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# Development of high throughput screening assay for detection of E2F mediated signaling pathways

Bakalářská práce

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Vedoucípráce: Mgr. Pawel Znojek, Ph.D.

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V Olomouci, 20.5.2015 Podpis _____

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Abstrakt

E2F jsou transkripční faktory regulující buněčný cyklus různými mechanismy. Tato skupina transkripčních faktorů se skládá z osmi členů E2F1 – E2F8. Narušení signální dráhy E2F je jednou z mnoha příčin rozvoje rakoviny. Teoretická část shrnuje základní informace o transkripčních E2 faktorech, retinoblastomovém proteinu, buněčném cyklu a jeho kontrole. Dále jsou popsány cesty k buněčné smrti – apoptóze. Experimentální část je zaměřena na testování buněčné linie 293H transfekovány E2F-luciferázou. Z této buněčné linie byl vybrán klon s nejlepšími vlastnostmi pro validaci homogenních vysocevýkonných screeningových testů, které detekují expresi E2F-zprostředkované genové exprese. S využitím této modifikace byly testovány biologicky aktivní látky knihovny LOPAC. Výsledky, diskutovány v závěru, hodnotí aktivaci či represi exprese E2F ve vztahu k buněčnému přežití.

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Abstract

E2Fs are transcription factors which regulate the cell cycle by a different mechanism. The E2F family contains eight members E2F1 – E2F8 and the disruption in the E2Fs signaling pathways is one of the reasons of cancer development. Theoretical part summarizes basic information about E2Fs, retinoblastoma protein, cell cycle and its control. It is also focused on the ways that cell death – apoptosis can be mediated by E2Fs. The experimental part is focused on testing three clones of cell line 293H transfected with E2F-luciferase construct. Clones with the best properties were selected for development and validation of homogenous high-throughput screening assays able to detect expression of E2F-mediated gene expression. Using this set up assays were validated on biologically active compounds of LOPAC library. Results summarized in the conclusion evaluate the role of activation or repression of E2F in relation to cell survival.

# Content

## Goals

The goal of the presented work was to validate clones of cell line 293H stably transfected with the E2F-luciferase response element. For this purpose starved cells were cultured in medium containing various amount of fetal bovine serum to stimulate cell E2F-dependent gene expression. The purpose of validating 293H clones was to select the clone with the best performance in luciferase assay.

Subsequently one of these clones was used for luciferase assay. This method was set up to make homogenous assay ina 384 well plate format. The goal was optimization of the luciferase assay kit to be high-throughput screening test compatible.

Validate HTS luciferase assay was used for testing collection of compound library of unknown activity from the LopacPhizer library.

# Theoretical part

## 1 Introduction

E2F transcription factorsplay key role in cell growth, differentiation stress response and apoptosis. The whole E2F family consists of eight members, divided into two classes: E2F1-E2F3a are “activators” which drive cells from G0 to the cell cycle and E2F3b-E2F8 are “repressors” involved in quiescent and differentiated cells(1). E2 factors are able to bind special exact sequences in gene and create binding site in the target promoter sequences.

Their transcriptional activity depends on binding with dimerization protein Dp family and pocket proteins - retinoblastoma protein pRb, p107 and p130 (2). pRb activity is influenced by phosphorylation mechanism mediated by complexes of cyclin and cyclin dependent kinases (cdk). Corresponding cdk phosphorylates pRb after its activation with cyclin. This interaction depends upon growth factors binding to receptors.

While pRb becomes phosphorylated by complex cyclin/cdks E2F is released from the binding trap. “Free” E2F is now ready to activate genes implicated in cell cycle progression also in apoptosis.E2Fs also coordinate progression through the important G1/S checkpoint.Whereas dephosphorylated retinoblastoma protein detaches E2F which leads to the inhibition of gene expression thus Rb represses transcription genes. Rb protein is presented all the time in cells and its phosphorylated and dephosphorylated state alternates. During the S, G2, M phase,pRb stays in phosphorylated deactive form(3).

Deregulation of Rb/E2F complex included in growth control can result in cell death – apoptosis. There is a link between Rb-mediated growth control of the cell cycle and p53-dependent apoptosis demonstrated by the first member of E2Fs – E2F1. Inactivation on Rb results in E2F1-induced apoptosis pathway. DNA damage induces activation of signaling molecules such as ATM and Chk2 in the E2F1 apoptosis pathway. Thus, E2F1 activate p53 through DNA damage response factors and kill cells(4).

## 1.1 The Cell cycle

The cell cycle is a sequence of events occurring in living cells, covering the period between the interphase and M phase. The reason of the cell cycle is division and replacement of dead cells. Cell division is a primary condition for every organism growth.

Theinterphase is the stage of cell between two mitotic duplications. The interphase consists of G1, S and G2 phase of the cell cycle.During the G1 phase, the cell is growing and enzymes for DNA replication are synthesizing. Cells in the G1 phase contain diploid number of chromosomes. During the S phase DNA is replicated. The DNA is repaired in G2 phase and checked by enzymes for mistakes. The cell is grows and prepares for its division. Now the cell is ready to go to the M phase of the cell cycle called mitosis.

Cells can exit the cell cycle and stay in a period called G0 phase. This state is a nonproliferative stage of the cell. There is an absence of signals for mitosis but genes related to cell metabolism are expressed(5). Cells may enter G0 phase from the G1 checkpoint(6).

The M phase is the stage in cell cycle that consists of karyokinesis – division of the nucleus and cytokinesis – division of cytoplasm. During mitosisthe cell divides into two identical daughter cells, so these new cells receive an exact copy of genetic information. The first period of mitosis is prophase. During this phase chromosomes condense, nuclear membrane dissolves and centrioles move to the opposite poles of the cell. There are also special fibers called spindle fibers, which consist of microtubules forming at the opposite poles of the cell. During the second phase – metaphase – chromosomesare pulled into an equatorial line in the middle of the cell. Next phase of the mitosis called anaphase begins when the chromatides of each chromosome are split to the opposite sides of the cell toward the spindle fibers. They are divided in the centromere. In the last one phase – telophase – chromosomes are at the opposite poles decondensing. The cell membrane rise up the same as the nuclear envelope and the spindle fiber dissembles. Cytokinesis begins when cytoplasm of two daughter cells detaches. Nuclear and nuclear membrane are modulated and outer membrane connects to the endoplasmatic reticulum. Chromosomes are released and used for transcription(7).

### 1.1.1 Control of cell cycle

The cell cycle control is pivotal matter because its disruption can lead from normal to cancer cell. Control system is presentedby cyclins which mediate specifity rate and activity of cyclin dependent kinases (cdks). Meaning that cdks activity depends on cyclinappereance. Thesecomplexestrigger phosphorylation of pRb and inactivate it. Checkpoints present another controlling system during the cell cycle(8).

The first and critical control point of the cell cycle is the G1/S checkpoint responsible for correct growth of the cell. This point decides if the cell cycle will continue or stop. Here comesthe information of whether a cell should divide or a cell division is arrested or if the cell has to enter the G0 phase. The G2 phase is the second G2/M checkpoint where the correct synthesis of genetic material is controlled. If there is no mistake the cell is able to enter mitosis. The last M checkpoint is localized in metaphase during mitosis. After cytokinezis the cell can again enter the next G1 phase.There are numerous of external factorsinfluencing a cell cycle. These factors include growth factors, size of the cell, nutritional state andhormones. Rate and quantities of the cell cycle depends on type of the cell (9).

Additionally, there is one more control point called restriction point (R point)in the G1 phase. Rather, G1 phase is divided into two parts: G1-postmitotic and G1-pre-S. The R point is probably located between these two parts(10).

### 1.1.2 Cyclins and Cyclin-dependent kinases

The major role in controlling cell cycle is played by cyclins-dependent kinases (cdk) and cyclins, which fulfill their role by phosphorylation of target proteins during the cell cycle. These kinases are made up from a small and a big subunit. The small N-terminal subunit contains β sheet conformation and the big C-terminal subunit contains α helix configuration. Parts of cdks are an ATP- activated site which accepts ATP molecule and cyclin-activated site which accepts cyclin that activates a molecule. Cyclins are proteins of helical structure containing cyclin box. This part is necessary for cdk binding and activation(11).

A combination of cyclin/cdks complexes are formed during the cell cycle independently in distinct phases. Overexpression of some cdk complexes results in increasing phosphorylation of pRb, which lead to the release of active E2F factors to drive G1 phase to S phase entry(9).

### 1.1.3 Cdks and the cell cycle

Proliferation activity is strictly controlled. Regulation is established by cyclins and their corresponding cdks as mentioned in section 1.1.2. There are many different types of these complexes throughout the cell cycle.

Progression from quiescent cells or cells after previous mitotic phase to the G1 phase is associated with D type cyclin/ cdk complexes.(12)

In the G1/S phase,there are present cyclins D and E. Three types of cyclins D exist– D1, D2, D3 that are typical for binding cdk4 or cdk6 and two types of cyclin E – E1 and E2.Quantity of cyclins D is dependent on mitogenic signals. During the G1 phase complex cyclinD/cdk4, 6 is responsible for phosphorylating Rb and release E2F transcription factors.Free E2F is now ready to active genes responsible for switching cells from G1 to S phase (9).

