goniothalamin has been shown to be a potential inhibitor of CRM1 nuclear export proteins (J. Y. Wach, Guttinger, Kutay, & Gademann, 2010). The structural similarity of goniothalamin and klavuzon thus led to the hypothesis that klavuzon would also constitute inhibitors of nucleocytoplasmic transport ledding to potent anti-poliferative effects. In this study, we analyzed klavuzon derivatives as inhibitors of nucleocytoplasmic transport in vivo transport assay and compared with goniothalamin.

Immunocytochemistry (ICC) assay was performed which is a sensitive method to examine localization of protein in individual cell. Technique is based on detecting antigens, for example; proteins, in cells by exploiting the principle of antibodies binding specifically to antigens in cell. Polyclonal mouse RIOK2 antibody and polyclonal goat anti-mouse antibody were used to determine nuclear localization of RIOK2 proteins which is carried with CRM1 nuclear transport receptor from nucleus to cytoplasm in cells.

Both goniothalamin and klavuzon have an α,β-unsaturated lactone ring which is a Michael acceptor. It is known that Michael acceptor can covalently bind themselves to the nucleophilic site of the target proteins. Because of that they can be considered as irrevisible inhibitors. Reversibility of the Michael acceptors might depend on pH of the reaction medium. Previously it was shown that at pH 8 additions of thiols to the Michael acceptor is totally reversible and it takes time to reach equilibrium. At low pH values the same reaction is totally irreversible and extremely slow (Shi & Greaney, 2005).

Among the intracellular cell compartment only mitochondria has a pH 8 which may trigger reversible Michael additinol reactions thiols. On the other hand, the pHs of the nucleus containing CRM1 transport proteins have a pH 7.2 (Casey, Grinstein, & Orlowski, 2010). At this pH addition of thiols to the Michael acceptor can be irreversible and quite slow. To test that time and concentration dependent CRM1 inhibition by goniothalamin and klavuzon derivatives were studied.

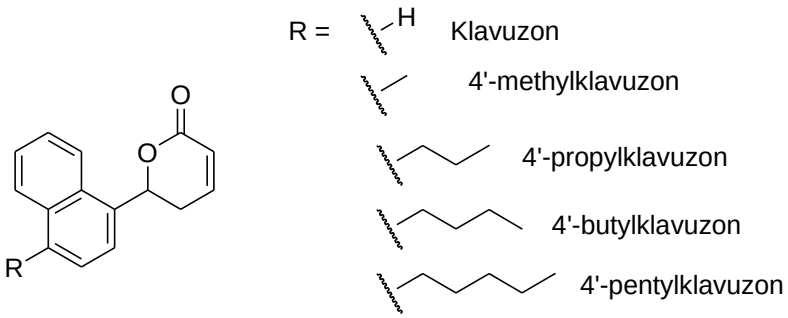


Figure 2.2. Structure of 4'-alkylklavuzon derivatives

In ICC experiments, basically cells were treaded with goniothalamin which is used for positive control, and different derivatives of klavuzon. The ICC assays of five compounds were examined on HeLa cell line. These compounds are: goniothalamin (1), 4'-methylklavuzon (2), 4'-propylklavuzon (3), 4'-butylklavuzon (4) and 4'-pentylklavuzon (5). These compouds were applied different concentration of 20, 10, 5, 1, 0.5, 0.2, 0.1 and 0.05 μM on HeLa cell line. For all images A, B and C refer to respectively secondary antibody images, Dapi dye images and inverted images.

HeLa cells were treated with 4'-methylklavuzon and goniothalamin at different concentration of 50, 20, 10, 5, 1, 0.5, 0.2, and 0.1 μM. 4'-methylklavuzon demostrated strong inhibition of CRM1 nuclear export protein (Figure 2.2.). It fully bloked export of human Riok2 proteins at 0.1 μM. In contrast, goniothalamin started to inhibit CRM1 proteins at 0.5 μM. Additinally, goniothalamin exactly blocked CRM1 nuclear export proteins at 1 μM. When it is compared that goniothalamin and 4'methylklavuzon at concentration of 1 μM, 4'-methylklavuzon blocked CRM1 nuclear export proteins more strongly than goniothalamin. It is observed that both componds inhibbit CRM1 protein at concentration of 5 μM. At this concentration, it is demostrated that Riok2 proteins are existed in the nucleus more than cytoplasm. When we increased concentratin of both compounds at 10, 20 μM, it was determined that CRM1 nuclear export proteins are totaly blocked and Riok2 proteins obviously accumulated in the nucleus. Interestingly, when the concentration of both compounds increased, it is observed that HeLa cell nucleus become smaller compared to that of low concentrations.

To Show the time dependency of the Michael addition initiated inhibition of goniothalamin. Same experiments were repeated for three hours imcubation. (Figure 2.4.)

