CYCLIN DEPENDENT PROTEIN KINASES AS DRUG TARGETS – POTENTIAL IN CARDIOVASCULAR DISEASES

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Tiivistelmä – Referat – Abstract
Complications of atherosclerosis such as myocardial infarction and stroke are the primary cause of death in Western societies. The development of atherosclerotic lesions is a complex process, including endothelial cell dysfunction, inflammation, extracellular matrix alteration and vascular smooth muscle cell (VSMC) proliferation and migration. Various cell cycle regulatory proteins control VSMC proliferation. Protein kinases called cyclin dependent kinases (CDKs) play a major role in regulation of cell cycle progression. At specific phases of the cell cycle, CDKs pair with cyclins to become catalytically active and phosphorylate numerous substrates contributing to cell cycle progression. CDKs are also regulated by cyclin dependent kinase inhibitors, activating and inhibitory phosphorylation, proteolysis and transcription factors. This tight regulation of cell cycle is essential; thus its deregulation is connected to the development of cancer and other proliferative disorders such as atherosclerosis and restenosis as well as neurodegenerative diseases. Proteins of the cell cycle provide potential and attractive targets for drug development. Consequently, various low molecular weight CDK inhibitors have been identified and are in clinical development.
Tylophorine is a phenanthroindolizidine alkaloid, which has been shown to inhibit the growth of several human cancer cell lines. It was used in Ayurvedic medicine to treat inflammatory disorders. The aim of this study was to investigate the effect of tylophorine on human umbilical vein smooth muscle cell (HUVSMC) proliferation, cell cycle progression and the expression of various cell cycle regulatory proteins in order to confirm the findings made with tylophorine in rat cells. We used several methods to determine our hypothesis, including cell proliferation assay, western blot and flow cytometric cell cycle distribution analysis.
We demonstrated by cell proliferation assay that tylophorine inhibits HUVSMC proliferation dose-dependently with an IC_{50} value of 164 nM ± 50. Western blot analysis was used to determine the effect of tylophorine on expression of cell cycle regulatory proteins. Tylophorine downregulates cyclin D1 and p21 expression levels. The results of tylophorine's effect on phosphorylation sites of p53 were not consistent. More sensitive methods are required in order to completely determine this effect. We used flow cytometric cell cycle analysis to investigate whether tylophorine interferes with cell cycle progression and arrests cells in a specific cell cycle phase. Tylophorine was shown to induce the accumulation of asynchronized HUVSMCs in S phase. Tylophorine has a significant effect on cell cycle, but its role as cell cycle regulator in treatment of vascular proliferative diseases and cancer requires more experiments *in vitro* and *in vivo*.

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Tiivistelmä – Referat – Abstract

Ateroskleroosin erilaiset komplikaatiot, kuten sydäninfarkti ja aivohalvaus, kuuluvat yleisimpiin kuolinsyyn aiheuttajiin länsimaissa. Ateroskleroottisten plakkien kehittymistä edistävät monet eri prosessit kuten LDL-kolesterolin kertyminen verisuonten seinämiin, tulehdustila, endoteelisolujen toimintahäiriö, soluväliaineen muutokset ja verisuonten sileiden lihassolujen proliferaatio, jota säätelee useat solusyklin säätelyproteiinit. Sykliinistä riippuvaiset kinaasit (cyclin dependent kinase, CDK) ovat tärkeitä solusyklin säätelijöitä. Ne muodostavat aktiivisen kompleksin sykliinien kanssa ja fosforyloivat kohdeproteiinejaan, jotka osaltaan edistävät solusykliä. Sykliinien lisäksi CDK:n toimintaa säätelevät CDK:n inhibiittoriproteiinit, aktivoiva ja estävä fosforylaatio, proteolyysi ja monet transkriptiotekijät. Solusyklin tarkka säätely on välttämätöntä. Solusyklin häiriintynyt säätely onkin monien sairauksien, kuten syövän, ateroskleroosin, restenoosin ja aivojen rappeumasairauksien, taustalla. Lääkehoidon kohdistaminen solusyklin säätelyproteiineihin on noussut mielenkiintoiseksi lähtökohdaksi näiden sairauksien hoidossa. Kliinisissä kokeissa tutkitaankin useiden pienimolekyylisten CDK:n estäjien vaikutuksia.

Tyloforiini on fenantroindolitsidiinialkaloidi, jonka on osoitettu estävän useiden syöpäsolulinjojen kasvua. Sitä on käytetty perinteisessä intialaisessa lääkinnässä tulehduksellisten sairauksien hoidossa. Tutkimuksen tavoitteena oli selvittää tyloforiinin vaikutus ihmisen napanuoran suonen sileiden lihassolujen (HUVSMC) proliferaatioon, solusykliin ja solusyklin säätelyproteiinien ilmentymiseen. Tavoitteena oli osoittaa rotan soluilla tehtyjen kokeiden tulokset myös ihmisen soluilla. Käytettyjä menetelmiä olivat soluproliferaatiokoe, western blot -analyysi ja virtaussytometria.

Soluproliferaatiokoe osoitti, että tyloforiini estää solujen proliferaatiota pitoisuudesta riippuen (IC_{50} 164 nM ± 50). Western blot -analyysia käytettiin tutkittaessa tyloforiinin vaikutusta säätelyproteiinien ilmentymiseen. Tyloforiini vähentää sykliini D1:n ja CDK:n inhibiittoriproteiini p21:n ilmentymistä. Tulokset tyloforiinin vaikutuksesta p53 proteiinin fosforylaatioon eivät oleet yhtenäisiä. Tämän vaikutuksen tutkimiseen tarvitaan lisäkokeita herkemmillä menetelmillä. Virtaussytometriaa käytettiin määrittämään tyloforiinin vaikutus solujen jakautumiseen eri solusyklin vaiheisiin. Tyloforiini aiheutti synkronoimattomien solujen akkumuloitumisen solusyklin S-vaiheeseen. Tyloforiinin vaikutus solusykliin on ilmeinen. Lisätutkimuksia kuitenkin tarvitaan selvittämään sen todellinen merkitys solusyklin säätelyssä sekä syövän ja verisuonten proliferatiivisten sairauksien hoidossa.

Avainsanat – Nyckelord – Keywords

solusykli, CDK, sykliini, ateroskleroosi, tyloforiini

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ABBREVIATIONS

Akt	Protein kinase B
AD	Alzheimer's disease
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
CAK	CDK activating kinase
CDK	Cyclin dependent kinase
CIP/KIP	CDK interacting protein/Kinase inhibitory protein
CKI	Cyclin dependent kinase inhibitor
DES	Drug eluting stents
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
E2F	E2 transcription factor
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorter
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine triphosphate
HUVSMC	Human umbilical vein smooth muscle cells
INK4	Inhibitor of CDK4/CDK6
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase, ERK
MDM2	Murine double minute 2
mRNA	Messenger ribonucleid acid
mTOR	Mammalian target of rapamycin
Myc	Myelocytomatosis oncogene cellular homolog
PAA	Polyacrylamide
PBS	Phosphate buffered saline
PDGF	Plateled-derived growth factor
PI3K	Phosphatidylinositol 3'kinase
PTCA	Percutaneous transluminal coronary angioplasty
Rb	Retinoblastoma protein
RNA	Ribonucleid acid

SDS-PAGE	Sodim dodecyl sulphate polyacrylamide gel
Ser	Serine
SMC	Smooth muscle cell
TBS-T	Tris-buffered saline containing Tween 20
TGF-β	Transforming growth factor-beta
Thr	Threonine
TRIS	Trihydroxymethylaminomethane
VSMC	Vascular smooth muscle cell

LITERATURE REVIEW

CYCLIN DEPENDENT PROTEIN KINASES AS DRUG TARGETS – POTENTIAL IN CARDIOVASCULAR DISEASES

1 INTRODUCTION

Complications of atherosclerosis are the primary cause of death in Western societies (Lusis 2000). Atherosclerotic lesions contribute to development of thrombosis, which result in myocardial infarction or stroke. The development of atherosclerotic lesions is a complex process and a number of questions remain incompletely answered. However, vascular smooth muscle cell (VSMC) proliferation is one of the processes contributing to lesion formation. Various cell cycle regulatory proteins control this proliferation.

The mammalian cell division is a series of events that are tightly regulated by numerous regulatory proteins. Cell cycle is divided into four phases: G1, S, G2 and M phase (Figure 1) (Nurse 2000). During the first gap phase (G1), cells are preparing for the DNA replication in S phase (synthesis). In the second gap phase (G2), cells are preparing for the division between two identical daughter cells in M phase (mitosis). The first three phases are called interphase. Most of the cells in an adult organism are not dividing, thus they are in a quiescent phase called G0.

Each cell cycle phase and transition is controlled by protein kinases called cyclin dependent kinases (CDKs) (Malumbres and Barbacid 2005). Various CDKs are orderly activated at specific phases of the cell cycle. Despite CDKs, cyclins and cyclin dependent kinase inhibitors (CKIs) are crucial for proper cell cycle progression. This tight regulation of cell cycle is essential; thus its deregulation is connected to the development of cancer and other proliferative disorders such as atherosclerosis and restenosis as well as neurodegenerative diseases.

Proteins of the cell cycle provide potential and attractive targets for new drug development in treatment of these proliferative disorders. Consequently, various low molecular weight CDK inhibitors have been identified and are in clinical development (Diaz-Padilla et al. 2009). Most of these studies of CDK inhibitors have focused on treatment of cancer, although the relevance of cardiovascular and neurodegenerative diseases have increased.

2 CELL CYCLE AND CELL PROLIFERATION

2.1 Progression through the cell cycle

Progression through the cell cycle requires cells to advance through the specific checkpoints (Hartwell and Weinert 1989; Elledge 1996). The best defined checkpoints are DNA damage, DNA replication and spindle-assembly checkpoints. This checkpoint concept blocks the cell cycle for instance, when the cells have not successfully completed the previous phase or DNA is damaged. These transitions are tightly controlled by various regulatory proteins, of which CDKs play a major role (Figure 1) (Malumbres and Barbacid 2005; Morgan 1997). CDKs are inactive without binding to their regulatory subunits, cyclins. Activated CDK-cyclin complexes contribute cell cycle by phosphorylating serine-threonine amino acids of their substrate proteins.

The initiation of cell cycle depends on extracellular cues such as mitogens and nutrient availability as well as intracellular cues (Malumbres and Barbacid 2005; Morgan 1997). For example, platelet-derived growth factors (PDGFs) are mitogens involved in development of various cell types (Andrae et al. 2008). The expression of D-type cyclins, regulators of G1 initiation and progression, depends on mitogenic stimulation. Before entering the cycle, cells must pass certain checkpoints for example to ensure their homeostatic size to be sufficient.

Retinoblastoma protein (Rb) is an important regulator of cell cycle progression from G1 to S phase (Cobrinik 2005; Weinberg 1995). Rb and its relatives p107 and p130 are tumour suppressor proteins that comprise the family of so-called pocket proteins. The loss of *Rb* was originally identified in rare pediatric eye tumour, retinoblastoma (Friend et al. 1986). Furthermore, it was found to be mutated in various cancers such as osteosarcoma, breast cancer and small cell lung cancer (Friend et al. 1986; Harbour et al. 1988; Lee et al. 1988). Rb plays a fundamental role in cellular regulation (Cobrinik 2005; Weinberg 1995). Its phosphorylation by CDK-cyclin complexes is required for cell cycle progression.

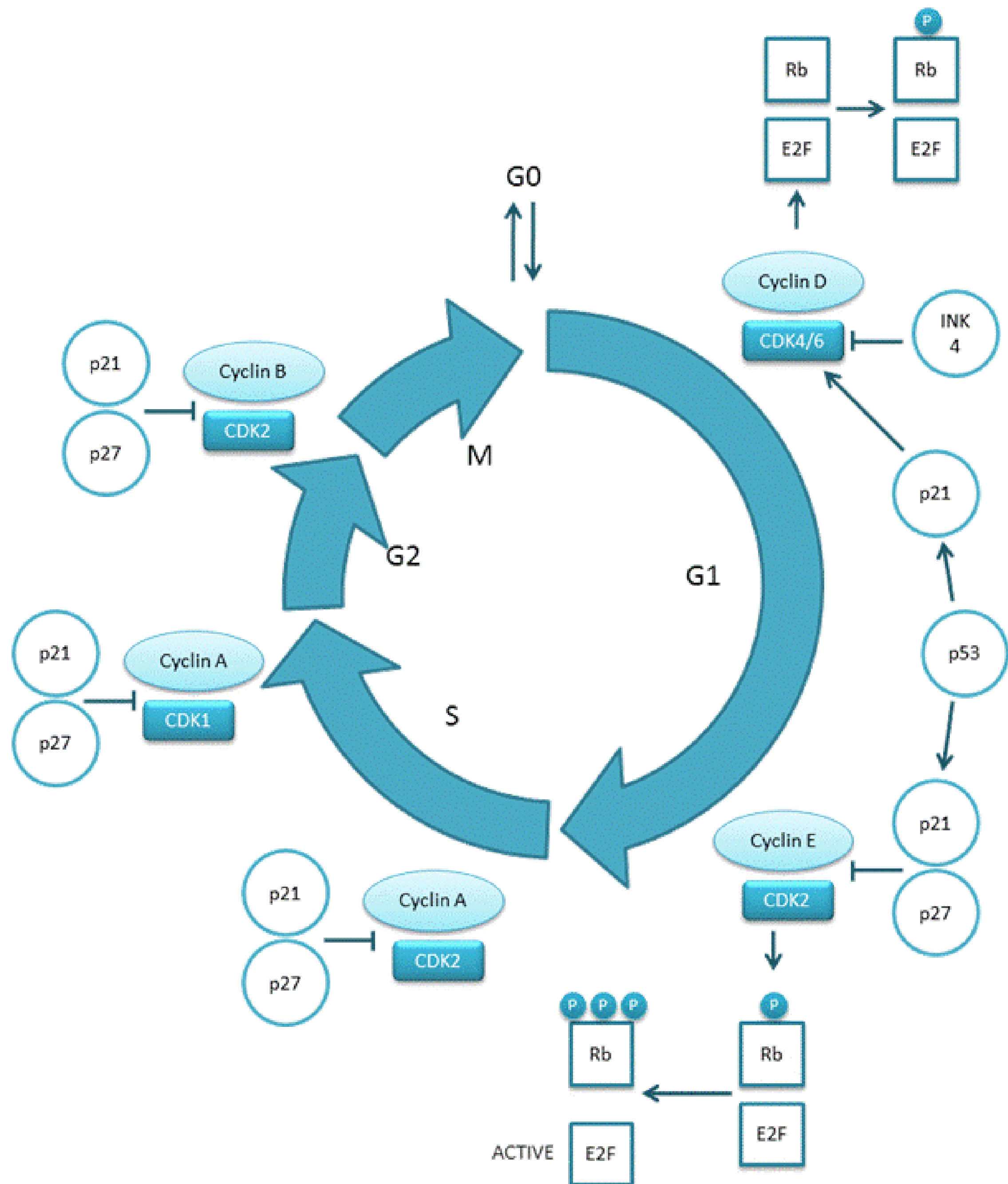

Figure 1. The mammalian cell cycle is divided into four phases: G1, S, G2 and M. G0 is a quiescent phase. The initiation of cell cycle in G1 is regulated by cyclin D-CDK4/6 complexes, which start Rb phosphorylation that is continued by cyclin E-CDK2 complexes. Rb becomes hyperphosphorylated, and releases the E2F transcription factors, which promotes the transcription of genes that contribute to cell cycle. Cyclin A-CDK2/1 and cyclin B-CDK2 complexes regulate the subsequent G2-M phase. Cyclin dependent kinase inhibitors INK4, p21 and p27 control the activation of cyclin-CDK complexes. p53 directly activates p21 (adapted from Boehm and Nabel 2001, Malumbres and Barbacid 2001).