Mitotic cyclins A andB mediate progression through three phases – S/G2/M. Cyclin A1 is expressed during meiosis and cyclin A2 in proliferating somatic cells(9).CyclinB1 starts to accumulate in G2 phase and it’s found in cytoplasm creating cyclinB1/cdk1 complex activated in early prophase. Complexstays in cytoplasm focused on duplicated chromosomes outside of the nucleus as they begin to separate. Cyclin B activates cdk1 and enable M phase enter.CyclinA binds cdk2 creating key complex for S phase enter. Cyclin A/cdk1 is a part of G2/M state (7). Regulation of the cell cycle by cyclin/cdks and phosphorylation state of pRb shows *Figure 1.*

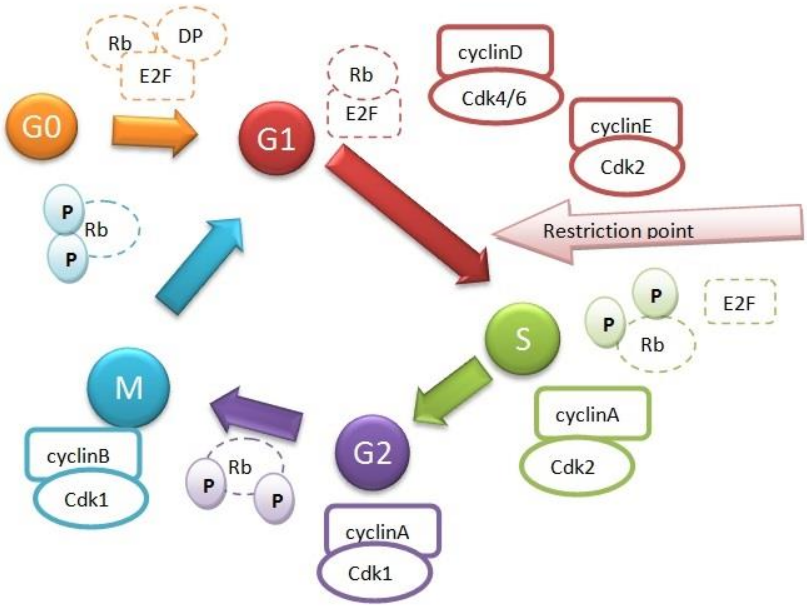


**Figure 1Rb, E2F and cyclin/cdks during the cell cycle Rb and the cell cycle** Rb present unphosphorylated and p,p-Rb phosphorylated retinoblastoma protein. In G1 phase cyclins D and E phosphorylate Rb and inactivate it. In late G1 phaseRb release E2F protein needed for start gene transcription. Once Restriction point is passed the cell cycle continues. Cyclin A stimulates DNA replication. Cyclin B initiates mitosis.

### 1.1.4 Cyclin dependent kinases inhibitors CDKI

Cyclin dependent kinases inhibitors control dephosphorylation and activation of pRb through inactivation cyclin/cdks. This mechanism results in cell cycle arrest in pRb-dependent manner.

CDKIs are largely classified into two families – INK4 family(p15, p16, p18, and p19) and the CIP/KIP family (p21, p27, and p57) on the base of their structures and affinities for cyclin/cdk complexes. Members of INK4 play key role during G1 phase. They compete with cyclinD for binding to cdk4 or cdk6. Meanwhile CIP/KIP (cdk inhibitor protein/kinase inhibitory protein) inhibit cdk2 present in the late G1 phase and S phase. These CKIs (cyclin kinase inhibitors) may bind cyclins or cdk individually, however the affinity is higher to cyclin/cdk complex (13). Expression of p21 is induced in p53-dependent also p53-independent manner (14) in contrast to p27 expressed after mitogenic stimulation in quiescent cells.

Both p27 and p21 are strong inhibitors of cyclin E/ cdk2 on the other hand are less effective in blocking of cyclin D/cdk4. In quiescent cells p27 suppress cyclin E/cdk2 activity meanwhile cyclin D1/cdk4 do not form. After mitogen stimulation cyclin D1 induces assembly with cdk4, cyclin E/cdk2 become active, simultaneously p27 become blocked. Both cdks then cooperate in Rb phosphorylation approaching G1/S transition. Inhibition of G1 cdks by p27 returns Rb proteins to their hypophosphorylated active state and cell exit the cell cycle(15).

## 1.2 Role of pRb and E2F proteins in cell cycle

### 1.2.1 Retinoblastoma (Rb) protein family

pRb is a product of the retinoblastoma gene first identified in a malignant tumor of retina. Its pivotal role is negative control of the cell cycle and in tumor progression. The pRb family members creating an interaction with E2F transcription factors family and in this state control expression of E2F- dependent genes (9). pRb passes through several phosphorylation states during the cell cycle caused by different combination of cyclin/cdk complexes(16).

Accordingly, Rb – E2F complex repress transcription once is stacked to E2F-regulated promoters. Mechanism of gene transcription regulation is through chromatine structure modification – preferentially histone deacetylases and methyltransferases. It was also shown that Rb is transcriptional repressor of those genes encoding RNA polymerases I and III (17).

Inactivation of pRB function thus deregulation pRB/E2F pathway may lead to cell cycle deregulation, inhibition of differentiation and apoptosis. In the view of chromosomal mutations conducing to gene inactivation of pRB is one of the reasons of retinoblastoma tumor progress. Several human inherited tumors confer mutations and deletions of the Rb genes (12).

Rb gene family includes three members Rb/p105, Rb2/p107 and Rb2/p130. These three factors share similar structures of their functional domain thus they are also known as "pocket proteins" (18). The pocket region is characterized by two domains – A and B separated by spacer region, which differs among the three Rb relatives *(Figure 2)* (19). Mentioned proteins interact with cyclin D/cdk4 or cdk6 in early G1 phase. In the middle to late G1 phase are cyclin E and cyclin A in complex with cdk2 and specially bind Rb/p105, Rb2/p107 and Rb2/p130. These phosphorylation steps through cyclin/cdks pathway are pivotal for inactivation of pRb family members and release E2F(9).

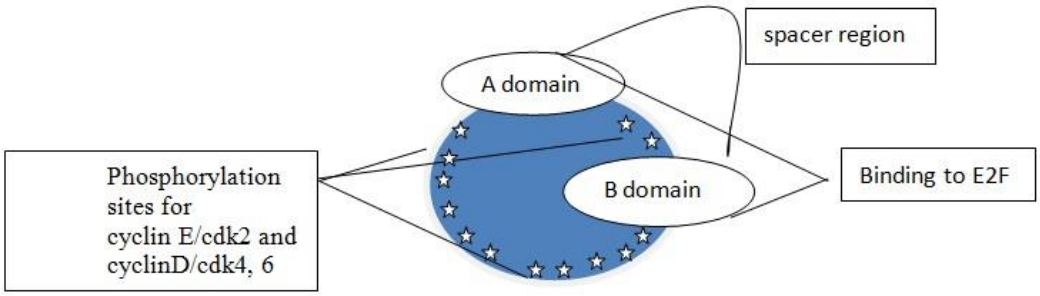


**Figure 2 Schematic structure of the pRb.** A and B domain refers to "pocket region" separated by space. They are responsible for most protein-protein interaction.

More precisely, as same as pRb, p107 phosphorylation depends on cyclin D1/ cdk4, 6. Rb2/p130is phosphorylated by cyclin D3/cdk4 complex. All three pocket proteins are also phosphorylated by cyclin E/cdk2. Rb2/p107 and Rb2/p130 but not Rb/p105 are phosphorylated by cyclinA/cdk2 (9).

In G1/G0 transition, when cells leave the cell cycle to quiescent stage, level of p130 and E2F/p130 highly accumulate (12). Rb2/p130 is highly expressed in quiescent and differentiated cells and its level decrease when cells enter the cell cycle. Rb/p105 level is approximately constant in both quiescent and proliferative cells.Rb2/p107 level rises when quiescent cells stimulate to proliferate (18).

### 1.2.2 E2F protein family

Successful cell division requires complete DNA duplication, which coordinate E2F transcription factors. E2F activate not only genes in G1/S phase that encode gene replication but also genes in G2/M include mitotic activities(20). E2Fs both activate and repress gene expression – function in DNA replication, mitosis, mitotic checkpoint, DNA-damage checkpoints, DNA repair, differentiation, development and apoptosis(21).

The E2F family consists of eight members (E2F1-8) subdivided into two classes based on their transcriptional activity: "activator" (E2F1-3a) drive quiescent cells to the cell cycle and the "repressors" E2Fs (E2F3b-8) appearing in gene silencing of quiescent or differentiated cells(22). E2F3a and E2F3b are closed to each other with difference of use another promoter. All family members contain DNA-binding domain. E2F1-5 have transactivation domain consists of short amino acid part mediates binding of pRB enables activation also repression of gene expression. E2F1-6 have dimerization domain required for interaction with DP1-4. This interaction enables binding DNA. E2F7-8 do not heterodimerize with DP and bind DNA as homodimers or together as heterodimers (21).

E2F1, E2F2, E2F3 preferentially binds to pRb. In addition, these factors contain cyclin– binding site and form complex with cyclin A/cdk2. This interaction regulates the DNA– binding ability of E2F/DP by phosphorylation. Thus E2F transcription activity in G1/S phase decrease. E2F4 and E2F5 lack this cyclin – binding site(23). Rb2/p130 exclusively binds to E2F4, E2F5 in G0 phase. Rb2/p107 binds E2F4 in G1 and Rb/p105 associated with E2F4 in S phase(18). Other repressors: E2F6-8 seems to exist without pocket protein interaction domain (9).

Activator E2Fs bind to pRB in quiescent cells. Mitogen stimulus promote phosphorylation of pRb by cyclin D/cdk4,6 which prevents binding pRB from E2Fs. Genes repressed by pRb-E2F complexes are derepressed and released E2Fs activate expression of genes which encode proteins involved in DNA replication and cell cycle progression such as DNA polymerases, cyclin E e.g. Overexpression of E2F1-3a induces quiescent immortalized cells to enter S phase.

Different activator E2Fs has different roles. E2F1 and E2F3 are required for cell cycle entry but only E2F3 is required for continued cell proliferation. Combined loss of E2F1-3 discontinues cell cycle proliferate – exhibit activation of p53 (21).

There is also a different E2Fs expression during cell cycle. E2F3 level increase in early and mid-G1 meanwhile E2F1, E2F2 increase in G1/S state. E2F4 together with E2F5 are expressed along the cell cycle (9).