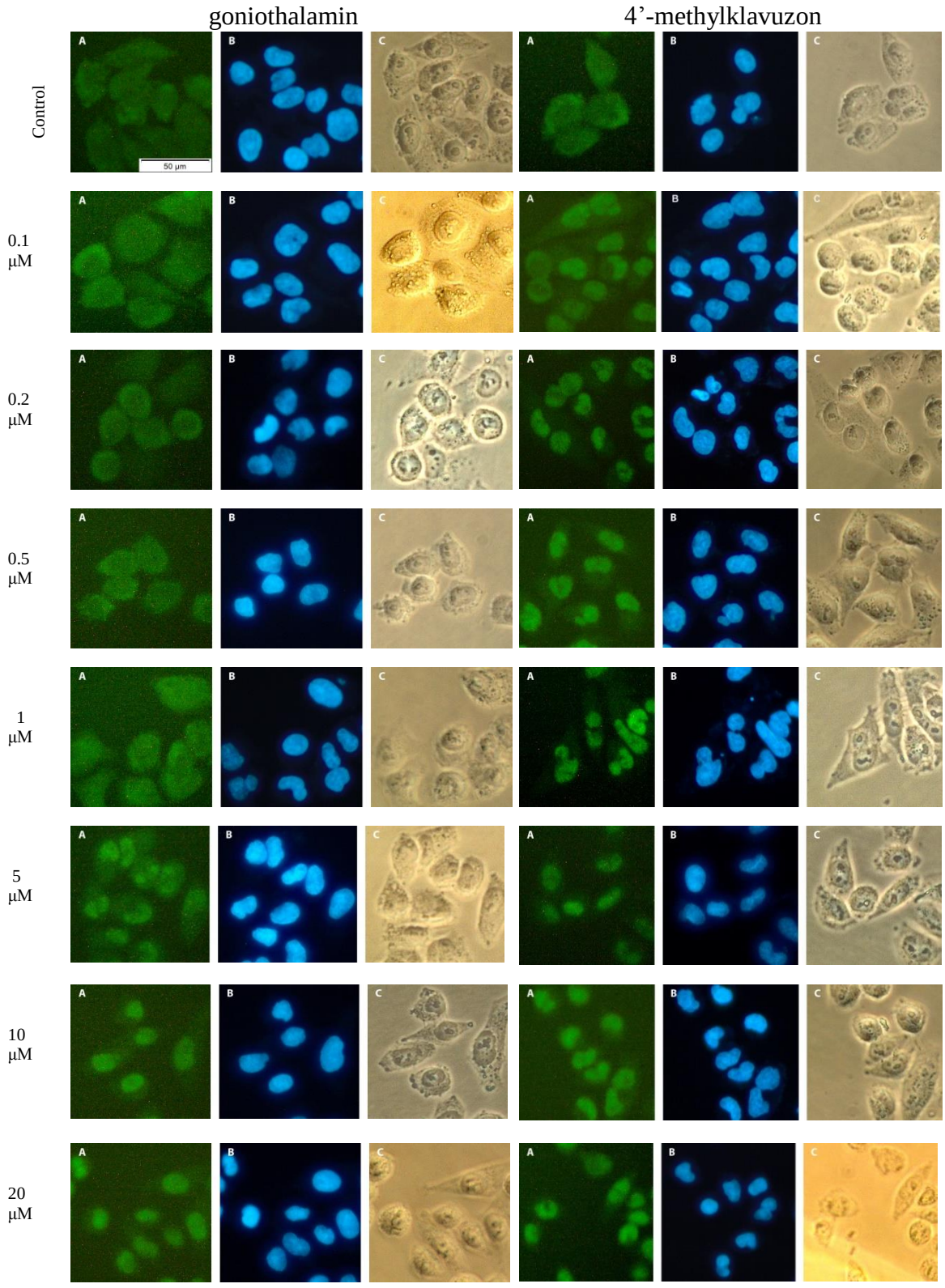


Figure 2.3. Inhibition of CRM1 by goniothalamin and 4’-methylklavuzon on HeLa cell line at one and a half hours

