UNIVERSITY OF LJUBLJANA
FACULTY OF PHARMACY

NIKOLA DOMINKOVIĆ

MASTER'S THESIS

UNIFORM MASTER'S STUDY OF PHARMACY

Ljubljana, 2015

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NIKOLA DOMINKOVIĆ

THE INFLUENCE OF Hsp90 SILENCING ON GALECTIN-3 EXPRESSION

VPLIV UTIŠANJA IZRAŽANJA PROTEINA Hsp90 NA IZRAŽANJE PROTEINA GALEKTIN-3

UNIFORM MASTER'S STUDY OF PHARMACY

Ljubljana, 2015

Magistrsko nalogo sem opravljal na Fakulteti za farmacijo Univerze v Ljubljani in Farmacevtsko-biokemijski fakulteti Univerze v Zagrebu pod mentorstvom prof. dr. Janja Marc, mag. farm. ter prof. dr. Jerka Dumić, dipl. ing. med. biokem.

Zahvala

Na tem mestu bi se najprej rad zahvalil prof. dr. Janji Marc za vso strokovno pomoč in podporo ter za to, da sem imel možnost opravljati praktični del diplome v Zagrebu v sklopu CEEPUS izmenjave. Velika zahvala gre tudi somentorici Jerki Dumić za vso pomoč, koristne nasvete ter predvsem potrpežljivost. Rad bi se zahvalil tudi celotnemu zavodu za Biokemijo in molekularno biologijo pod vodstvom Jerke Dumić za topel sprejem in pripravljenost priskočiti na pomoč kadarkoli je to bilo potrebno.

Posebna zahvala gre kolegici Tamari Zorbaz, za nesebično pomoč pri pridobivanju rezultatov. Dobro je vedeti, da obstajajo takšni ljudje na tem svetu.

Najlepša hvala tudi vsem sošolcem in prijateljem, ki so poskrbeli za nepozabna študentska leta.

Nazadnje bi se iskreno zahvalil še svojim staršem in sestri za nenehno podporo, spodbudo in prenašanje med izpitnimi obdobji. Na vaši vzgoji in podpori temelji vse moje šolanje in s tem tudi ta zaključek.

Izjava

Izjavljam, da sem magistrsko nalogo samostojno izdelal pod mentorstvom prof. dr. Janja Marc, mag. farm.

Nikola Dominković

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ABBREVIATIONS

APS - Ammonium persulfate

ADP - Adenosine diphosphate

Akt - Protein kinase B

ATP - Adenosine triphosphate

BCA - Bicinchoninic acid assay

Bcl-2 - B-cell lymphoma 2 protein

BSA – Bovine serum albumin

CCD - charge-coupled device

Cdk - Cyclin-dependent kinase

CRD - Carbohydrate recognition domain

dsRNA – Double stranded RNA

ESR - Environmental stress response

FDA - Food and Drug Administration

HER-2 - Human epidermal growth factor receptor 2

HGNC - HUGO Gene Nomenclature Committee

HRP – Horseradish peroxidase

HSE - Heat shock element

HSR – Heat shock response

Hsf - Heat shock transcription factors

Hsp – Heat shock protein

IgG – Immunoglobulin G

kDA – Kilodaltons

mAb - Monoclonal antibody

NC RNA – Negative control RNA (scrambled siRNA)

NSCLC - Non-small-cell lung carcinoma

NTD – N-terminal nucleotide binding domain

MD – Middle domain

CTD - C-terminal dimerization site

pAb – Polyclonal antibody

PAGE - Polyacrylamide gel electrophoresis

PBS - Phospate-buffered saline

PPIase - Prolyl isomerase

PVDF - Polyvinylidene fluoride

RISC - RNA-induced silencing complex

RNA - Ribonucleic acid

RNAi - RNA interference

SDS – Sodium dodecyl sulphate

SHR – Steroid hormone receptors

siRNA – Small interfering RNA

TBS - Tris-buffered saline

TBST - Tris-buffered saline-Tween

TEMED - Tetramethylethylenediamine

THP–1 - Human monocytic cell line derived from patient with acute monocytic leukaemia

POVZETEK

Proteini toplotnega šoka (angl. Heat Shock Proteins, Hsp) oz. stresni proteini so evolucijsko visoko ohranjeni proteini, ki jih najdemo tako pri evkariontih kot pri prokariontih. Kljub temu, da se njihovo izražanje poveča pod vplivom raznih stresorjev, so značilno izraženi tudi v fizioloških pogojih in opravljajo vrsto različnih pomembnih bioloških funkcij kot na primer: preprečevanje agregacije proteinov pri stresu, pomoč pri pravilnem zvijanju na novo sintetiziranih proteinov in denaturiranih proteinov, pomoč pri transportu proteinov med celičnimi organeli in funkcije v imunskem odzivu. Iz njihove vloge v organizmu sledi, da lahko igrajo pomembno vlogo pri preprečevanju oz. napredovanju patofizioloških procesov. Predvsem se raziskuje njihov vpliv na razvoj rakavih obolenj, saj je veliko proteinov, ki koristijo "usluge" proteinov toplotnega šoka klienti proteini) hkrati tudi onkogenov. Povišano izražanje Hsp lahko namreč zaščiti mutiran ali previsoko izražen protein pred razgradnjo in tako posledično pomaga pri razvoju tumorske celice. Izražanje stresnih proteinov regulirajo transkripcijski faktorji HSF (angl. Heat Shock Factors), med katerimi je najbolj pomemben HSF-1.

Hsp90 je eden najbolj pomembnih stresnih proteinov in je hkrati tudi eden izmed najbolj zastopanih proteinov pri evkariontih. Pomembni sta dve izoformi: Hsp90α, katerega ekspresija se poveča predvsem pod vplivom stresa, ter Hsp90β, ki je predvsem konstitutivno izražen. Za Hsp90 je značilno tudi to, da izraža določeno selektivnost proti določenim tipom proteinov, med katere sodi tudi veliko proteinov, ki so potrebni za razvoj raka (npr. Akt in Her-2). Ravno zato danes ocenjujejo, da bi Hsp90 lahko predstavljal potencialno tarčo pri zdravljenju rakavih obolenj. Raziskave so namreč pokazale, da inhibicija tega proteina vodi v degradacijo klientnih proteinov po ubikvitin-proteasomski poti. Po prvih odkritih inhibitorjih (geldanamicin in radicicol) je sledilo veliko novih derivatov, ki so bili bolj ali manj uspešni. Glavni problem predstavlja toksičnost inhibitorjev, saj Hsp90 opravlja pomembno funkcijo v telesu, vendar se z izboljšanjem farmakokinetičnih in farmakoloških lastnosti inhibitorjev ter z novimi dostavnimi sistemi ta problem zmanjšuje. Pomembno je določiti vpliv inhibicije Hsp90 na ekspresijo ostalih proteinov tudi z namenom ugotavljanja kompenzacijskih mehanizmov. Tako lahko določimo potencialne tarče za zdravljenje s kombinacijami različnih inhibitorjev (npr. inhibitor Hsp90 skupaj z inhibitorjem Hsp70).

Zanimalo nas je, kako bi lahko odsotnost aktivnosti proteina Hsp90 kot posledica utišanja gena vplivala na ekspresijo galektina-3. Galektin-3 je član družine lektinov, proteinov z veliko specifičnostjo do sladkorjev, ki ima pomembno vlogo pri različnih procesih kot so npr.: apoptoza, celični cikel, celična rast, adhezija, diferenciacija, aktivacija in kemoatrakcija. Njegova funkcija kot anti-apototični protein je bila še posebej zanimiva za nas, saj smo predvidevali, da bi lahko inhibicija proteina Hsp90 lahko vodila do povišanja izražanja galektina-3 ravno zaradi njegove anti-apoptotične aktivnosti.

Odločili smo se, da bomo gen za Hsp90 utišali s transfekcijo celic s siRNA. siRNA so RNA molekule, ki se razširjeno uporabljajo v raziskavah funkcije genov, pa tudi v raziskavah novih terapevtskih pristopov. Prednost siRNA je predvsem v tem, da lahko z njihovo pomočjo utišamo praktično vsak gen, slabost pa v tem, da je učinek prehoden, kar se najbolj izrazi pri hitro delečih celicah. To slabost se da zaobiti z uporabo ekpresijskih vektorjev za želeno siRNA. Perspektivna je tudi uporaba siRNA v terapevtske namene, saj je z napredkom farmacevtske tehnologije postalo možno dostaviti siRNA ciljano v tarčne celice, kar ima določene prednosti predvsem z vidika redkejših stranskih učinkov.

Poleg vpliva na izražanje galektina-3 smo želeli ovrednotiti tudi vpliv utišanja Hsp90 na še dva pomembna stresna proteina Hsp70 in Hsp27, za katera se je že pokazalo, da sta potencialni tarči za zdravljenje v kombinaciji s HSP90. Poleg tega smo ovrednotili tudi vpliv na izražanje Cdk proteinov (Cdk-1, Cdk-2, in [pTyr15]Cdk-1), ki so znani proteini klienti Hsp90. Pričakovati je, da bo zmanjšanje delovanja stresnega proteina Hsp90 vodila v razgradnjo njegovih proteinov klientov. Raziskavo smo izvajali na celični liniji THP-1, humani monocitni celični liniji, pridobljeni iz bolnika z akutno monocitno levkemijo, ki smo jo tretirali z siRNA proti Hsp90α in »scrambled« siRNA kot negativno kontrolo. Proteine smo ločili s SDS-PAGE elektroforezo, nato pa izražanje ovrednotili z Western blot metodo. Slike pridobljene z Western blot metodo smo kvantificirali in rezultate normirali glede na izražanje β-aktina.

Najprej smo preverili vpliv na izražanje obeh izoform (α in β) proteina Hsp90. Po pričakovanjih je bila ekspresija Hsp90β nespremenjena, saj je naš siRNA bil usmerjen samo pri α izoformi, medtem ko se je ekspresija Hsp90α zadovoljivo zmanjšala. Kljub temu je potrebno pri interpretaciji rezultatov vzeti v obzir to, da je v celicah tudi po tretiranju z siRNA ostala občutna količina proteinov Hsp90β, ki so še vedno normalno opravljali svojo funkcijo. Prav tako je bil opazen porast ekspresije stresnega proteina Hsp70, ki je reguliran z istim faktorjem Hsf-1. Porast v ekspresiji Hsp70 nakazuje na to, da

je prišlo do aktivacije Hsf-1. Prav tako je možno, da je višja koncentracija proteina Hsp70 del kompenzacijskega mehanizma, ki poskuša nadomestiti primanjkljaj proteina Hsp90. To dognanje podpira dejstvo, da se ekspresija Cdk proteinov, z izjemo rahlega znižanja v ekspresiji Cdk-1, praktično ni spremenila, kar je v nasprotju s pričakovanji.

Zaradi zgoraj navedenih rezultatov, je potrebno biti previden pri interpretaciji vpliva na ekspresijo galektina-3. Res je, da ni bilo vidne statistično značilne spremembe, vendar je najprej potrebno z dodatnimi raziskavami izločiti vpliv ostalih dejavnikov preden bi lahko nedvoumno ovrgli hipotezo, da je lahko tudi galektin-3 del kompenzacijskega odziva na odsotnost delovanja Hsp90. Je pa bil opazen zanimiv porast galektina-3 po 24-ih urah v vzorcu obdelanem z siRNA proti Hsp90α kot tudi v slepem vzorcu. To nakazuje na to, da transfekcija predstavlja neko novo stresno okolje za celice, na katero se celica adaptira tudi s pomočjo galektina-3. Vsekakor so potrebne dodatne raziskave za potrditev teh domnev.

ABSTRACT

Heat shock protein 90 (Hsp90) is one of the most important heat shock proteins (Hsps), a group of proteins that perform various essential biological functions (*e.g.* stopping protein aggregation under influence of physical stressors, assisting the folding of newly synthetized proteins and altered proteins). They are particularly important under stress conditions but also have significant role under the physiological conditions. Hsp90 has a number of client proteins which are responsible for the development of the cancer and are studied as a possible drug target, since it has been shown that Hsp90 inhibition leads to client protein degradation. We studied the effect of Hsp90 silencing on the expression of the galectin-3 in order to determine if the galectin-3 is included in the compensatory mechanisms that follow the inhibition of Hsp90 action. The Hsp90 silencing was achieved with the siRNA transfection by lipofection of the THP-1 cell line. The expression of the Hsp90, its client proteins (Cdk-1, Cdk-2, in [pTyr15]Cdk-1), and two other important Hsps (Hsp70 and Hsp27) was determined using the Western blot method. Results showed that there was no effect of Hsp90 inhibition on galectin-3 expression. Cdks expression with the exception of the Cdk-1 was unaffected. Hsp70 expression was considerably induced which suggests that it can compensate for the lack of the Hsp90 action. Hsp27, on the other hand, showed no difference in the expression. The results also imply that the lipofection itself could have impact on the expression of the Hsp90 and galectin-3. Further researches are needed to indisputably confirm these results, since the silencing with the siRNA presented only a partial inhibition of the Hsp90.

1 INTRODUCTION

1.1 HEAT SHOCK PROTEINS

Heat shock proteins (Hsps) are a highly conserved class of proteins that are present in prokaryotes and eukaryotes alike. They can be found in different subcellular compartments (1). This class of proteins was discovered by accident, when puffs of salivary glands of *Drosophilla melanogaster* were treated with higher temperature (heat shock) (2) – hence the name *Heat shock proteins*.

Although it is true that the expression of the Hsps may be induced by various stressors (*e.g.* elevated temperature, ischemia, ethanol, heavy metals, microbial infection, and cancer), they are also constitutively expressed and perform different important, even essential, biological functions in both stressful and physiological conditions (1,3,4). Hsps are one of the most abundant intracellular proteins. Their share of total cellular proteins, when constitutively expressed, amounts to approximately 5-10% - that share can easily jump up to approximately 15% when induced by different stressors.

