Targetomics, application of *in vitro* screening methods for the identification of new drugs

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Kurzfassung

Die Wirkung zahlreicher Naturstoffe und chemischer Verbindungen an ihren Targets ist häufig unbekannt. Um eine Target-orientierte Wirkstoffsuche effizient durchzuführen, benötigt man Targets und einen entsprechenden Test. Diese Tests beruhen meistens auf bindenden oder katalytischen Funktionen, die meist über optische Verfahren visualisiert werden. Die Wirkstofftests können dann zell- oder enzymbasiert erfolgen. Vielfach sind diese Tests aufwendig und teuer. Um dies zu optimieren, wurde ein hoch miniaturisierter Microarray-basierter Test für Proteine entwickelt, der mit sehr geringen Materialmengen durchführbar ist. Gereinigte Proteine sind dann die Targets für die Wirkstoffe, dabei wird die Bindungseigenschaft der Targets für Liganden oder ähnliches ausgenutzt, um daran mögliche kompetitive Wirkstoffe und ihre chemisch optimierten Derivate zu testen. Targets unterschiedlicher Proteome wie Hsp90, Hsp70 u.a. als Signaturen von zellulärem Stress oder Pathogenien können so hinsichtlich Wirkstoffsuszeptibilität oder Diagnostik erforscht werden. Um die Bindung und die Kompetition zu visualisieren wurde fluoreszenzmarkiertes ATP eingesetzt. Hierbei konnte gezeigt werden, dass die Position des Fluoreszenzmarkers am ATP die Bindung an die Hitzeschockproteine Hsp90 und Hsp70 unterschiedlich beeinflusst. Der Einfluss einer hausinternen Wirkstoffbibliothek (~150 Inhibitoren) wurde im Kompetitionstest mit Geldanamycin als Leitstruktur auf die ATP-Bindetasche an verschiedenen Hitzeschockproteinen 90 (Hsp90) untersucht. Es war möglich, Gda-Derivate zu identifizieren, die sehr hohe Affinitäten für menschliches Hsp90 im nanomolaren Bereich aufweisen, aber mit geringeren Affinitäten für Hsps von *Leishmania braziliensis*. Die Bindungsaffinitäten wurden zuerst als IC_{50}-Werte mittels Mikroarray-Verfahren bestimmt, sowie orthogonal über ITC und zellbasierte Tests bestätigt. Weiterhin war es durch die Verwendung von Hsps in voller Länge möglich, die Wechselwirkungen von L1CAM, einem Zell-Zell Adhäsionsprotein, mit verschiedenen Proteinen wie Hsp90, Hsp70, L1CAM oder CsgA (Curli) Mikroarray-basiert nachzuweisen. Neben der Möglichkeit Protein-Ligand Wechselwirkungen Mikroarray-basiert zu testen, kann man die Technik auch nutzen, um Protein-Protein Interaktionen zu bestimmen.

Stichwörter: Hitzeschockprotein, Geldanamycin, Miniaturisierter Protein-Mikroarray, *Leishmania braziliensis*, Fluoreszenz-markiertesATP

Abstract

The effect of various natural products and chemical compounds to their corresponding molecular target is often unidentified. Drugs are effective by binding to a molecule (the target) and inhibiting its function. In order to perform an efficient target-oriented drug discovery, target and related tests are needed. These tests are mostly based on a binding or catalytic function, which are usually visualized by optical methods. Subsequently, the cell- or enzyme-based drug tests can be done. In many cases, these tests are complex, expensive and time-consuming. To optimize these obstacles, a highly miniaturized microarray-based test for proteins has been developed which can be carried out with very small amounts of material. Purified proteins are the target of the active compounds, while the binding property of the targets for the ligand and possible drugs and their optimized derivatives are tested. Targets of different proteomes such as heat shock proteins 90 (Hsp90) and Hsp70, a cellular stressed or pathogenic, are studied with regard to drug susceptibility or diagnostic potential. Fluorescent ATP was used to visualize binding of the natural ligand and competition by potential drugs. It could be shown that the position of the fluorescent marker on the ATP influences the binding to the heat shock proteins Hsp90 and Hsp70 differently. The influence of an in-house drug library (~150 inhibitors) was investigated in the competitive assay with geldanamycin (Gda) as a lead structure for the ATP binding pocket on various heat shock proteins 90 (Hsp90). It was possible to identify Gda derivatives that bind very affine on human Hsp90 with K_d in the nanomolar range while exhibiting lower affinities for Hsp90 from *Leishmania braziliensis*. Compounds determined by microarray techniques were further confirmed by ITC and cell-based assays. Furthermore, the use of full-length Hsps in microarray-based assay made it possible to measure the interaction of L1CAM, a cell-cell adhesion protein with various proteins such as Hsp90, Hsp70, L1CAM and CsgA (Curli). In addition to the ability of microarray-based assays in testing the protein-ligand interactions, it has been also documented that it is possible to determine protein-protein interaction by this technique.