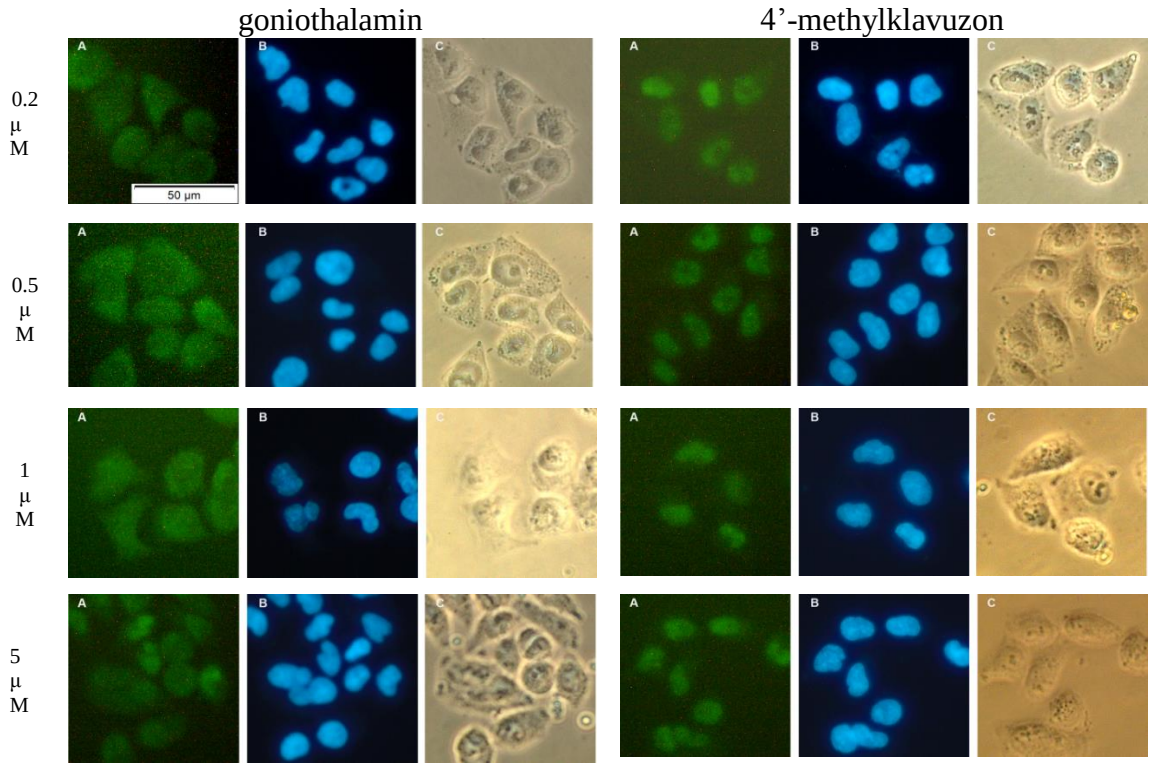


Figure 2.4. Inhibition of CRM1 by goniothalamin and 4’-methylklavuzon on HeLa cell line at three hours.

Both goniothalamin and 4’-methylklavuzon at concentrations of 0.2, 0.5, 1, 5 µM treated cells were incubated for three hours. Congruently, it is determined that goniothalamin started to block CRM1 nuclear export proteins at concentration of 1 µM and 4’-methylklavuzon bloked at concentration of 0.2 µM. Strong inhibition was observed at the concentrations of 5 µm for both compounds. When incubation time was increased, it was determined that nuclear export bloking concentratinos did not change for both goniothalamin and 4’-methylklavuzon.

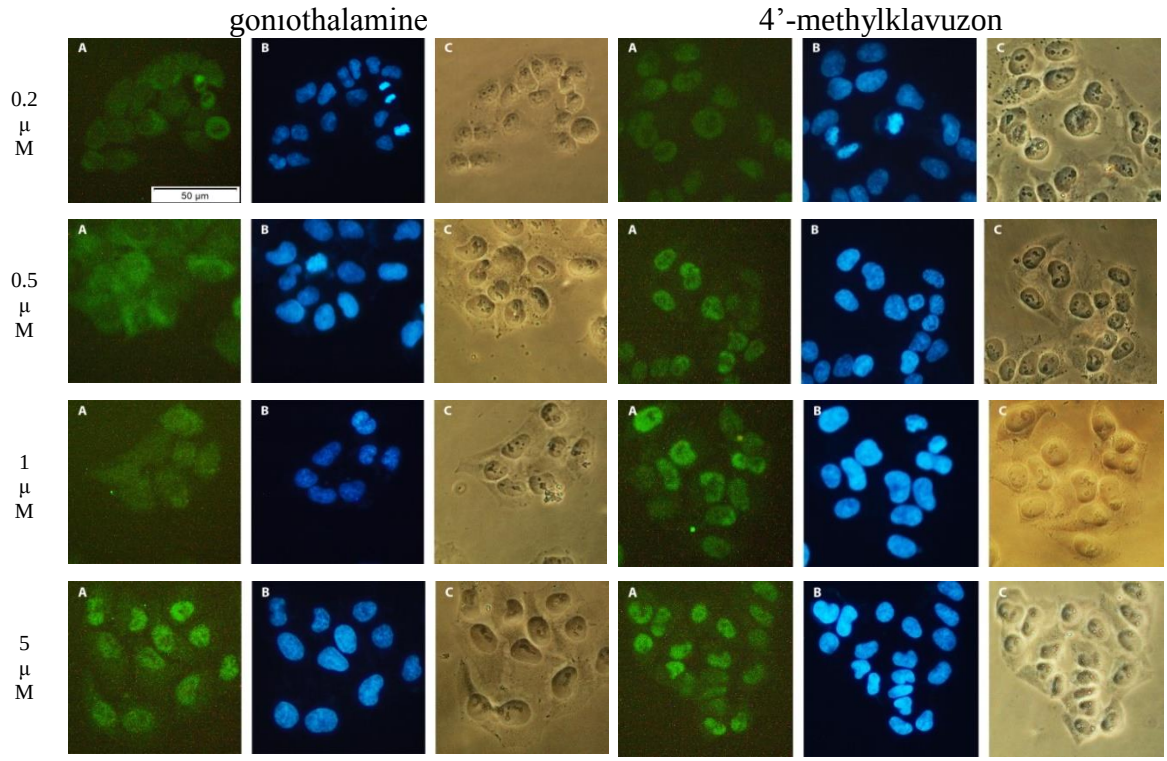
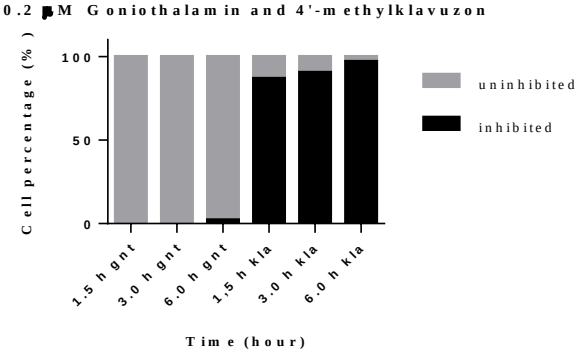