In G0 cells, Rb is unphosphorylated or hypophosphorylated and interacts with transcription factors of E2F family (Flemington et al. 1993; Helin et al. 1993). It represses transcription of genes that are important for cell cycle progression by binding and inhibiting E2Fs. This repression of E2F regulated genes mediates G0-G1 cell cycle arrest. In humans, several E2Fs act in transcriptional regulation (Trimarchi and Lees 2002). E2F1, E2F2 and E2F3 are potent transcriptional activators inhibited by Rb, whereas E2F4 and E2F5 act as transcriptional repressors that interact with p107 and p130.

In G1 phase, cyclin D-CDK4/6 and later cyclin E-CDK2 complexes increasingly phosphorylate Rb (Cobrinik 2005). Hyperphosphorylated Rb releases repression of E2F, which promotes transcription of genes, including cyclin E, cyclin A and cyclin B. A positive feedback loop is created, when E2F induce cyclin E, which pairs with CDK2 and further phosphorylates Rb. This stage in late G1 has been defined as the restriction point, after which cells are able to undergo cell division without mitogenic stimulus. Furthermore, in late G1, cyclin E-CDK2 complex phosphorylates also other substrates required for the S phase entry.

In S phase, the amount of E-type cyclins decreases and CDK2 binds to A-type cyclins (A1 and A2) (Malumbres and Barbacid 2005; Morgan 1997). This complex phosphorylates various proteins involved in exit from S phase. In G2 phase, the level of cyclin A starts to decrease and the amount of B-type cyclins (B1 and B2) increases. They associate with CDK1 and together form a complex called M phase promoting factor during G2 and M phase. CDK2 and CDK1 maintain Rb in a hyperphosphorylated form until the end of M phase. In addition, Cyclin C-CDK3 complex may promote G0-G1 transition by stimulating the phosphorylation of Rb (Helin et al. 1993).

2.2 Cyclin dependent kinases

The first CDK was originally identified in cell division cycle mutation studies in budding yeast *Saccharomyces cerevisiae* and in fission yeast *Schizosaccharomyces pombe* (Hartwell et al. 1974; Nurse et al. 1976). In both studies, one gene was found to

be essential for cell cycle progression: *Cdc28* in *S. cerevisia* and *Cdc2* in *S. pombe*. The human homolog of the proteins encoded by these genes was identified and later named CDK1 (Lee and Nurse 1987). Over ten genes encoding CDKs have been identified in the mammalian genome (Malumbres and Barbacid 2005; Lapenna and Giordano 2009).

Cell cycle progression has been assumed to be one of the major roles of CDKs (Table 1) (Malumbres and Barbacid 2005). However, only five CDKs have been directly implicated in this function. Generally, it is believed that CDK1 is involved in mitosis, whereas CDK2-CDK4 and CDK6 play roles in interphase.

Table 1. Major CDKs and cyclins in humans (adapted from Morgan 1997, Malumbres and Barbacid 2005).

CDK	**Cyclin partner (preferred partner underlined)**	**Function**
CDK1	Cyclin B, Cyclin A, Cyclin E, Cyclin D	Cell cycle: M
CDK2	Cyclin A, Cyclin E, Cyclin B, Cyclin D	Cell cycle: G1, S, G2
CDK3	Cyclin E, Cyclin A, Cyclin C	Cell cycle: G0, G1, S
CDK4	Cyclin D	Cell cycle: G1
CDK5	p35, p39	Neuronal differentiation
CDK6	Cyclin D	Cell cycle: G1
CDK7	Cyclin H	CAK, transcription
CDK8	Cyclin C	Transcription
CDK9	Cyclin T, Cyclin K	Transcription, cell cycle: G2, M
CDK10	-	Transcription, cell cycle: M
CDK11	Cyclin L, Cyclin D	Transcription

Regulation of transcription is another significant function of CDKs. CDK7 and cyclin H form CDK activating kinase (CAK) (Fisher 2005). This complex regulates the activity of other CDKs during cell cycle. In addition, it is a component of TFIIH (transcription initiation factor IIH). Furthermore, CDK8-Cyclin C and CDK9-Cyclin T phosphorylate the carboxyterminal domain of the large subunit of RNA polymerase II, thus regulating transcription (Garriga and Graña 2004; Rickert et al. 1996). CDK8-Cyclin C also regulates transcription by phosphorylating cyclin H leading to inhibition of TFIIH (Akoulitchev et al. 2000). CDK5 differs from other CDKs and is activated by non-

cyclin proteins, p35 and p39 (Dhariwala and Rajadhyaksha 2008). It plays a major role in regulation of central nervous system by phosphorylating a number of proteins involved in transcription, neuronal function, migration and synaptic transmission.

New knowledge gathered from knock-out animals has challenged some previous hypotheses of cell cycle progression (Malumbres and Barbacid 2005; Sherr and Roberts 2004). Genetic studies in mice lacking a specific CDK have revealed that not all CDKs are strictly essential for the cell cycle progression. In addition, CDKs can, at least partly, compensate for each other's function. However, some CDKs have been shown to be essential for development of specific types of cells or tissues.

CDK2 has been assumed to be a crucial regulator of S phase. However, mice lacking CDK2 are viable and healthy but infertile (Berthet et al. 2003; Ortega et al. 2003). These results suggest that CDK2 is dispensable for the mitotic cell cycle but essential for meiosis. The loss of CDK2 activity might be compensated by CDK1 associating with A- or E-type cyclins (Santamaría et al. 2007). Thus, CDK1 seems to be essential for cell cycle progression.

CDK4 and CDK6 have been shown to be dispensable for cell cycle of most cell types (Malumbres et al. 2004). Accordingly, mice lacking CDK4 or CDK6 are viable, but these ablations affect the proliferation of certain specialized cells. Mice lacking CDK4 are small, and most males and all females are sterile (Rane et al. 1999; Tsutsui et al. 1999). In addition, these mice develop insulin-dependent diabetes, since loss of CDK4 inhibits the postnatal proliferation of pancreatic β-cells. Ablation of CDK6 results in mild defects in hematopoiesis indicating the role of CDK6 in hemocytes (Malumbres et al. 2004).

2.3 Cyclins

Cyclins were originally identified from fertilized sea urchin eggs, as proteins that are constitutively synthesized and destroyed at each cleavage division (Evans et al. 1983). Consequently, cyclins are rather unstable proteins, whose expression fluctuates during

the cell cycle, whereas CDKs are expressed relatively constantly through the cell division (Morgan 1997). Owing to the changes in cyclin levels, different CDK-cyclin complexes are activated in the specific phase of the cell cycle.

Such as CDKs, only certain cyclins are essential for cell cycle progression and the essentiality of different isoforms of cyclins varies between cell types (Sherr and Roberts 2004). Mice lacking cyclin D1 are viable, but they are small and exhibited defective development of retina and breast tissue (Fantl et al. 1995; Sicinski et al. 1995). Mice lacking all three D-type cyclins develop until mid or late gestation, but die due to severe anemia combined with a cardiac output failure (Kozar et al. 2004). However, mice develop normally to midgestation, suggesting that cell cycle without D-type cyclins is possible. Mice expressing only one D-type cyclin (D1, D2 or D3) die before or soon after birth (Ciemerych et al. 2002). During early embryonic development the residual cyclin is upregulated in most of the tissues. However, some tissues, in which cyclin was not upregulated, exhibited severe abnormalities later in gestation. For example, the loss of cyclin D1 in retinas was not compensated by cyclin D2 or D3.

Mice lacking one cyclin E gene (E1 or E2) are viable, but loss of both genes leads to lethality at late embryonic stage on account of defective endoreplication of trophoblasts (Geng et al. 2003). Loss of cyclin A2 leads to embryonic lethality, whereas cyclin A1 is required for meiosis in male mouse, but not for mitosis (Liu et al. 1998; Murphy et al. 1997). Cyclin B1 is essential for cell cycle, since its ablation leads to embryonic lethality, whereas mice lacking cyclin B2 develop normally (Brandeis et al. 1998).

3 REGULATION OF CDK ACTIVITY

The cell cycle control system involves complex series of biochemical signals and processes, which all contribute or inhibit the cell cycle progress (Morgan 1997). For instance, besides cyclins, CDK activity is modulated by various means including activating and inhibitory phosphorylation, transcription factors, proteolysis and

endogenous CKIs. However, the initiation of cell cycle also requires the stimulative signals outside the cell.

3.1 Signalling pathways

Extracellular signalling pathways involve mitogens that stimulate cell division (Malumbres and Barbacid 2005; Sherr and Roberts 2004). Most of them, for instance PDGF and epidermal growth factor (EGF), act broadly stimulating various cell types. Mitogens interact with many different receptor tyrosine kinases and G protein-coupled receptors, thus activating multiple intracellular signalling pathways, which further trigger the entry and progression through the cell cycle. Major signalling pathways are mediated through Ras oncogene or lipid kinase, phosphoinositol 3'kinase (PI3K) (Massagué 2004).

Various Ras signalling pathways play an important role in initiation of cell cycle (Massagué 2004; Sears and Nevins 2002). Mitogenic stimulation activates cell-surface receptors leading to phosphorylation of their tyrosine residues on the cytoplasmic domain. This activation creates docking sites for adaptor proteins that trigger effectors such as Ras to the membrane. Binding to GTP activates Ras leading to recruitment of other effectors. Phosphorylation of various effectors contributes to signal downstream cascades. One of these cascades is Ras-Raf-MAPK (mitogen activated protein kinase) (Campbell et al. 1998). Furthermore, MAPK phosphorylates c-Myc on Ser-62 leading to its stabilization (Sears et al. 2000).

c-Myc is an important transcription factor contributing the entry to the cell cycle (Adhikary and Eilers 2005). It belongs to Myc family of transcription factors that regulates a significant number of genes involved in cell proliferation, growth and apoptosis. They induce the expression of D-type cyclins by binding to their partner protein, Max (Myc-associated factor x) (Bouchard et al. 1999). In addition, they suppress the expression of CDK inhibitors, p21 and p15, in association with another transcription factor, Miz1 (Myc-interacting protein 1), thus stimulating the entry to G1

(Seoane et al. 2002; Staller et al. 2001). They have also been indicated in activation of RNA polymerases (Gomez-Roman et al. 2003; Grandori et al. 2005).

Another signalling pathway is mediated through PI3K. It is recruited to the cell membrane due the activation of cell-surface receptor or substrate, such as insulin-like receptor substrate (Vivanco and Sawyers 2002). In addition, activation of Ras can trigger PI3K to the membrane. PI3K binds to phospho-tyrosine sites of receptors or substrates and generates PIP3 (phosphatidylinositol-3,4,5-triphosphate) in the membrane. PIP3 functions as a second messenger to activate downstream pathways by triggering cell cycle regulatory proteins such as mTOR (mammalian target of rapamycin) and Akt (protein kinase B).

Akt is a serine/threonine kinase acting as an important regulator of many biological processes such as proliferation and apoptosis (Datta et al. 1999). For example, activation of Akt contributes CDK activity by inhibiting glycogen synthase kinase-3β (GSK-3β) (Cross et al. 1995). GSK-3β phosphorylates cyclin D1 on Thr-286, which triggers a rapid cyclin D1 degradation by ubiquitin-dependent proteolysis (Diehl et al. 1998). In addition, GSK-3β redirects cyclin D1 subcellular localization from the nucleus to the cytoplasm. Therefore, GSK-3β inhibition by Akt leads to cyclin D1 accumulation contributing cell cycle progression. Furthermore, GSK-3β phosphorylates c-Myc on Thr-58 facilitating its proteolysis by the ubiquitin pathway and colocalizes with c-Myc in the nucleus (Gregory et al. 2003).

Extracellular signals can also prevent cell cycle initiation and cell proliferation, thereby maintaining tissue homeostasis (Massagué 2004). Transforming growth factor-β (TGF-β) is a cytokine, which in mature cells leads to cytostasis or apoptosis (Massagué 1998; Shi and Massagué 2003). TGF-β activates membrane-receptor serine/threonine kinases that phosphorylate SMAD proteins. The founding member of the SMAD family was first identified as the product of the *Drosophila* gene *Mad* (*mothers against dpp*) (Sekelsky et al. 1995). Shortly, three *Mad* homologues were identified in *C. elegans* as cytoplasmic signal transducers and called *sma-2*, *-3*, and *-4* (Savage et al. 1996). Many

homologues were then defined in vertebrates and named SMADs (for SMA/MAD related) (Massagué 1998).

Proteins of the SMAD family are tumour suppressors that play an important role in the transduction of receptor signals to target genes in the nucleus (Massagué 1998; Shi and Massagué 2003). Activated SMAD proteins accumulate in the nucleus and form transcriptional complexes. These complexes target different genes contributing G1 arrest by various mechanisms. For example, the activation of TGF-β increases expression of CDK inhibitors, p21 and p15 (Reynisdottir et al. 1995). TGF-β also inhibits Myc, thus inhibiting gene transcription that contributes to the cell cycle (Seoane et al. 2001).

3.2 Ubiquitylation and proteasomal degradation of CDKs

Protein degradation through ubiquitin-mediated proteolysis plays an important role in cell cycle regulation targeting various cell cycle regulatory proteins, including cyclins and CKIs (Reed 2003; Weissman 2001). Ubiquitylation is a covalent conjugation of ubiquitin to target proteins for degradation by a proteasome. It is a multistep process that involves several ATP-dependent enzymes including an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin protein ligase (E3). Ubiquitylation is mediated through two strategies. Proteins can be degraded via activation of the target substrate itself or activation of the ubiquitin protein ligase that transfers ubiquitin to a particular class of target.

Target-activated degradation is most commonly carried out by E3 ligase SCF (Skp/Cullin/F-box protein) and depends on the regulatory context of individual target molecules (Reed 2003; Weissman 2001). F-box proteins, such as SKP2 and Cdc4, recognize phosphorylated substrates, thus acting as substrate-specificity factors of SCF complexes. For example, phosphorylated p21 and p27 are recognized by F-box protein SKP2 leading to their degradation (Bornstein et al. 2003; Carrano et al. 1999). Furthermore, Cdc4 binds to cyclin E contributing to its ubiquitin-mediated proteolysis (Strohmaier et al. 2001).