Keywords: Heat shock protein, Geldanamycin, Miniaturized protein-microarray, *Leishmania braziliensis*, Fluorescence-labeled ATP

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1. Introduction and objective

According to the report of the World Health Organization, more than 30 million among the 56.9 million deaths worldwide in 2016 were due to the fatal diseases namely heart disease, cancer, tuberculosis, Alzheimer and other dementias-causing diseases as well as infections like leishmaniasis and malaria, etc[1–5].

Diseases are based on changes that happen at the cellular level. Molecular chaperones are involved in many constitutive cellular processes, folding, processes for cellular homeostasis, intracellular transport, and degradation of proteins as well as in assisting cell signaling[6,7].

Organisms must remain alive among diverse stresses and extreme conditions. Stress may damage the cellular structures and interfere with important functions[8]. Heat shock proteins belong to the molecular chaperone family and play a major role in the protein folding and prevent the formation of protein aggregates. In another word, they present a sophisticated protection mechanism[8,9]. Heat shock proteins are essential for the survival of many organisms. In many different investigations of cancers, the high expression of Hsps was reported which demonstrate the heat shock proteins as a promising target to fight cancer[10,11]. Hsps are associated with several signs of cancer in particular cell proliferation, invasion, and metastasis. Thereby Hsps support the development of a tumor. Hence by inhibiting Hsps, prosperous therapeutic consequences have been achieved[10]. Hsp90 and Hsp70 are the most important heat shock proteins which are found in all prokaryotes and in cellular compartments of eukaryotic organisms[12,13].

By finding specific small molecules to inhibit these chaperones, they would be no longer contributing to the stabilization of the tumors and the development of other diseases. The high-throughput screening (HTS) method is necessary to test the numerous compounds and their bindings in the fastest possible time. A miniaturized protein microarray-based assay provides a powerful approach to test the functions of the proteins as well as identifying novel inhibitors in a quick, efficient and affordable way[14]. The Hsps are immobilized on a single microarray chip and are analyzed in parallel with a few amounts of potential inhibitors. The miniaturized protein microarray-based technique is based on competitive binding of fluorescence-labeled ATP and potential inhibitors to the ATP-binding pocket of Hsps[14]. Other applications of the microarray system are the determination of protein-protein interactions.

1. Introduction and objective

The aim of the present study is to use a highly miniaturized microarray-based screening method for the identification of novel compounds specific for the ATP-binding site of Hsps with respect to the diversity of Hsps. The purified Hsp proteins from human, bacteria, protozoa as well as plant have been used as targets.

The present study consists of three individual projects; One project is focused on patho-targetomics which is an approach for the identification of new targets specific in pathogenic cells such as cancer as well as leishmaniasis[15]. The next project is based on ATP and potential Hsp70/DnaK inhibitors binding tests on a highly miniaturized microarray, as well as the effect of the fluorescence label on ATP[16].

The further investigation is led to the great results of the effect of two novel labdane diterpenoids and one new β-lactam derived from the aerial parts of *Roylea cinerea* on Hsp 90[17].

The potential inhibitors have been kindly provided by the Institute of Organic Chemistry (OCI) of Leibniz University of Hannover.

2. Theoretical background

2.1. Disease marker in the proteome and their physiological impact

According to the World Health Organization (WHO), over half (54%) of the deaths worldwide in 2016 were due to the ten leading causes of global deaths including Ischaemic heart disease and stroke which are the world's main killers in the last 15 years, Lower respiratory infections, chronic obstructive pulmonary disease, lung cancer, diabetes, Alzheimer disease, diarrhoeal diseases, tuberculosis and road injuries respectively[5,18].