Figure 2.5**.** Inhibition of CRM1 by goniothalamin and 4’-methylklavuzon on HeLa cell line at six hours.

HeLa cells were incubated with 0.2, 0.5, 1, 5 µM concentrations of goniothalamin and 4’-methylklavuzon for six hours. Very interesting results were obtained for these trials. When incubation times increased 4 times, still 4’-methylklavuzon inhibits CRM1 nuclear export protein more than goniothalamin doses. Six hours data showed that goniothalamin was started to inhibit again at concentration of 0.5 µM and 4’-methylklavuzon very strongly inbited CRM1 nuclear export proteins concentration of 0.2 µM. When the cells were incubated with goniothalamin, the concentration of nuclear export inhibition did not change and it did not depend on time. Also 4’-methylklavuzon continued to inhibit nuclear export at all concentrations.

A



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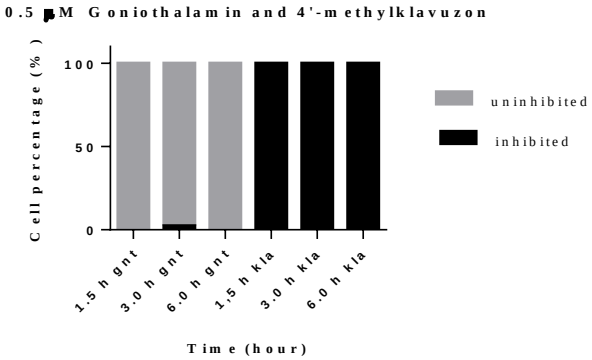

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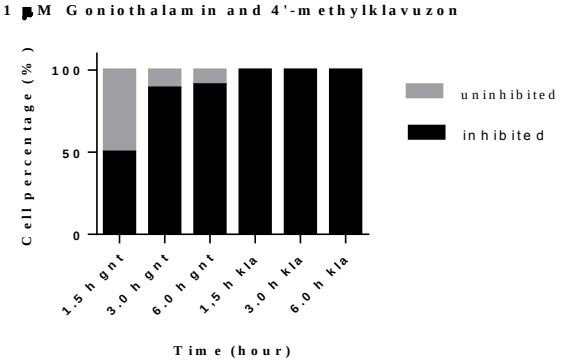


Figure 2.6. Percentage of HeLa cells having CRM1 inhibition after 1.5, 3, and 6 hours incubation with goniothalamin and 4’-methylklavuzon

HeLa cells were incubated with 4’-methylklavuzon and 4’-propylklavuzon at concentration of 0.05, 0.1, 0.2, 0.5, 1, 5, 10 µM. After six hours incubation, it is observed that both compounds started to inhibite nuclear export at concentration of 0.05 µM. At this concentration, when 4’-methylklavuzon powerfully inbited CRM1 nuclear inhibited weakly. It is observed that effect of 4’-propylklavuzon increased export proteins, 4’-propylklavuzon throughout higher concentrations. It determined that 4’-propylklavuzon at concentrations of 0.05, 0.1, 0.2, 0.5 and 1 µM had some cells which