On the contrary, APC/C (anaphase-promoting complex/cyclosome) is responsible for ligase activation, which enables total degradation of target molecule populations (Reed 2003; Weissman 2001). Activation of APC/C requires the activity of two alternative co-factors: Cdc20 (cell division cycle protein 20) or Cdh1 (Cdc20 homolog 1). Cdc20 is synthesized in late cell cycle. Cdh1 is expressed constitutively, but it is negatively regulated by phosphorylation; for example, by CDKs. Both of these co-factors are involved in degradation of cyclin A and cyclin B (Sudakin et al. 1995).

3.3 p53

p53 protein is the major regulator of stress response pathways in cells, and thus an important regulator of cell cycle arrest and apoptosis (El-Deiry 1997; Levine 1997; Ryan et al. 2001). Besides its role in cell cycle arrest and apoptosis, its activation can induce several other responses in cells, including differentiation, senescence, DNA repair and inhibition of angiogenesis. Many types of cellular stress can activate p53, including DNA damage, hypoxia, oncogene activation, oxidative stress and change in normal growth and survival signalling.

p53 is a transcription factor that activates numerous target genes (Kruse and Gu 2009; Laptenko and Prives 2006). For example, in order to induce cell cycle arrest in G1, it directly activates the cyclin dependent kinase inhibitor p21 (Deng et al. 1995; El-Deiry et al. 1993). In contrast, G2 arrest requires multiple effector target genes (Taylor and Stark 2001). p53 induces apoptosis by regulating multiple downstream targets both in the mitochondrial and in the death-receptor-induced apoptotic pathways (Ryan et al. 2001). For example, it mediates the induction of pro-apoptotic members of the Bc1-2 family, including Bax (Bcl-2 associated X protein) and PUMA (p53 upregulated modulator of apoptosis) (Miyashita and Reed 1995; Nakano and Vousden 2001). These proteins trigger cytochrome c release leading to activation of APAF1 (apoptotic protease activating factor 1) and further to apoptotic cascade (Srinivasula et al. 1998). p53 also induces the expression of death receptors, such as Fas (TNF superfamily receptor 6) and Killer/DR5 (death receptor 5) or mediates their relocalization to the cell

surface contributing to apoptosis (Bennett et al. 1998; Owen-Schaub et al. 1995; Wu et al. 1997).

Since p53 is such a potent inhibitor of cell growth, there are several regulatory mechanisms to control its stability, subcellular localization, activity, transcription and translation (El-Deiry 1997; Levine 1997; Ryan et al. 2001).

One of the major regulators of p53 is MDM2 (murine double minute 2) protein, responsible for its degradation by various mechanisms (Brooks and Gu 2006). MDM2 is E3 ubiquitin ligase that target p53 leading to its ubiquitylation and further degradation by the proteasome (Haupt et al. 1997; Honda et al. 1997). In addition, MDM2 can inhibit transcriptional activity of p53 and direct its nuclear export. However, MDM2 activation is also regulated by several mechanisms (Brooks and Gu 2006). DNA-damage-induced checkpoint kinases Chk1 and Chk2 phosphorylate p53, which inhibits MDM2 to bind p53 and thus prevents the degradation of p53 (Shieh et al. 2000). Tumour suppressor protein ARF (alternative reading frame) is another inhibitory mechanism of MDM2 activity (Pomerantz et al. 1998). After oncogene activation ARF protein binds and inhibits MDM2 directly.

p53 function depends on its nuclear localization, which is tightly regulated by nuclear import and export mechanisms (Ryan et al. 2001). Microtubule network and dynein is involved in its nuclear import (Giannakakou et al. 2000). p53 also contains leucine-rich nuclear export signal located in its C-terminal region regulating its nuclear import (Stommel et al. 1999).

In addition, p53 is regulated by a number of post-translational modifications both during normal homeostasis and in stress-induced responses (Kruse and Gu 2009). These modifications include phosphorylation, acetylation, methylation and sumoylation. For instance, phosphorylation of p53 on Ser-46 controls its ability to induce expression of the apoptotic target gene p53AIP1 (p53 apoptosis inducing protein 1) (Oda et al. 2000).

3.4 Cyclin dependent kinase inhibitors

Endogenous CKIs are classified into two families: INK4 (inhibitors of CDK4) family and CIP/KIP (CDK interacting protein/Kinase inhibitory protein) family, based on their structure and target CDKs (Sherr and Roberts 1999). The INK4 family, including p16, p15, p18 and p19, binds specifically to monomeric CDK4 and CDK6. The CIP/KIP family, including p21, p27 and p57, binds more broadly to various cyclin and CDK subunits (Table 2). CIP/KIP family members inhibit the activity of CDKs by binding to the adenosine triphosphate (ATP) binding site, therefore blocking the ATP binding. The INK4 family members bind both N- and C-terminal lobes of CDK inducing allosteric changes. These modifications block cyclin binding and also reduce the binding of ATP.

Initially, CKIs were considered only as negative regulators of cell cycle, but paradoxically CIP/KIP proteins act also as positive regulators (Cheng et al. 1999; Labaer et al. 1997). CIP/KIP proteins inhibit the activity of CDK2, but activate CDK4 and CDK6. Cyclin D-CDK4/6 complexes bind and sequester CIP/KIP proteins. This interaction stabilizes and directs the transport of cyclin D-CDK4/6 complexes into the cell nucleus and relieves cyclin E-CDK2 from CIP/KIP constraint, thereby increasing their activation (Sheaff et al. 1997; Vlach et al. 1997). Furthermore, when the level of effective CIP/KIP proteins is decreased enough, cyclin E-CDK2 can trigger the degradation of p27, thus facilitating its own activation (Carrano et al. 1999). When p27 is phosphorylated by cyclin E-CDK2, it is degraded through ubiquitylation by SCF. When mitogenic stimulation ceases, cyclin D levels fall and the tethered CIP/KIP proteins are released (Sherr and Roberts 1999). They bind and inhibit the activity of cyclin E-CDK2 complexes leading to G1 arrest. Accumulation of INK4 proteins can also disrupt cyclin D-CDK complexes and release CIP/KIP proteins, and when overexpressed even lead to cell cycle arrest in G1.

Regulation of CKIs is complex and they are induced in response to various cellular processes (Sherr and Roberts 1999). For instance, p15 is induced by TGF-β contributing to G1 phase arrest (Hannon and Beach 1994). p27 expression increases in absence of extracellular mitogens or in response to anti-mitogenic signalling, which contribute to

cell cycle arrest by inhibition of cyclin E-CDK2 (Blain et al. 2003). p21 is activated by tumour suppressor protein p53 (Chen et al. 1995; Harper et al. 1993). Activation of p53 after DNA damage leads to transcriptional induction of p21, which inhibit the activity of CDK2 leading to cell cycle arrest in G1 or apoptosis. Besides its role as an effector of p53, p21 has been implicated in numerous biological processes. For instance, p21 can bind and inhibit PCNA (proliferating-cell nuclear antigen), which is a subunit of DNA polymerase-δ, thus involved in DNA replication (Waga et al. 1994). This inhibition suppresses the DNA synthesis until DNA repair is finished.

Table 2. CDK inhibitors and their targets (adapted from Sherr and Roberts 1999).

	Synonyms	Targets
INK4 family		
p16	INK4a	CDK4 CDK6
p15	INK4b	
p18	INK4c	
p19	INK4d	
CIP/KIP family		
p21	Cip1, Waf1	CDK1, CDK 2, CDK 4, CDK 6 Cyclin A, Cyclin B, Cyclin D, Cyclin E
p27	Kip1	
p57	Kip2	

3.5 Phosphorylation

The activity of CDKs is also regulated by activating and inhibitory phosphorylation (Morgan 1997). CAK, consisting of CDK7 and cyclin H, is essential for the activity of most CDKs by phosphorylating a threonine residue (Thr-172 in CDK4, Thr-160 in CDK2) (Fisher and Morgan 1994; Lolli and Johnson 2005). On the contrary, the activity of CDKs is inhibited by phosphorylation on threonine and tyrosine residues by regulatory kinases such as Myt1 (myelin transcription factor 1) (Mueller et al. 1995). Cdc25 (cell division cycle 25) phosphatases can dephosphorylate these residues and thus trigger entry into mitosis.

4 CDKS IN HUMAN DISEASES

4.1 Cancer

The role of CDKs and their regulators has been extensively studied in cancer (Sherr 1996; Lapenna and Giordano 2009; Malumbres and Barbacid 2009). Alterations particularly in CDKs have been reported quite infrequently but in a wide variety of tumours. However, other cell cycle regulators are frequently mutated in human cancer leading to misregulation of CDK activity. These alterations result in uncontrolled cell proliferation and aberrant cell division. Predictably, proteins that drive the cell cycle, such as cyclins, are often overexpressed in primary tumours, whereas proteins that inhibit cell cycle, including CKIs, are inactivated. Cell cycle regulators interact together in extensive and complex network; thus in most cases, expression of more than one protein is altered. Consequently, the primary cause of cancer or the significance of a single regulatory protein is difficult to assess.

CDK4 is mutated in a few cases of familial melanoma by a miscoding mutation that blocks binding of INK4 inhibitors (Wolfel et al. 1995). In addition, CDK4 is amplified or overexpressed in several malignancies, such as breast cancer tumours, sarcomas and glioblastomas (An et al. 1999; Khatib et al. 1993; Schmidt et al. 1994). CDK6 is overexpressed in some lymphomas in response of nearby translocations (Corcoran et al. 1999; Hayette et al. 2003).

Most human tumours carry mutations or alterations in regulators of CDKs including D- and E-type cyclins and CDK inhibitors (Malumbres and Barbacid 2001; Malumbres and Barbacid 2009). In addition, mutations that activate mitogenic signalling pathways are common in cancer. For instance, mutations that cause hyperactivation or overexpression of Ras or Myc proteins, promote cell growth, proliferation and further development of cancer (Downward 2003; Nilsson and Cleveland 2003).

Cyclin D1 is one of the most prevalent cell cycle regulators implicated in the development of various cancer types (Deshpande et al. 2005; Tashiro et al. 2007). Its gene amplification, rearrangement or overexpression has been described in breast, non-small-cell lung, colorectal and esophageal cancer (Arber et al. 1996; Gillett et al. 1994; Jiang et al. 1992; Reissmann et al. 1999). In fact, cyclin D1 gene amplification occurs in 15-20% of human mammary carcinomas and cyclin D1 is overexpressed in 50% of breast cancers (Deshpande et al. 2005). In addition, E-type cyclins are frequently overexpressed in human tumours, for example in breast cancer and gastric cancer (Akama et al. 1995; Keyomarsi et al. 1994).

Tumour suppressor proteins p53 and Rb are commonly altered in human cancers. The gene encoding p53 is mutated in 50-55% of human cancers, and it is the most mutated gene in human cancers (Levine 1997; Sherr 1996; Sherr and McCormick 2002). Its inactivation inhibits its major role in apoptosis and cell cycle arrest in response to DNA damage. In addition, mutations in gene encoding Rb are common in various human cancers. Its hyperphosphorylation releases E2F transcription factors to activate genes required for S phase entry. The loss of *Rb* was originally identified in retinoblastoma, and thereafter in osteosarcoma, breast cancer and small cell lung cancer (Friend et al. 1986; Friend et al. 1986; Harbour et al. 1988; Lee et al. 1988).

4.2 Neurodegenerative diseases

CDK5 is an unusual member of CDK family; it has only a little role in cell cycle regulation and it is not activated by cyclins (Dhariwala and Rajadhyaksha 2008; Dhavan and Tsai 2001). Instead, Cdk5 is highly expressed in the nervous system and it seems to be essential for neuronal survival and development.

Like other CDKs, for activation CDK5 requires its regulatory partners, non-cyclin proteins, p35 or p39 (Tang et al. 1995; Tsai et al. 1994). CDK5-p35 and CDK5-p39 phosphorylate numerous substrates involved in transcription, neuronal function, migration and synaptic transmission (Dhariwala and Rajadhyaksha 2008; Dhavan and

Tsai 2001). Most of them are associated with the cytoskeletal elements as signalling molecules or regulatory proteins.

CDK5 plays an essential role in the development of central nervous system and neuronal differentiation (Chae et al. 1997; Ohshima et al. 1996). Mice lacking CDK5 die just before or after birth and their brains display variety of neuronal positioning defects in cortex, hippocampus and cerebellum. In addition, mice lacking p35 display severe cortical lamination defects and suffer from seizures and sporadic adult lethality.

Various neurotoxins lead to calpain-mediated conversion of p35 to p25 (Lee et al. 2000; Patrick et al. 1999). In contrast to p35, p25 is stable and has a much longer half-life. It is a neurotoxin that accumulates in neurons and constitutively activates Cdk5. Hyperactive CDK5-p25 complex phosphorylates cytoskeletal proteins leading to neuronal cell death. For instance, level of p25 is remarkably increased in brains of Alzheimer's disease (AD) patients (Patrick et al. 1999).

Deregulation of Cdk5 has been implicated in several neurodegenerative diseases, including AD, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease and acute neuronal injury (Dhariwala and Rajadhyaksha 2008; Dhavan and Tsai 2001).

The typical pathological feature of AD is brain atrophy caused by neuronal loss and formation of β-amyloid plaques and neurofibrillary tangles (Cruz and Tsai 2004; Tsai et al. 2004). In AD and several other neurodegenerative diseases, microtubule binding protein Tau is abnormally hyperphosphorylated. This hyperphosphorylation of Tau leads to its intracellular aggregation, called neurofibrillary tangles, which reduce Tau's ability to associate with microtubules (Baumann 1993; Patrick et al. 1999; Paudel et al. 1993). CDK5-p25 potently phosphorylates Tau, whereas CDK5-p35 poorly phosphorylates it.

Another mechanism of AD pathology is the extracellular accumulation of fibrillogenic β-amyloid peptides that form amyloid plaques (Cruz and Tsai 2004; Selkoe 1999). This accumulation results from systematic cleavage of β-amyloid precursor protein (APP) by

secretases enzyme complex. CDK5 has been implicated to phosphorylate APP on Thr-668 in the cytoplasmic domain (Iijima et al. 2000). Increased phophorylation of Thr-668 is associated with APP processing and amyloid pathology.

4.3 Cardiovascular diseases

4.3.1 Atherosclerosis

Cardiovascular diseases, with atherosclerosis as the underlying cause, are the most common reason for death in Western countries (Dzau et al. 2002; Glass and Witztum 2001; Falk 2006). Atherosclerosis is a chronic progressive inflammatory disease that can convert into an acute clinical event by plaque rupture or thrombosis. In atherosclerosis; lipids, immune cells, fibrous elements and VSMCs accumulate into the intima of large and medium-sized arteries forming lesions. Atherosclerosis involves multifactorial processes including endothelial cell dysfunction, inflammation, extracellular matrix alteration and VSMC proliferation and migration.