One of the first things noticed while reading the literature on Hsps are inconsistencies in nomenclature of this proteins. Growing number of members of various Hsp families along with these inconsistencies results in confusion – different names for same gene product or very similar names for different gene products. *HUGO Gene Nomenclature Committee (HGNC)* issued a more consistent nomenclature which is summarized in review by Kampinga et al. (5). The most commonly used nomenclature is based on naming the Hsps according to their molecular weight (in kilodaltons) and grouping the ones with similar size (*e.g.* Hsp70 and Hsp90 family). This is the nomenclature that will be suitable for the needs of this master's thesis even though ever growing number of new members discovered asks for the naming of the proteins in question after the name of their gene.

Heat shock transcription factors (Hsfs) are a primary way of modulation of increased HSP expression. Among Hsfs the Hsf1 plays the most important role in transcriptional regulation of Hsp expression (the vertebrates and plants have evolved four members: Hsf1-4) (6). Hsps are bound to Hsf1 when cell is in unstressed situation, maintaining the HSF1 in a monomeric (inactive) state. When cell is in a situation which requires elevated expression of Hsps (stress), Hsp/Hsf1 complex dissociates. The unbound Hsf1 is now free to translocate to the nucleus and convert to trimeric complex, which gives it DNA – binding ability. Hsf1, now transcriptionally competent, activates heat shock element (HSE)

in promoter region of the *HSP* genes. Evidences suggest that even though this is the primary way of Hsp expression regulation, other regulatory mechanism exist (7,8).

To understand the role Hsps play in the cells, it is essential to know that these proteins are part of one of the most evolutionary conserved protective mechanisms – the Heat shock response (HSR), which is a part of environmental stress response (ESR) (6,7). Hsps are defined as molecular chaperones that non-covalently bind exposed hydrophobic surfaces of non-native proteins and are as such responsible for a wide variety of biological functions. General functions include: (a) stopping protein aggregation under influence of physical stressors; (b) assisting the folding of newly synthetized proteins and altered (denaturated) proteins; (c) serving as molecular chaperones in protein transport between cell organelles; and (d) roles in immunological processes (1, 4). These functions can be divided in ones that are part of HSR and ones that Hsps perform during non-stressful situations. As part of HSR they deal with fully or partially denaturated proteins. If damage is reversible, the proteins are assisted in refolding to previous state. If not, the Hsps assist the degradation of these proteins via the ubiquitin-proteasome pathway. Additionally, HSR has the ability to modulate immune/inflammatory response. Hsps as a component of the HSR, especially the most highly heat inducible member Hsp70, evolved into both pro- and anti-inflammatory molecules. As anti-inflammatory molecules, the Hsps modulate cytokine signal transduction and gene expression. Also, the Hsps indirect role in maintaining epithelial gut barrier has important anti-inflammatory effects (prevents movement of endotoxins into circulation). Necrotic and non-necrotic release of Hsps into the extracellular space is a pro-inflammatory effect of HSR (7). Hsps assistance in protein transport and folding of newly synthetized proteins are one of the most important roles in non-stressful situations.

As we can see, it can be assumed that Hsps can play an important role in preventing or promoting pathophysiological process. Even though they maintain the homeostasis of the cell and limit the damage of the cellular stress, it is easy to see how they could actually do harm while fulfilling their purpose. The Hsps can actually be involved in pathogenesis of the disease, if the Hsp client proteins are responsible for the development of the disease, since the Hsps can prevent their degradation and stabilize their structure. This is the reason why Hsps are considered to be potential targets for therapy in various diseases (especially cancer, since there are many oncoproteins which are Hsp client proteins).

1.1.1 Hsp90

Hsp90, the 90 kDa sized chaperone as the name suggests, is one of the most abundant proteins in eukaryotic cells. Even in non-stressful conditions it can represent 1-2% of total cellular proteins – a share which can grow up to 4-6% under stress (9). Table I shows the Hsp90 analogues with names recommended by HGNC and names usually found in literature.

Table 1-I The Hsp90 nomenclature
The Hsp90 analogues with recommended names, commonly used names and their location in cell (5)

Common name	Recommended name	Gene name	Location
Hsp90α1 (inducible)	**HspC1**	***HSPC1***	**Cytosol**
Hsp90α2 (inducible)	**HspC2**	***HSPC2***	
Hsp90β (constitutive)	**HspC3**	***HSPC3***	
Endoplasmin / Grp-94	**HspC4**	***HSPC4***	**Endoplasmic reticulum**
TNF Receptor - Associated protein 1 / Hsp75	**HspC5**	***HSPC5***	**Mitochondrion**

Among those found in cytosol, two major isoforms are important: Hsp90α and Hsp90β. While Hsp90β is constitutively expressed, Hsp90α is mostly and highly inducible. It is of notice that Hsp90β, even though as constitutive, can also be induced by stress. They are considered the result of a gene duplication (10). On the Figure 1.1, which shows the structure of Hsp90 protein, the homodimer, we can see the domain architecture that all of the Hsp90 homologs share: N-terminal nucleotide binding domain (NTD), a middle domain (MD) and a C-terminal dimerization site (CTD).

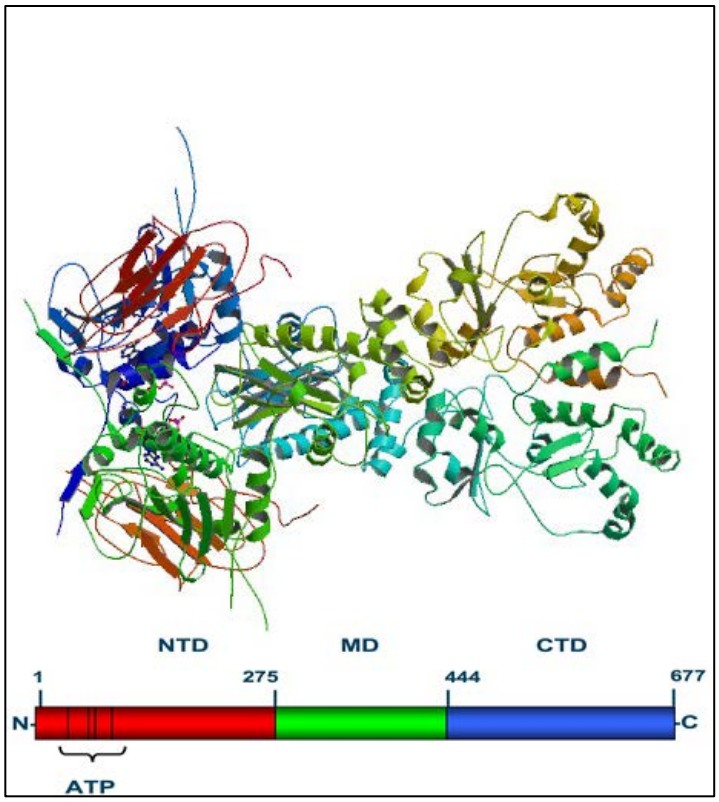


Figure 1.1 The structure of Hsp90 protein in closed state *(RCSB protein data bank)*

A highly conserved ATP binding domain is located near N-terminus and is essential for chaperoning activity of Hsp90. Binding of ATP alters the conformation state of the protein – contact formation is induced which results in closed and twisted dimer. Hsp90 client proteins interact with chaperone while it is in conformational change in order to reach stabilization of unfavourable conformations. Closed Hsp90 state is essential for hydrolysis of ATP, which results in separation of N-domains and release of ADP. Figure 1.2 displays the more detailed model of Hsp90 mechanism, shown on example of steroid hormone receptor (SHR), which is one of the most studied group of Hsp90 client proteins (the other being protein kinases). On this figure we can see other members of chaperone machinery that are also crucial: Hop/STIP1, PPIase, Hsp70, Hsp40 and p23. One of the functions of this machinery is stabilizing the SHR in a state in which they can bind steroid. Review by Eckl and Richter presents the more comprehensive review of protein processing by Hsp90 (11).

Hsp90 differs from other chaperons in that it exhibits certain selectivity toward certain kinds of proteins. Most of its client proteins are either transcription factors involved in signal transduction, or protein kinases. To name the few: previously mentioned SHR, tyrosine kinases (*e.g.* $p185^{erbB2}$), serine/threonine kinases (*e.g.* Cdk-1), and mutant transcription factors (*e.g.* p53) (9). Hsp90 also stabilizes the tertiary structure 26S proteasome, which means that its non-direct function also lies in degradation of the proteins trough ubiquitin-proteasome pathway.

Hsp90 can be considered as an evolutionary advantage, since its expression makes the accumulation of the mutated proteins possible and thus influences genetic diversity. This

fact in turn makes the Hsp90 potential target for cancer treatment since many of the Hsp90 client proteins are known to promote tumour growth when overexpressed or mutated. Hsp90 is known to stabilize different types of proteins that are required for tumour development: growth factor receptors (*e.g.* Her-2) and signal transduction proteins (e.g. Akt), which are overexpressed in tumour cells, and mutated proteins (*e.g.* v-src, Bcr/Abl, and p53) (9). HSP90 inhibition is explained in more detail in chapter *1.1.2. Hsp90 as a therapeutic target*

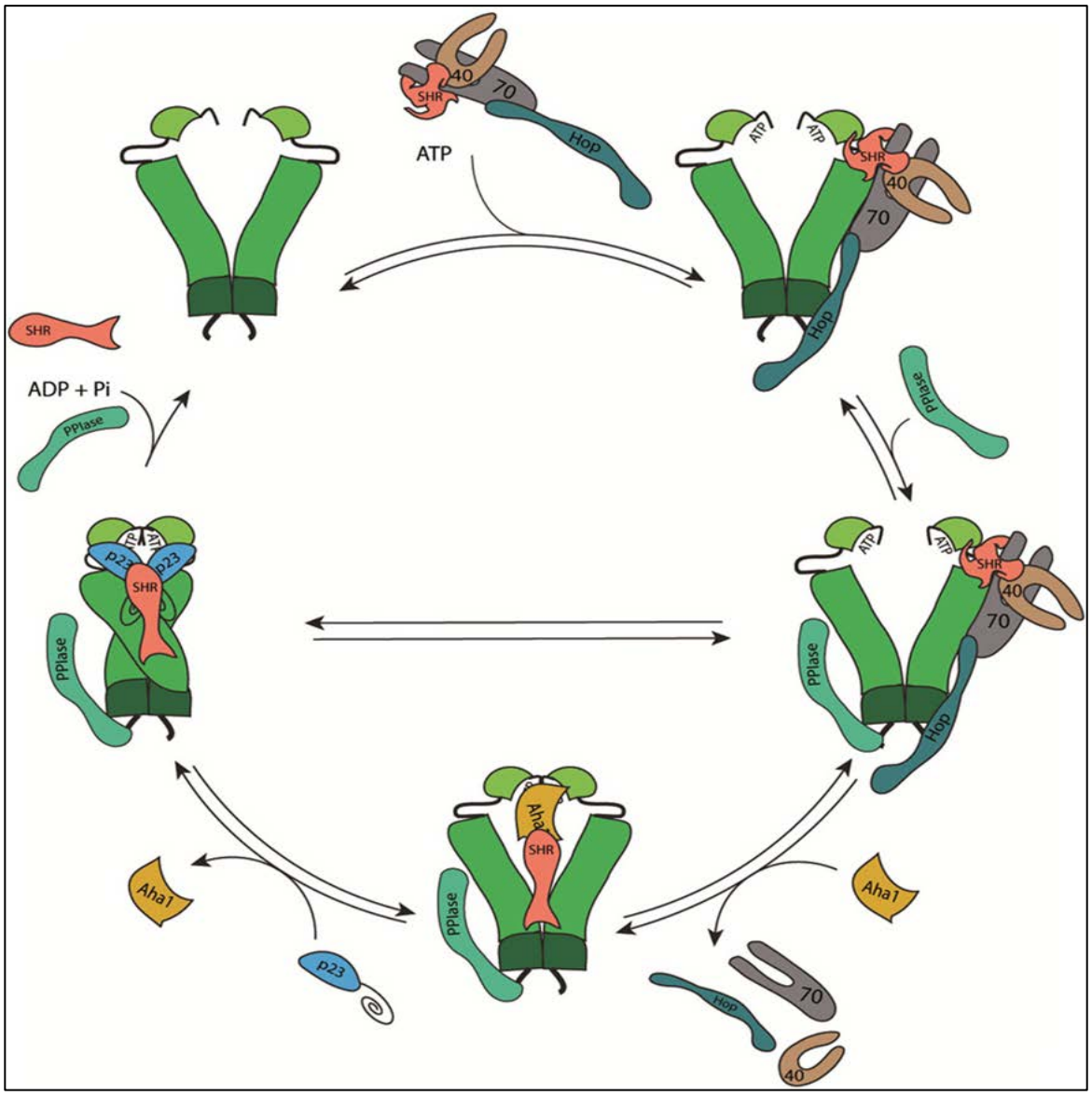


Figure 1.2 Model of Hsp90 mechanism
Activation of steroid hormone receptor. At different stages of SHR activation different co-chaperones assist the Hsp90.

1.1.2 Hsp90 as a therapeutic target

Hsp90 is exploited by cancer cells for at least a) stabilizing oncoproteins which are translocated, overexpressed or mutated and b) buffering stresses caused by "malignant lifestyle" (*e.g.* hypoxia) (12). Therefore, as mentioned before, it is of interest to use Hsp90 as a therapeutic target. It is easy to see why this idea, when it was being promoted in early 1990s, was met with scepticism. Targeting a protein that is normally expressed in cells and that is considered a housekeeping protein could result in unexpected and dangerous side effects. In later years this opinion has changed. As of 2013 thirteen Hsp90 inhibitors with different modes of action are undergoing clinical trials (13), that is an evidence of great interest of pharmaceutical industry in inhibiting Hsp90 function. This can be attributed to improvement of pharmacological and pharmacokinetic properties of drugs and development of targeted drug delivery.