E2F1 can induce or inhibit apoptosis depending on cell type, development stage and apoptotic stimulus. When normal cell is stimulates to grow, E2F1 activity is required for initial entry into the cell cycle meanwhile E2F1-induced apoptosis is blocked (21).

E2F1 induces $p14^{ARF}$ an alternate reading frame protein encoded by p16 (INK4a). This protein represses Hdm2 following p53 block. Hdm2 is negative regulator of transcription and catalyze p53 ubiquitination and degradation (15).

E2F1 is the most investigated factor from E2F family. E2F1 has dual fiction – stimulating proliferation or inducing apoptosis. Its activity is controlled during the cell cycle and is regulated by numerous of post-translational modification as described in *Figure 4.* The inhibition of E2F1 activity occurs through interaction with pRb. The consequence is G1 arrest.

E2F1 underlies phosphorylation, acetylation, methylation and ubiquitinationas shown in *Figure 3*. In the case of phosphorylation is E2F1 protect from interaction with pRb. E2F1 is positively indirectly regulated by cyclin D and cyclin E through their interaction with pRb and negatively regulated with cyclin A. E2F1 binds to cyclin A/cdk2 during S phase what allows DP1 phosphorylation. DNA damage-responsive elements phosphorylated E2F1 are ataxia telengiectasia mutated and ataxia telengiectasia mutated Rad3-related (ATM/ATR) kinases(16).

TopBP1 (DNA topoisomerase IIβbinding protein 1) binds E2F1 during DNA damage and repress E2F1-dependent apoptosis. The 14-3-3τ, family member of 14-3-3 proteins, interacts with E2F1 phosphorylated by ATM. This interaction inhibits ubiquitination and stabilizes E2F1. The 14-3-3τ is also implicated in pro-apoptotic genes expression, such as p73 and Apaf1 during DNA damage (24).

Histone acetyltransferases PCAF and p300 mediate E2F1 acetylation on domain lying close to DNA binding domain (*Figure 4*). E2F1 acetylation gives enhanced DNA binding ability (16).Both, DNA-damage caused acetylation and phosphorylation of E2F1 lead to its stabilization. E2F1 degradation is mediated by ubiquitin-proteasome pathway during S/G2 phase.

Histone methyltransferase Set9 mediates E2F1 methylation within DNA binding domain. Methylation E2F1 prevents pro-apoptotic activation and also triggers ubiquitination and subsequent degradation. Demethylation by lysine demethylase LSD1 restores E2F1 apoptotic activity *(Figure 3*) (16).

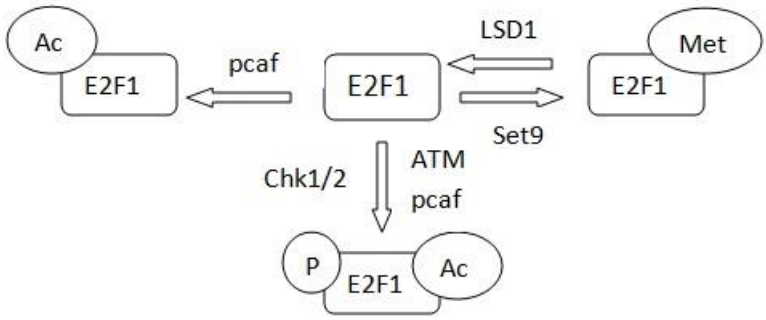


**Figure 3 E2F1 pathways** E2F1 methylation (Met) mediates Set9. This process leads to ubiquitylation following degradation. Acetylation (Ac) mediates pcaf and leads to activating transcription of genes requires for S phase enter. E2F1 acetylation through pcaf and methylation through Chk1/2, ATM lead to its stabilization and accumulation. This way are induced pro-apoptotic genes.

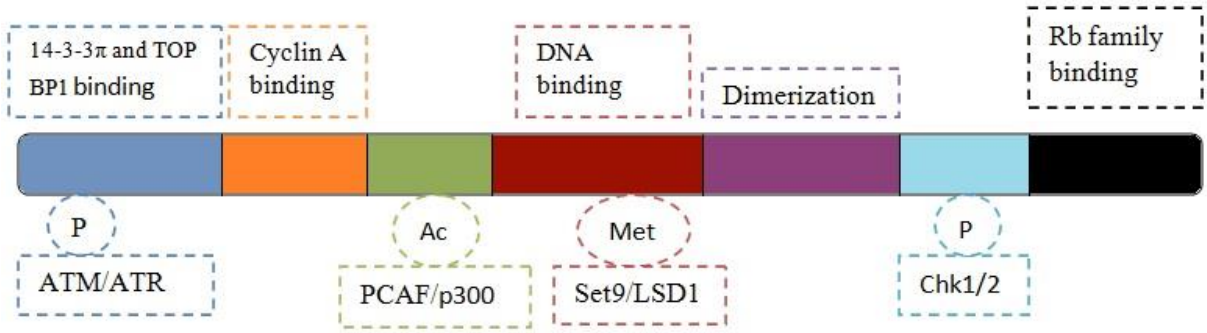


**Figure 4 Post-translational domains E2F1** Transducers ATM and ATR phosphorylate (P) E2F1, PCAF and p300 are able acetylation (Ac) of E2F1 and Set9 and LSD1 methylation (Met) of E2F1.

## 1.3 E2F1 and apoptosis

DNA damage signaling pathway is a high sophisticated response to viral infection, radiation, chemical exposure, replication errors or chemotherapeutic agents. This pathway initiates a cascade of phosphorylation, methylation or acetylation processes direct by many kinases, transducers or effectors. Key role has E2F1, ATM and ATR which transfer signals to checkpoint kinases Chk1 and Chk2 and also p53.

Other E2F- E2F3 has been shown to induce apoptosis if it is dependent to E2F1. E2F3 is able to transactivate E2F1 gene. Other E2Fs might not induce apoptosis directly. E2F1 induces apoptosis trough p53-dependent and p53-independent pathways (21).

Transcription factor p53 is encoded by gene TP53 and act as an apoptosis inductor. Its synthesis takes place in the cytoplasm where occurs accumulating during G1 phase. During G1/S transition is located in the nucleus and after beginning of S phase is returned back to cytoplasm (25).

### 1.3.1 Initiation of apoptosis

Activation of p53 by E2F1 occurs due to its phosphorylation and stabilization by DNA damage sensors. Except E2F1 all activator E2Fs are able to directly bind and stabilize p53 through cyclinA binding domain. Cyclin A commonly prevents E2F1 from interacting with p53. Meanwhile in response to DNA damage, levels of cyclinA decrease simultaneously with increase E2F1-p53 complex.

When DNA is damaged protein kinase ATM become activated and checkpoint kinase Chk2 activate and stabilize p53 transcription factor. One of the possibilities how to arrest cell cycle in checkpoints is through the CIP/KIP p21. Its way leads through inhibition cyclin E/cdk2 or cyclin A/cdk2. Following, pRb inhibits E2 factor release and mediate G1/S arrest. The second opportunity is inhibition of cyclin D/cdk4, 6 through INK4a – p16. Again, E2 factor is blocked and cell cycle is arrested in G1. At both case checkpoint arrest offers DNA repair or cell death – apoptosis (*Figure 5*) (26).

Another kinase – ATR activate Chk1 what initiates CDC25 C and CDC25 A. CDC25 C inhibits phosphorylation of cyclin B/cdk1 leading to G2/M arrest. Meanwhile, CDC25 A inhibits phosphorylation of cyclin A/cdk2 or cyclin E/cdk2 leading to G1/S checkpoint arrest (*Figure 5*).Additional, phosphorylation of CDC25 A leads to its ubiquitination and degradation (27).

Defects of p53 signaling pathways called genotoxic signaling pathway lead to malignant tumor (28).Therefore, ATM is activated predominantly by double-strand breaks by ionizing radiation meanwhile ATR responds to replication stress, single-strand breaks, UV radiation or stuck replication forks. After ATR activation is induce Chk1 phosphorylation mechanism. It has been shown that the E2F7 and E2F8 cooperate together to repress E2F1-dependent apoptosis (22).

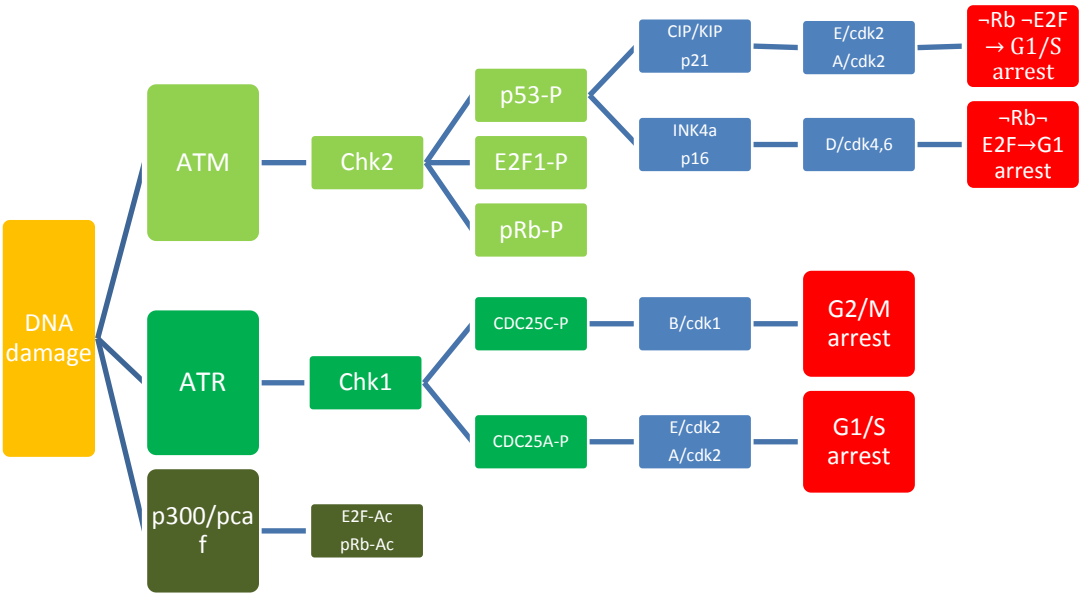


**Figure 5 Schema of DNA damage responsive pathways** After DNA damage ATM and ATR become activated and phosphorylate Chk2 and Chk1. Chk2 is able to phosphorylate p53, E2F1 or pRb (shows as P in schema). p53 initiates G1/S arrest through p21 which inhibits phosphorylation Rb by cyclinE/cdk2 or A/cdk2. UnphosphorylatedRb inhibits E2F release and thus G1/S transition. p53 can also mediates G1 arrest through p16 inhibition. Following, phosphorylation of Rb by D/cdk4, or cdk6 is inhibited. Unreleased E2F arrest cell cycle in G1. Chk1 phosphorylates CDC25 C and CDC25 A (shows as p in schema). These mediators inhibitscyclincyclin B/cdk1 leading to G2/M arrest; A/cdk2 or cyclin E/cdk2 leading to G1/S arrest. E2F acetylation (shows as Ac in schema) by p300/pcaf increase DNA-binding ability. DNA damage-response acetylation of pRb releases E2F1.