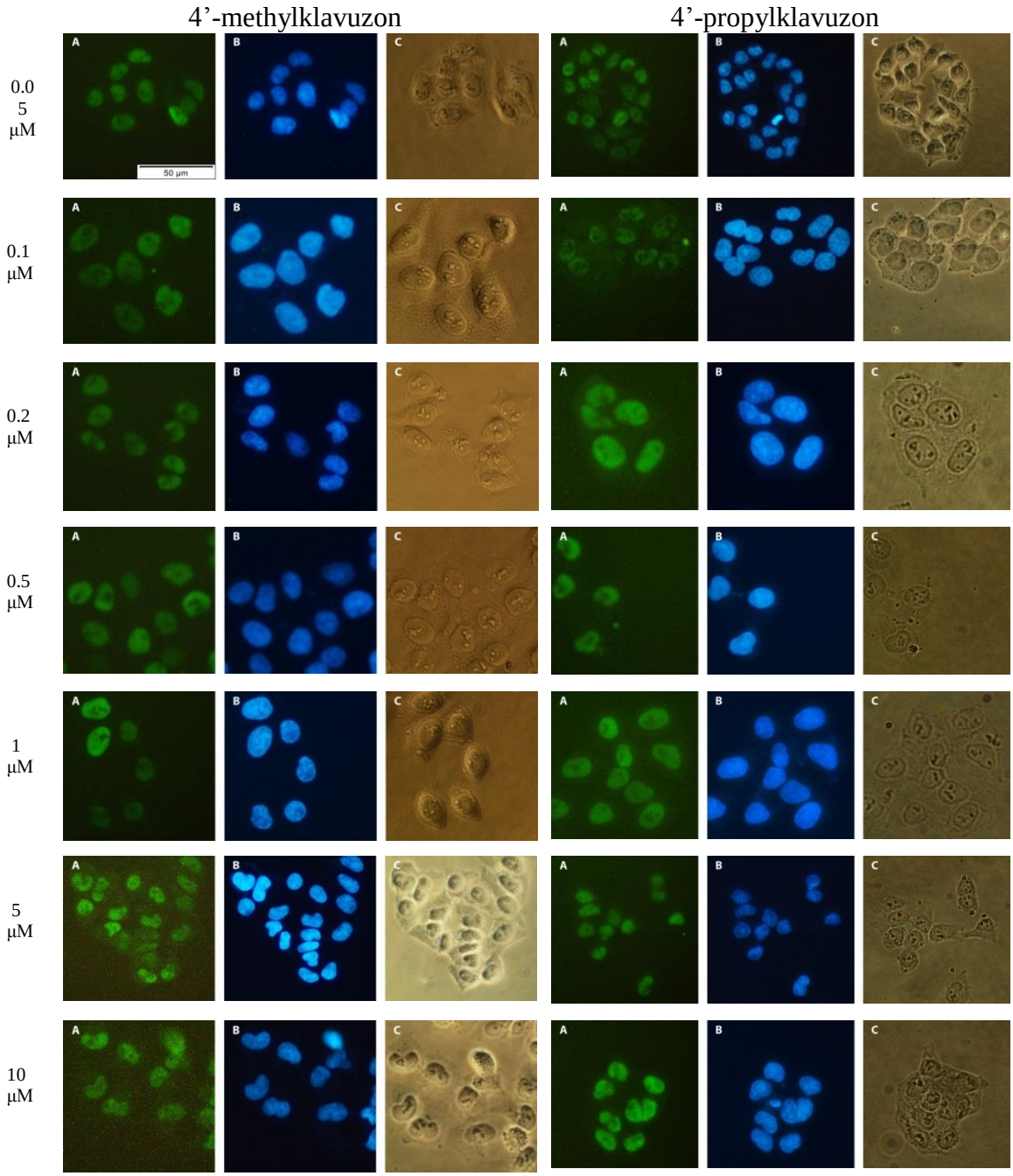


Figure 2.7. Inhibition of CRM1 by 4’-methylklavuzonand 4’-propylklavuzon on HeLa cell line at six hours

did not have any inhibition of CRM1 nuclear export proteins. ICC data of both compounds demostrated that. 4’-methylklavuzon more powerfull inhibitor of CRM1 nuclear export proteins compered to 4’-propylklavuzon.

HeLa cells were incubated with 4’-butylklavuzon and 4’-pentylklavuzon at concentrations of 0.05, 0.1, 0.2, 0.5, 1 µM for six hours. Both compounds did not have nuclear export inhibition at concentration of 0.1 µM. Riok2 proteins were observed in

the cytoplasm. At 0.2 µM concentration of 4'-butylklavuzon inhibition of CRM1 nuclear export protein was determined. Riok2 proteins were accumulated in the nucleus.