Inhibition of Hsp90 results in client protein degradation via ubiquitin-proteasome pathway. First inhibitors discovered were natural products Geldanamycin (benzoquinone ansamycin antibiotic) and Radicicol (macrocyclic lactone antibiotic). They were shown to mimic the structure which ATP adopts in binding pocket of Hsp90 which blocks the ATP binding and hydrolysis (14, 15). Even though Geldanamycin and Radicicol turned out to be overly toxic and unstable, they established the foundation for the development of other therapeutic agents. Some of the most important drugs that entered the clinic are 17-AAG (tanespimycin), 17-DMAG (alvespimycin), IPI-504 (retaspimycin) and STA-9090 (ganetespib) with some of them reaching phase 2 and 3 clinical studies. As one could expect, the most promising results were shown in trials on cancers, which are most dependent on Hsp90 clients. Efficacy of the inhibitors was shown in the Her-2+ breast cancer and NSCLC (non-small-cell lung carcinoma), with mutated Alk. Both Her-2 and Alk are Hsp90 clients as mentioned before. Multiple myeloma and chronic myelogenous leukaemia also showed to be sensitive to this kind of treatment (12, 13). Even though development of some of these drugs stopped because of various reasons like

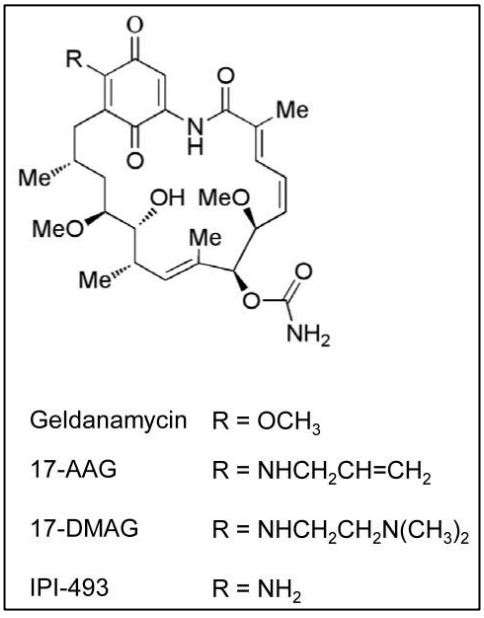


Figure 1.3 Structure of Geldanamycin and its derivatives

toxicity (16) or even patent expiration problems (17), the progress is being made and inhibiting the Hsp90 action is still a promising way of treating cancer.

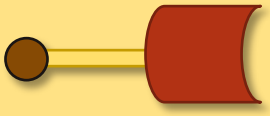
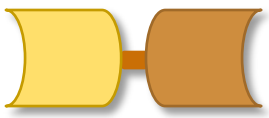
The use of single molecule, highly specific to one target (*e.g.* Hsp90), can lead to clinically ineffective treatments because of either the redundancy in targeted pathway or compensatory changes that take place in cells due to drug exposure. Establishing the effects inhibition of Hsp90 action has on expression of other proteins is of great importance, since it can show us the compensatory mechanisms which take place following the inhibition. This kind of studies could provide us with the knowledge needed to determine possible new therapeutic targets that could lead to new molecules which could increase cancer cell sensitivity to Hsp90 inhibition (synergistic action). There was some progress in this field and researches have already shown that silencing the expression of other chaperons (HSP70 and Hsp72), Hsf-1, and co-chaperons (*e.g.* p23) leads to greater sensitivity to Hsp90-targeted drugs (12).

1.2 GALECTIN-3

Galectin-3 is a member of lectin family, which are carbohydrate-binding proteins with high specificity for sugar moiety. Galectins, as the name suggests, have an affinity towards β-galactoside structure of glycoconjugates.

Table 1-II Basic galectin structure forms.

Dimeric galectins are made of two identical CRDs, chimera galectins have one CRD linked with proline, glycine, and tyrosine rich domain, and tandem galectins are made of two different CRDs linked with short peptide domain.

Structure			
Type	Dimeric (prototype)	Chimera	Tandem
Galectin	-1, -2, -5, -7, -10, -11, -13, -14, -15	-3	-4, -6, -8, -9, -12

Currently, 15 mammalian galectins have been identified, and they all contain at least one CRD (carbohydrate recognition domain), which enables specific binding of β-galactosides. CRD is composed of about 130 amino acids, which are highly conserved – a fact that suggests evolutionary importance of this class of proteins (18). Three different forms of galectins structure have been identified: dimeric (also called prototype), tandem and chimera. Two identical galectins subunits associated together represent dimeric structure. Two distinctive CRDs linked with small polypeptide linker represent tandem structure. In the vertebrates, chimeric structure is only represented by galectin-3. Chimeric form of the galectins contains one CRD and a long domain rich with proline, glycine, and tyrosine, which ends with N-terminal domain. Both monomeric and multivalent complexes (association via non-lectin domain) of galectin-3 can exist (19, 20). Table 1-II shows the forms of galectin structure.

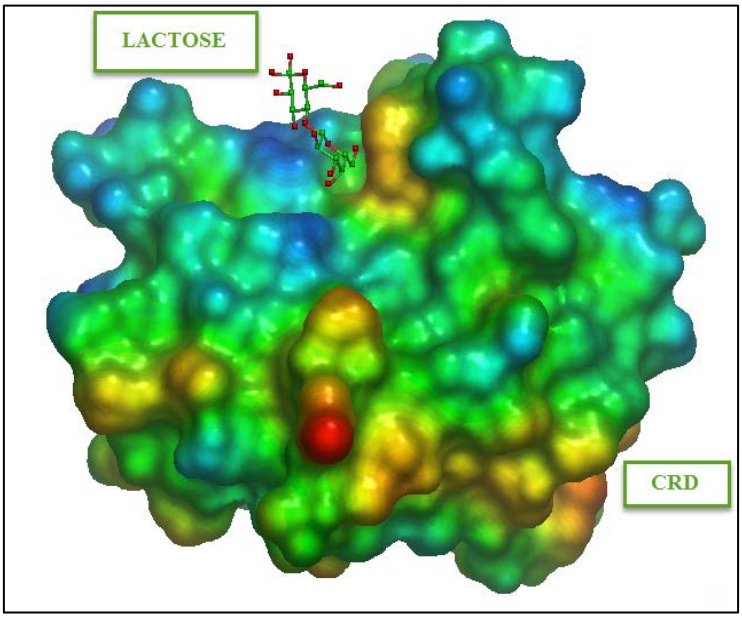


Figure 1.4 Strucutre of galectin-3 CRD domain with bound lactose
Taken and modified from PDBe

Galectin-3 is about 30 kDa sized protein, coded by a gene *LGALS3* (19). Structure can be divided in two regions: C-terminal region with CRD (Figure 1.4) and N-terminal region. While CRD is directly responsible for lectin function of the galectin-3 by recognition of carbohydrate structures, the N-terminal region is of great importance for the correct function of galectin-3. It is responsible for the oligomerization and aggregation, for the secretion via exosomes, and intracellular localization (21-23).

Galectin-3 is present in practically all cellular compartments (nucleus, cytoplasm, mitochondrion, and cell surface) and extracellular space. Lately, numerous different ligands of galectin-3 with different spectrum of functions have been identified. Thus it comes as no surprise that this protein is involved in broad spectrum of biological processes: apoptosis, cell cycle, growth, adhesion, differentiation, activation and chemoattraction (19). This in turn leads to its involvement in various diseases

(cardiovascular diseases, cancer and fibrosis) and galectin-3 is being evaluated as possible drug target for fibrosis (24) and diagnostic marker for different cancers (19). Galectin-3 function as anti-apoptotic protein is explained in more detail in chapter 1.2.1, since it is of interest to us in this study. Review by Dumić et al. provides a detailed overview of other functions of this protein (19).

1.2.1 Anti-apoptotic activity of galectin-3

Intracellular galectin-3 is well known for is anti-apoptotic activity against various stimuli – the only member of the galectin family with this activity. However the exact mechanism is still not well described. One of the galectin-3 ligands is Bcl-2, an important anti-apoptotic protein. These two share some structural characteristics and have 28% identity and 48% similarity. In CRD of the galectin-3 amino acid sequence NWGR can be found – this sequence is found in Bcl-2 family and is shown to be responsible for anti-apoptotic activity (25). There are other anti-apoptotic proteins that are known galectin-3 ligands. Galectin-3 also downregulates caspase activation. It does so by translocation to mitochondrial membrane (from nucleus or cytosol), where it prevents mitochondrial damage and inhibits release of cytochrome c (19).

Anti-apoptotic activity can lead to increased resistance to the chemotherapy in cancers where galectin-3 is overexpressed, which was proven by various researches (19). This means that galectin-3 could be used as prognostic marker for efficiency of the cancer therapy.

Galectin-3 mechanism of regulation is still not well described in literature. Since inhibition of Hsp90 action can lead to apoptosis, there is a possibility of induction of anti-apoptotic mechanisms as a compensatory effect. With galectin-3 being a well-known anti-apoptotic protein, the rise of the expression of this protein could be one of the ways cells defend them self against the lack of chaperone activity.

1.3 SMALL INTERFERING RNA

HSP90 coding gene silencing will be achieved by the use of small interfering RNA (siRNA). This valuable tool for gene function validation and drug targeting has an important role in RNA interference (RNAi) since its main function is interfering with the expression of genes.

Structure of siRNA is well-defined: double-stranded RNA (dsRNA), 21-25 base-pairs long. Additionally, each strand of siRNA has hydroxylated 3’ end, phosphorylated 5’ end and two nucleotide overhangs (26) (Figure 1.5.).

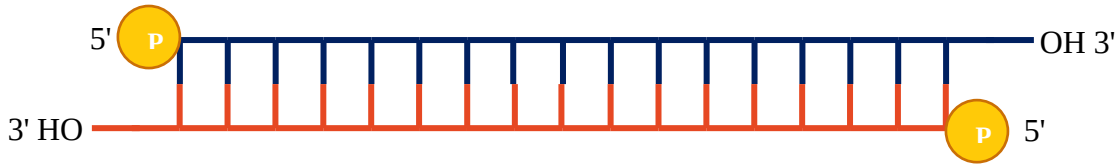


Figure 1.5 Scheme of a siRNA structure

The siRNAs are cleaved from longer dsRNAs by the enzyme called Dicer. These siRNAs are than unwound into the sense (passenger) strand, which is degraded, and the antisense (guide) strand, which is complementary to the target gene and is incorporated into RNA-induced silencing complex (RISC). The guide strand than leads the RISC to the mRNA of targeted gene – this leads to the degradation of mRNA and consequently to the gene silencing (27).

Since virtually any gene can be “knocked down” with delivery of the synthetic siRNA to the cell, the potential uses of this class of RNA is enormous. It is especially useful in the field of functional genomics – for defining gene and protein functions and for validation of potential drug targets. It may be also possible to use siRNA in therapy of various diseases – which is becoming even more promising with the evolution of novel drug delivery systems (*e.g.* lipid nanoparticles). There has even been first Phase 1 in-human trial with siRNA delivered through lipid nanoparticles for treatment of metastatic cancer in liver with clinical benefits (28). Lately, siRNAs have been assessed for use in treatment of Ebola and TKM-ebola is currently allowed by FDA to be used under expanded access in people infected with Ebola virus (29).

The downside of the transfection with exogenous siRNA is the fact that the effect is only temporary (2-4 days) and can be used only for short-term “knock-down”. The more rapidly the cells divide the shorter the effect is. Creating the expression vectors is a way of overcoming this shortcoming.

2 AIM OF THE STUDY

As shown in the Introduction, the Hsp90 is a very promising therapeutic target. It is important to determine the effect of Hsp90 silencing on expression of other proteins, since the existence of compensatory mechanisms is always a possibility. The purpose of our work is to investigate the effects of Hsp90 protein silencing (using siRNA) on expression of anti-apoptotic protein galectin-3 in human monocytic cell line THP-1. This can give us more information on how the inhibiton of Hsp90 expression affects cells and if the protein galectin-3 is potentially included in compensatory pathways. The increase of galectin-3 expression following Hsp90 silencing could indicate that galectin-3 inhibitors should be used in combination with Hsp90 inhibitors in order to achieve enhanced sensitivity of cancer cells to the latter. It could also shed some new light on the mechanism of the regulation of the galectin-3 expression, since it still poorly understood. Additionally, interrelation between Hsp90 family and Hsp70 and Hsp27 family of heat shock proteins will be investigated by measurement of the difference in the expression of these two proteins before and after the Hsp90 knock-down. It is believed that Hsp90 silencing will result in Hsf-1 activation which in turn would induce expression of other Hsps.

The efficiency of protein silencing will be estimated by determining the expression of both Hsp90 isoforms – Hsp90α and Hsp90β. Hsp90 client proteins expression will be determined as well, since the absence of the Hsp90 action leads to client protein degradation. The client proteins used will be cyclin-dependent kinase 1 and 2 (Cdk-1 and Cdk-2) and a phosphorylated Cdk-1 ([pTyr15]Cdk-1).

The THP-1 cell line will be transfected with siRNA targeted against Hsp90. Samples will then be collected from cell suspension after 24 h and after 48 h. Two types of control samples will be used – untreated THP-1 cell line (0 h, 24 h and 48 h) and THP-1 cell line transfected with scrambled siRNA (24 h and 48 h) in order to establish if transfection itself can change the expression levels of investigated proteins. Studied proteins will be separated using the SDS polyacrylamide gel electrophoresis and then analysed using the western blot method. The detection will be performed using the two-step technique – incubation with primary antibody, rinsing and then incubation with secondary antibody conjugated to HRP. Since we will compare relative expression levels in western blots, it is expected that there will be technical errors and inconsistencies that arise during sample

preparation, loading and transfer steps. In order to minimize the effect of these variations, normalization technique with housekeeping protein beta-actin will be used.