E2F1 switches on an expression of pro-apoptotic co-factors of p53 – ASPP1 and ASPP2 (apoptosis stimulating protein of p53). These co-factors influence p53 to activate pro-apoptotic genes and induce apoptosis. Moreover, E2F1 may bind p53 which stimulate its apoptotic function in response to DNA damage. This interaction between E2F1 and p53 is independent of E2F1 transcription activity (22).

E2F1 can also induce apoptosis in the absence of p53 by pro-apoptotic genes including Apaf1 (apoptotic protease activating factor 1), caspases, family member p53 – p73 etc. (21)

E2F1 was shown to activate transcription of p73 which stimulates gene expression of those genes common with E2F1 and p53. That mechanism constitutes cooperating p53, p73 and E2F1 which result in maximum induction of cell death (22).

### 1.3.2 "Activators" E2F's role in apoptosis

Recently studies revealed E2F3a as a substrate of the checkpoint Chk1 and Chk2. Both, E2F3a and E2F3b are identical except that the E2F3a protein contains chk ability permitting phosphorylation by DNA damage. Furthermore, E2F1 and E2F2 are transcriptionally induced upon DNA damage in an E2F3-dependent manner. Therefore, there is interdependency between E2F1 and E2F3 in activating each other after genotoxic stress. It appears that all three activator E2Fs are upregulated as a part of the DNA damage response machinery. E2F1 activity is the determinant of whether cells will undergo apoptosis. (22)

### 1.3.3 pRB's role in E2F1-mediated genotoxic stress

Defects of Rb tumor suppressor gene have been identified in almost all human tumors because it results in deregulation E2F and uncontrolled cell proliferation.

pRb's activity is dependent upon its ability to directly bind E2F members in dephosphorylated state and prevent them from promoting transcription of their target genes. Inhibition occurs through binding E2F transactivation domain within pRB pocket domain results in pRB-E2F complex. In the case of E2F1, pRB also binds the transcription factor- this secondary binding site within pRB inhibits E2F1-induced apoptosis (22).

Binding of E2F1 to this site is lost after DNA damage thus E2F1 is released without affecting the binding of pRB o other E2Fs. (21)

This domain become acetylated after DNA damage what results in release of E2F1 without influence the binding of pRB to other E2Fs. Consequence of DNA damage-induced acetylation of pRB, E2F1 can accomplish its tumor suppressor function by inducing the expression of apoptotic target genes. DNA damage cause not only dephosphorylation of pRB at cdk phosphorylation sites and linkage pRB to E2F, but also pRB phosphorylation by Chk1/2 in an ATM-dependent manner. This DNA damage phosphorylation is not associated with disruption of Rb-E2F1 (E2F2-3) complex but assumed that pRB is inactive by phosphorylation and its dephosphorylation restores pRB to active state. pRB seems to lose ability after DNA damage(22).

The mechanism of acetylation proceeds in following manner. E2F1 site is occupied with DP1-E2F1-pRb complex and histones. Linking HMGA2 to pRb triggers displacement HDAC1 from E2F1-target promoters (*Figure 6)*.Histone acetylation by pcaf/p300 activates transcription factors. Displacement of HDAC1 from pRb recruit histone acetyl transferase to the E2F1.Thus after histone acetylation occurs E2F1 acetylation equally phosphorylated pRb and HMGA2 are displaced *(Figure 7*) (29).

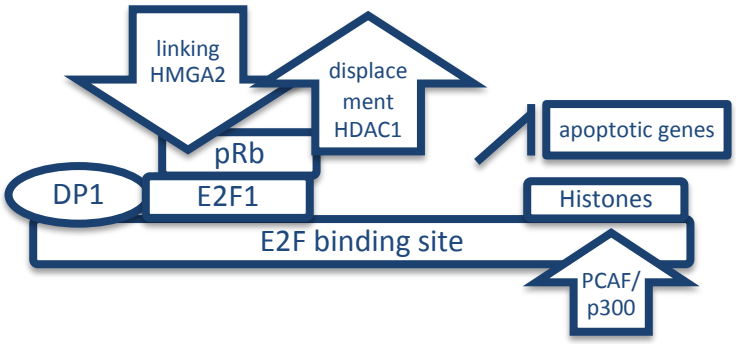


**Figure 6 E2F1 activation by HMGA2** HMGA2 binds to pRb which creates complex with E2F1 and HDAC1. After this interaction is HDAC1 displaced. Activation of apoptotic genes is blocked

Upon DNA damage pRb becomes phosphorylated between pocket domain A and B leading to stable pRB-E2F1-3 complex that inhibits E2F-dependent gene expression. Acetylation of E2F1 binding domain lead to the release of E2F1 without affecting E2F2-3 and free E2F1 transactivate apoptotic target genes.

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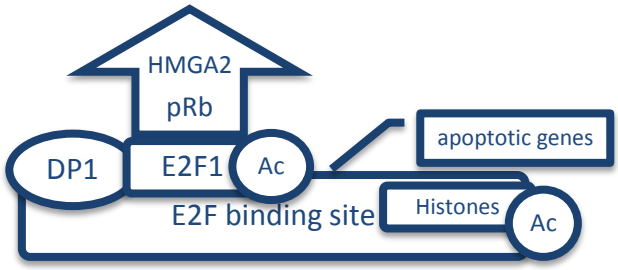


**Figure 7 Transactivation of apoptotic target genes** Histones are acetylated by pcaf/p300. Histone acetylase acetylate E2F1 what lead to stabilization of E2F1 itself and triggers apoptotic genes

## 1.3.4 E2F1 – not only apoptosis

E2F1 is also implicated in DNA damage-induce cell death– autophagy. (21). Autophagy is a cellular catabolic state, which mediates degradation of cytosolic proteins and organelles in specific lysozomes in response to low stage of nutrient, growth factors or oxygen. This degradation is used as a source of energy following cell survival (28). E2F1 has a key role in DNA damage-induced apoptosis as mentioned in 1.3. Therefore E2F1 may be hypothetical implicated in DNA damage- induced autophagy by transcription of autophagy genes (30).

#### *1.3.4.1 Positive-feedback in the Rb-E2F pathway*

E2F activates expression of cyclinE which together with cdk2 phosphorylate pRB thereby E2F is released from pRB grip. E2F-cyclin E-pRB interactions constitute a positive feedback circle. Expression of E2F1-3a is E2F- regulated constitutes another positive-feedback circle and culminate in G1 and early S phase (21). Since E2F1 transcriptionally regulates expression of these kinases, DNA damage triggers positive-feedback circle between E2F1 and ATM/Chk2(22).

#### *1.3.4.2 Negative-feedback in the Rb-E2F pathway*

Activity of activators E2Fs is influenced by cyclin A which is itself E2F regulated gene. Cyclin A in complex with cdk2 binds to E2F1-3a during S phase, leading to phosphorylating their dimerization partner (DP) and inhibition of E2F-DP DNA binding activity.E2Fs also regulate expression of Rb, Arf, p21,p27, which might function in negative-feedback circle that limit E2F level of transcription. Arf directs degradation of activator E2Fs, pRB and CDKi limit the activity of activator E2Fs.

# Experimental part

## 2 Materials and methods

## 2.1 Materials

### 2.1.1 Devices

Devices used during the experiments were: Multidropcombi (Thermo Scientific), Echo 550 liquid handler 500 series (LABCYTE), Vi-cell XR(Beckman coulter), microscopeOlympus IX81, flowbox (Thermo Scientific), automatical pipettes (Eppendorf), centrifuge (Eppendorf), Spectrophotometer (Envision)

### 2.1.2 Reagents

Chemicals used during experiments were: phosphate buffered saline (PBS), Tryptiline, dimethylsulfoxid (DMSO), Glutamine, EMEM (Eagle's Minimum Essentials Medium), fetal bovine serum (FBS), sodium salt XTT, Triton X100 (SIGMA-ALDRICH), Prionex (SIGMA-ALDRICH), Antifoam (SIGMA-ALDRICH), Tween 20 (SIGMA-ALDRICH), NP40 (SIGMA-ALDRICH), Lysis assay buffer 10ml (Promega), Lysis assay Substrate (Promega), Lopac Pfizer (SIGMA-ALDRICH)

## 2.2 Methods

### 2.2.1 Cell line

293H cell line goes from the human embryonic kidney (HEK) 293 cell line. HEK 293 are easy-to-transfect cells widely use an in vitro system for experiments. For the experiment three types of adherent cell line were used: 293H_5, 293H_9, 293H_24 prepared by Cignal Lenti E2F Reporter assay. Lentiviral particals were transduced to cell types for monitoring E2F-mediated pathway activity (31). Lentivirus is one of the viral vectors, more precisely subtype of retroviruses. They are used as a delivery system of genes to nondividing and dividing cells (32).