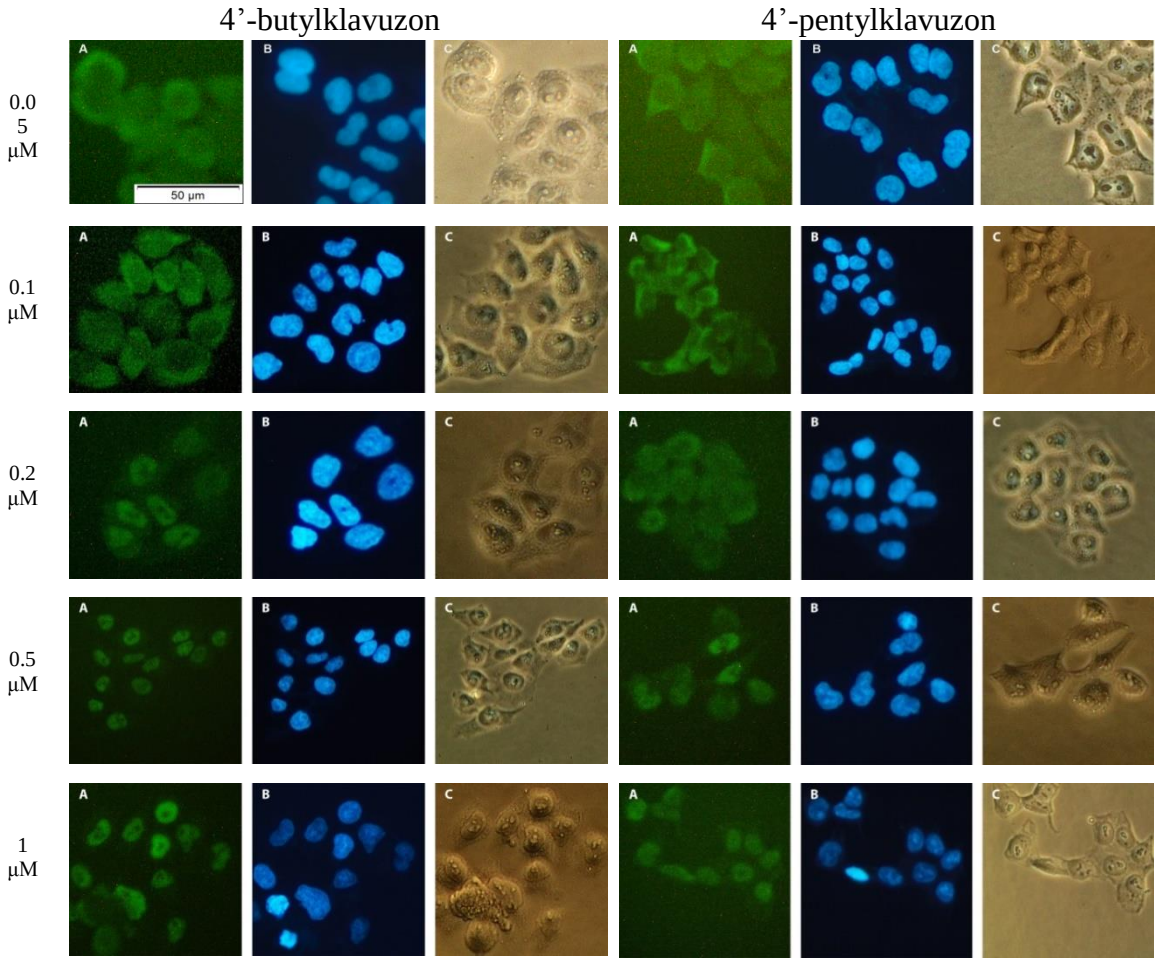
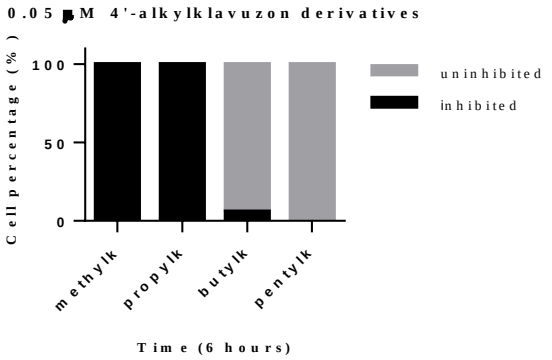


Figure 2.8. Inhibition of CRM1 by 4'-butylklavuzonand 4'-pentylklavuzon on HeLa cell line at six hours

On the other hand, at the same concentration of 4'-pentylklavuzon inhibition of CRM1 proteins were not observed. Riok2 proteins were still in the cytoplasm. Both 4'-butylklavuzon and 4'-pentylklavuzon have been demostrated as inhibition of CRM1 nuclear export protein. In the nucleus, Riok2 protein concentration was increased compered to cytoplasm at 0.5 and 1 µM concentrations. Obviously, 4'-butylklavuzon might be stronger inhibitor than 4'-pentylklavuzon. Percentage of HeLa cells counted with ImageJ softare. Data was determined that when 4'-alkylklavuzon derivatives chains are incrıase, inhibitory affect of 4'-alkylklavuzon derivatives are decrease (Figure 2.9.).