Silencing of the Hsp90 protein is expected to lead into lower expression of the Hsp90 client proteins Cdk-1, Cdk-2, and [pTyr15]Cdk-1. There is also the possibility of the higher expression of the Hsp70 and the Hsp27 family as a compensatory mechanism since they are all part of a complex heat shock response. Finally, the inhibition of Hsp90 activity as a chaperone protein and consequentially the loss of protection against environmental stress could lead to higher expression of known anti-apoptotic protein galectin-3 as a compensatory mechanism. These effects will depend on the efficiency of Hsp90 silencing.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Standard chemicals

Acrylamide/Bis-acrylamide mix 30%, 37.5:1 (*Sigma*)

Ammonium persulfate, APS, $(NH_4)_2S_2O_8$ (*Sigma*)

Bicinchoninic acid, BCA (*Sigma*)

Bovine serum albumin, BSA (*Sigma*)

Bromophenol blue (*Sigma*)

Copper (II) sulphate pentahydrate, $CuSO_4$ x $5H_2O$ (*Sigma*)

Disodium phosphate, Na_2HPO_4 (*Sigma*)

Foetal bovine serum (*Sigma*)

Glycerol (*Sigma*)

Glycine (*Sigma*)

Hydrochloric acid, HCl (*Sigma*)

Isopropyl alcohol (*Sigma*)

β-mercaptoethanol (*Sigma*)

Methanol, MeOH (*Kemika*)

Potassium chloride, KCl (*Sigma*)

Potassium dihydrogen phosphate, KH_2PO_4 (*Sigma*)

Sodium deoxycholate (*Sigma*)

Sodium dodecyl sulphate, SDS (*Sigma*)

Sodium chloride, NaCl (*Sigma*)

Tetramethylethylenediamine, TEMED (*Sigma*)

Tris(hydroxymethyl)aminomethane, Trizma base (*Sigma*)

Triton X-100 (*Merck*)

Trypan blue solution (*Sigma*)

Tween-20 (*Sigma*)

3.1.2 Specific commercial reagents and mediums

Amersham ECL™ Prime Western Blotting Detection Reagent (*GE Healthcare Life Sciences*)
Bicinchoninic Acid Protein Assay Kit (*Sigma*)
Lipofectamine RNAiMAX (*Life Technologies*)
OPTI-MEM Reduced Serum Medium (*Life Technologies*)
Pre-stained protein standard Novex® Sharp Protein Standard (*Life Technologies*)
Protease Inhibitors Cocktail Tablets - Complete, Mini, EDTA-free; (*Roche*)
RPMI 1640 Medium (*Life Technologies*)

3.1.3 Antibodies

Goat anti-rabbit IgG polyclonal antibody conjugated to horseradish peroxidase (*Sigma*)
Mouse anti-human Hsp27 IgG1 monoclonal antibody (*Enzo*)
Mouse anti-human Hsp90α IgG2a monoclonal antibody (*Enzo*)
Rabbit anti-human Hsp70/72 polyclonal antibody (*Enzo*)
Rabbit anti-human Hsp90β IgG polyclonal antibody (*Enzo*)
Rabbit anti-mouse IgG polyclonal antibody conjugated to horseradish peroxidase (*Sigma*)

3.1.4 Cell lines

THP-1 cell line (TIB-202™) - Human monocytic cell line derived from patient with acute monocytic leukaemia (*American Type Culture Collection*).

3.1.5 siRNAs

siRNA for Hsp90α: *Silencer*®Select Pre-designed siRNA – s6995 (*Ambion*)
scrambled siRNA: Silencer® Select Negative Control No. 2 siRNA (*Ambion*)

3.1.6 Solutions

Cell lysis buffer (pH 8.0)

Tris HCl	5 mM
NaCl	15 mM
Triton X-100	1%
Sodium Deoxycholate	0.5%
SDS	0.1%
Protease inhibitors cocktail	2 mM
in distilled water	

SDS-PAGE Sample buffer (pH 6.8)

Tris HCl	187.5 mM
Glycerol	20%
SDS	6%
β-mercaptoethanol	5%
Bromophenol blue	0.1%
in distilled water	

SDS-PAGE Running buffer (pH 8.3)

Glycine	92.0 mM
Tris HCl	25.0 mM
SDS	3.5 mM
in distilled water	

Transfer buffer – Cathode buffer (pH 9.4)

Tris HCl	25 mM
Glycine	40 mM
MeOH	20%
in distilled water	

Transfer buffer – Anode buffer I (pH 10.4)

Tris HCl	300 mM
MeOH	20%
in distilled water	

Transfer buffer – Anode buffer II (pH 10.4)

Tris HCl	25 mM
MeOH	20%
in distilled water	

Tris-buffered saline, TBS (pH 7.5)

Tris HCl	50 mM
NaCl	150 mM
in distilled water	

TBST buffer (pH 7.5)

Tween-20	0.1%
in TBS, pH 7.5	

Phospate-buffered saline, PBS (pH 7.4)

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	1.8 mM
in distilled water	

3.1.7 Electrophoresis gels

Stacking gel (5%)

Acrylamide/Bis-acrylamide mix 30%, 37.5:1	5%
Tris HCl, pH 6.8	250 mM
SDS	0.1%
APS	0.1%

TEMED	0.05%
in distilled water	

Resolving gel (12%)

Acrylamide/Bis-acrylamide mix 30%, 37.5:1	12%
Tris HCl, pH 8.8	375 mM
SDS	0.1%
APS	0.1%
TEMED	0.05%
in distilled water	

3.2 METHODS

3.2.1 Cell cultivation and treatment

THP-1 cell line (TIB-202™) was obtained from American Type Culture Collection (Manassas, VA, USA). All experiments were performed on cells between passages 5-10. After 15 passages, cells were discarded and replaced by frozen stocks.

THP-1 cells were asynchronously grown (37 ºC, 5% CO_2, relative humidity 95%) in RPMI 1640 medium supplemented with foetal bovine serum (10 %), penicillin G (100 U/mL), streptomycin-sulphate (100 μg/mL), amphotericin B (250 ng/mL) and plasmocin (2.5 μg/mL) and subcultured every 2-3 days to maintain cell concentration between 1 x 10^5 and 1 x 10^6 cells/mL. Cell viability was assessed by trypan blue (0.04%) exclusion dye using a haemocytometer, while the cell count was determined using *Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter).* For the silencing experiment, the cells were collected and resuspended in antibiotic-free medium to achieve concentration of 10^5/mL.

siRNA for Hsp90α and negative control, scrambled siRNA (*Silencer*®Select Pre-designed siRNA – s6995 and *Silencer*® Select Negative Control No. 2 siRNA, respectively) were purchased from Ambion, resuspended in water to 50 μM concentration and aliquots were stored at -20 °C. 120 μL of working siRNA dilution (10 μM) was gently mixed with 20 mL of OPTI-MEM medium, 200 μL of Lipofectamine RNAiMAX was added. The solution was then mixed and incubated at room temperature for 15 minutes. Following the incubation, the mixture was added to 100 mL of cell suspension, so that the final concentration of siRNA was 10 nM.

3.2.2 Sample homogenization – Ultrasonication

After 24 and 48 hours of cultivation, aliquots of the cell culture were centrifuged at 300*g* for 5 min. The cell pellet (4 x 10^6 cells) was washed twice with ice-cold PBS, resuspended in 0.15 mL cell lysis buffer and additionally homogenized by sonication with ultrasound disintegrator (*MSE, 100 W*) for 2 min (amplitude 5 μm, ν=22 kHz). Protein concentrations in cell homogenates were determined by the bicinchoninic acid assay.

3.2.3 Determination of total protein concentration

Principle

Bicinchoninic acid assay (BCA) was used for determination of total protein concentration. In alkaline solution peptide bonds in protein reduce Cu^{2+} ions to Cu^{+} (biuret reaction). Next, each Cu^{+} ion forms a purple-coloured complex with two molecules of BCA which absorbs light at wavelength of 570 nm. The amount of reduced Cu^{+2} is proportional to the amount of proteins in the solution, therefore is the absorption at 570 nm proportional to the concentration of proteins in the sample. It is advised to incubate BCA assay at higher temperatures since it reduces the deviations due to differences in amino acid composition (30).

Procedure

Bicinchoninic Acid Protein Assay Kit (*Sigma*) was used for BCA assay. First, series of standard BSA (bovine serum albumin) solutions were prepared for construction of calibration curve (0 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 0.90 mg/mL, 1 mg/mL). Next, working reagent was prepared by adding 1 part of Cooper solution (4% w/v $CuSO_4$) to 50 parts of BCA solution (BCA, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N NaOH, pH 11.25). For construction of calibration curve 100 μL of working reagent and 25 μL of each standard BSA solution was added to microtitre plate in two parallels. For determination of protein concentration in cell homogenates 23 μL of water and 2 μL of homogenate were added to 100 μL of working reagent in two parallels. Microtitre plate is incubated in 37 °C for 30 minutes afterwards.

Measurement of absorbance of each well was carried out at 560 nm on spectrophotometer *Victor 1420 Multilabel Counter* (*PerkinElmer*).

3.2.4 SDS-page

Sample preparation

After the protein concentration of the sample was determined with BCA assay, the samples were diluted to final concentration of 1 mg/mL with SDS-PAGE Sample Buffer and water. To 100 µL of each sample such an amount of Sample Buffer was added that it represented 1/3 of final volume. Final volume was calculated from desired final, protein concentration in the sample and the volume of each sample (100 µL). Next, samples are heated for 5 minutes at 96 °C in order to reduce disulphide linkages with β-mercaptoethanol.

Gel preparation

Gels were polymerized between two glass plates placed in a gel caster; 0.75 mm wide spacers, which determined the gel thickness, were placed between glass plates. Since we used discontinuous SDS-PAGE, two gels with different acrylamide concentrations and different pH value were prepared. First gel poured in between plates was Resolving gel (12%). Isopropanol was added in small amount to remove bubbles and make the surface smooth. When the Resolving gel polymerized, the Stacking gel (5%) was added. The comb was inserted at the top in order to create wells. After the polymerization of the Stacking gel, the comb was removed and wells were washed out with distilled water and dried.

Electrophoresis

Gel caster was placed in electrophoresis tube. The samples were loaded in wells (15 µL), which was followed by filling of the remaining volume in the wells with SDS-PAGE Running buffer. Additionally, 5 µL of Pre-stained protein standard Novex® Sharp Protein Standard (*Life Technologies*) was loaded on the gel for easier determination of the positon of analysed proteins on the gel. Running buffer was then poured inside the tank (enough to cover the bottom edge) and in the central area which enables retaining of the buffer above upper edge of the gel. The voltage used was 80 V in Stacking gel and 120 V in Resolving

gel. The samples were allowed to run until the dye front (bromophenol blue) reached the bottom of the gel.

3.2.5 Western blot

Transfer of the proteins to PVDF

After the electrophoresis, Stacking gel was carefully removed, Resolving gel was transferred to "transfer sandwich" for semi-dry transfer of proteins from gel to Polyvinylidene fluoride (PVDF) membrane (*Milipore*). The composition of sandwich from top to bottom (the direction of transfer) was as follows:

1. Three filter papers and gel soaked in Cathode buffer (pH 9.4)
2. PVDF membrane and one filter paper soaked in Anode buffer I (pH 10.4)
3. Two filter papers soaked in Anode buffer II (pH 10.4)

PVDF membrane was also soaked in methanol before soaking in buffer, since it is hydrophobic. This composition was put in Semi-Dry transfer cell *Semi-Dry Blotting System IMM 1-A* (*The W.E.P. Company*). Blotting was performed at current density of 0.8 mA /cm^2 of membrane for one hour.

Membrane incubation with specific antibodies

After blotting, membrane was cut into strips with proteins of specific molecular mass. Protein standard served as orientation. Blocking of non-specific binding was achieved by incubating each strip with 3% BSA in TBS for 1h. Each strip was then incubated over night with specific primary antibodies against analysed proteins. Primary antibodies were diluted with 3% BSA in TBS. Next day, membranes were rinsed three times for 15 minutes in TBST buffer. After rinsing, membranes were incubated with secondary antibodies linked to horseradish peroxidase (HRP), who were also diluted with 3% BSA in TBS, for one hour. Incubation was again followed by rinsing in the same manner as with primary antibodies. Antibodies used for each protein and their respective dilutions are shown in Table 3-I.

Table 3-I Antibodies used for each protein and their respective dilutions

Protein	Primary antibody	Dilution	Secondary antibody	Dilution
Hsp90α	mouse mAb	1 : 1000	rabbit pAb, HRP linked	1:100 000
Hsp90β	rabbit pAb	1 : 1000	goat pAb, HRP linked	1:10 000
Hsp70/72	rabbit pAb	1 : 1000	goat pAb, HRP linked	1:10 000
Hsp27	mouse mAb	1 : 1000	rabbit pAb, HRP linked	1:100 000

Visualization

Visualization was performed using chemiluminescence. Reaction between hydrogen peroxide and luminol is catalysed by HRP on secondary antibodies. The product of this reaction, 3-aminophthalate, then emits light at 428 nm. Amersham ECL™ Prime Western Blotting Detection Reagent (*GE Healthcare Life Sciences*) was used as reagent. It was prepared immediately before application to the membrane by mixing the same amount of Peroxide Solution and Luminol Enhancer Solution. Enhancer solution contains modified phenols which enhance the light emitted up to 1000-fold. 0.07 mL of reagent per cm^2 of membrane was applied in dark room and was incubated for 5 minutes afterwards. Chemiluminescence was detected using Amersham™ Imager 600 (*GE Healthcare*). Quantification of bands was carried out with ImageQuant™ 5.0 software.

3.2.6 Statistical processing

The quantification of the bands was followed by statistical processing of the results using the *IBM SPSS Statistics 20* and *Microsoft Excel* software. Independent samples t-test was used for the comparison of the groups. Significantly different results were considered those with the $p < 0.05$. Figures 4.4 to 4.11 show the charts of the expression analysis of the proteins in question. The results are shown as relative values for easier interpretation. The results of the proteins analysed (including β-actin) in the untreated THP-1 cells (0 h) were assigned with the relative value of 1.0. All the other results were than calculated relative to the results of the proteins in the untreated THP-1 cells (0 h). Each result was afterwards normalized according to the expression of the β-actin, since it was assumed that β-actin expression does not change during the experiment.

4 RESULTS AND DISCUSSION

4.1 PROTEIN CONCENTRATION DETERMINATION

Protein concentration were determined using the bicinchoninic acid assay, explained in more detail in chapter 3: *Materials and methods.* Figure 4.1 shows the calibration curve obtained from the standard BSA solutions.