The vascular wall is composed of three layers (Figure 2) (Lusis Aldons 2000). Intima, the first layer from lumen, is mainly composed of extracellular matrix, such as collagen and proteoglycans. Intima is lined with a monolayer of endothelial cells on the luminal side and internal elastic lamina on the peripheral side. The middle layer, media, consists of smooth muscle cells (SMCs). The third layer, adventitia, is formed of connective tissues as well as fibroblasts and SMCs.

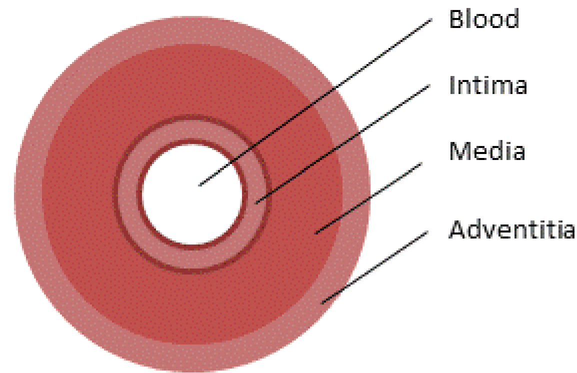

Figure 2. Structure of a normal large artery. Artery consists of three layers: intima, media and adventitia (adapted from Lusis 2000).

Atherosclerotic lesions form preferably in specific arterial regions, such as branches and curvatures, where the blood flow is disturbed and pulsatile causing mechanical stress to the vessel wall (Cunningham and Gotlieb 2005; Gimbrone Jr. et al. 2000). This shear stress modulates endothelial structure and function for instance by increased endothelial permeability to lipoproteins, increased expression of adhesion molecules and reduced production of endothelial nitric oxide synthase (eNOS). All these alterations promote atherosclerotic lesion formation.

The primary initiating event in atherosclerosis is the accumulation of lipids, mostly low density lipoproteins (LDL), into the intima, where they are modified by oxidation, lipolysis, proteolysis and aggregation (Lusis 2000; Ross 1999). Particularly, oxidation plays a critical role in atherogenesis (Steinberg 1997). Oxidatively modified LDL is found in lesions of atherosclerosis (Ylä-Herttuala et al. 1989). The accumulation of minimally oxidized LDL stimulate endothelial cells to produce various pro-inflammatory molecules including growth factors and adhesion molecules such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (Nakashima et al. 1998). Furthermore, these molecules contribute to monocyte migration into the intima, where they proliferate and differentiate into macrophages. Oxidized LDL is recognized by scavenger receptors and taken up into macrophages (Suzuki et al. 1997). These inflammatory responses contribute to the development of atherosclerosis.

Lipid-filled macrophages are called foam cells (Lusis 2000; Ross 1999). Foam cells and T lymphocytes form so-called fatty streaks, which are the earliest type of lesion and common already in infants and young children. Fatty streaks do not cause symptoms but they may further progress into more advanced, atherosclerotic lesions. They form the core of the lesion, which is surrounded by a fibrous cap. This cap contains VSMCs from media and collagen-rich matrix. Lesions continue to grow in response to VSMC proliferation and migration, extracellular matrix production and further lipid accumulation (Doran et al. 2008; Gerthoffer 2007; Glass and Witztum 2001). In addition, apoptosis of macrophages and VSMCs contribute the formation of the necrotic core. Development of advanced lesions is also contributed by inflammatory cytokines, matrix-degrading proteases and adhesion molecules. In addition, activated mast cells are found in atherosclerotic lesions (Kaartinen et al. 1994). They trigger matrix degradation and participate in the destabilization of the lesion, thus contributing to acute coronary events.

Advanced lesions may contribute to plaque rupture and thrombosis, which lead to acute cardiovascular events such as myocardial infarction and stroke (Davies et al. 1993; Lee and Libby 1997). These complications occur generally in shoulder regions of the plaque, more likely in lesions with thin fibrous caps, large necrotic cores and high concentrations of lipid-filled macrophages. Other typical features of advanced lesions are neovascularisation and calcification influencing on the stability of lesion (Folsom et al. 2008; Moreno et al. 2006).

4.3.2 Restenosis

Restenosis is renarrowing of an artery dilated by percutaneous transluminal coronary angioplasty (PTCA). PTCA is the most common revascularization procedure for coronary artery disease (Bennett and O'Sullivan 2001; Weintraub 2007). Despite the pharmacological and technical development, it limits the long-term success of PTCA. Restenosis occurs in 30-50% of patients after balloon angioplasty and in 10-30% of patients received an intravascular stent. Luminal renarrowing leading to restenosis involves various biological processes, including elastic recoil, reorganization of

thrombus, inflammation, VSMC migration and proliferation, neointimal hyperplasia and vascular remodelling. After angioplasty, restenosis results primarily from vascular remodelling, whereas after stenting, neointimal formation seems to play a major role.

Elastic properties of the vessel wall cause elastic recoil in response to the mechanical dilatation and overstretch (Rensing et al. 1991). It occurs straight after PTCA resulting in nearly 50% loss of luminal areas. This acute recoil can be reduced by stenting (Haude et al. 1993).

Angioplasty and stenting cause mechanical stress that induces endothelial denudation, rupture of internal elastic lamina and medial dissection contributing to thrombus formation (Ip et al. 1990). Human studies of stented arteries have shown activation of platelets, fibrin and acute inflammatory cells early after stenting (Farb et al. 1999; Grewe et al. 2000; Komatsu et al. 1998). Activation of platelets leads to their adhesion and cross-linking by fibrinogen promoting their aggregation. Inflammatory cells, primarily macrophages, are found in the site of stent struts indicating the role of inflammation in restenosis. Consequently, mural thrombus forms on denuded arterial intima.

Neointimal formation after injury occurs due to VSMC proliferation and migration and synthesis of extracellular matrix and collagen (Bennett and O'Sullivan 2001; Weintraub 2007; Zargham 2008). Platelets, endothelial cells, macrophages and VSMCs release mitogens, chemoattractants and cytokines that promote VSMC proliferation after arterial injury. For example, PDGF, TGF-β, angiotensin II and EGF play important roles in intimal proliferation and matrix accumulation (Braun-Dullaeus et al. 1998). They also stimulate the proliferation of VSMCs. In addition VSMCs undergo phenotypic transition from contractile to synthetic, which facilitates their migratory activity (Zargham 2008).

VSMCs seem to play a major role in restenosis after stenting (Moreno et al. 1999). SMC content was larger and proliferation index was higher in restenotic tissue after stenting than in restenotic tissue after PTCA. A significant change in neointimal

thickness has been observed three months after stenting with only a little change at six months (Asakura et al. 1998). When compared six months to three years after procedure, neointimal thickness was decreased significantly. In addition, thrombus occurred more often early after stenting and was not observed after three years.

Vascular remodelling plays also an important role in development of restenosis (Bennett and O'Sullivan 2001). Remodelling is a normal process to maintain an appropriate lumen size, particularly in response to changes in blood flow, either constrictive (negative) or expansive (positive) remodelling. Already in early atherosclerosis, vessels are dilated in order to compensate the increasing intima (Glagov et al. 1987). Vessel wall remodelling has been determined to induce the majority of the lumen loss after angioplasty (Mintz et al. 1996). Remodelling may be caused by changes in extracellular matrix properties (Lafont et al. 1999). Angioplasty causes acute changes in extracellular matrix synthesis and degradation, resulting in increased collagen synthesis.

5 CELL CYCLE AS DRUG TARGET IN CARDIOVASCULAR DISEASES

Vascular proliferative diseases involve multifactorial and complex processes (Andrés 2004; Braun-Dullaeus et al. 1998; Dzau et al. 2002). Cell cycle is the final pathway for signalling cascades that contribute cell proliferation. Accordingly, cell cycle regulators are more favourable target to inhibit cellular proliferation than a single mitogenic factor in upstream cascades. However, cell cycle regulation is very similar among various cell types, which complicates the specific targeting only at arteries. Cell cycle inhibition is possible to achieve by pharmacological agents, brachytherapy or gene therapy. So far, the therapeutic application of cell cycle inhibition has focused on inhibition of stent restenosis and bypass graft failure.

Vascular gene therapy is a possible approach to modulate the cell cycle by various means such as antisense oligonucleotides, overexpression of specific genes or gene transfer (Bennett and O'Sullivan 2001; Dzau et al. 2002). For instance, transcription factor decoy oligonucleotides bear the consensus binding sequence of a specific

transcription factor, for instance E2F, whose function they can competitively prevent (Morishita et al. 1995). This modulation of gene expression inhibits smooth muscle proliferation and vascular lesion formation.

Brachytherapy (β- and γ-radiation) is another approach to prevent cell proliferation. It has been shown to prevent neointimal formation and cell proliferation in stented vessels (Leon et al. 2001; Teirstein et al. 1997). Catheter-based intracoronary radiotherapy after coronary stenting substantially reduced the rate of restenosis (Teirstein et al. 1997). In addition, in another study the effects of brachytherapy were shown to persist for three years (Teirstein et al. 2000).

The emphasis of pharmaceutical approach is to block the cell cycle progression without inducing cell death, thus to develop cytostatic molecules (Diaz-Padilla et al. 2009; Ivorra et al. 2003). Direct inhibition of the cell cycle by CDK inhibitors is a promising method to inhibit cell proliferation. The development of CDK inhibitors has mostly focused on treatment of cancer, but they might also be potent in treatment of vasculoproliferative diseases. Accordingly, the mechanisms behind the deregulation of cell proliferation in cancer and vasculoproliferative diseases are similar. In addition, indirect inhibition of cell cycle progression by inhibiting other regulatory mechanisms involved in cell proliferation is shown to be beneficial. For instance, both rapamycin and paclitaxel inhibit cell cycle indirectly.

Systemic delivery of antiproliferative treatment is problematic, since it affects also nonvascular tissue (Sriram and Patterson 2001). Therefore, local delivery is preferred. For instance, drug eluting stents (DES) were developed for local intracoronary drug delivery. In contrast, especially CDK inhibitors have also been investigated for systemic delivery.

5.1 Small molecular weight compounds as CDK inhibitors

CDK inhibitors are low molecular weight compounds that compete with ATP for binding to ATP-binding site of the CDKs (Diaz-Padilla et al. 2009; Knockaert et al.

2002). This binding site differs substantially from that of other kinases, thus enabling high specificity to CDKs. CDK inhibitors are flat, hydrophobic heterocycles. They can inhibit the function of all CDKs without any selectivity or selectively inhibit the function of some specific CDK groups, for example CDK1, CDK2 and CDK5 or CDK4 and CDK6. In treatment of vasculoproliferative diseases, absolute selectivity might not be the best approach. Therefore, targeting multiple steps in the same pathway or multiple pathways with similar downstream effectors might be more beneficial.

Table 3. Selectivity of pharmacological CDK inhibitors. IC_{50} values of each compound are provided in micromolar concentrations (adapted from Knockaert et al. 2002, Ivorra et al. 2003).

CDK inhibitor	CDK1 – cyclin B	CDK2 – cyclin A, E	CDK4 – cyclin D	CDK5 – p25	GSK-3β
Olomoucine	7	7	> 1000	3	100
R-Roscovitine (Seliciclib, CYC202)	0.45	0.7	> 100	0.16	130
Flavopiridol	0.4	0.1	0.4	-	0.45
Indirubin	10	2.2 (A), 7.5 (E)	12	5.5	0.6
Indirubin-3'-monoxime	0.180	0.44 (A), 0.25 (E)	3.33	> 100	0.009
Indirubin-5-sulfonate	0.055	0.04 (A), 0.15 (E)	0.3	0.065	0.28

On cultured cells CDK inhibitors might lead to cell cycle arrest in either G1 or G2 phase depending on the model and conditions such as concentration and cell line (Ivorra et al. 2003). Various families of pharmacological CDK inhibitors have been identified including purines, flavonoids and indirubins (Table 3).

5.1.1 Purines

Substituted purines were discovered in systematic screenings of CDK1 inhibitors (Meijer and Raymond 2003). The first selective and potent CDK inhibitors were cytokinin analogues: olomoucine, roscovitine and purvalanols. Purine-based CDK inhibitors have been reported to inhibit CDK1, CDK2, CDK5, CDK7 and CDK9, but

exhibit no inhibitory activity against CDK4 and CDK6. Purine portion of these inhibitors binds to ATP-binding site of CDKs preventing the binding of ATP.

R-roscovitine (CYC202, Seliciclib), a trisubstituted purine derivative, is a potent and broad-spectrum inhibitor of CDKs competing for the ATP binding site of the kinase (Meijer et al. 1997). It has been reported to inhibit CDK1, CDK2 and CDK5. Besides CDK inhibition, it has several additional effects. It inhibits phosphorylation of the carboxyl-terminal domain of RNA polymerase II possibly via its inhibitory effects on CDK7 and CDK9 (MacCallum et al. 2005; Whittaker et al. 2004). It has also been reported to decrease Rb phosphorylation at multiple sites in human colon cancer cells (Whittaker et al. 2004). In addition, it induces the accumulation of p53 (Ljungman and Paulsen 2001). In phase I trial in patients with refractory advanced solid tumours, higher doses of R-roscovitine induces unexpected toxicity (Benson et al. 2007). Hence, further studies will need careful optimization of the dose and schedule.

5.1.2 Flavonoids

Flavopiridol is a semisynthetic flavonoid derived from rohitukine, an alkaloid isolated from *Dysoxylum binectariferum* (Dai and Grant 2003; Diaz-Padilla et al. 2009). It was the first small molecular CDK inhibitor to enter clinical trials. Flavopiridol has been demonstrated to inhibit cell proliferation in various human tumour cell lines arresting cells in G1-S and G2-M phases. *In vitro* and *in vivo* studies have demonstrated that it inhibits several protein kinases, with the greatest activity against CDKs including CDK1, CDK2 and CDK4 (Carlson et al. 1996; Losiewicz et al. 1994).

Flavopiridol has been indicated in inhibition of RNA polymerase II transcription by inactivation of CDK9-cyclin T1 (positive transcription elongation factor b) (Chao and Price 2001). In addition, it downregulates the activity of cyclin D1. It has been shown to inhibit the proliferation of human aortic SMCs *in vitro* and also the proliferation of rat carotid artery SMCs after balloon injury when administered orally (Ruef et al. 1999). Downregulation of cyclin D1 by flavopiridol has also been reported in breast cancer (MCF-7) and human osteosarcoma (U2OS) cell lines (Carlson et al. 1999).

Despite promising results from preclinical trials, generally flavopiridol has disappointed in clinical trials (Schwartz et al. 2001; Shapiro et al. 2001). Nevertheless, an optimization of administration schedule of flavopiridol led to clinical activity in chronic lymphocytic leukemia (Byrd et al. 2007).

5.1.3 Indirubins

Indirubin is the active component of a Quing Dai (indigo naturalis) that is called Danggui Longhui Wan in traditional Chinese medicine (Hoessel et al. 1999). This mixture of plants was used to treat chronic diseases such as leukaemia and inflammation. Indirubin has poor water solubility and low bioavailability, thus several derivatives with enhanced pharmacokinetic and pharmacologic properties were synthesized, including indirubin-3'-monoxime and indirubin-5-sulfonate.