A



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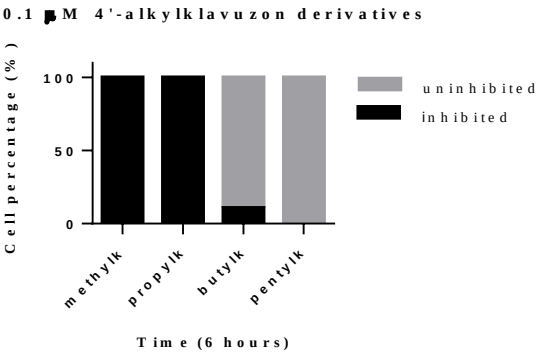

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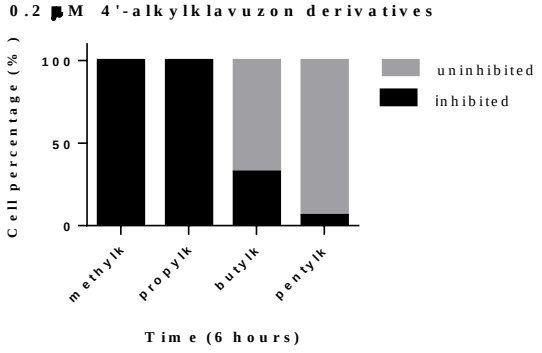

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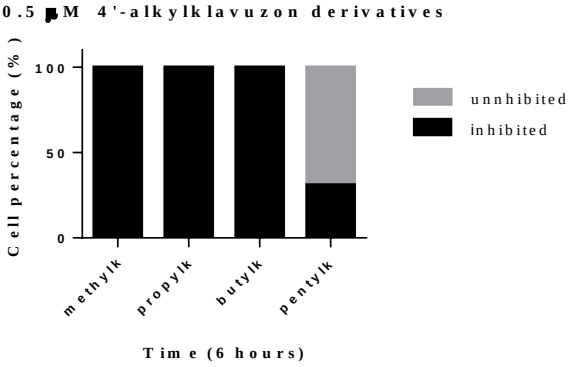

Figure 2.9. Percentage of HeLa cells having CRM1 inhibition after 6 hours incubation with 4’-alkylklavuzon

CHAPTER 3

EXPERIMENT

3.1. Thawing Cell

Vial of frozen HuH-7/HeLa cells was taken from -80 ^{0}C and transferred to 37 ^{0}C water bath for thawing 1-2 minutes. After thawing, cells were transferred to falcon tube and added 5 ml of new media and centrifuged at 800 rpm for 5 minutes. Then the supernatant was removed very carefully and pellet was dissolved with new media and pipetting can be necessery, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fatal bovine serum, 1% penicillin-streptomycin and 1% non-essential amino acids. HuH-7/HeLa cells were added to T75 flask and incubated to grow at 37 ^{0}C in 5% CO_2 incubator.

3.2. Passaging Cell

Firstly, passage of HeLa/HuH-7 cells, 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 with steril glass Pasteur pipette and flask was washed once with 3 ml medium to remove any residual and medium was removed again. Then 3 ml of trypsin solution was added to flask and waited at 37 ^{0}C in 5% CO_2 incubator for 3-4 minutes. After detachment of cells from flask 9 ml of media was added to inhibit the activity of trypsin solution and cells were transferred to falcon tube to centrifuge at 800 rpm for 5 minutes. Supernatant was removed very carefully with steril glass pipette and pellet was dissolved with 5 ml fresh media and transferred to new flask which has 10 ml fresh media and incubated at 37 ^{0}C in 5% CO_2 incubator.

3.3. Cell Counting

Cell counting is a process to determine the density of the cell suspension in the 1 ml of cell solution. 180 µl trypan blue mixed with 20 µl cell suspension in 1.5 µl

centrifuge tube. About 40 µl of mixture was transferred to Neubauer counting chamber and covered with coverslip. The chamber has two individual site and each side has four counting squares. Totally it has eight square and they were counted and total number of cells divided in eight; calculation was done according to dilution factor and chamber area as written below:

Cells/ml = (average count/square) x dilution factor x 10^4 (Chamber factor)

3.4. Freezing Cells

Cells were trypisined from flask as described above and transferred to a falcon tube and centrifuged at 800 rpm for 5 minutes. After that, supernatant was removed and pellet was dissolved in the freezing medium (70% DMEM, 20%FBS, 10% dimethylsulfoxide (DMSO)) and aliquoted in 1 ml cryopreservation tubes. Then HuH-7/HeLa cells were stored at -80 ^{0}C.