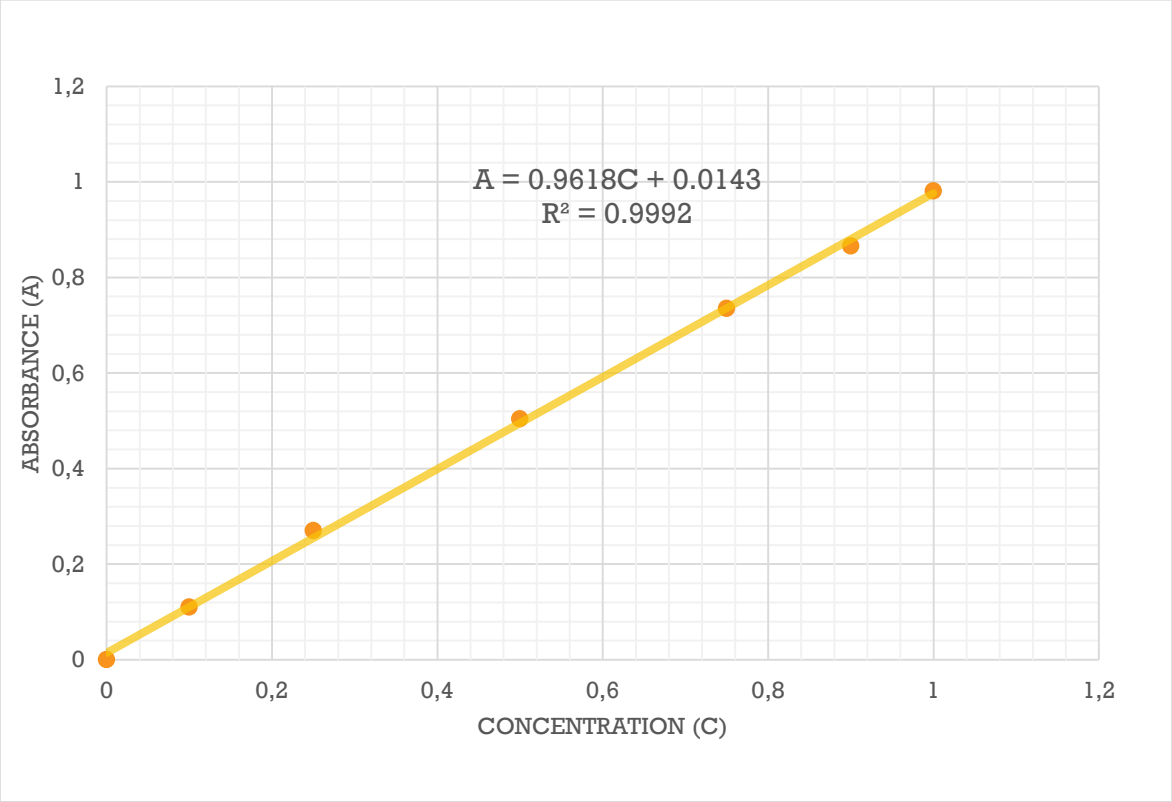


Figure 4.1 Calibration curve used for determination of total protein concentration
Standard BSA solutions (concentrations: 0 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 0.90 mg/mL, 1 mg/mL) were used.

Total protein concentrations in the cell homogenates were calculated from the equation obtained from the calibration curve (A = 0.9618C + 0.0143) and absorbance of the homogenates. Table 4-I shows the results obtained from the individual samples and the calculated concentrations. The desired final loading concentration was 1 mg/mL and was achieved by diluting with SDS-PAGE Sample Buffer and water.

Table 4-I Absorbance of each homogenate with calculated concentrations.

The dilution of homogenate had to be considered so the real concentration of the homogenate is the concentration of the solution multiplied with 12.5. Each sample was analysed in two parallels.

sample	A	A-0	concentration of solution (mg/mL)	concentration of homogenate (mg/mL)
THP-1 (0 h)	0.417	0.357	0.36	4.45
THP-1 (0 h)	0.403	0.343	0.34	4.27
siRNA (24 h)	0.433	0.373	0.37	4.67
siRNA (24 h)	0.458	0.398	0.40	4.98
NC RNA (24 h)	0.407	0.347	0.35	4.32
NC RNA (24 h)	0.454	0.394	0.39	4.93
THP-1 (24 h)	0.426	0.366	0.37	4.58
THP-1 (24 h)	0.426	0.366	0.37	4.57
siRNA (48 h)	0.459	0.399	0.40	5.00
siRNA (48 h)	0.463	0.403	0.40	5.05
NC RNA (48 h)	0.459	0.399	0.40	5.00
NC RNA (48 h)	0.465	0.405	0.41	5.08
THP-1 (48 h)	0.464	0.404	0.41	5.07
THP-1 (48 h)	0.427	0.368	0.37	4.59

4.2 PROTEIN EXPRESSION

Expression of the proteins was analysed with the Western blot method, using the specific antibodies and chemiluminescence for visualization. The method is described in more detail in the chapter 3: *Materials and methods.*

The samples of the untreated THP-1 cells were loaded in one parallel, while the samples of the THP-1 cells treated with the scrambled siRNA and siRNA against Hsp90 were loaded in two parallels in order to fully exploit the number of possible samples analysed in one blot (one well for the protein standard and 11 wells for samples).

Results of the Western blot analysis are images, which were obtained using the automated chemiluminescence imager *Amersham Imager* 600 with CCD (charge-coupled device) camera. Exposure time used varies since the *auto exposure* function (imager determines the most optimal exposure for each blot) was used in order to achieve the most optimal results. Typical exposure times ranged from 30 seconds to 7 minutes. The intensity of each band was then quantified using the *ImageQuantTM 5.0.* Variations in the results which occur due to inevitable errors like unequal loading, sampling irregularities, different

exposure time and uneven protein transfer across a membrane, were normalized using the housekeeping protein β-actin. This allowed us to compare results of different blots, which would otherwise be impossible.

Figures 4.2 to 4.4 show the examples of the images obtained by the Western blot analysis. It should be mentioned that this are the most representable images obtained and that each protein was analysed in multiple parallels. Also, not all proteins could be analysed on one blot due to similarities in molecular weight (*e.g.* Hsp90α and β) so different blots were used for analysis of different combinations of proteins.

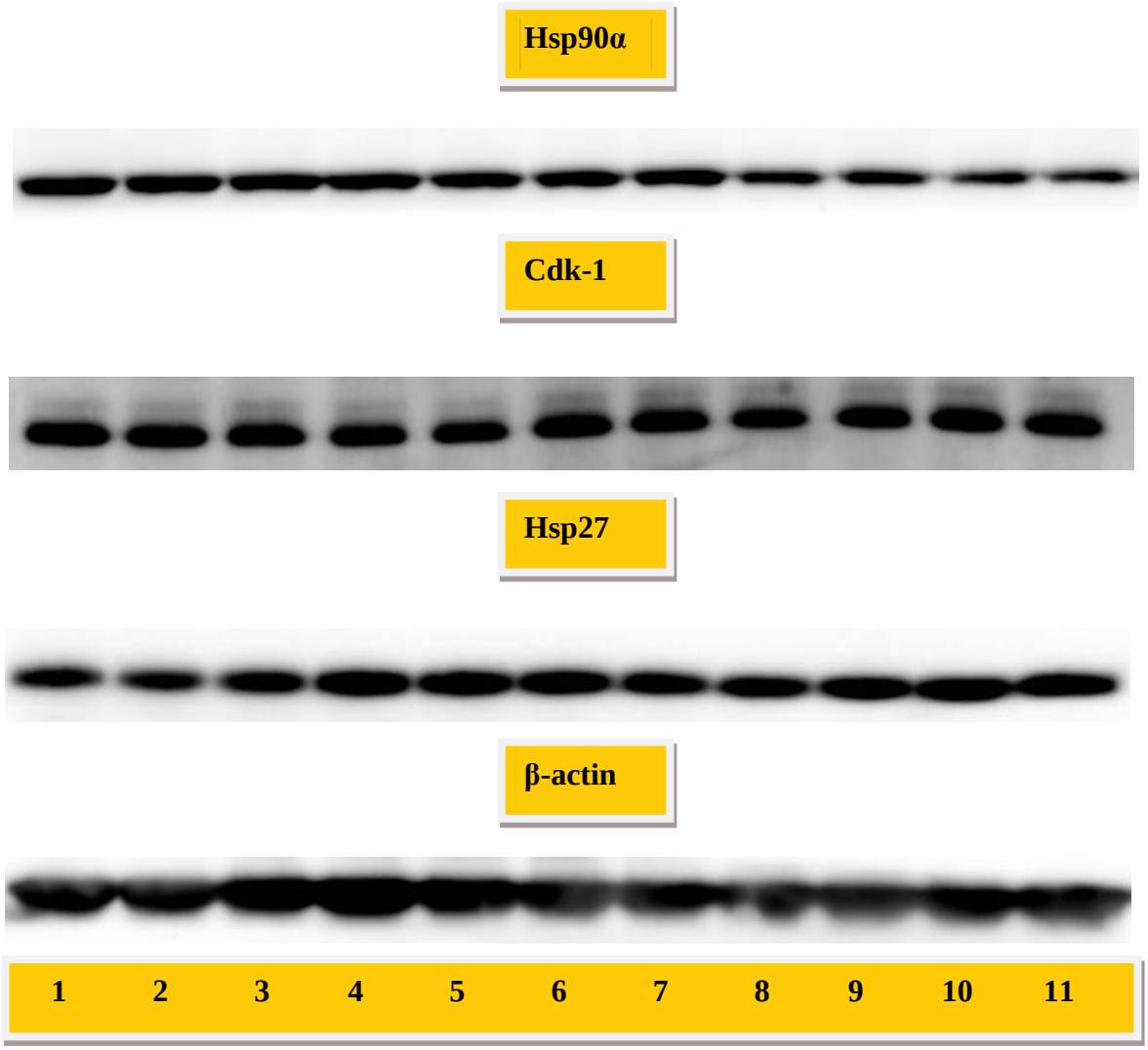


Figure 4.2 Example of western blot analysis of Hsp90α, Hsp27, Cdk-1, and β-actin
Samples were loaded in following sequence:
1-3: Untreated THP-1 cell line (0 h, 24 h, 48 h)
4-7: THP-1 cell line treated with scrambled siRNA (24 h, 24 h, 48 h, 48 h)
8-11: THP-1 cell line treated with siRNA against Hsp90 (24 h, 24 h, 48 h, 48 h)
β-actin was analyzed for normalization of the results

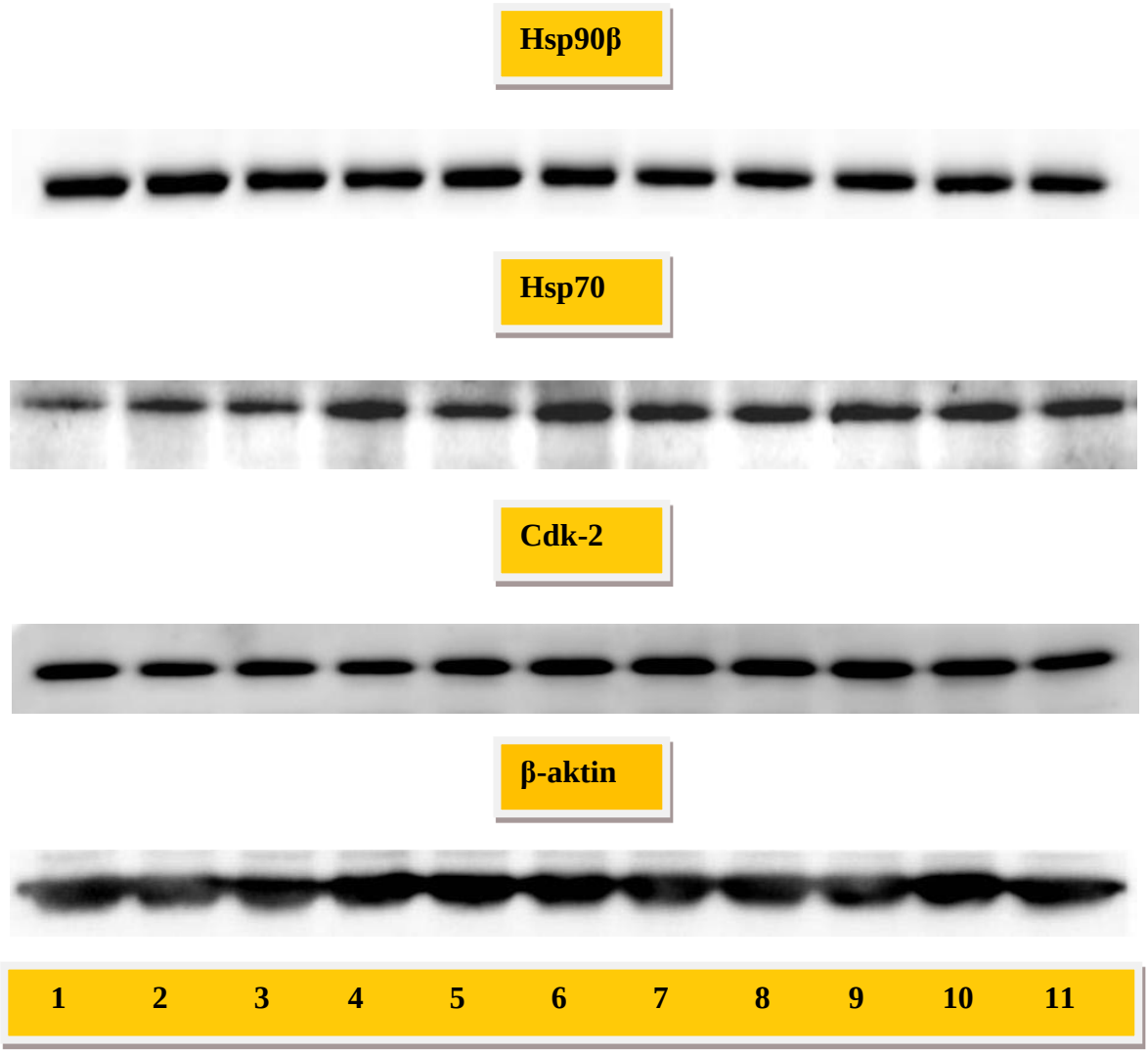


Figure 4.3 Example of western blot analysis of Hsp90β, Hsp70, Cdk-1, and β-actin

Samples were loaded in following sequence:

1-3: Untreated THP-1 cell line (0 h, 24 h, 48 h)

4-7: THP-1 cell line treated with scrambled siRNA (24 h, 24 h, 48 h, 48 h)

8-11: THP-1 cell line treated with siRNA against Hsp90 (24 h, 24 h, 48 h, 48 h)