They are reported to inhibit potently CDK1 and CDK2 by binding to the ATP binding pocket of CDKs (Hoessel et al. 1999; Marko et al. 2001). This inhibition has led to the cell cycle arrest in G1-S or G2-M phase depending on cell type and concentration range. Indirubin derivatives have also been shown to inhibit GSK-3β (Leclerc et al. 2001). In addition, indirubin-3'-monoxime inhibited the phosphorylation of tau suggesting a beneficial effect in the prevention of AD (Leclerc et al. 2001). Indirubin-3'-monoxime has also been reported to inhibit VSMC proliferation and neointima formation *in vivo* (Schwaiberger et al. 2010).

5.1.4 Future prospects

Despite a significant effect on cell cycle of CDK inhibitors, there are several deficiencies regarding to their pharmacokinetic and pharmacologic profile (Diaz-Padilla et al. 2009; Malumbres et al. 2008). For example, it has been problematic to define an appropriate administration schedule with an acceptable toxicity profile. Hence, more potent and more selective molecules with preferable inhibition of certain subtypes of

CDKs are in development. These so-called second-generation CDK inhibitors are promising, but still in their early stages of clinical development.

One attractive approach is to induce a synergistic effect by combination of a CDK inhibitor and a cytotoxic drug (Diaz-Padilla et al. 2009; Malumbres et al. 2008). Synergistic effect is highly dependent on administration schedule and sequence as observed in studies with flavopiridol together with antineoplastic agents (Bible and Kaufmann 1997). More pronounced synergy was reported when these agents were administered before flavopiridol. In another study, when administered after paclitaxel, flavopiridol was shown to enhance paclitaxel-induced caspase activation in the human gastric and breast cancer cell lines (Motwani et al. 1999). This potentiation was highly sequence dependent, thus pretreatment with flavopiridol antagonized the paclitaxel effect. In addition, flavopiridol combined with irinotecan was shown to be tolerable and safe with clinical activity against advanced solid tumours (Shah et al. 2005). On the contrary, flavopiridol combined with docetaxol in patients with metastatic breast cancer was reported to be unfeasible due to dose-limiting neutropenia (Tan et al. 2004).

5.2 Pharmacological inhibitors in drug-eluting stents

Bare-metal stents have been used in prevention of restenosis after angioplasty (Costa and Simon 2005; Kukreja et al. 2008). The major problem of bare-metal stents is that they are foreign bodies and act as a stimulus for cell proliferation and neointimal hyperplasia. DES coated with immunosuppressive or antiproliferative drugs were developed to improve clinical outcomes of coronary angioplasty. Rapamycin and paclitaxel were the first antiproliferative compounds used in DES.

However, the major problem of DES seems not to be neointimal hyperplasia, but the delay in formation of protective endothelial cell layer to cover the stent (Finn et al. 2007). Thus, the endothelial coverage is a powerful predictor of late stent thrombosis. In fact, DES were associated with an increased risk of late stent thrombosis and also increased rate of death as compared with bare-metal stents (Lagerqvist et al. 2007; Stettler et al. 2007). These results have stimulated the research to develop alternative

polymer coatings for permanent polymer such as biocompatible and bioabsorbable polymers (Kukreja et al. 2008).

5.2.1 Rapamycin

Rapamycin (sirolimus) is a natural macrocyclic lactone with potent immunosuppressive properties (Costa and Simon 2005; Kukreja et al. 2008). It was isolated from *Streptomyces hygroscopicus* in the mid 1970s. Rapamycin binds to FKBP12 (FK506-binding protein 12), which is upregulated in human neointimal SMCs (Bjornsti and Houghton 2004; Schmelzle and Hall 2000). The FKBP12-rapamycin complex binds to mTOR inhibiting its activation. This inhibition blocks cell cycle progression at G1-S transition in VSMC (Marx et al. 1995). TOR is a serine/threonine kinase that plays a central role in regulation of cell growth and proliferation (Bjornsti and Houghton 2004; Schmelzle and Hall 2000). PI3K-Akt signalling pathway regulates the kinase activity of mTOR. Rapamycin has also been indicated in other cellular processes. For example, it has been reported to stabilize p27, which inhibits CDK2 kinase activity (Nourse et al. 1994).

Rapamycin has been shown to be effective in DES (Holmes Jr. et al. 2004; Morice et al. 2002). It reduces neointimal proliferation, restenosis and associated clinical events compared with standard stents one year after implantation. It is now used in the sirolimus-eluting Cypher® stent. However, early and late stent thrombosis has been observed with sirolimus-eluting stents (Daemen et al. 2007). Especially diabetes was a strong predictor for stent thrombosis.

Several rapamycin analogues including everolimus and zotarolimus have been developed for DES (Fajadet et al. 2006; Grube et al. 2004). Everolimus-eluting stent with bioabsorbable polymer was shown to effectively reduce neointimal hyperplasia and restenosis without observations of stent thrombosis (Grube et al. 2004; Serruys et al. 2009). However, both of these studies had small numbers of patients with standard-risk lesions. Zotarolimus-eluting stent was also reported to reduce rates of clinical and angiographic restenosis without late stent thrombosis, but it was associated with a

significantly greater amount of neointimal hyperplasia compared with sirolimus-eluting stent (Fajadet et al. 2006; Miyazawa et al. 2008).

5.2.2 Paclitaxel

Paclitaxel is a diterpenoid compound, which was isolated from the bark of the Western yew tree in 1970s (Costa and Simon 2005; Kukreja et al. 2008). It binds specifically to the β-tubulin subunit of microtubules (Rowinsky and Donehower 1995). Microtubules form the mitotic spindle during cell division and are also important in other cell functions, including maintenance of cell shape, motility and intracellular transport. This inhibition results in accumulation of microtubule bundles with aberrant structures arresting cells in G2-M phase. Whether the effect is cytostatic or cytotoxic, depends on drug concentrations and duration of cell exposure (Kukreja et al. 2008).

The paclitaxel-eluting stent Taxus® was shown to markedly reduce the risk of clinical and angiographic restenosis as compared with the bare-metal stent in nine months follow-up (Stone et al. 2004). However, paclitaxel-eluting stents have also been connected to stent thrombosis (Daemen et al. 2007).

6 SUMMARY AND CONCLUSIONS

The cell cycle is a complex process that involves numerous regulators either contributing or inhibiting the cell cycle progress. Its deregulation is connected to the development of various diseases including cancer, vasculoproliferative and neurodegenerative diseases.

Cell cycle regulation has been extensively studied. However, it is not completely understood and new kinases involved in cell cycle regulation are identified. The main regulators include CDKs, cyclins, CKIs and their upstream regulators. Furthermore, the total function of CDKs is still under investigation. Besides cell cycle regulation, they play an important role in transcription and neuronal differentiation. On the other hand,

the role of CDKs in cell cycle regulation has been questioned not to be essential. Genetic studies in mice lacking a specific CDK or cyclin have revealed that not all CDKs are strictly essential for the cell cycle progression and that they can, at least partly, compensate for each other's function. For instance, CDK2 has been shown to be dispensable for cell cycle progression. In addition, some CDKs have been shown to be essential for development of specific cell types or tissues and dispensable from others.

The regulation of cell cycle, especially CDKs, has become a potent approach in treatment of cancer, cardiovascular and neurodegenerative diseases. Entry and progression through the cell cycle is one of the key events in vascular proliferative diseases. Hence, cell cycle regulation represents a promising but challenging target to treat these diseases. Few small molecular weight CDK inhibitors have reached to clinical trials in treatment of cancer but so far, none of them have been approved for commercial use.

When developing CDK inhibitors to target cell proliferation, attention has to be paid to the activity of these compounds against other CDKs. It might be difficult to evolve truly selective CDK inhibitors for the cell cycle due the high homology of the ATP binding domains for example in CDK1, CDK2 and CDK9. In addition, the dispensability and compensability of CDKs must be taken into account in development of CDK inhibitors.

Despite the promising results from pre-clinical trials, the clinical activity of CDK inhibitors has been limited with remarkable toxicity. In addition, many CDK inhibitors show *in vitro* and *in vivo* effects that can not be explained by the inhibition of CDKs, indicating other cellular functions. The aim is to design more potent and selective CDK inhibitors with inhibition of certain subtypes of CDKs. In addition, the pharmacokinetic and pharmacodynamic properties of existing CDK inhibitors have to be improved. Synergistic effect was observed when CDK inhibitors were administered sequentially with cytotoxic chemotherapy. Therefore, combination therapies might be effective in treatment of vasculoproliferative diseases.

More profound understanding of the complexity of cell cycle and its regulatory mechanisms is needed for its proper modulation. This knowledge may also reveal novel potential therapeutic targets. Careful investigation of existing CDK inhibitors benefits the development of more potent and more selective molecules with preferable pharmacokinetic profile.

EXPERIMENTAL PART

INFLUENCE OF TYLOPHORINE
ON HUMAN UMBILICAL VEIN SMOOTH MUSCLE CELL PROLIFERATION

1 INTRODUCTION

1.1 Tylophorine

Tylophorine belongs to phenanthroindolizidine alkaloids; a small group of plant natural compounds (Figure 3) (Li et al. 2001). It is the main active component of *Tylophora indica (Asclepiadaceae)*, which was used in Ayurvedic medicine to treat allergic and inflammatory disorders.

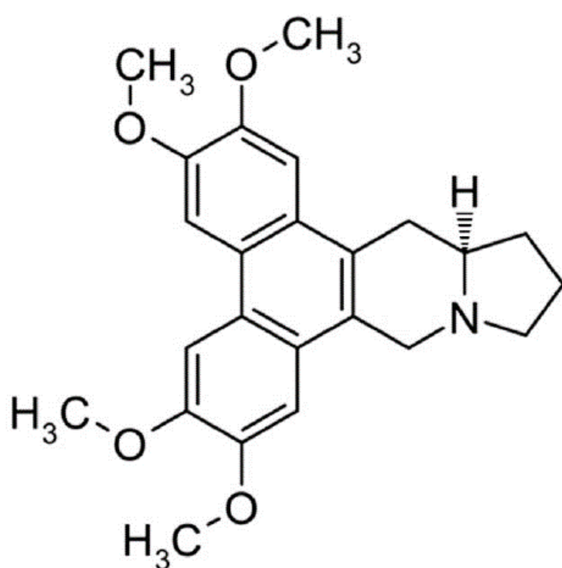

Figure 3. Chemical structure of (-)-R-tylophorine (adapted from Alexis Biochemicals, Axxora, 2011).

Clinical trials of tylocrebrine, a positional isomer of (-)-R-tylophorine, as an anti-cancer agent failed in 1960s due to central nervous system toxicity, including ataxia and disorientation (Steerk et al. 2002). This disappointing result delayed further investigations of tylophora alkaloids. However, various analogues of tylophorine have recently been shown to inhibit the growth of several human cancer cell lines (Gao et al. 2004; Wei et al. 2006; Wei et al. 2007). They showed potent cytotoxic activity against human hepatocellular carcinoma (HepG2), prostate (DU-145), breast (ZR-751) and both wild type (KB) and multidrug resistant (KB-Vin) nasopharyngeal cell lines (Gao et al. 2004; Wei et al. 2006).

Tylophorine has recently been shown to arrest HepG2, human gastric (HONE-1), and human nasopharyngeal (NUGC-3) carcinoma cells in G1 phase by downregulating cyclin A2 expression (Wu et al. 2009). However, research has focused on tylophorine's effect on cancer cells and there are no publications of tylophorine's effect on VSMCs. Since tylophorine inhibits the growth of various cancer cells, it might be also promising in inhibition of VSMC proliferation.

1.2 Background and aim of the work

My supervisor Helge Joa has previously investigated the effect of (-)-R-tylophorine on rat aortic VSMCs. According to his results, tylophorine inhibits rat aortic VSMC proliferation concentration-dependently in calf serum stimulated cells with an IC_{50} of 128 nM (± 44 nM) (Joa 2011). He has also shown that tylophorine downregulates cyclin D1 and p21 expression levels without affecting their mRNA levels. It has no effect in total p53 expression levels. Additionally, he has shown that tylophorine is a G1 phase arrestor in PDGF stimulated rat aortic VSMCs.

All the experiments were performed in rat aortic VSMCs. Therefore, in order to exclude species specific effects of tylophorine, we tested its activity on human cells. The aim of my study was to investigate the effect of tylophorine on human umbilical vein smooth muscle cells (HUVSMCs). The main purpose of my study was to confirm the findings made with tylophorine in rat cells in HUVSMCs. Therefore, we were interested in the effect of tylophorine on HUVSMC proliferation, cell cycle progression and the expression of various cell cycle regulatory proteins.

Additionally, my supervisor Joa has received the results from Pepchip™ kinase array. This kinase array was performed by the cooperation partners from Pepscan in the Netherlands. Hereby, whole cell extracts of tylophorine-treated and DMSO-treated VSMCs were examined with regard to their potential to phosphorylate selected peptide sequences (spotted on a chip), which were specific for a certain protein. The output data of this assay resulted in a list of proteins, in which the phosphorylation of a specific amino acid residue was modulated by tylophorine (in comparison to the DMSO-treated

control cells) as well as the list of suggested kinases responsible for these phosphorylations (APPENDIX). The data analysis revealed a 43-fold induction of p53 phosphorylation on serine 9 by tylophorine. Therefore, one part of my work was to confirm this effect of tylophorine on p53 phosphorylation of serine 9 and also on other phosphorylation sites of p53 in cultivated HUVSMCs.

2 MATERIALS AND METHODS

2.1 Materials

(-)-R-tylophorine was originally obtained from Department of Pharmaceutical Biology, University of Düsseldorf and later from Alexis Biochemicals. 1 µM tylophorine was used in all experiments except in the cell proliferation assay.

All chemicals were obtained from Sigma Aldrich, Fluka or Carl Roth unless otherwise stated. Antibodies were mainly obtained from New England Biolabs and cell culture medium and supplements from Invitrogen and Lonza Group. All the chemicals were obtained from the following companies.