As quoted by Rudolph Virchow, the father of modern pathology, all diseases are disturbances and alterations that occur at the cellular level[19]. In diseases like cancer, the cells have a specific proteome that is different in comparing to the proteome of the normal cells. The random mutations in cancer cells which trigger the development of genomic instability allow them to propagate, proliferate and disseminate. The genetic changes in malignant cells result in the tumor progression and survival[20]. The mutation in genome leads to the misfolding, unfolding and aggregation of the proteins. Protein folding errors may enhance various diseases as well as many problems in cells. As a matter of fact, the failure of proteins to fold properly is the origin of malfunctioning of living systems and a broad diversity of pathological situations. Therefore most diseases are folding diseases. Earlier was shown that heat shock proteins are induced by different stresses and are essential for eukaryotic cells[21,22].

A failure in protein folding leads to several known diseases. When the proteins do not fold properly, there are two problems happen in the cells.

One type of problems is called "loss of function" which occurs when the insufficient of a certain protein folds properly. For example, a protein is responsible for metabolizing sugar in order to be used as energy. If the particular protein is not enough in its functional state, the cells will grow gradually due to the lack of energy. This condition affects the cells and makes it sick because of the deficiency of the specific properly folded functional protein. Cystic fibrosis, Tay-Sachs and some types of cancers are the best examples of this kind of problem.

The second type of the problems happens when the proteins do not fold properly into their functional state. Misfolded proteins which lead to devastating human disease[23,24]

may stick together and form the clumps called “aggregates”. In the high aggregate rates, it will kill the cells. In several neurological ailments, including Alzheimer, Parkinson, Huntington, and amyotrophic lateral sclerosis (ALS) diseases, etc, accumulation of misfolded proteins in the other word aggregated proteins plays a significant role[25].

The best ways to control cellular activity are folding and unfolding. Some mechanisms are directly dependent on folding and unfolding such as the immune response and regulation of the cell cycle, translocation across the membranes and secretion. Recently it was shown that the active presence of a kinase is under the control of Hsp90[26]. Molecular chaperones have the main role in helping the newly synthesized proteins in order to adapt their biologically active conformations mediate refolding of misfolded proteins and break down the protein aggregations [27,28].

As mentioned before, there is a correlation between diseases and the changes in protein expression levels[29]. Therefore by investigating deeply on the different cellular conditions including normal and diseased states, proteomics present a better understanding of the pathophysiological basis of protein target identification and validation for intervention and therapeutic approaches. Moreover, proteomics has substantially provided for the detection of targets for preventive or chemotherapeutic treatment, likewise biomarkers that can apply for the diagnosis of the various diseases. The markers may be utilized as protein signatures in order to screen new chemical entities for target organ toxicity in preclinical trials and in the development of clinical trials, certify their efficiency in the diagnosis and prognosis of diseases[29,30].

A biomarker is a measurable indicator of some biological states. It is the presence of a biological molecule which is a sign of normal or pathogenic processes, and also pharmacologic responses to therapeutic remedy. For example, the occurrence of an antibody may indicate an infection. Precisely, a biomarker demonstrates a change in expression of a protein that correlates with the risk or development of a disease. They are applied as analytical tools for a fast and comprehensive therapeutic analysis in order to early diagnosis and the development and assessment of novel therapies. An advantageous marker needs to fulfill some characteristics such as:

I) Easy to be measured
II) Being specific to the target
III) Expressed as early as possible in order to have initial diagnosis[31]

It has already proofed that in order to hinder protein misfolding, the molecular chaperones are required. As a result of that, the concentrations of many of these species considerably rise during cellular stresses. In fact, the designation of many as heat shock proteins (Hsps) confirms this fact that the increased cellular level of misfolded proteins is in correlation with the increasing concentration of Hsps[28,29]. This strongly illustrates that the increased level of intracellular Hsp in cells can serve as a cellular stress marker. Numerous diseases emerging from folding errors are recognized including different types of cancer, neurodegenerative diseases, cystic fibrosis, Alzheimer's and Parkinson's diseases, retinitis pigmentosa or Huntington´s chorea and more other[11,32]. The functions of Hsps have been studied in various conditions and pathologies where they accumulate, such as many kinds of cancer. Some members of the diverse family of Hsps share several features that make them eligible to be as cancer biomarkers. It sounds that Hsps do not distinguish different cancer types. But this characteristic may be assumed as the significant advantage of Hsps that make them be as a potential universal family of cancer biomarkers[31].