β-actin was analyzed for normalization of the results

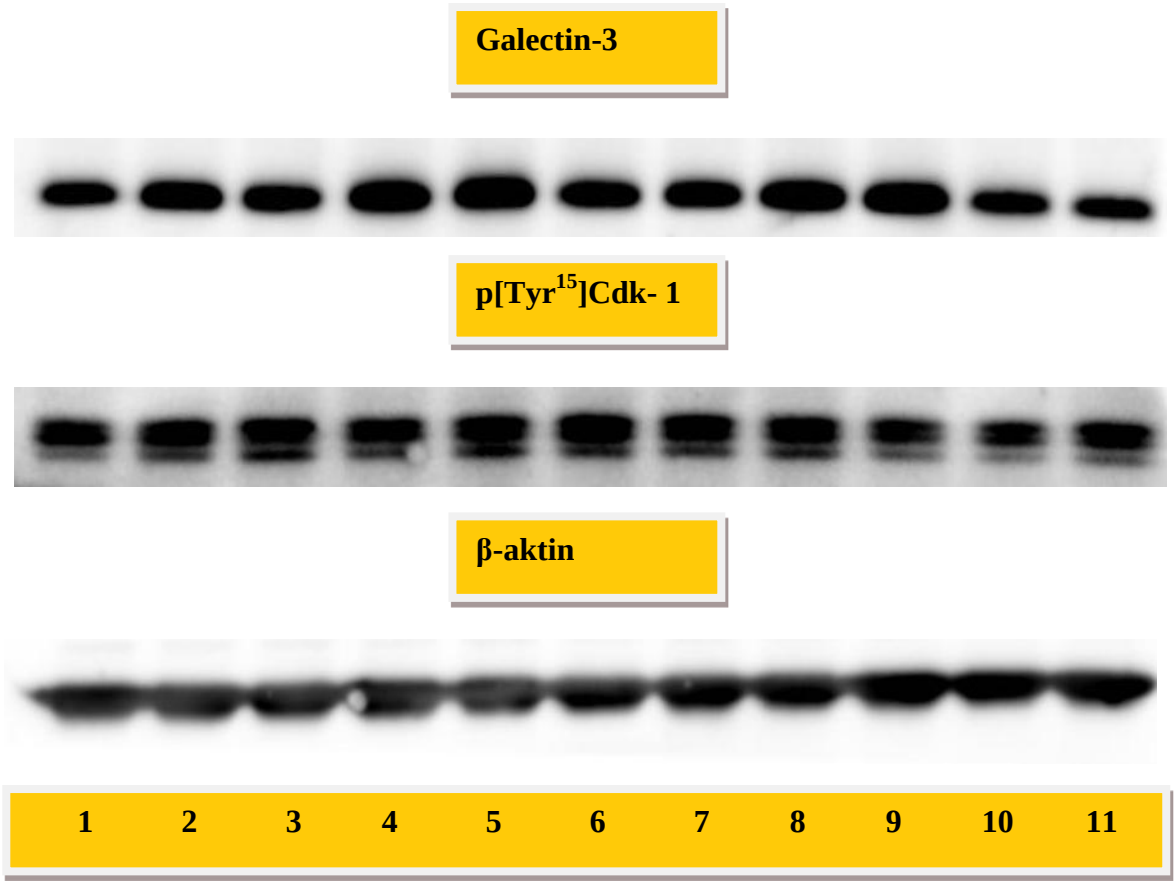


Figure 4.4 Example of western blot analysis of galectin-3, p[Tyr15]Cdk-1, and β-actin

Samples were loaded in following sequence:

1-3: Untreated THP-1 cell line (0 h, 24 h, 48 h)

4-7: THP-1 cell line treated with scrambled siRNA (24 h, 24 h, 48 h, 48 h)

8-11: THP-1 cell line treated with siRNA against Hsp90 (24 h, 24 h, 48 h, 48 h)

β-actin was analyzed for normalization of the results

4.2.1 Silencing efficiency

First of all, we wanted to determine to what extent, if at all, was the silencing of the Hsp90 achieved. The expression of Hsp90α was reduced considerably (Figure 4.5). Protein levels after the 48 h were reduced for approximately 60% relative to the control group. Transfection with the siRNA is only transient and the fact that the THP-1 cell lines have the doubling time of about 35 h – 50 h could be the reason that the reduction in the expression of the Hsp90α was not more efficient. There is also the possibility that the siRNA transfection of the THP-1 cell line by lipofection could be taken as a form of stressful condition, which would mean that the Hsp90α expression could be induced by the transfection itself, since it is highly inducible by stress. This could explain the significant rise in HSP90α expression after the transfection with scrambled siRNA (NC RNA) for 48 h when compared to the untreated cells ($p = 0.007$) and could be another explanation for the lower than expected silencing of the Hsp90α. On the other hand, the expression of the other Hsp90 isoform Hsp90β was practically unaffected (Figure 4.6), which was expected since our siRNA was directed only towards Hsp90α isoform. Since Hsp90β is not a highly inducible form, the expression of this proteins is not induced by the transfection itself – there are no observable differences before and after the transfection.

Even though there are differences in function between both isoforms, the majority of studies have been carried out on Hsp90 isoforms mixture since biological separation of them is considered difficult. This means that functions of the Hsp90 are not usually attributed to specific isoform and that both isoforms should be taken into account when determining silencing efficiency. Additionally, it is worth mentioning that in normal conditions HSP90β (constitutive form) expression is usually higher than Hsp90α (highly inducible) expression (10). Even if we consider the transfection with siRNA as a stressful situation which would induce the Hsp90α expression, it is safe to assume that only a minor fraction of the overall Hsp90 expression was effected with siRNA silencing. It is important to have this in mind when interpreting other results.

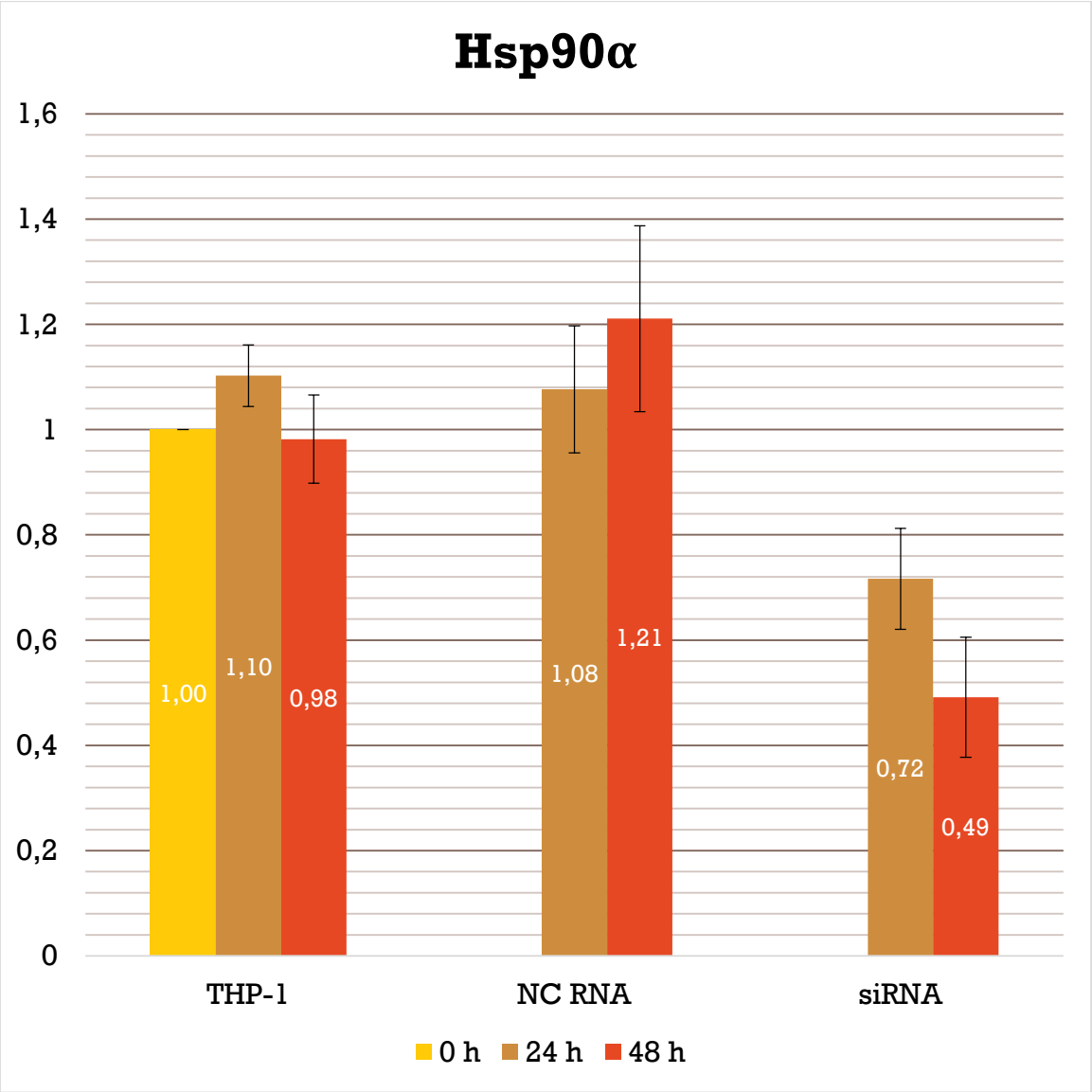


Figure 4.5 Clustered column chart of relative quantitative analysis of Hsp90α expression

Results are shown as an average value ± SD

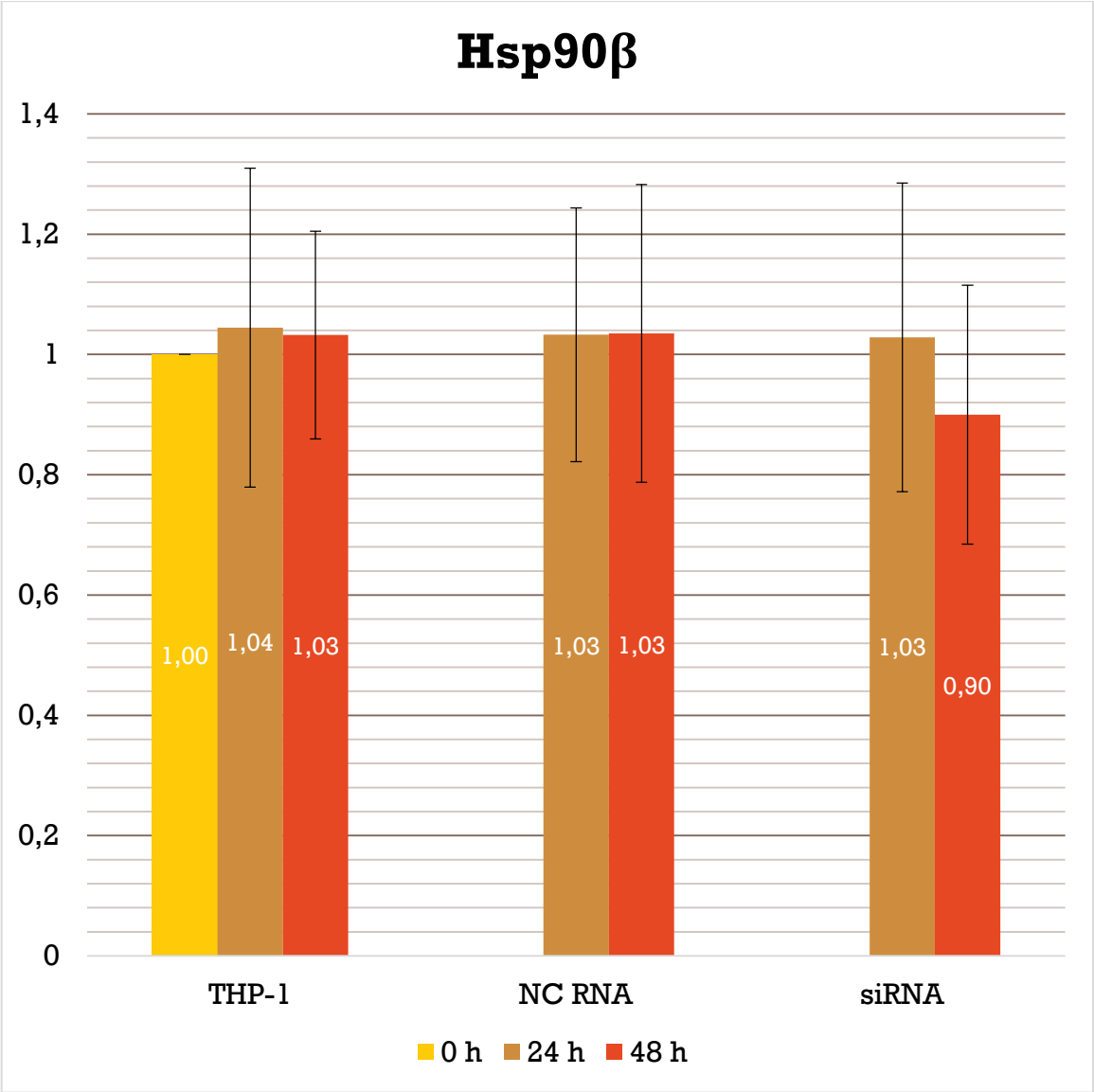


Figure 4.6 Clustered column chart of relative quantitative analysis of Hsp90β expression

Results are shown as an average value ± SD

4.2.2 Cdk expression

In order to further evaluate the efficiency of the Hsp90 silencing, effect on the expression of some of the Hsp90 client proteins was investigated. As mentioned in the introduction, it was previously shown that inhibition of Hsp90 action results in degradation of the client proteins via the ubiquitin-proteasome pathway (7, 14). We decided to assess the effect on expression of Cdk proteins, which are known Hsp90 client proteins (9). Cdks are protein kinases with an important role in regulating cell cycle. They form complexes with cyclins and only cyclin-Cdk complex has a kinase activity. Cdks role in cell cycle is the reason why they are considered as potential drug targets in cancer therapy (31). Evaluating the effects of Hsp90 silencing on client proteins could, in addition to helping us assess silencing efficiency, give more information on pathways, which lead to cancer cell death after interfering with Hsp90 action.