After Cignal Lenti Pathway Reporter transduction cells were cultured with a puromycin. This antibiotic inhibits protein synthesis through early chain termination during translation by peptide transferaze blocking. Selection of puromycin resistant cells leads to stably transduced cell line (33). Lentiviral particals express a transcription of luciferase reporter gene.

### 2.2.2 Maitenance of cell cuture

Cell cultures are basic materials for defined cell lines experiments isolated from pathological or physiological tissue. Their cultivation is based on keeping in characterized medium containing nutrients, which should mimic the environment in organism. Cell cultures are kept and grow in various tissue culture flask in incubator.

#### *2.2.2.1 Conditions for cultivation*

Cultivation in vitro is not exactly the same as in human organism. Cells cannot communicate with other cell types and gene expression is changed. Working with cells requires sterile conditions so we use laminar flow box.

For cell survival and proliferation we have to keep right conditions. Most of cell lines grow on flask's surface – we called them adherent. After specific time the cell expanse on whole surface and create monolayer. Their density rise up, nutrients in medium run down so we have to use a method called passage for density reduction.

Incubating proceeds in conditions: 37°C;5% $CO_2$; 19,7% $O_2$ and define humidity. Also it is important to avoid contamination with bacteria. Optimal environment for cell culturing allow cultivation medium comprises of fetal bovine serum.The reason of its using is present of growth factors, which support cell proliferation. Then antibiotics - preferentially is used penicillin and streptomycin. Generally, antibiotics are used for avoid contamination, glucose, amino acids, stabilizators of pH ($CO_3^{2-}$/$HCO_3^-$), fatty acids, lipids and phenol red. This dye indicates nutrition and pH state of culturing cells. In optimal conditions medium is red but during pH decrease and nutrient depletion becomes pink even orange.

#### *2.2.2.2 Passaging*

When cells create monolayer and expand to whole cultivation flask bottom, the space for next dividing is missing, so we need to passage. This method keeps cells in appropriate conditions for experiments.

We take fresh medium stored in cold room, FBS from freezer, antibiotics and streptomycin. Mostly medium already contain L-glutamin. After all components reach laboratory temperature, antibiotics (penicillin 250µl), streptomycin (2,5ml), FBS (50ml) are added to medium and filter. Then cultivation medium is kept in the fridge.

Passaging cells is following: medium empty out and cells washed by PBS along sidewallsof the flask. PBS is used for washing cells from FBS. Subsequently, tryptilineis added because of cells bottom release and cultivation flask put into incubator approximately 5 minutes. New medium is added and part of amount put to new flask and to incubator. After centrifuge (1200 speed, 5min) medium empty out and new medium free of tryptiline is added.

Cells after passage start to attach the bottom of flask and divide. Passaging is not everlasting. Cultivation is limited depending on cell line.

#### *2.2.2.3 Counting cells*

Important operation is cell counting not only for culturing but also for experiments. We use Bürker chamber cell counter or solution of trypan blue (34).

We sample 0,5ml cells to small tube and put to the device Vi-cell XR (Beckman coulter). Counting began by reaction with trypan blue. The software offers information about cell count- viable cells and total cells.

#### *2.2.2.4 Freezing cells*

Cultivation cells are possible to freeze in deep freeze fridge (-80°C). Since cell culture may be destroyed or contaminated this advantage gives opportunity work with new cell line.

Consist of freezing medium depends on the cell line. We prepare this medium by mixing 1ml 10% FBS with 1ml 10% DMSO and 8ml medium. This solution is added to passage and centrifuge (1200 speed, 5min) cells and keep in freezing vials (35).

Into cultivation medium we add DMSO so that cells will not be destroy by water crystals. Freezing process should be slower on the other hand working during de-freezing should be very fast (34).

De-freeze cells from freezing vials put to cultivation tube with medium and centrifuge. Staying medium is emptied out and new medium is added to pellet of cells. After shuffle is medium with cells pipetting to cultivation flask.

#### *2.2.2.5 Seeding cells*

Since experiments are done on any type of microplateit is an advantage to use high throughput dispense of solutions we tested. The device for seeding cells to platesis Thermo Scientific MultidropCombi (*Figure 8)*. We are able to set parameters for different types of microplates (6 - 1536 wells) and dispensing (0,5-2500µl). At the first we insert standard cassette and rinse plastic tubes by ethanol pressing button PRIME. Itis necessary to think about sterile background. Storing end of small tubes put into bottle with medium and cells. After setting parameters for filling up we can start seeding by press START button(36).

**Figure 8 Thermo Scientific MultidropCombi**
(**Passed**http://www.thermoscientific.com/content/dam/tfs/LPG/LCD/LCD%20Product%20Images/Liquid%20Handling%20and%20Dispensing/F62197~p.eps/jcr:content/renditions/cq5dam.thumbnail.450.450.png 20.3.2015)

## 2.2.3 Compound dispension

Compounds used in this work were dispensedwith the aid ofEcho 550 liquid handler 500 series with Software Echo Cherry Pick. Pharmacologically active compounds are transported from source plate to the destination plate (already fill up with verifying cells). This transmission is based on acoustic droplet ejection uses sound energy. Droplet of precisely volume is moved to define wells on destination plate(37).

## 2.2.4 LOPAC library

Library of Pharmacologically Active Compounds is used for high-throughput screening assay.Lopac library is a collection of pharmacologically active compounds of high purity consists of more than 25 commercial drugs and more than 60 highly characterized literature compounds. Biological active compounds pre-solubilized at 10mM in DMSO comprise inhibitors, receptors ligands, drugs impact signaling pathways.

## 2.2.5 Luciferase assay

### *2.2.5.1 Principle*

Luciferase reporter gene is commonly used in biomedical researchto study gene expression. Luciferase is an enzyme generating light through the release chemical energy of oxidative decarboxylation substrate luciferin to oxoluciferin. Luciferase uses adenosine triphosphate (ATP)-$Mg^{2+}$(*Figure9*). After enzyme and substrate combined, the light is rapidly generated. The assay requires only few seconds per sample and due to highest efficiency of chemiluminescent reaction is very sensitive. For increased light intensity is incorporated coenzyme A. Luciferase assay system includes one vial of lyophilized luciferase assay substrate, 10ml of luciferase assay buffer and 30ml luciferase assay cell culture lysis reagent 5x concentrated. Both, luciferase assay buffer and substrate should be stored in -20°C meanwhile lysis buffer at room temperature. Luciferase assay reagent is prepared by adding luciferase assay buffer to the vial with luciferase assay substrate already kept at room temperature (38).


**Figure9Bioluminiscent reaction catalyzed by enzyme Luciferase**
**(**Passedhttp://www.promega.com/~/media/images/resources/figures/1300-1399/1399ma03_6a.jpg?la=en 2.5.2015)

#### *2.2.5.2 Promega luciferase assay protocol*

Growth medium is removed from cultured cells, following cells are rinsed by PBS once. This step should be done carefully so cells are not dislodged from the bottom of the plate. As muchas possible volume of PBS is removed. 96 well plate is filled 20µl/well with lysis buffer (5xconcentrated). Consequently, the plate is filled with luciferase assay reagent 20µl/well and light production is measured.

Promega protocol requires few steps. That is not suitable for HTS so assay was adapted by exchange lysis buffer for solution consist of 100 µl triton X100, 100 µl tween20, 100 µl NP40, 200 µl prionex, 200 µl antifoam in luciferase kit (10ml luciferase assay buffer in luciferase assay substrate). Subsequently, 20µl of this solution was added to each of well and luminescence was measured.Protocol

Luciferase assay was adapted for HTS by exchange Lysis buffer for mixture of 100 µl triton X100, 100 µl tween20, 100 µl NP40, 200 µl prionex, 200 µl antifoam in luciferase kit (10ml luciferase assay buffer in luciferase assay substrate). Subsequently, 20µl of this solution was added to each of well and luminescence was measured.NP40 and Triton X100 act as non-ionic surfactants. TWEEN 20 is a non-ionic detergent used as an emulsifying reagent for the preparation of stable oil-in-water emulsions. Prionexis protein stabilizingreagent. Antifoam is a silicone-based product used to prevent or minimize foaming(39).

### 2.2.6 XTT assay

#### *2.2.6.1 Principle*

The XTT assay is based on reduction of yellow salt XTT (*Figure 10*) to orange Formazan dye, which occurs only in active cells. Formazan is water soluble and directly quantified spectrophotometrically. In living cells the reaction is catalyzed by mitochondrial dehydrogenases. Following, with increasing number of living cells increases activity of mitochondrial dehydrogenases what correlate to amount of formazan orange salt.

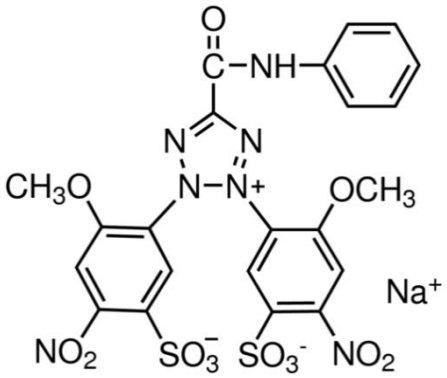


**Figure 10 XTT** (Passed http://www.sigmaaldrich.com/content/dam/sigma-aldrich/structure4/104/mfcd00083517.eps/_jcr_content/renditions/mfcd00083517-large.png 2.5.2015)

This method became widely used due to its sensitiveness and rapidness also for high-throughput assays.