3.4. Cell Viability Assay (MTT Assay)

For viability analysis of HuH-7 cells, 95 µl of cell suspension with the inclusion of 2000 cells was supplemented to all wells of 96 well plate (Corning® Costar®) and incubated for 24 hours at 37 ^{0}C in 5% CO_2 incubator. Next day different concentrations of the tested compounds were dissolved in steriled DMSO and then filtered. 5 µl of diluted samples were added into wells. Then, plates were incubated at 37 ^{0}C in 5% CO_2 incubator throughout 24 and 48 hours. At 24 hours data sets were incubated both 4'-methylklavuzon and thymidine. At 48 hours data set, cells were incubated 4'-methylklavuzon for 24 hours and then medium was removed and fresh medium containing thymidine was added. Then cytotoxicity of compounds were determined by MTT (Sigma) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) based on colorimetric analysis. 10 µl of MTT solution (5 mg/ml in PBS) was put in to all well and waited for 4 hours at 37 ^{0}C in 5% CO_2 incubator. After that plate was centrifuged at 1800 rpm for 10 minutes and then supernatant was very carefully removed. 100 µl DMSO added to all well to solve formazan crystals and kept on shaker at 130 rpm for 15 minutes. Finally absorbance was measured at 540 nm. All experiment was

determined with two separed triplicate experiments, and results were analyzed by GraphPad Prism 6 software.

3.5. Immunocytochemistry Assay

25.000 HeLa cells were seeded in to 24 cell culture plate with 760 µl media and incubated overnight for attaching surface of the plate. Growth medium was removed and cells were washed with 1X PBS, three times and fixed with 4% PFA which was prepared in PBS for 20 minutes and incubeted +4 ^{0}C in the fridge. The cells were rinsed with 1X PBS with three times. Then, cells were permabilized with 0.5% Triton-X100 (Sigma Aldric) that is diluted in 1X PBS for 12 minutes. Then it was quickly rinsed with 1X PBS for five minutes. Next step was blocking step. At this step, the cells were incubated with bloking solution which is included 5% BSA (sigma Aldric) and 0.5% Tween-20 (Sigma Aldric) in 1X PBS and this solution was added 125 µl per well for 24-well plate for 60 minutes. Then, bloking solution was removed and washed 1X PBS one time.

Second step was primary antibody (Polyclonal Mouse Riok2 Antibody Nnovusbio) step. Primer antibody was added desired concentration and primary antibody was diluted in blocking buffer and incubated overnight at 4°C in the frıdge. In this step, the cells were rocked gentlyl and amonth of primer antibody solution was 125 µl per well. After that the cells were washed three times with 1X PBS for 10 minutes.

Third step was application of the secondary antibody (Polyclonal Goat anti-Mouse IgG, H/L Chains Antibody DyLight 488-novusbio) step. Secondary antibody was added desired concentration and secondary antibody was diluted in blocking buffer and the cells were incubated for over night at +4 ^{0}C in the fridge. Then the cells were washed four times with 1X PBS for 15 minutes. For nucleus staining, DAPI dye was used and added 125 µl 300 nM DAPI (Sigma-Aldric) and incubated for 10 minutes. The cells were washed with extra pure water one time. After that 1000 ml 1X PBS was added per well. The cells were determined with Olmypus CKX41 fluorescence microscope with Olmypus SC100 camera and Cell Sense Sofware.

CHAPTER 4

CONCLUSSION

Previously it was demonstrated that goniothalamin is a naturally occurring inhibitor of CRM1 nuclear export proteins. It is the pioneering compound of 4'-methylklavuzon showing increased cytotoxic activity over cancer cell lines compared to both goniothalamin and klavuzon. In the first part of this thesis, any posible recovery of HuH-7 cells from thymineless death caused possible thymidylate synthase inhibition. 5-Fluorouracil is an effective thymidylate synthase inhibitor and it acts by an irreversible Michael addition of thymidylate synthase enzyme to 5-fluorouracil. Such Michael acceptor also exists in the structure of both goniothalamin and klavuzon derivatives, so it has expected that these compounds can also react with thymidylate synthase by Michael addition reaction and inhibits its activity. By means of this, thymidine systhesis stop and cell goes to die. Addition of supplemental thymidine growth medıum can help to cell to recover from thymidineless death. Unfortunately all trials showed that mechanism of action of 4'-methylklavuzon is not a thymidineless death.

In the second part, effect of 4'-methlyklavuzon and other 4'-alkylklavuzon derivatives were investigated on HeLa cell line to determined nucleocytoplasmic transport mechanism. Inhibitory properties of goniothalamin and 4'-methylklavuzon and 4'-alkylklavuzon derivatives compared with each other. Data showed that 4'-methylklavuzon and derivatives inhibit CRM1 nuclear export protein. They are very strong inhibitors compared to goniothalamin. Additionally, 4'-methylklavuzon is powerful inhibitor of CRM1 nuclear export proteins than other 4'-alkylklavuzon derivatives.

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