Results were not promising. Out of the three Cdks analysed (Cdk-1, p[Tyr15]Cdk-1, and Cdk-2) only the sample after 48 h showed slight but statistically significant ($p = 0.020$) reduction in Cdk-1 expression when compared to scrambled siRNA control sample (Figure 4.7). This is in accordance with the fact that the same sample (siRNA, 48 h) showed observable (but statistically insignificant) reduction in expression of Hsp90β (Figure 4.6).

Nakai and Ishikawa showed in their study that reduction in only Hsp90α isoform leads to Cdk-2 instability (32) but our study showed not similar results – in fact the Cdk-2 expression rose in samples treated with scrambled siRNA (after 48h) and siRNA against HSP90 (after 24 h and 48 h) when compared to the untreated cell line (Figure 4.9), which actually suggests that the transfection with siRNA itself could cause the rise in Cdk-2 expression. *Nakai and Ishikawa* used different approach in reducing the Hsp90 expression – by generating cell lines lacking HSFs (32), which are the most important means of Hsp induction, as mentioned in the introduction.

There was similar but less obvious rise in the expression of Cdk-1 ($p = 0.037$) after the transfection with scrambled siRNA for 48 h (Figure 4.7). This is interesting because Cdks expression is usually constant and their regulation based more on the post-translational modifications (phosphorylation, cyclin binding and Cdk inhibitors). Even though this phenomenon could be a consequence of the analytical error, further studies could provide us with more information on Cdks regulation during the transfection with siRNA.

The reason why expression of the Hsp90 client proteins Cdks was not reduced as expected could lie in the fact that Hsp90β expression remained unchanged and that there

was a compensatory induction of the Hsp70 expression, which is discussed in more detail in the next chapter.

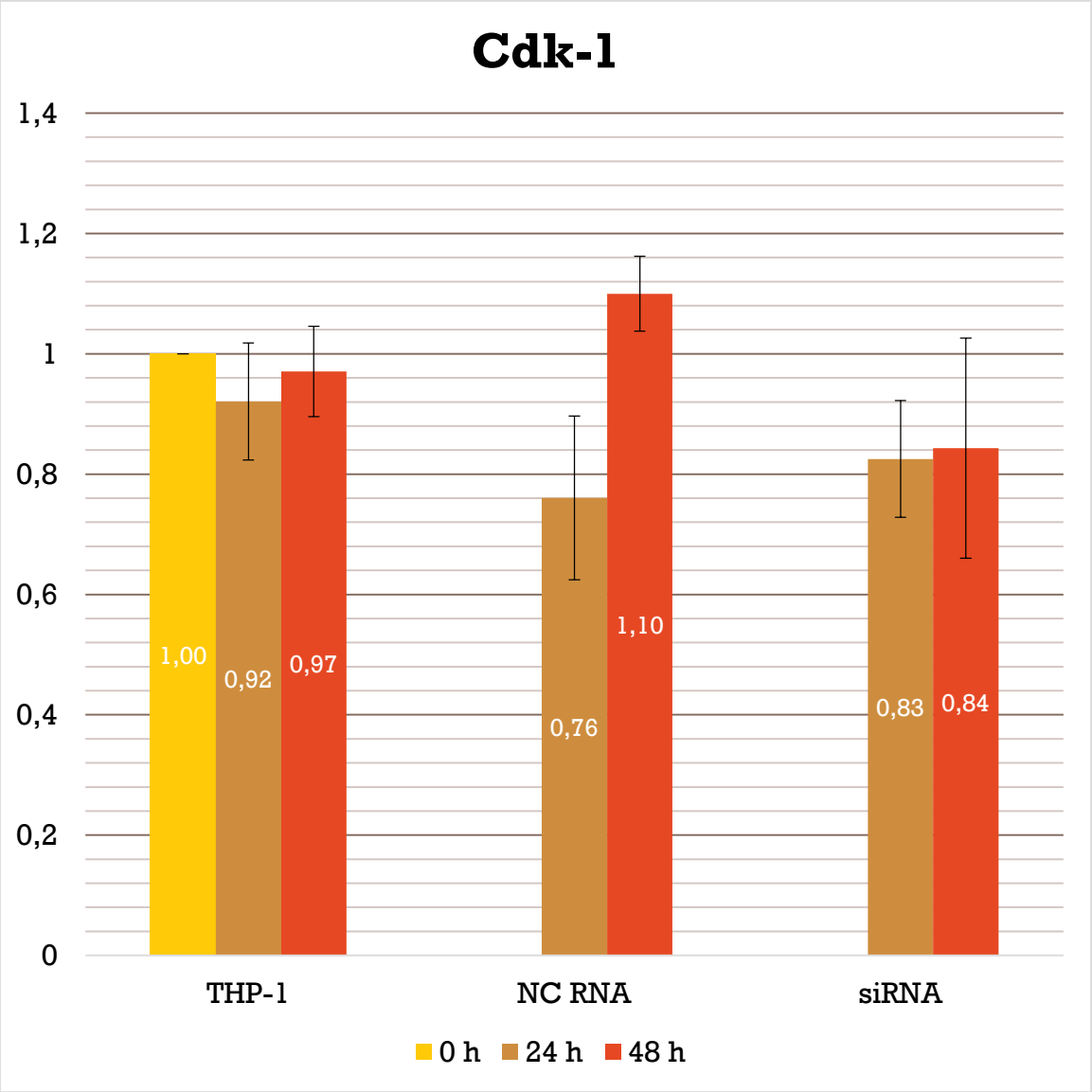


Figure 4.7 Clustered column chart of relative quantitative analysis of Cdk-1 expression

Results are shown as an average value ± SD

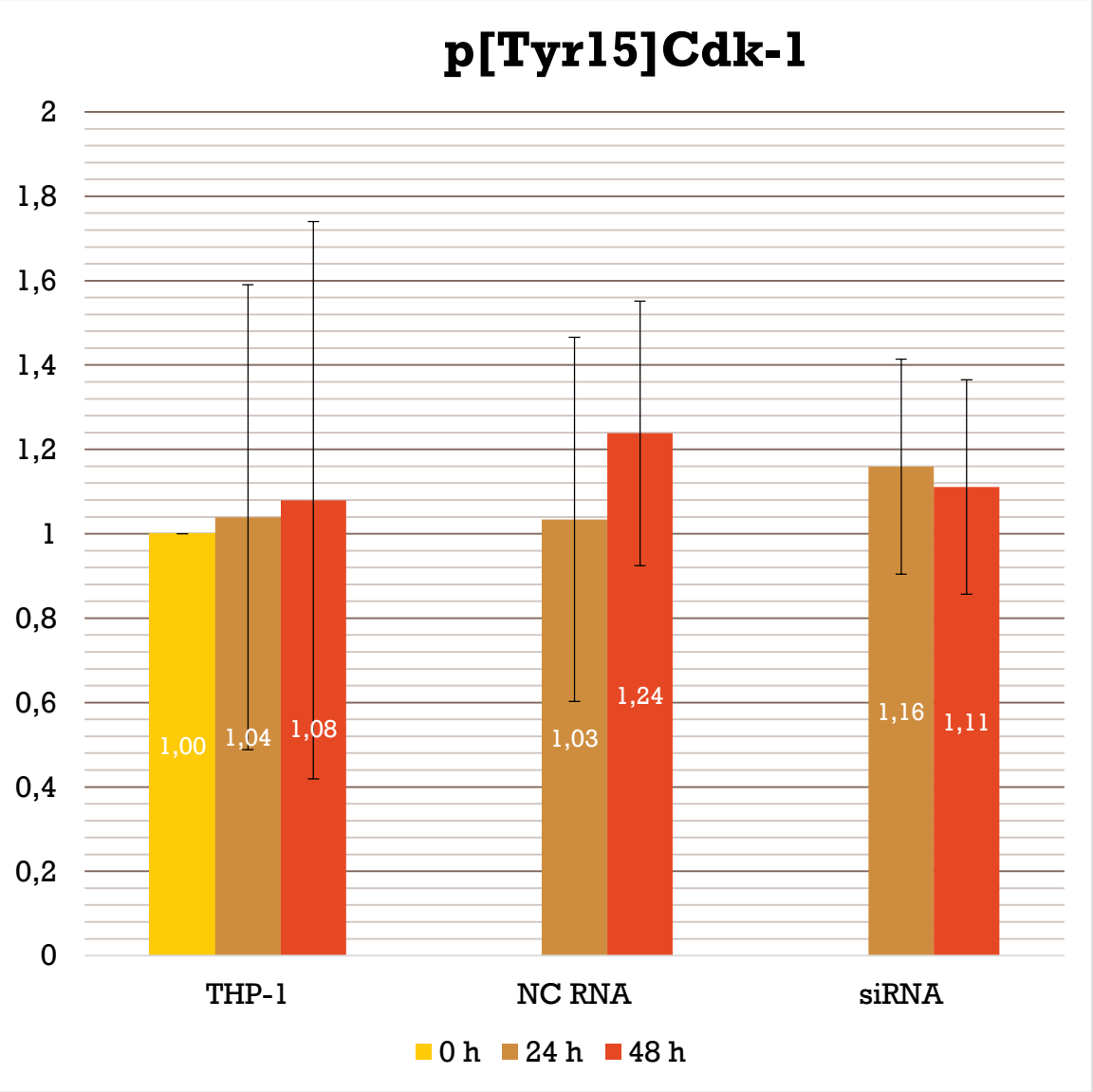


Figure 4.8 Clustered column chart of relative quantitative analysis of p[Tyr^{15}]Cdk-1 expression

Results are shown as an average value ± SD

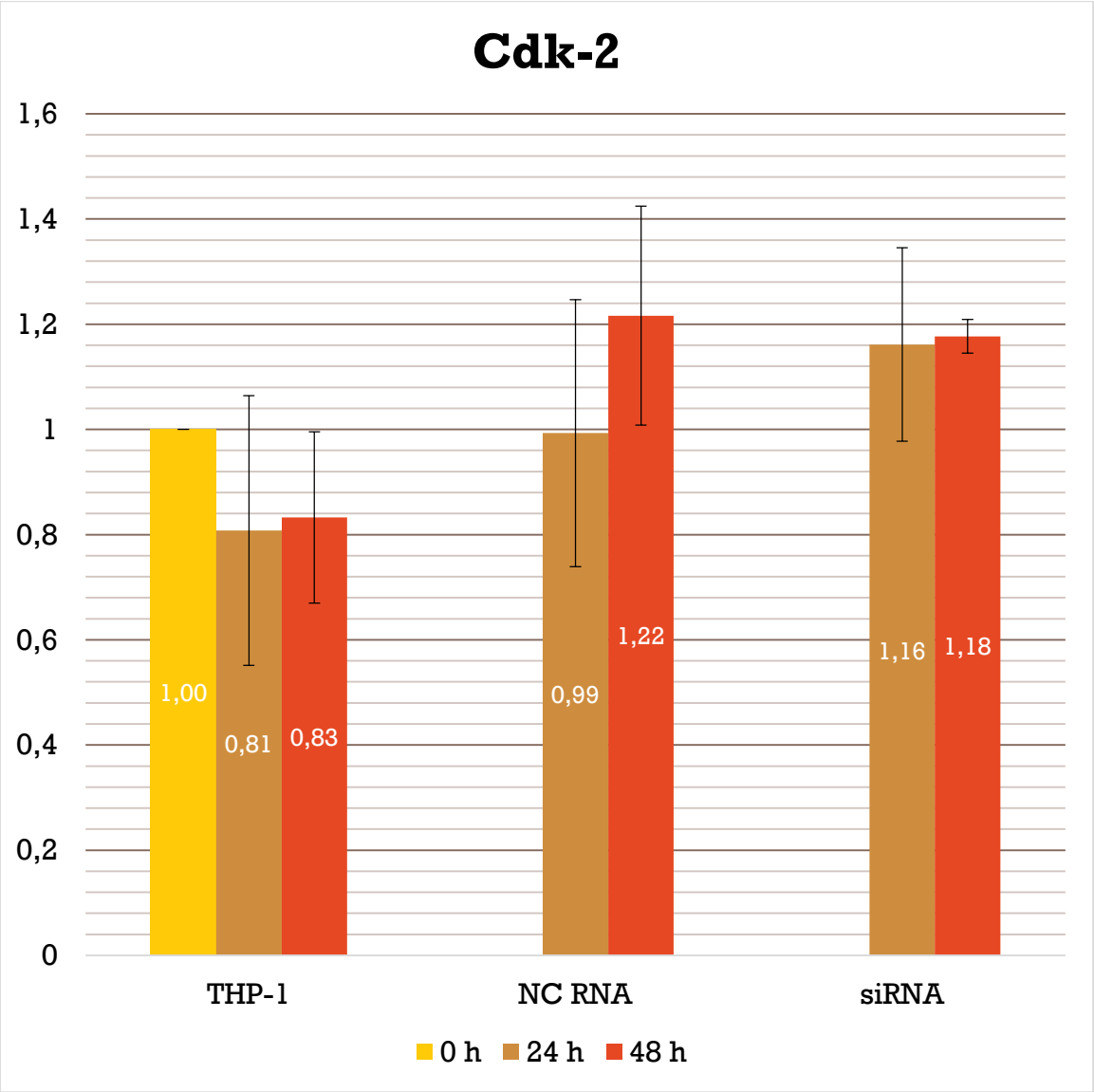


Figure 4.9 Clustered column chart of relative quantitative analysis of Cdk-2 expression

Results are shown as an average value ± SD

4.2.3 Expression of galectin-3

Our main aim of this study was to assess the effect of Hsp90 silencing on the galectin-3 expression. We theorized that galectin-3 expression would rise, since it is a well-known anti-apoptotic protein. Our results showed no such connection. According to our western blot analysis of the galectin-3 expression there is no significant difference between samples treated with scrambled siRNA and ones against Hsp90 (Figure 4.10). This suggests that there is no correlation between the Hsp90 silencing and galectin-3 expression and thus disproving our hypothesis.