XTT reagent is stored at -20°C but for experiment is heating to 37°C necessary. Solution of XTT is provided at a 3x concentration. It was used 0,9mg/ml concentration in RPMI – medium without phenol red. Immediately before use is XTT activator added. This activator is also stored at -20°C. Moreover both, XTT reagent and XTT activator must be kept at dark.

XTT activator – PMS is added for efficient reduction of XTT as an electron coupling reagent. We use 100µl to 5ml of XTT reagent. XTT working solution is added to cells few minutes after preparation.

#### *2.2.6.2 XTT assay protocol*

First day cells 293H_24 were seeded by Multidropcombi (Thermo Scientific) to 384 well plate format in concentration 2000 cells per welland for 24 hours kept in incubator. After appointed time drugs from LOPAC Pfizer was added in different concentrations for 72 hours. XTT working solutionwas added after three days cultivation. Before own signal measure plates were spun downin centrifuge, following orange formazansolution was formed. Quantification of formazan was spektrophotometrically quantified on Spectrophotometer (Envision)

# 3 Results

## 3.1.1 Validation of 293H clones transfected with E2F-luciferase response element

Cell line 293H was prepared by Mgr. Jiří Řehulka at Ústavmolekulární a translační medicíny při Lékařské Fakultě Univerzity Palackého v Olomouci. Three clones 293H_5, 293H_9 and 293H_24 were selected as resistant to antibiotics. Those clones were followed up to validate which of 293H clones has the best properties in E2F-luciferase response element expression. Subsequently, the best clone was used for next experiments.

All three clones of 293H: 293H 5, 293H 9, 293H 24 were plated to 96 well plate format in concentration 20 000 cells per well in medium EMEM with 10% FBS and kept in incubator. This condition was changed after 24 hours. Cells get rid of staying 10% medium, washed by PBS and incubated in 0, 5% medium for next 24 hours *(Table 1).* Subsequently, medium was aspirated again, cells washed by PBS and conditions arranged for medium with different concentration (*Table 2*). After 6 hours of incubation was medium supplemented with 100µl mixture of 100 µl triton X100, 100 µl tween20, 100 µl NP40, 200 µl prionex, 200 µl antifoam in luciferase kit (10ml luciferase assay buffer in luciferase assay substrate) was added. Following, luminescence was measured. This assay was repeated three times for each clone.

| | 293H_5 | | | 293H_9 | | | 293H_24 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | | |
| | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | | |
| | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | | |
| | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | | |

**Table 1 Experimental condition for validation of 3 clones of 293H cell line**

| | 293H_5 | | | 293H_9 | | | 293H_24 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | 0.5% | | |
| | 1% | 1% | 1% | 1% | 1% | 1% | 1% | 1% | 1% | | |
| | 5% | 5% | 5% | 5% | 5% | 5% | 5% | 5% | 5% | | |
| | 10% | 10% | 10% | 10% | 10% | 10% | 10% | 10% | 10% | | |

**Table 2 Assay layout for 293H clone**

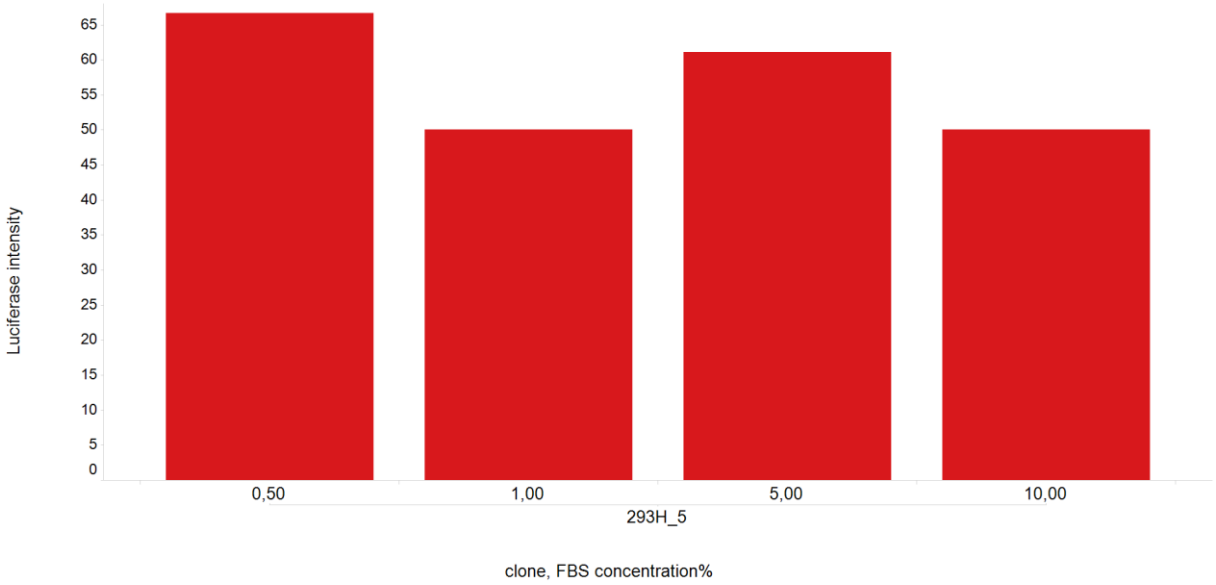


**Figure 11 Validation of 293H_5 clone**

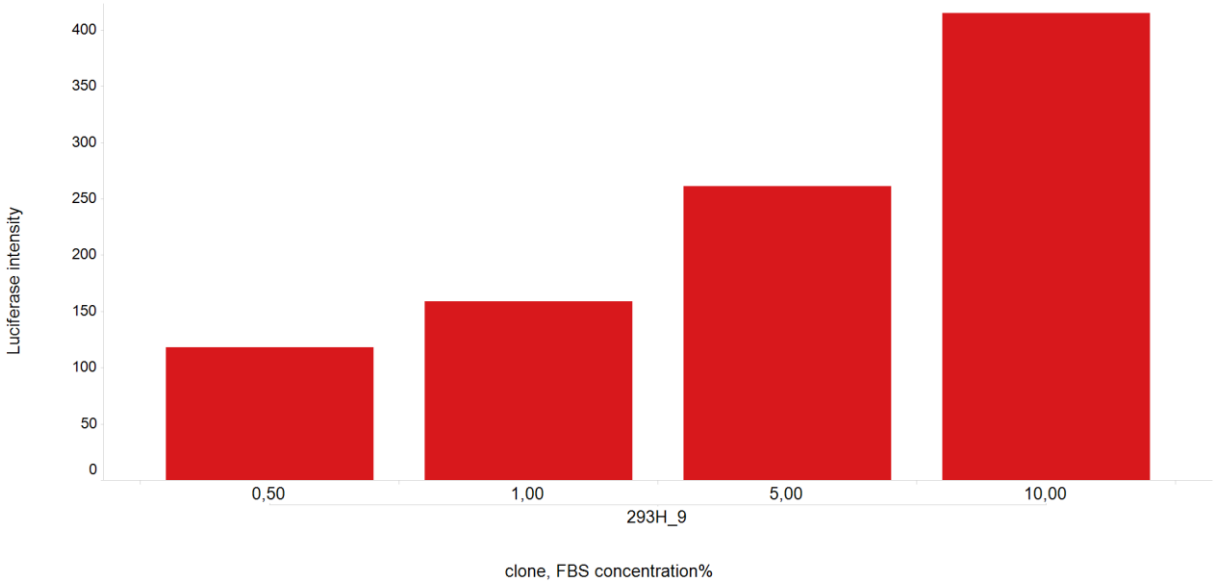


**Figure 12 Validation of 293H_9 clone**

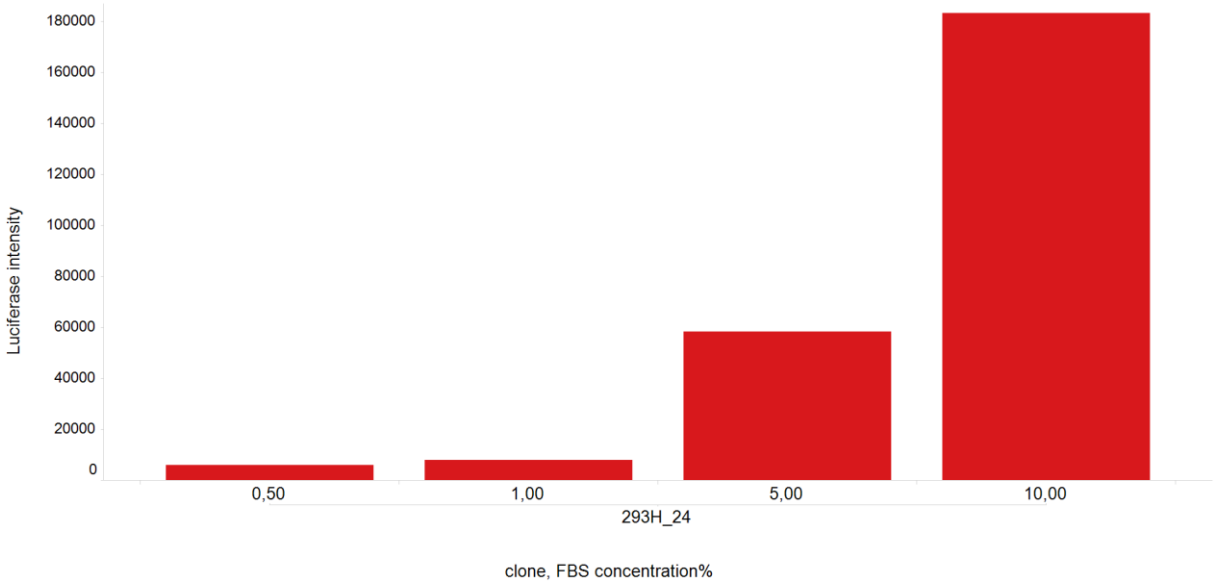


**Figure 13 Validation of 293H_24 clone**

Principle of this method is measuring light emission of enzyme luciferase that is characteristic for each cell line we used.According to the*Figure 13*the highest luminescence is noticed by 293H_24 cell clone. Simultaneously with increasing FBS concentration% increases signal of luminescence. The most considerable signal is presented by 293H_24 cells in 10% medium.So, increase concentration of FBS, which stimulate cell division by growth factors results in directly increase luciferase intensity.