Alexis Biochemicals (Switzerland)
BIO-RAD Laboratories (Hercules, CA, USA)
Carl Roth (Karlsruhe, Germany)
New England Biolabs (Beverly, USA)
Fluka (Buchs, Switzerland)
Invitrogen (Carlsbad, CA, USA)
Lonza Group Ltd. (Basel, Switzerland)
Promega (Madison, USA)
Roche Diagnostics, Penzberg, Germany
Santa Cruz (Santa Cruz, USA)
Sigma Aldrich (St. Louis, MO, USA)

2.1.1 Solutions and reagents

Culture medium

M231 (Invitrogen)	500 ml
SMGS (Invitrogen)	25 ml

Wash medium

RPMI 1640 (Sigma Aldrich)	500 ml
Benzylpenicillin/Streptomycin (Lonza Group Ltd.)	5 ml

Stop medium

RPMI 1640	500 ml
Benzylpenicillin/Streptomycin	5 ml
Calf serum (Lonza Group Ltd.)	50 ml

Starvation medium

DMEM (Lonza Group Ltd.)	500 ml
Benzylpenicillin/Streptomycin	(100 U/ml potassium penicillin / 100 µg/ml streptomycin sulphate)
L-glutamine (Lonza Group Ltd.)	200 mM
Calf serum	500 µl

PBS pH 7.4

NaCl	36.0 g
Na_2HPO_4	7.4 g
KH_2PO_4	2.2 g
ddH_2O	ad 5000 ml

Lysis buffer (pH 7.5)

Stock solution	HEPES (50 mM)	25 ml
	(Hydroxyethylpiperazineethanesulfonic acid)	
	NaCl (50 mM)	25 ml
	NaF (50 mM)	1.05 g
	$Na_4P_2O_7$ (10 mM)	2.23 g
	EDTA (5 mM)	25 ml
	(Ethylenediaminetetraacetic acid)	
	Na_3VO_4 (1 mM)	91.95 mg
	ddH_2O	ad 430 ml
Prior to use	Stock solution	430 µl
	PMSF (0.1 M in isopropanol)	5 µl
	(Phenylmethanesulphonylfluoride)	
	Triton X-10 (10% in ddH_2O)	50 µl
	Complete™ 25x	20.2 µl
	One tablet (Roche Diagnostics)	
	dissolved in 2 ml ddH_2O)	

SDS

Stock solution	Tris-HCl (0.5 M, pH 6.8)	37.5 ml
	SDS	6.0 g
	Glycerol	30.0 ml
	Bromphenol blue	15.0 mg
	ddH_2O	ad 100 ml
Prior to use	Stock solution	85.0 ml
	β-Mercaptoethanol	15.0 ml

Resolving gel 10%

Freshly prepared	30% PAA	5 ml
	Tris-HCl (1.5 M, pH 8.8)	3.75 ml
	SDS 10%	150 µl
	ddH_2O	6.1 ml
Prior to use	Temed (Tetramethylethylene diamine)	15 µl
	Ammonium persulfate 10%	75 µl

Stacking gel

Freshly prepared	30% PAA	1.28 ml
	Tris-HCl (1.25 M, pH 6.8)	750 µl
	SDS 10%	75 µl
	ddH_2O	5.25 ml
Prior to use	Temed	15 µl
	Ammonium persulfate 10%	75 µl

Electrophoresis buffer

Stock solution (10x)	Tris-Base	30 g
	Glycine	144 g
	SDS	10 g
	ddH_2O	ad 1000 ml
Prior to use	Stock solution (10x)	100 ml
	ddH_2O	ad 1000 ml

Blotting buffer

Stock solution (5x)	Tris- Base	15.2 g
	Glycine	72.9 g
	ddH_2O	ad 1000 ml
Prior to use	Stock solution (5x)	200 ml
	Methanol	200 ml
	ddH_2O	ad 1000 ml

TBS-T pH 8.0

Stock solution (10x)	Tris- Base	3.0 g
	NaCl	11.1 g
	Tween 20	1 ml
	ddH_2O	ad 1000 ml
Prior to use	Stock solution (10x)	100 ml
	ddH_2O	ad 1000 ml

Home-made ECL solution

Stock solution	H_2O	4.5 ml
	TrisBase (1 M, pH 8.5)	500 µl
	Luminol (0.25 M in DMSO)	25 µl
	p-coumaric acid (90 mM in DMSO)	11 µl
Prior to use	ddH_2O_2	3 µl

FACS (Fluorescence-activated cell sorter) buffer (pH 7.4)

NaCl	40.6 g
KH_2PO_4	1.3 g
Na_2HPO_4	11.75 g
KCl	1.4 g
LiCl	2.15 g
NaN_3	1.0 g
Na_2EDTA	1.8 g
ddH_2O	ad 5000 ml

Hypotonic fluorochrome solution

Triton X-100 (in PBS)	0.1% (V/V)
Sodium Citrate	0.1% (W/V)
Propidium iodide 0.2% (W/V)	0.005% (W/V)

2.1.2 Antibodies

Primary antibodies

Antibody	Source	Dilution	Provider
Cyclin D1	Mouse	1:2000	New England Biolabs
Cyclin E	Rabbit	1:1000	Santa Cruz
CDK4	Rabbit	1:1000	Santa Cruz
p21	Mouse	1:1000	Promega
p53	Rabbit	1:1000	New England Biolabs
phospho-p53 (Ser-6)	Rabbit	1:1000	New England Biolabs
phospho-p53 (Ser-9)	Rabbit	1:1000	New England Biolabs
phospho-p53 (Ser-15)	Rabbit	1:1000	New England Biolabs
phospho-p53 (Ser-20)	Rabbit	1:1000	New England Biolabs
phospho-p53 (Ser-37)	Rabbit	1:1000	New England Biolabs
phospho-p53 (Ser-46)	Rabbit	1:1000	New England Biolabs
phospho-p53 (Ser-392)	Rabbit	1:1000	New England Biolabs
α-tubulin	Mouse	1:2000	Santa Cruz

Secondary antibodies

Antibody	Source	Dilution	Provider
Rabbit	Goat	1:2000	New England Biolabs
Mouse	Goat	1:2500	Santa Cruz

2.1.3 Technical equipment and software

Olympus CKX 31 Light Microscope	Olympus Europe GmbH, Hamburg, Germany
Vi-Cell™ XR Cell viability analyzer and cell counter	Beckmann Coulter, Fullerton, CA, USA
TECAN Sunrise™ 96-well-plate reader	TECAN, Mannedorf, Switzerland
LAS-3000™ Luminescent image analyzer	Fujifilm, Tokyo, Andpan
FACSCalibur™	BD Biosciences Pharmingen, San Diego, Ca, USA
AIDA™ (Advanced Image Data Analyzer) version 4.06	Raytest GmbH, Straubenhardt, Germany
GraphPad PRISM™ version 5.02	GraphPad Software, Inc., San Diego, Ca, USA
LAS-3000™ Image Reader version 2.0	Fujifilm, Tokyo, Andpan
Vi-Cell™ XR 2.03	Beckman Coulter, Fullterton, Ca, USA
Light Microscope Olympus CKX 31	Olympus Europe GmbH, Hamburg, Germany
Mini-PROTEAN™3 Cell	BIO-RAD Laboratories Hercules, CA, USA
Power Pac™ HC power supply	BIO-RAD Laboratories Hercules, CA, USA
Mini Trans-Blot™ Electrophoretic Transfer Cell	BIO-RAD Laboratories, Hercules, CA, USA

2.2 Methods

2.2.1 Cell culture

HUVSMCs were kindly provided by Prof. Dr. David Bernhard (Medical University of Vienna, Vienna General Hospital). They were isolated from umbilical cords. Fresh cells at passage 4 were stored in aliquots of one million cells in liquid nitrogen at -196°C.

A cryovial containing approximately one million cells was taken out of liquid nitrogen and thawed in a 37°C water bath. The content of the vial was added into 75 cm^2 plastic flask containing 15 ml pre-warmed culture medium, which was changed next day.

HUVSMCs were cultured in 75 cm^2 or 150 cm^2 plastic flasks, containing 15 or 30 ml culture medium, respectively, at 37°C and 5% CO_2. They were passaged twice a week. Passages 3 to 8 were used.

For passaging, all solutions were warmed in a 37°C water bath prior to use. Culture medium was discarded from the flask, cells were washed with wash medium and trypsinized with Trypsin/EDTA (Invitrogen) (0.05% / 0,02% in PBS) solution for 3 minutes at 37°C. Trypsinization was stopped with stop medium and solution was centrifuged at 1200 rpm for 4 minutes. The supernatant was discarded and culture medium was added to the cells. Cell viability was counted from 500 µl sample with a Vi-CELL cell viability analyzer (Beckmann Coulter). The cells were seeded into 75 cm^2 ($1.5x10^6$ cells/ml) or 150 cm^2 ($3.0x10^6$ cells/ml) plastic flasks for further cultivation. For experiments, cells were seeded into different culture dishes as stated below.

2.2.2 Cell proliferation assay

Resazurin conversion assay was used for assessment of cell proliferation. The metabolic activity of living cells was measured using resazurin, which is converted to fluorescent resofurin by viable cells. Therefore, the fluorescence is direct proportional to the amount of resazurin converting cells due to assumption that the metabolic rate is in all cells identical.

HUVSMCs were seeded at 5000 cells/well in 96-well plates in culture medium. After 24 h cultivation, the cells were treated with different concentrations of tylophorine or 0.1% DMSO (Fluka). After 48 h incubation at 37°C, they were washed with starvation medium and then incubated in starvation medium containing 10 µg/ml resazurin (Sigma Aldrich) for 2 h. The samples were measured by monitoring the increase in fluorescence at wave length of 580-610 nm using excitation wavelength of 540-570 nm in a 96-well plate reader (TECAN).

2.2.3 Western blot

Proteins involved in proliferative signalling pathways were analysed by western blotting. Western blot is a common method used for analysing alterations in protein expression levels. In addition, changes in the phopshorylation status of a protein are possible to investigate by using phospho-specific antibodies.

Cells were seeded after passaging in 60 mm dishes (0.3 x 10^6 cells/dish) and cultivated 48 h. They were treated with tylophorine (1 µM) or vehicle (0.1% DMSO) for the indicated periods of time (15-120 min). The cells were placed on ice, washed with ice-cold PBS and 100 µl ice-cold lysis buffer was added to the cells. After 5 minutes, the cells were scraped and lysates were transferred to labelled tubes. Samples were centrifuged at 13000 rpm for 10 minutes at 4°C. For protein quantification, 5 µl of supernatants were diluted in 45 µl H_2O. To prepare protein samples, 90 µl of supernatants were mixed with 45 µl loading buffer and heated at 95°C for 5 min. All samples were stored at -20°C.

Protein concentrations were determined by using the Bradford method (Bradford 1976). 10 µl of samples were transferred to 96-well plate in triplicate and 190 µl of Bradford reagent, 1:5 dilution of Roti®-Quant (Carl Roth) in H_2O was added to each well. Absorbance at 595 nm was measured with a TECAN Sunrise™ microplate reader.

The protein samples were separated by discontinuous SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) (Laemmli 1970). For SDS-PAGE, resolving gel was first prepared and transferred into the gel cassette (BIO-RAD Laboratories) and stacking gel was prepared approximately after 30 minutes and transferred above resolving gel. A 15-well comb was inserted and the gel was allowed to polymerize at least for one hour.

The gels were placed into the electrophoresis chamber filled with electrophoresis buffer and the comb was removed. Precision Plus™ protein standard (BIO-RAD Laboratories) and equal amounts of the proteins (25 µg) were added into the wells of stacking gel.

Proteins were separated by using a Mini-PROTEAN™ 3 Cell system (BIO-RAD Laboratories) connected to a Power Pac™ HC power supply (BIO-RAD Laboratories). Electrophoresis was performed at 110 V for 21 minutes or until the end of stacking gel and 200 V for 36 minutes.

The proteins were transferred to Immuno-blot™ PVDF (polyvinylidenefluoride) membrane (BIO-RAD Laboratories) using Mini Trans-Blot™ Electrophoretic Transfer Cell system (BIO-RAD Laboratories) at 100 V for 90 minutes. The membranes were blocked with 5% fat free milk powder (Carl Roth) in TBS-T for one hour. After washing with TBS-T three times for 10 minutes, the membranes were incubated with specific primary antibodies in 5% fat free milk powder or in 5% BSA (Carl Roth) at 4°C overnight. The membranes were washed with TBS-T (3 x 10 min) between incubations and detection. Then, they were incubated with respective horseradish-peroxidase conjugated secondary antibodies for 2 hours at room temperature. A home-made ECL solution was added to the membranes and proteins were detected with LAS-3000™ luminescent image analyser (Fujifilm). Bands were quantified with AIDA™ software (Raytest).

2.2.4 Flow cytometric cell cycle distribution analysis

Flow cytometry was used to quantitate the DNA content of individual cells to assess the cell cycle distribution within cell populations. For this experiment, cells were stained with propidium iodide, a fluorescent dye, which binds to DNA (Riccardi and Nicoletti 2006).

Cells were seeded in 6-well plates (3 x 10^5 cells/well) and treated with 1 µM tylophorine or 0.1% DMSO after 24 h cultivation. After the indicated time points (24-120 h), supernatant of each well was transferred into the correspondingly labelled FACS tubes to collect also the cells that have detached during the incubation period. Attached cells were washed once with PBS, trypsinized with Trypsin/EDTA solution for 3 minutes at 37°C. Trypsinization was stopped with stop medium and the cells were transferred into the tubes, which were centrifuged at 1400 rpm for 4 minutes at 4°C.

Supernatants were discarded and PBS was added into each tube. Tubes were vortexed thoroughly and centrifuged again as before. Supernatants were discarded and hypotonic fluorochrome solution buffer was added and tubes were incubated for 2 h at 4°C. 10 000 cells were analysed by flow cytometry on a FACSCalibur™ (BD Bioscience Pharmingen).

3 RESULTS

3.1 The effect of tylophorine on cell proliferation and cell cycle phases

Resazurin conversion assay was performed to examine the effect of tylophorine on HUVSMC proliferation and to determine the concentration for 50% proliferation inhibition. Tylophorine inhibits HUVSMC proliferation dose-dependently with an IC_{50} of 164 ± 50 nM (Figure 4). 1 µM tylophorine blocked the proliferation completely.

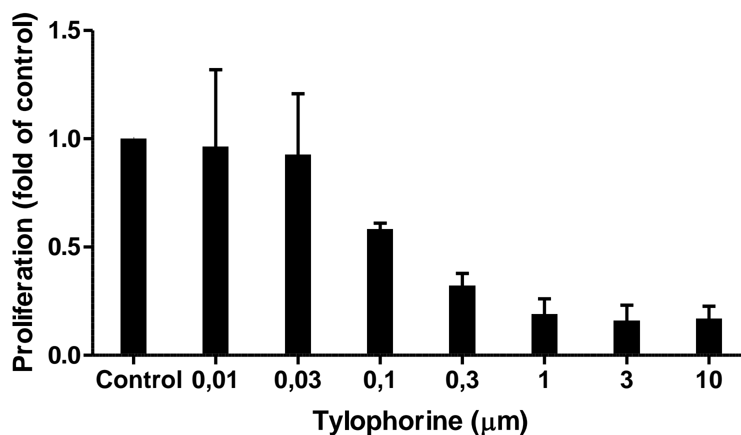

Figure 4. The effect of tylophorine on serum-stimulated HUVSMC proliferation. Cells were seeded one day before treatment with indicated concentrations of tylophorine or 0.1% DMSO (control). After 48 h, cells were treated with resazurin for 2 h. Samples were measured by monitoring the increase in fluorescence at a wave length of 580-610 nm using excitation wavelength of 540-570 nm in a 96-well plate reader (mean ± SD, n=3).