For the sake of developing a faster diagnostic test procedure and providing therapeutic indications at the same time, the study of biomarkers would be an extremely important approach to diseases diagnosis.

2.1.1. Heat shock proteins (Hsps) as stress regulators in pathogens and diseases

Stress or heat shock proteins (Hsps) are one of the largest components of the cytoplasmic network which present in all cells of all organisms since the thermo regulation of a proper temperature area is essential for survival. Large deviations from this area cannot tolerate for a longer time, but for thermal adaptation, different heat shock proteins exist. Moreover, they have an important role in all cellular stress response. Their expression is induced by exposure to different types of physiological and environmental stresses including physical, chemical and biological insults. Radiation, pH changes, lack of nutrients, oxidative stresses as well as alcohol are some examples of the physical and chemical stresses. Whereas the biological stresses contain high temperature, infection, cold, inflammatory processes, various diseases including cancer, cardiac and neurodegenerative diseases, which increase heat shock protein production in the cells[31,33,34].

Due to their protective function, the proteins are produced as a response to the stress and ensure a lasting existence of the cell. Hsps are essential for the survival of many organisms. By reason of the involvement of these proteins in numerous diseases, they could gain the attention of medical and molecular investigations[35–37].

Heat shock proteins have strong cytoprotective effects and behave as molecular chaperones for other cellular proteins thus they play a critical role in the proper folding of proteins, assembly of multiprotein complexes, transport and trafficking of proteins into correct subcellular compartments, cell-cycle control, signaling, and protection of cells against stress/apoptosis[38]. Hsps take a role as a rescue pathway of many damaged or unfolded client proteins by restoring the function of proteins without the necessity for the *de novo* synthesis of the unfolded proteins[11]. They assist remarkable part of the proteome by three different types of function such as foldases (assist in folding and refolding), holders (preventers of unfolding and misfolding), and disaggregates (involved in solubilizing aggregates)[39]. Many diseases, for instance, cystic fibrosis and some types of cancer result from the improper folding of proteins. In some other diseases including Alzheimer's and Parkinson's diseases, the spongiform encephalopathies and diabetes type II, which are exactly involved with the deposition of such aggregates in brain, heart and spleen tissues respectively, the proteins aggregate within cells or more commonly in the extracellular area. Since the probability of misfolding is usually higher in mutational variants, several such disorders are hereditary[28]. The increased cellular level of misfolded proteins also increases the concentration of Hsps.

The heat shock proteins are typically classified into five main and broadly conserved families by their average molecular weight: Hsp100s, Hsp90s, Hsp70s, Hsp60s, and small heat shock proteins (sHsps). Hsp70 and Hsp90 families are known as the best instance of foldases whereas the small Hsp families and Hsp100 chaperones are the outstanding examples of holders and disaggregate respectively[40,41].

The interaction of heat shock transcription factor (HSF) and heat shock element (HSE) is providing the transcription of HSP genes. Four different HSFs have been determined: HSF1, HSF2, HSF3 and HSF4. Only HSF1 regulates the HSP synthesis whereas all HSFs are induced during development and adaptation of the cells. When the cell is exposed to stress, HSF1 is activated and regulates the expression of HSP genes[39,41,42]. In the most molecular chaperones like Hsp during the folding procedure in order to function effectively, they need ATP as energy[28].