Transduction of plasmid with E2F to cell clone 293H_5 genom was not successful as show *Figure 11.* Intensity of luciferase show very low values. This clone was not able to use in next experiments.

Clone 293H_9 in compare to 293H_5 embodies better luminescence signal as show *Figure 12.* With growing FBS concentration simultaneously grows luciferase intensity. On the other hand, in comparism 293H_9 to 293H_24 is this signal still low.

On the base of these results was 293H_24 clone used for next experiments due to its properties in stably transfected transduction of E2F.

### 3.1.2 The effect of Lopac library compounds on survival of 293H cells

XTT assay is used for cell viability detection. In viable cells mitochondrial enzyme dehydrogenase catalyzes reduction of yellow XTT to orange soluble formazan dye spectrophotometrically quantified.

Cells 293H_24 were in CellCarrier 384 well plate andincubated 24 hours. After appointed time compounds from Lopac library was addedin renge of concentration from 100 uM to 0.002 uMfor 72 hours. After this period_XTT working solution was added, plate was incubared for 3 hoursand absorbance was measured.

This method was used for detection of cell viability with 90 pharmacologically active compounds from LOPAC Pfizer libraryResults were exported to Dotmaticsdatabase which automatically analyzes curves and IC50 values. Based on curve fitting 29active compounds from 90 tested compounds were picked up*Figure 14*.

IC50 values indicate concentration of compounds which cause cell death in 50%. Compounds are the most active with low IC50 –cause cell death. Meanwhile high IC50 indicate poor ability to cause cell death.

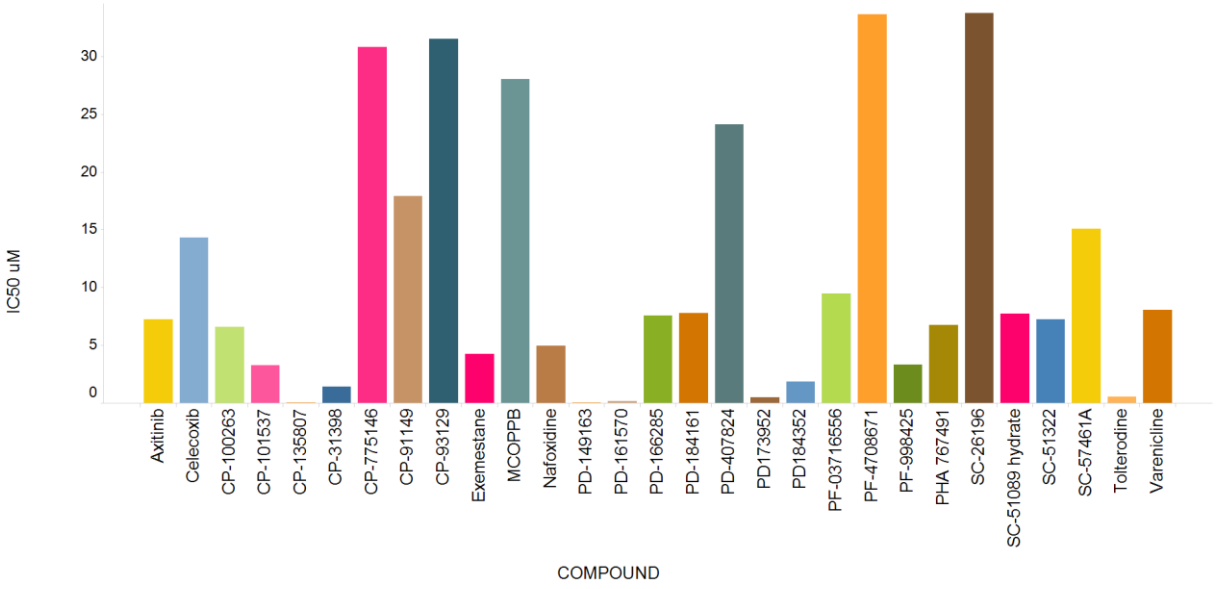


**Figure 14 Effect of LOPAC compounds to 239H_24**

Compounds with 30uM concentration are considered as deactive due to their very high IC50 values. The best activity have compounds under 10uM, better under 5 uM.

The most active compounds are:

CP-135807 with IC50 0,03

This compound is classified as neurotransmitter. This drug binds to 5-humanT1D receptor and act as serotonine agonist.

PD-161570 with IC50 0,14

This drug act as inhibitor of the human enzyme fibroblast growth factor 1, FGF-1, because inhibits tyrosine kinase activity.

PD-173952 with IC50 0,51

Src family kinase inhibitor.

CP-31398 with IC50 1,39

This drug is connected to cell cycle, respectively apoptosis. In tumor cells is mutation of p53 detacable in 50%. Its main target is p53 stabilization. Precisely, CP-31398 regenerates p53 tumor suppressor function in cancer cells.

Tolterodine with IC50 0,55

Tolterodine acts on muscarinic cholinergic receptors. This drug is already used for treat overactive bladder and is commercial available as Detrol®.

| COMPOUND | MOL WEIGHT | CLASS | TARGET | ACTION | SELECTIVITY | DESCRIPTION | IC50 |
|---|---|---|---|---|---|---|---|
| CP-101537 | 198,26 | Metalloproteinase | Enzyme | Inhibitor | MMP | Non selective matrix metalloproteinase (MMP) inhibitor. | 3,28 |
| CP-135807 | 351,4 | Neurotransmitter | Receptor | Agonist | 5-HT1D | Selective 5-HT1D serotonin receptor agonist. | 0,03 |
| SC-51089 hydrate | 459,33 | Lipid Signaling | Receptor | Antagonist | EP1 | Selective EP1 prostanoid receptor antagonist. | 7,71 |
| PD-149163 | 943,91 | Neurotransmitter | Receptor | Agonist | NTR1 | Neurotensin (8-13) analog; Neurotensin NTR1 receptor agonist. Brain penetrating with pro-cognitive, antipsychotic and anxiolytic effects. | 0,00 |
| PD-161570 | 532,51 | Tyrosine Kinase | Enzyme | Inhibitor | FGFR-1 | Inhibits FGF-1 receptor tyrosine kinase activity. | 0,14 |
| PF-03716556 | 394,47 | Ion Channels | Ion Channel | Antagonist | H,K-ATPase | Potent, selective and reversible acid pump antagonist (H, K-ATPase). | 9,49 |
| SC-26196 | 423,55 | Lipid Signaling | Enzyme | Inhibitor | Delta6D | Delta6 fatty acid desaturase (Delta6D) inhibitor. Blocks conversion of linoleic acid to arachidonic acid. | 33,75 |
| Varenicline | 361,35 | Neurotransmitter | Receptor | Antagonist | nAChRa7 | Partial a4ß2 nicotinic acetylcholine receptor agonist and a7 full agonist. | 8,07 |
| SC-57461A | 363,88 | Lipid Signaling | Enzyme | Inhibitor | LTA4 | Inhibitor of leukotriene A4 hydrolase. | 15,08 |
| PD-407824 | 328,32 | Ser/Thr Kinase | Enzyme | Inhibitor | Wee1/Chk | Wee1/Chk1 inhibitor. | 24,14 |
| PHA 767491 | 213,24 | Ser/Thr Kinase | Enzyme | Inhibitor | cdc7/cdk9 | Selective ATP-competitive dual inhibitor cdc7/cdk9. Blocks DNA helicase phosphorylation. | 6,74 |
| Exemestane | 296,4 | Growth Factor | Enzyme | Inhibitor | Aromatase | Steroidal antiestrogen and irreversible aromatase inhibitor. Prevents the conversion of androgens to estrogens. | 4,25 |
| PD-184161 | 557,56 | Ser/Thr Kinase | Enzyme | Inhibitor | MEK | MEK inhibitor. | 7,79 |
| PF-4708671 | 390,41 | Ser/Thr Kinase | Enzyme | Inhibitor | S6K1 | Selective p70 ribosomal S6 kinase (S6K1) inhibitor. No activity against RSK and MSK kinases. | 33,66 |
| MCOPPB | 518,01 | Neurotransmitter | Receptor | Agonist | ORL1 | Potent non-peptide nociceptin/orphanin FQ peptide (NOP)-receptor full agonist. Potent oral anxiolytic activity in mice. | 28,04 |
| Axitinib | 386,47 | Tyrosine Kinase | Enzyme | Inhibitor | VEGFR 1, 2, 3 | Tyrosine kinase inhibitor, blocks VEGF-induced endothelial cell tube formation. | 7,23 |
| PD173952 | 482,36 | Tyrosine Kinase | Enzyme | Inhibitor | Src | Src family kinase inhibitor. | 0,51 |
| Nafoxidine | 462,02 | Gene Regulation | Receptor | Antagonist | Estrogen Receptor | Derivative of Tamoxifen. Potent estrogen receptor antagonist with anti-proliferative properties. | 4,95 |
| Celecoxib | 381,37 | Cyclooxygenase | Enzyme | Inhibitor | COX-2 | COX-2 selective inhbitor with 10-20 fold selectivity over COX-1. Antiinflammatory. | 14,31 |
| PF-998425 | 269,26 | Growth Factor | Receptor | Antagonist | Androgen receptor | Nonsteroidal androgen receptor antagonist. | 3,31 |
| PD184352 | 478,66 | Ser/Thr Kinase | Enzyme | Inhibitor | MEK | Highly selective non-competitive inhibitor of MEK (MKK1). Suppresses the ERK pathway. | 1,86 |
| Tolterodine | 475,57 | Neurotransmitter | Receptor | Antagonist | CHRM3 | Bladder-specific muscarinic cholinergic receptor antagonist. | 0,56 |
| CP-93129 | 288,17 | Neurotransmitter | Receptor | Agonist | 5-HT1B | Potent and selective 5-HT1B agonist. | 31,53 |
| CP-31398 | 435,39 | Apoptosis Cell Cycle | Tumor suppressor | Stabilizer | p53 | p53 stabilizer; apoptosis inducer. | 1,40 |
| SC-51322 | 457,93 | Lipid Signaling | Receptor | Antagonist | EP2 | Selective EP1 prostanoid receptor antagonist. | 7,24 |
| PD-166285 | 585,35 | Tyrosine Kinase | Enzyme | Inhibitor | Src, FGFR | Broad spectrum protein tyrosine kinase inhibitor. | 7,57 |
| CP-775146 | 423,54 | Lipid Signaling | Receptor | Agonist | PPARa | Potent and selective PPARa agonist. | 30,83 |
| CP-91149 | 399,87 | Phosphorylase | Enzyme | Inhibitor | glycogen phosphorylase | Selective glycogen phosphorylase inhibitor. | 17,92 |
| CP-100263 | 369,33 | Neuropeptides | Receptor | Antagonist | NK-1 | Neurokinin receptor antagonist. | 6,58 |