There were however some interesting results. There was significantly higher expression of galectin-3 after 24 h of transfection with scrambled siRNA ($p = 0.018$), which than returned to normal after 48 h. There was a similar difference between 24 h and 48 h sample treated with siRNA against Hsp90. This suggests that there were some processes during the transfection with siRNA (both scrambled and directed against Hsp90) that affected the galectin-3 expression. One possible conclusion is that transfection with siRNA represents a new environment for the THP-1 cells, which than need to get adapted to it. There are many possible ways the cells can adapt to environment changes (*e.g.* hyperplasia, hypertrophy, atrophy) and further studies would be needed to determine if there was any adaptation at all, and if there was – if galectin-3 is included in the process of adaptation. This conclusion is in compliance with the previously mentioned hypothesis that transfection with siRNA represents a form of stress for the cell line. Other possible explanations are that a part of THP-1 cell line underwent spontaneous differentiation to macrophages or that cells were extensively proliferating at the 24 hour mark. Galectin-3 is known to be involved in both differentiation to macrophages of THP-1 cells and proliferation of various cell lines (19).

There were no similar researches that studied the connection between Hsp90 silencing and galectin-3 expression, so we are not able to evaluate our results on the basis of results obtained by other authors. This is why further researches are needed to confirm our findings.

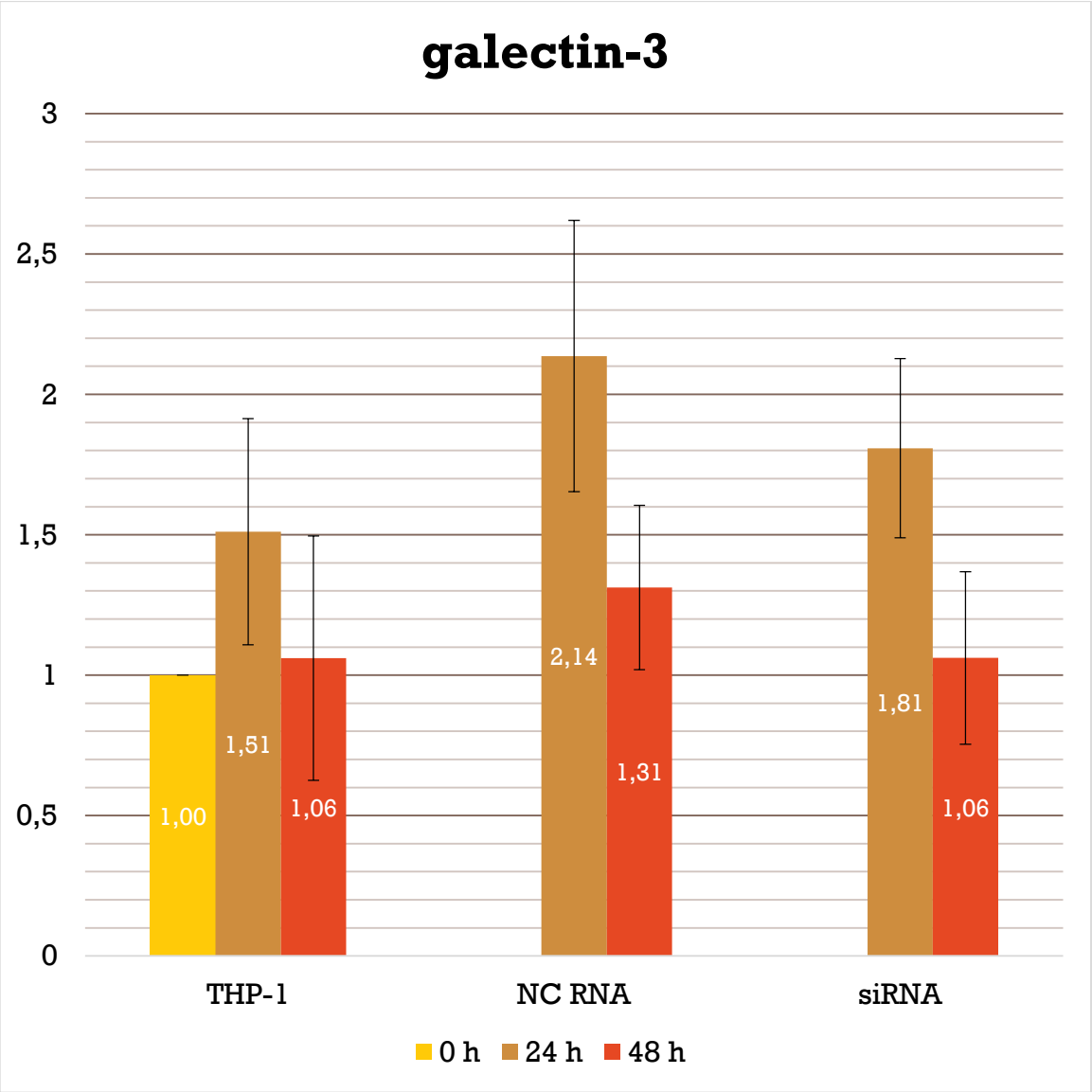


Figure 4.10 Clustered column chart of relative quantitative analysis of galectin-3 expression

Results are shown as an average value ± SD

4.2.4 Expression of Hsp70 and Hsp27

To further evaluate the compensatory mechanism resulting from Hsp90 silencing we analysed the expression of two other very important Hsps: Hsp70 and Hsp27. The Hsp90 binds to inactive form of Hsf-1 which means that silencing of the Hsp90 expression could result in Hsf-1 activation and consequentially in induction of Hsp70 and Hsp27. This occurrence has already been reported during the clinical evaluation of Hsp90 inhibitors and is considered a disadvantage in terms of cancer treatment since it protects cancer cells from apoptosis (12). We wanted to see if Hsp90 silencing had the same effect on our cell line.

Hsp70 was our primary Hsp of interest since it forms chaperone machinery together with Hsp90 and is a known anti-apoptotic protein (33). The Hsp70 is also upregulated in tumours and is considered as a possible drug target for use in a combination therapy with Hsp90 inhibitors (12, 34). Analysis of Hsp70 expression (Figure 4.11) showed that there was indeed a significant rise in Hsp70 expression for samples treated with siRNA against Hsp90 relative to the control group treated with scrambled siRNA for both 24 h ($p = 0.011$) and 48 h ($p = 0.006$) samples. It is possible that higher Hsp70 expression could compensate for Hsp90 function, which would explain why there was no significant change in Hsp90 client proteins (Cdk-1, p[Tyr15]Cdk-1, Cdk-2). The idea that the induction of Hsp90 co-chaperons could compensate for the lack of Hsp90 action was already hypothesized by Munje et al. (34) but further researches are needed to confirm this.

We previously postulated that the transfection with siRNA itself could represent a form of stress for the cell line. This should be observable on Hsp70 expression, since Hsp70 is extremely induced by stress (1). However our results did not show any transfection related induction in Hsp70 expression. This means that further research is needed in order to prove or disprove this postulation.

Hsp27 is also considered a possible drug target for combination therapy with Hsp90 inhibitors since it is also upregulated in response to activation of Hsf-1 following Hsp90 inhibition. Hsp27 silencing showed increase in cells sensitivity to Hsp90 inhibition (12). Our results, on the other hand, showed no increase in Hsp27 expression following Hsp90 silencing (Figure 4.12). This raises some questions given the fact that there was significant rise in Hsp70. It could be possible that rise in Hsp70 expression was not a consequence of the Hsf-1 activation but of some other pathway. Other possibility is that rise in Hsp70 expression inactivated Hsf-1 before Hsp27 expression could be induced. It would certainly

be interesting to investigate further how rapidly after activation of Hsf-1 is Hsp70 induced and how rapidly can the rise in Hsp70 inactivate Hsf-1.

Idea that Hsp90 co-chaperons could compensate for the lack of Hsp90 action and the fact that the expression of at least some of them is induced following Hsp90 silencing or inhibition could be the reason why induction in galectin-3 expression is not observed. Further investigations with silencing Hsp90 (both isoforms), Hsp70 and Hsp27 could shed some more light on the relationship between heat shock proteins and galectin-3.

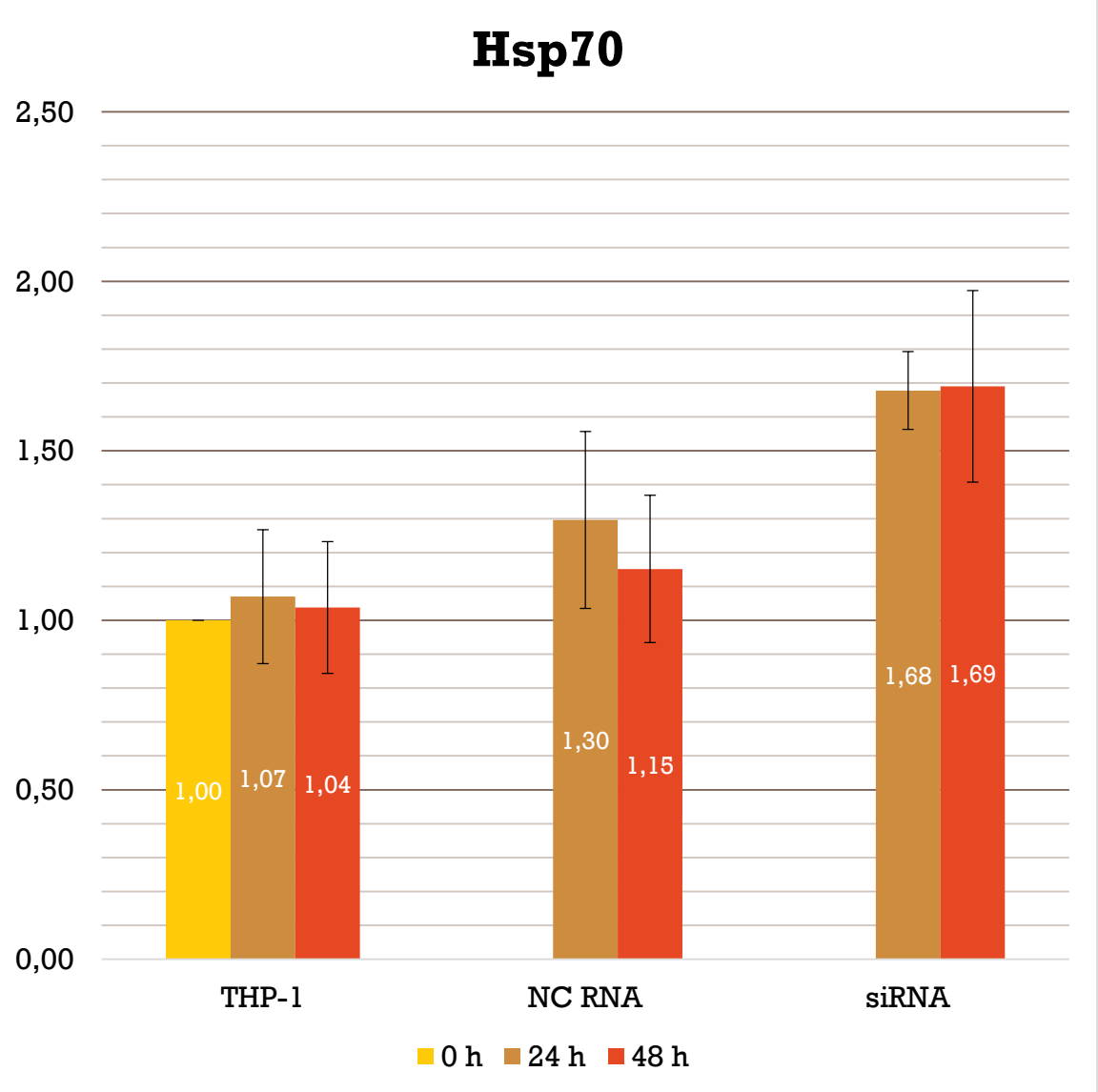


Figure 4.11 Clustered column chart of relative quantitative analysis of Hsp70 expression

Results are shown as an average value ± SD

4.3 IDEAS FOR FURTHER RESEARCH

If we would really want to eliminate galectin-3 as a possible drug target for co-treatment with Hsp90 inhibitors, we should do some further researches. We could improve the silencing of the Hsp90 (both isoforms) by creating an expression vector for the siRNA and thus overcoming the effects of transfection itself and the transient effect of siRNA transfection – creating a vector provides a long lasting knockdown. Another interesting possibility would be to try to silence all three Hsps investigated in this study and see if there are any effects on galectin-3 expression. There is also the possibility of silencing the Hsf-1 and thus eliminating the activation of this protein that follows Hsp90 silencing. Further investigation in functions of Hsp70 and Hsp27 in Hsp90 absence could provide us with the answer if they could really compensate for the lack of the Hsp90 action.

Looking at the changes in mRNA and miRNA after the Hsp90 silencing could provide us with information that is needed in order to evaluate which other protein expression is affected by Hsp90 silencing. It could provide us with hints on proteins that are compensatory induced even if there are no clear connections between them and Hsp90.

Since novel drug delivery systems are being developed it would be interesting to investigate the possibility of delivering the combination of siRNAs against Hsp90, Hsp70 and Hsp27 directly to cancer cells via lipid nanoparticles and thus overcoming the downsides of conventional chemotherapy.

5 CONCLUSION

Our results showed that the silencing of the Hsp90α can only be partially achieved by the siRNA transfection, because of the transient effect of siRNA silencing and doubling time of THP-1 cell line. The results also pointed out that the expression of Hsp90α is potentially induced by transfection by lipofection. The expression of Cdk-1 is only partially affected by Hsp90 silencing, while the Cdk-2 and Cdk-1 ([pTyr15]Cdk-1) are not affected.

The man aim of this study was to assess the effects of Hsp90 silencing on galectin-3 expression. Galectin-3 expression is not affected by partial silencing of the Hsp90α which suggests that galectin-3 is not regulated with this pathway and is not potentially included in the compensatory pathways that follow the inhibition of Hsp90 action. There were however some indications that the galectin-3 expression is potentially induced by transfection by lipofection itself.

The Hsp70 expression is induced with the Hsp90α silencing, which suggests that Hsp70 can compensate for the lack of Hsp90 action and can be used in the combination therapy with Hsp90 inhibitors. Expression of Hsp27 on the other hand is not correlated with the inhibition of the Hsp90 action.

To fully confirm the results of this study, further researches are needed. More effective silencing of both Hsp90 isoforms is needed in order to completely dismiss the connection between the Hsp90 and galectin-3 expression.

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