The potent antiproliferative action of tylophorine also becomes evident when observing cell density under the microscope over time (Figure 6). Whereas DMSO-treated cells almost reach confluence after 120 h, tylophorine-treated cells do not gain in density throughout the entire observation period.

In order to investigate whether tylophorine interferes with cell cycle progression and arrests cells in a specific cell cycle phase, we performed flow cytometric cell cycle analysis. Figures 5 and 7 show the accumulation of HUVSMCs in S phase after tylophorine treatment at 24, 48 and 72 h when compared with DMSO treatment. With tylophorine treatment, cells accumulated in S phase over time, whereas after treatment with DMSO, cells accumulated in G1 phase. This accumulation of DMSO-treated cells was obvious after 72 h and presumably due to contact inhibition. Effect of tylophorine on cell density is clearly seen in microscopic pictures on Figure 6.

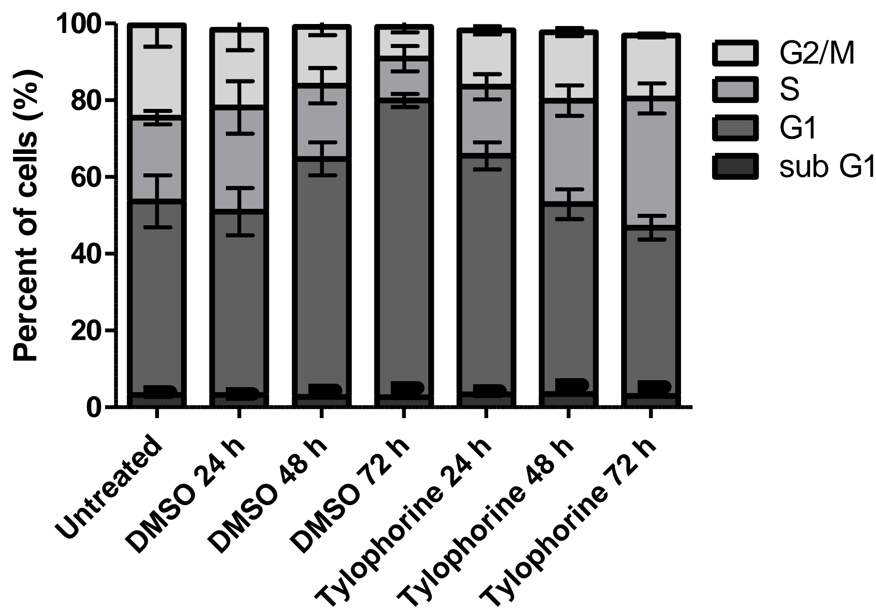

Figure 5. Effect of tylophorine on cell cycle distribution. Cells were seeded one day before treatment with 1 µM tylophorine or 0.1% DMSO. Cells were stained with propidium iodide after 24, 48 or 72 h and analysed by flow cytometry (mean ± SD, n=3).

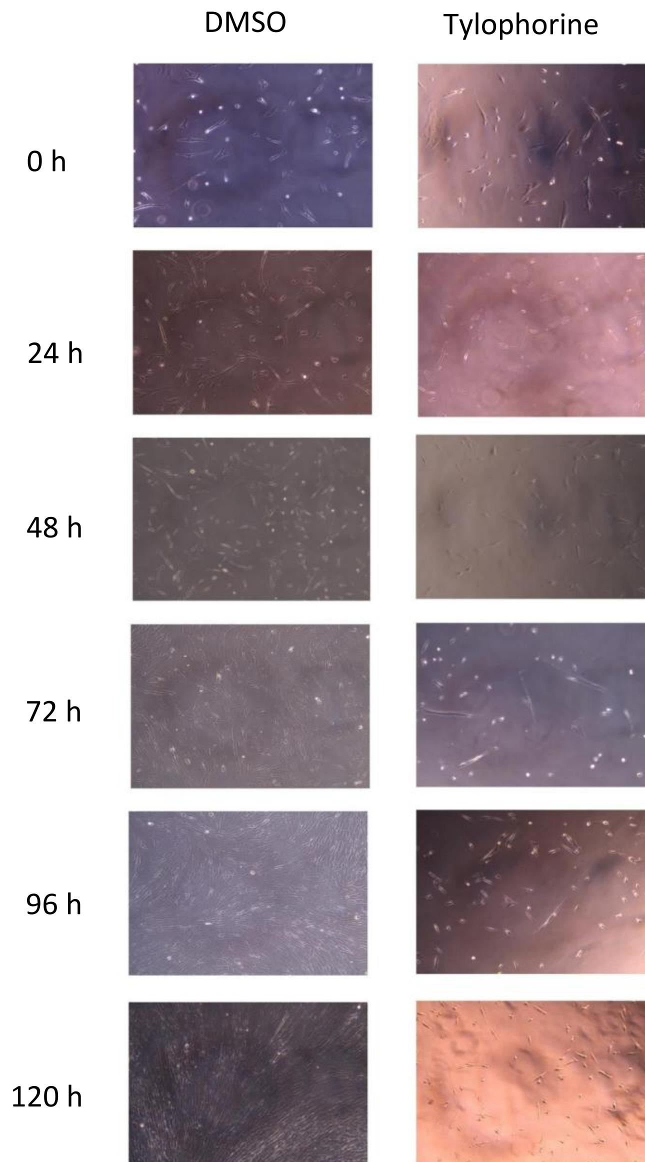

Figure 6. Light microscopic analysis of tylophorine-treated HUVSMCs. Asynchronized serum-stimulated HUVSMCs were seeded one day before treatment with 1 μM tylophorine or 0.1% DMSO. Light microscopic pictures were taken every 24 h.

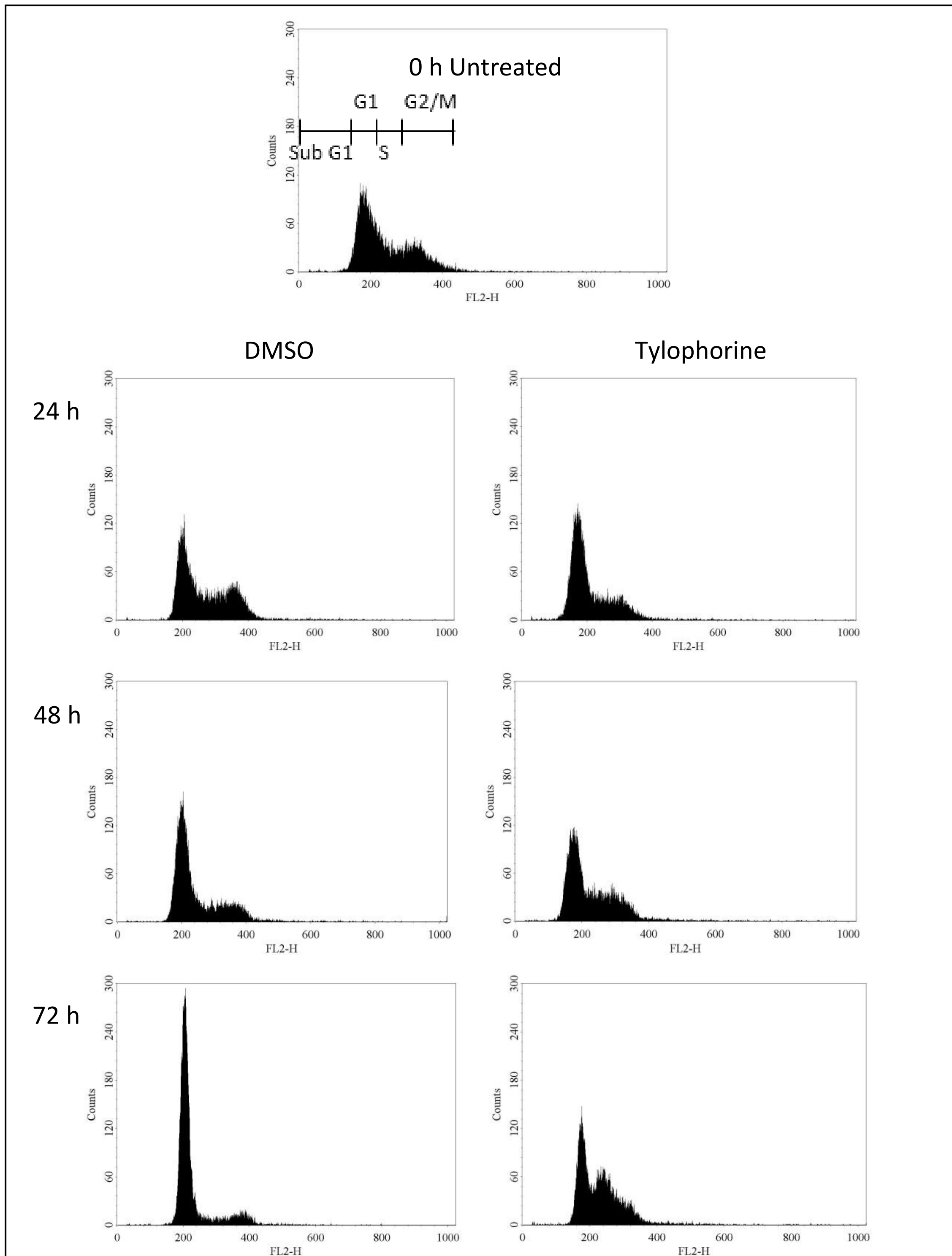

Figure 7. Cell distribution in asynchronized HUVSMCs. Cells were seeded one day before treatment with 1 µM tylophorine or vehicle (0.1% DMSO). Cells were stained with propidium iodide after 24, 48 or 72 h and analysed by flow cytometry. Results from one analysis are shown. Comparable results were obtained in three different experiments.

3.2 The effect of tylophorine on cell cycle regulatory proteins

In order to confirm the downregulation of cyclin D1 by tylophorine observed in rat aortic VSMCs, we performed western blot analysis of protein samples obtained from HUVSMCs. Tylophorine treatment led to rapid degradation of cyclin D1 with first effects already seen 15 minutes after treatment (Figure 8). In addition, western blotting was used to determine the effect of tylophorine on several other cell cycle regulatory proteins. p21 expression levels were downregulated by tylophorine treatment, as demonstrated in Figure 8. The level of cyclin E and CDK4 did not change significantly (data not shown). Overall, tylophorine elicited comparable changes in the expression of cell cycle regulators in rat and human SMCs.

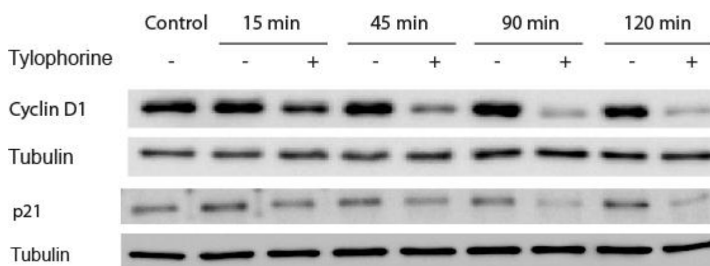

Figure 8. Western blot analysis of tylophorine's effect on cyclin D1 and p21 expression. HUVSMCs were seeded two days before treatment with 1 µM tylophorine or DMSO (0.1%) in indicated time points. As a control was a sample of HUVSMCs without treatment. One representative blot is shown. Consistent results were obtained in three separate experiments.

3.3 The effect of tylophorine on p53 phophorylation

The results of the kinase array led to the hypothesis that tylophorine treatment of VSMCs induces the p53 phosphorylation on serine 9 approximately 43-fold. Western blotting was used to confirm this outcome. Figure 9 demonstrates that p53 phosphorylation on serine 9 is hardly detectable and not upregulated 43-fold by tylophorine as indicated in kinase array. Thus, we were not able to confirm the result of the kinase array in our cell system.

Furthermore, we performed western blot analysis on several other phosphorylation sites of p53. Tylophorine induces a reproducible dephosphorylation of p53 on serine 15. Results of other phosphorylation sites were not consistent (data not shown).

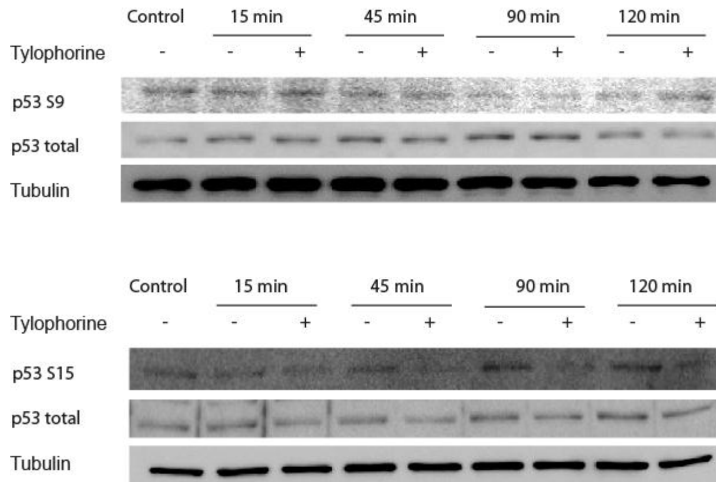

Figure 9. Western blot analysis of tylophorine's effect on phosphorylation of p53 on serine 9 and 15. HUVSMCs were seeded two days before treatment with 1 µM tylophorine or 0.1% DMSO in indicated time points. As a control was a sample of HUVSMCs without treatment. One representative blot is shown. Consistent results were obtained in three separate experiments.

4 DISCUSSION

4.1 The effect of tylophorine on cell proliferation

Our experiments demonstrated that tylophorine inhibits HUVSMC proliferation dose-dependently (IC_{50} 164 nM ± 50 nM). Tylophorine has been shown to inhibit the growth of several cancer cell lines with IC_{50} values shown in Table 4 (Chuang et al. 2006; Steerk et al. 2002; Wu et al. 2009). Most of the studies have investigated the effect of (+)-S-tylophorine and tylophorine analogues. Only Steerk et al. (2002) showed the antiproliferative effect of (-)-R-tylophorine on cancer cells as we showed in HUVSMCs. The inhibitory concentrations of tylophorine are in a similar range in cancer cells and HUVSMCs. In addition, these concentrations are comparable to the

IC_{50} value obtained in rat aortic VSMCs (IC_{50} of 128 nM ± 44 nM) (Joa 2011). To conclude, tylophorine has a significant inhibitory effect on cell proliferation.

Table 4. IC_{50} values of tylophorine in several cancer cell lines. Wu et al. and Chuang et al. have used (+)-S-tylophorine whereas Steerk et al. (-)-R-tylophorine.

Cell line		IC_{50} (nM) (mean ± SD)	Reference
HepG2	human hepatocellular carcinoma	237 ± 32	Wu et al. 2009
HONE-1	human nasopharyngeal carcinoma	114 ± 6	
NUGC-3	human gastric carcinoma	134 ± 9	
MCF-7	human breast carcinoma	489 ± 45	Chuang et al. 2006
NCI-H460	human lung carcinoma	584 ± 39	
SF-268	human central nervous system carcinoma	1764 ± 105	
KB-3-1	drug sensitive human nasopharyngeal carcinoma	214 ± 60	Steerk et al. 2002
KB-V1	multidrug resistant human nasopharyngeal carcinoma	173 ± 45	

4.2 The effect of tylophorine on cell cycle regulatory proteins

4.2.1 Cyclin D1

We revealed that tylophorine reduces cyclin D1 expression levels. A small decrease in cyclin D1 level can already be seen after 15 minutes of tylophorine treatment, which indicates that tylophorine induces a fast downregulation of cyclin D1.