**Table 3 Validated LOPAC Pfizer active compounds**

### 3.1.3 The effect of Lopac library compounds on E2F- mediated gene expression

The goal of this experiment was to find out which of biological active compounds from the LOPAC library enable activation or inhibition of E2F expression.

For HTS the 293H_24 cells were seeded by Multidropcombi (Thermo Scientific) to 384 white OptiPlatein concentration 2000 cells per well. Incubation period took 24hours. After appointed time Echo 550 liquid handler 500 was used to transfer LOPAC Pfizer compounds from source plate in defined volume 10µM to destination plate with cells and incubate 72 hours. Third day plates for 6 hours were incubated with20µl luciferase assay reagent suplemented with triton X100, tween20, NP40, prionex and antifoam. Plates were spun down and luminescence was measured on spectrophotometer.

Results are described in *Figure15*. The highest E2F expression of 293H_24 cells was detected in compound Neflinavir at 4946373,33 luminescence value. PD-180970 also embodies high E2F expression at 4847013,33luminiscence value. Comparatively high luminescence values indicate Bosutinib, PD-161570 and PD-407824.

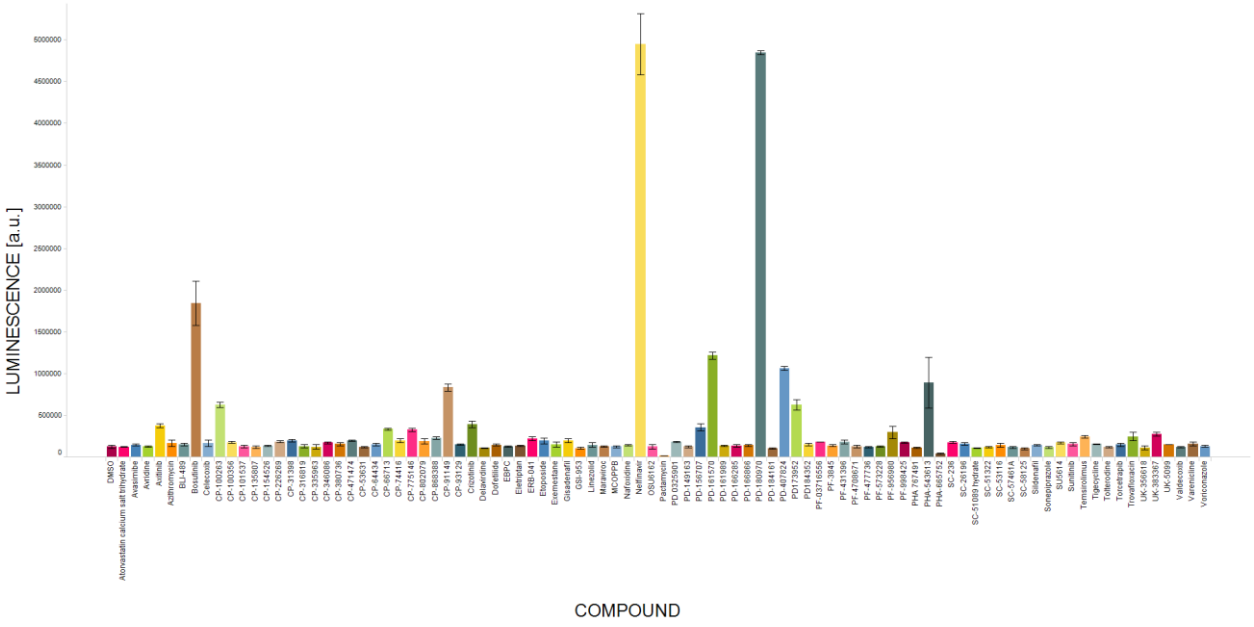


**Figure15 LOPAC compounds affecting E2F expression of 293H_24 cells**

DMSO serves as control compound. Its luminescence value is 122431a.u. It were compared compounds with higher luminescence value. The highest lumiscence embodied Neflinavir (4946373,33a.u.) and PD-180970 (4847013,33a.u.).Nelfinavir is protease inhibitor. This drug used for treating viral infections with the human immunodeficiency virus HIV. PD-180970 has been shown as inhibitor of Bcr-Abl. This drug acts as inducor of apoptosis in Bcr-Abl-expressing leukemic cells. Also serves as treatment for chronic myelogenous leukemia. Very high luminiscence values of these two compounds and lack of cell cytotoxicity cused by thsees compounds may indicate E2F-independent cell death.Luminiscence of compound PD-161570 is higher than DMSO control value 1214773a.u. and with compare to its low IC50 – 0,13 (Figure 14) E2F is assumedto be involved in cell death.Luminiscence of compound CP-100263 is 624200 and with compare to its IC50 – 6, 5 E2F is also assumed to be involved in cell death. Luminiscence of compound PD-173952 is 623653 and with compare to its IC50 – 0, 5 E2F is also assumed to be involved in cell death.

Similar but still higher luminescence of DMSO embodied compounds: CP-31398 (194547 a.u.), PD-184352 (149333 a.u.) and PF-998425 (169893 a.u.). In compare with their IC50 values: CP-31398 (1, 3), PD-184352 (1, 8) and PF-998425 (3, 3) is also assumed that E2F may influence cell death in these compounds.

However, results of of this experimentmay be biased due to lack ofcontrol showing how LOPAC Pfizer compounds influence only luciferase activity.

# 4 Discussion and Conclusion

First goal was validate cell line 293H stably transfected with luciferase reporter gene for monitoring E2F-mediated pathway activity. Depending on luminescence measuring cell clone 293H_24 was considered has optimal properties for next high-troughput screening test (section 3.1) .

Luciferase assay was optimalized for HTS. Lysis buffer was exchanged for mixture of triton X100, tween20, NP40, prionex, antifoam in luciferase kit (10ml luciferase assay buffer in luciferase assay substrate) to make homogenous assay. Assay became cheaper and amount of reagents were reduced in this manner.

Consequently, compounds of LOPAC Pfizer library were tested to investigate cell survival (section 3.2).29 compounds were validated as active based on dose response curve fitting. Next all lopaccompounedswere tested for their effect on E2F-mediated gene expression (3.3). The highest E2F gene expression exhibit compounds Neflinavir and PD-180970 which should be hence investigated but not for E2F-dependent cell death.

Compounds were compared to DMSO as control luminescence value. Higher luminescence than DMSO was shown: PD-161570 - compare to its low IC50 – 0,13; CP-100263 - compare to its IC50 – 6, 5; PD-173952 - compare to its IC50 – 0, 5 E2F is also assumed to be involved in cell death.

Next compounds: CP-31398 (194547 a.u.), PD-184352 (149333 a.u.) and PF-998425 (169893 a.u.) in compare with their IC50 values: CP-31398 (1, 3), PD-184352 (1, 8) and PF-998425 (3, 3) is also assumed that E2F may influence cell death in these compounds.

Summarising these data may suggest possible mechanism involving E2F-mediated gene expression in cell death.

## Seznamzkratek:

| | |
|---|---|
| Apaf1 | Aapoptotic protease activating factor 1 |
| ASPP1 | Apoptosis stimulating protein of p53 1 |
| ASPP2 | Apoptosis stimulating protein of p53 2 |
| ATM | Ataxia telengiectasia mutated |
| ATP | Adenosine triphosphate |
| ATR | ATMRad3-related |
| CDK | Cyclin dependent kinases |
| CDKI | Cyclin dependent kinases inhibitor |
| Chk1 | Checkpoint kinase 1 |
| Chk2 | Checkpoint kinase 2 |
| DMSO | Dimethylsulfoxide |
| DP | Dimerization protein |
| E2F | E2 factor |
| EMEM | Eagle's Minimum Essentials Medium |
| FBS | Fetal bovine serum |
| HDAC1 | Histone deacetylase 1 |
| HMGA2 | High mobility group A |
| LOPAC | Library of of Pharmacologically Active Compounds |
| PBS | Phosphate buffered saline |
| Pcaf | K(lysine) acetyltransferase 2B |
| PMS | N-methyldibenzopyrazine methyl sulfate |
| pRB | Retinoblastoma protein |
| RPMI | Roswell Park Memorial Institute |
| XTT inner salt | 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide |
| LSD1 | lysine specific demethylase |
| TopBP1 | DNA topoisomerase IIβbinding protein 1 |

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