My supervisor Joa has previously shown that tylophorine downregulates Cyclin D1 in rat aortic VSMCs (Joa 2011). In addition, he determined by qPCR (quantitative polymerase chain reaction) analysis that mRNA levels of cyclin D1 were unaffected in rat aortic SMCs after tylophorine treatment. This result suggests that the downregulation of cyclin D1 is not due to transcriptional inhibition but rather due the post-transcriptional regulation of cyclin D1. Furthermore, the experiments with proteasome inhibitor MG132 demonstrate that it blocks the downregulation of cyclin

D1 levels by tylophorine (Joa 2011). This indicates that tylophorine leads to downregulation of cyclin D1 via proteasomal degradation in rat aortic VSMCs. In addition, Joa later confirmed this result in HUVSMCs (Joa 2011).

Cyclin D1 is a key regulator of G1 phase and initiation of cell cycle (Fu et al. 2004; Stacey 2003). Extracellular signals mediate its function as a gatekeeper of cell cycle from early to mid-G1 phase. In the initiation of S phase, cyclin D1 levels decrease automatically, which is necessary for DNA synthesis. It is a proto-oncogene. Its overexpression is known to correlate with the early onset of cancer and it is overexpressed in various cancer types including breast, bladder and lung cancer (Knudsen et al. 2006). For example, cyclin D1 has been determined to be overexpressed in breast cancer at high frequency without an increase in cyclin D1 gene copy number (Gillett et al. 1994). Therefore, its deregulation may be explained by other mechanisms including its defective regulation at the post-translational level such as deregulated degradation (Alao 2007).

Cyclin D1 is highly unstable with a short half-life (~24 min) (Diehl et al. 1997; Diehl et al. 1998). It is mainly degraded by ubiquitin-dependent proteasomal pathway. This degradation depends on cyclin D1 phosphorylation on Thr-286, which was determined to be phosphorylated by GSK-3β. GSK-3β also triggers the relocalization of cyclin D1 from the nucleus to the cytoplasma further contributing its inactivation.

Various therapeutic agents that modulate the expression level of cyclin D have been studied primarily for anti-cancer therapy (Alao 2007). Only few of these agents have been considered for treatment of vasculoproliferative diseases.

Rapamycin has been shown to inhibit cell proliferation, besides its other cellular mechanisms, by downregulating cyclin D1 expression in fibroblast (NIH3T3) and breast cancer (MCF-7 and MDA-MB-468) cell lines (Dong et al. 2005; Hashemolhosseini et al. 1998). Rapamycin affects both transcriptional and post-transcriptional regulation of cyclin D1. It delays the accumulation of cyclin D1 mRNA levels and affect cyclin D1 ubiquitin-dependent degradation by stimulating the activation of GSK-3β.

Expression of cyclin D1 has also been shown to be decreased by flavopiridol in breast cancer (MCF-7) and human osteosarcoma (U2OS) cell lines (Carlson et al. 1999). This downregulation was observed by 6 hours and followed by the decline in CDK4 activity and the induction of retinoblastoma hypophosphorylation. Cyclin D1 decline was at least in part explained by decrease in cyclin D1 promoter activity that contribute to decreased cyclin D1 mRNA levels.

We demonstrated that tylophorine induce rapid downregulation of cyclin D1 in HUVSMCs. As my supervisor Joa demonstrated, tylophorine leads to inhibition of proteasomal degradation of cyclin D1 in rat aortic VSMCs as well as in HUVSMCs (Joa 2011). However, further investigations are needed to determine the exact mechanism underlying the tylophorine-induced degradation of cyclin D1.

4.2.2 p21

According to our results, tylophorine decreases the expression of p21. p21 is an important CDK inhibitor and regulator of cell cycle (Chen et al. 1995; Harper et al. 1993). For example, it mediates p53-dependent G1 arrest. However, p21 acts also as a positive regulator of the cell cycle (Labaer et al. 1997). Most of the cellular cyclin D1 -dependent kinase activity is associated with binding to p21, although p21 was shown to inhibit cyclin D1-CDK4 kinase activity when expressed at higher concentrations. In addition, p21 has been determined to promote nuclear accumulation of cyclin D1-CDK complexes via inhibition of GSK-3β-triggered nuclear export of cyclin D1 (Alt et al. 2002; Cheng et al. 1999). Therefore, p21 is essential for the activation of cyclin D1-CDK complexes rather than inhibiting them.

Such as cyclin D1, p21 is also a very unstable protein that is degraded by ubiquitin-dependent proteasomal pathway (Bornstein et al. 2003). Phosphorylation of p21 at Ser-130 stimulates its degradation. In addition, p21 is degraded in an ubiquitin-independent manner (Sheaff et al. 2000)

My supervisor Joa showed that p21 gene mRNA levels were not significantly altered by tylophorine, indicating that it has no influence on the transcription of p21 (Joa 2011). Therefore, these results may indicate that tylophorine affects both cyclin D1 and p21 leading to their downregulation, which further interferes with the formation of an active cyclin D1-CDK4 complex.

Tylophorine analogue DCB-3503 has been demonstrated to decrease the expression of cyclin D1, p21 and p53 in HepG2 cells (Wang et al. 2010). Earlier, it was shown to inhibit the growth of these cells (Gao et al. 2004). DCB-3503 induces rapid downregulation of cyclin D1, already after 15 minutes of treatment without affecting mRNA levels (Wang et al. 2010). These downregulations were suggested to result from translational modulations such as reduction in the rate of polypeptide chain elongation. Our results cannot completely rule out this as underlying mechanism for the tylophorine-mediated downregulation of cyclin D1 and p21 in VSMCs or HUVSMCs.

Overall, tylophorine was identified to downregulate both cyclin D1 and p21 levels post-translationally in a proteasome-dependent manner. The detailed mechanism underlying the decrease in both proteins needs further detailed investigation.

4.2.3 Phosphorylation sites of p53

Based on the kinase array, we assumed that p53 phosphorylation on serine 9 is 43-fold upregulated. However, we were not able to confirm this result in our cell system. Phosphorylation of p53 was hardly detectable. In addition, our other results of tylophorine's effect on phosphorylation sites of p53 were not consistent. The only effect of tylophorine that was displayed was the slight decrease in the phosphorylation of p53 on serine 15.

One reason for our unfavourable results could be the inadequate amount of the protein. In addition, extremely low levels of phosphorylated p53 with inadequately sensitive antibodies may explain our unsatisfying results. Hence, it is possible that we were not able to detect the effect of tylophorine on p53 phosphorylation on serine 9, which was

predicted by the kinase array as upregulation. In order to completely clarify the issue of p53 phosphorylation at serine 9 and its susceptibility to tylophorine, more experiments are needed with more sensitive methods.

p53 protein is an important regulator of cell cycle inducing cell cycle arrest and apoptosis (Levine 1997). Phosphorylation of p53 is associated with DNA damage and induction of apoptosis (Higashimoto et al. 2000; Mayo et al. 2005). My supervisor Joa showed by annexin V staining assay that tylophorine does not induce apoptosis (Joa 2011). Our results from western blot analysis are unreliable, but also suggesting that tylophorine is not apoptotic. However, more experiments are needed to confirm this data.

4.2.4 Other cell cycle regulatory proteins

We investigated the effect of tylophorine on cyclin E and CDK4 expression levels but they were not changed. Consequently, there are only few publications on the effect of tylophorine on cell cycle regulatory proteins. Wu et al. (2009) investigated the effect of tylophorine on expression levels of various cell cycle regulatory proteins including cyclin A2, cyclin B1, cyclin E, CDK1, CDK2, CDK4 and p27 in asynchronized and synchronized HepG2, HONE-1 and NUGC-3 cells. They determined that tylophorine treatment downregulates cyclin A2 expression levels, whereas the level of other proteins did not change significantly. Furthermore, in synchronized cells arrested in G1-S phase with double-thymidine, tylophorine treatment led to a reduction of cyclin A2 level linearly over 24 h and also the mRNA level of cyclin A2 was significantly decreased. In addition, they determined that cyclin A2 promoter activity was decreased by tylophorine suggesting that it downregulates cyclin A2 expression through transcription. On the contrary, Joa determined that tylophorine treatment did not downregulate cyclin A in VSMCs (Joa 2011). This effect was not addressed in HUVSMCs.

To conclude, tylophorine has a significant effect on several cell cycle regulators. However, the exact mechanism behind these effects needs to be examined.

4.3 The effect of tylophorine on cell cycle phases

We investigated the effect of tylophorine on cell cycle distribution in asynchronized cells. Our experiments indicated an accumulation of HUVSMCs in S phase in the presence of tylophorine. Light microscopy observations indicated contact inhibition in DMSO treated cells after 120 h (Figure 6). It was also observed that there were no increase in density of tylophorine treated cells over 120 h, thus they were not dividing. These results indicate that tylophorine may retard the progression through S phase.

FACS analysis upon propidium iodide staining alone is an inadequate method to precisely define the stage of an apparent cell cycle arrest. The amount of DNA in S phase cells varies and hence may be falsely added to G1 phase or G2-M phase. It is quite difficult to properly assign the gates for each cell cycle phase unless one is very experienced with this kind of analyses. Therefore, more profound analyses are needed to confirm the results.

Joa had also investigated the effect of tylophorine on cell cycle distribution (Joa 2011). He showed an accumulation of quiescent PDGF-BB-stimulated VSMCs in G1 phase in the presence of tylophorine. This result was also confirmed by analysing the phosphorylation of Rb, which was completely blocked by tylophorine. In addition, he analysed asynchronized serum-stimulated VSMCs, which were shown to accumulate in S phase. The distribution of cells in G1, S and G2 phase was comparable to the distribution in HUVSMCs.

Similar results were demonstrated by Wu and his colleagues (Wu et al. 2009). They investigated the effect of 2 µM tylophorine on cancer cell cycle by using both asynchronized and synchronized cells. According to their results, tylophorine arrests HepG2, HONE-1 and NUGC-3 carcinoma cells in G1 phase. Asynchronized cells accumulated in S phase. In synchronized cells, they used double-thymidine and thymidine-nocodazole to block the cells in G1-S and G2-M arrest, respectively. After release from G1-S or G2-M arrest, treatment with tylophorine led cells to arrest in G1

phase. They proposed that tylophorine inhibits carcinoma cell growth by dominantly arresting cells in G1 phase rather than retarding S phase progression.

Gao et al. (2004) analysed the effects of (+)-S-tylophorine and its analogue DCB-3503 on cell cycle progression in cancer cells. They showed a consistent increase in S phase in KB cells but no specific cell cycle arrest was observed in HepG2 cells.

These results suggest that tylophorine retards the progression of S phase and arrest cells in G1 phase. It would be interesting to investigate cell cycle progression also in synchronized HUVSMCs whether tylophorine induces G1 arrest as observed in VSMCs and various cancer cells (Wu et al. 2009, Joa 2011).

5 CONCLUSIONS

The aim of this thesis was to evaluate the effect of tylophorine on HUVSMC proliferation and on expression of cell cycle regulatory proteins. We were able to demonstrate that tylophorine inhibits HUVSMC proliferation dose-dependently. Tylophorine downregulates cyclin D1 and p21 expression levels. This decrease in cyclin D1 was already seen after 15 minutes of tylophorine treatment. Effect of tylophorine on p53 phosphorylation sites remains unresolved and requires more sensitive methods to be completely unravelled. In our experiments, tylophorine was shown to induce the accumulation of HUVSMCs in S phase.

There are several unanswered questions in tylophorine’s diverse effects on cell cycle and the underlying mechanism of action. However, tylophorine has significant effect on cell proliferation and cell cycle distribution. According to Joa’s results, the effect of tylophorine on cyclin D1 and p21 might be explained by proteasome-mediated degradation (Joa 2011). A number of investigations are needed in order to determine the specific mechanism of this degradation. In addition, the significance of tylophorine as cell cycle regulator and in treatment of vascular proliferative diseases and cancer requires more experiments *in vitro* and *in vivo*.

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APPENDIX

Results from Pepscan Pepchip™ kinase array

PROTEIN	PSITE	Upstream kinase	Fold activation
p53	S9	ATM + Casein_kinase_1,_alpha_1	43.70
SMAD5	Y128	nd	3.57
STAT5B	Y679	c-Src	2.35
Metabotropic glutamate receptor 1	T695	Protein_kinase_C_alpha	2.09
Erythropoietin receptor	Y368	Janus_kinase_2	2.08
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	T475	Protein_kinase_C_alpha	1.87
p47-phox	S348	Casein_kinase_II,_alpha_2 + Casein_kinase_II,_beta	1.78
RGS2	S46	Protein_kinase__cGMP-dependent_type_I	1.78
SPIB transcription factor	S146	Casein_kinase_II,_alpha_1 + Casein_kinase_II,_alpha_2	1.77
Regulator of G protein signaling 19	S151	ERK2	1.72
Synuclein alpha	Y136	SYK	1.62
Transcription factor IIA, 1	S280	Casein_kinase_II,_alpha_2 + Transcription_factor_IID	1.62
Stomatin	S10	-	1.59
Formyl peptide receptor 1	S328	G_protein_dependent_receptor_kinase_2	1.56
AFX 1	S196	AKT1	1.56
HLA-A	S359	Protein_kinase_C_alpha	1.55
Polo like kinase	T210	-	1.55
FAS associated factor 1	S289	Casein_kinase_II,_alpha_1	1.53
CTD phosphatase, subunit 1	S575	Casein_kinase_II,_alpha_2 + Casein_kinase_II,_beta	1.48
TBK1	S172	-	1.45
ERK5	T218	-	1.42
Protein phosphatase inhibitor 2	S87	Casein_kinase_II,_alpha_1 + Casein_kinase_II,_alpha_2	1.42
E2F transcription factor 1	S403	Cyclin_dependent_kinase_7 + TFIIH_62_kDa_subunit	1.42
ZAP70	Y319	ZAP70	1.41
Syntrophin alpha 1	S193	MAPK12	1.39
Ribosomal S6 kinase 1	S230	3_Phosphoinositide_dependent_protein_kinase_1 + Pyruvate_dehydrogenase_kinase,_isoenzyme_1	1.37
Phosphatidylinositol 3-kinase, regulatory gamma subunit	Y341	-	1.35
Eukaryotic translation initiation factor 4E	S209	Protein_kinase_C,_beta_1 + Protein_kinase_C,_gamma	1.34
p21-activated kinase 7	S573	p21_activated_kinase_7	1.33
LNK	Y273	Lck + ZAP70	1.33
SHP2	S591	Protein_kinase_C,_beta_1 + Protein_kinase_C,_eta	1.31