According to the intracellular or extracellular location of Hsps, they have a double task. The membrane-bound or extracellular Hsps has immunological functions. By means of natural or adaptive immune system, they can evoke an immune response modulated. In contrast, the Intracellular Hsps have a protective role that survives the cells from fatal conditions by their anti-apoptotic characteristics. Hsp90, Hsp70 and Hsp27 can directly interact with different proteins that are related to programmed cell death machinery, thereby they can block caspases activation and apoptotic cell death. For example, Hsp27 binds to cytochrome and Hsp70 or Hsp90 binds to the apoptotic protease activating factor 1 (Apaf-1). Moreover, Hsp70 associating with apoptosis-inducing factor (AIF), can inhibit the caspase-independent cell death[43]. Therefore, the cytoprotective functions of Hsps are essential in maintaining the survival of the malignant cell. The expression and/or activity of heat shock proteins (Hsp90, Hsp70 or Hsp27) are abnormally high in cancer cells, and this overexpression is linked with poor prognosis, increase in tumor growth, metastatic potential and high resistance to chemotherapy and the anticancer treatment. The inhibition of Hsps may lead to reducing the size of tumors and prevent the relapsing. Thus, the policy of blocking Hsps may provide benefit to cancer patients[33].

As it was mentioned before, molecular chaperones, for instance, Hsps have an important role in maintaining protein homeostasis. They are induced by various stress signals but they promote cell survival in adverse conditions[31]**.** They are also involved in different pathogenesis processes in intercellular protozoa. Being infected by the pathogens is stressful for both parasites and its host. Due to this issue, the molecular chaperones and specially Hsps are produced by the parasites to make an adaptation[39,44,45].

In recent years the heat shock protein 90 (Hsp90) and heat shock protein 70 (Hsp70) have been recognized as main targets in many diseases especially in cancer cells and they are under intensive studies.

2.1.1.1. Heat shock protein 90 (Hsp90)

Hsp90 belongs to a molecular chaperone family which is the most ubiquitous and abundant cytosolic proteins that have an essential role in various cellular processes including cell cycle and survival, hormones and signaling pathways. It is the main player in keeping the cellular homeostasis. The heat shock protein 90 constitute up to 1% of the total soluble protein under physiological conditions, whereas stress increases

the cellular Hsp90 protein level up to 4-6% of cellular proteins[46,47]. In the last decades, it has emerged as a major therapeutic target for cancer as well as increasing interest in targeting Hsp90 in neurodegenerative diseases, antiviral and anti-protozoan infections[46,48–50].

The Hsp90 family can be subdivided into five subfamilies: cytosolic Hsp90s (Hsp90α and Hsp90β), Endoplasmic Reticulum-localized Grp94 (*glucose-regulated protein*), mitochondrial TRAP1 (*tumor necrosis factor receptor-associated protein 1)* as well as bacterial HtpG and chloroplast Hsp90[48,51].

The Hsp90 chaperone is a homodimer and the individual monomer is composed of three specific domains with different functions:

i) N-terminal Domain (NTD) (25 kDa), has an adenine binding pocket which is essential for the ATPase activity of the protein. Furthermore, this binding site is the target of many drugs (e.g., geldanamycin and radicicol) which can inhibit Hsp90 activity.

ii) Middle Domain (MD) (55 kDa) stabilizes the protein is a binding site for some co-chaperones such as Aha1, p23.

iii) C-terminal Domain (CTD) (10 kDa) which is responsible for being dimer. There is also an unstructured region tetratricopeptide repeat (TPR) motif recognition site, the conserved MEEVD pentapeptide, containing co-chaperones such as the immunophilins, Hsp70/Hsp90-organizing protein (Hop) and protein phosphatase 5 (PP5) which interacts with the Hsp90 and specifically regulates ATP activity[52–54].

Between the Middle and the N-terminal domain is an unstructured linker region. The N-domain binds to ATP / ADP in the ATP binding pocket by a conformational change of the nucleotide cap (lid) and together with the M-domain catalyzes ATP hydrolysis[35,55] (Fig. 1). Hsp90 is an ATP-dependent molecular chaperone and its activity depends on the ability to bind and hydrolyze ATP which involve in various conformational changes in proteins. When ATP molecule is bound and hydrolyzed in the adenine binding pocket, the conformation of the protein changes and it becomes to the closed status which is an active form. After hydrolysis, ADP + Pi (phosphate) is released and the protein returns to its open initial state. Hsp90's ATPase activity is associated with its chaperone function. Co-chaperones, as well as posttranslational modifications, are important for regulating its ATPase activity[56,57].

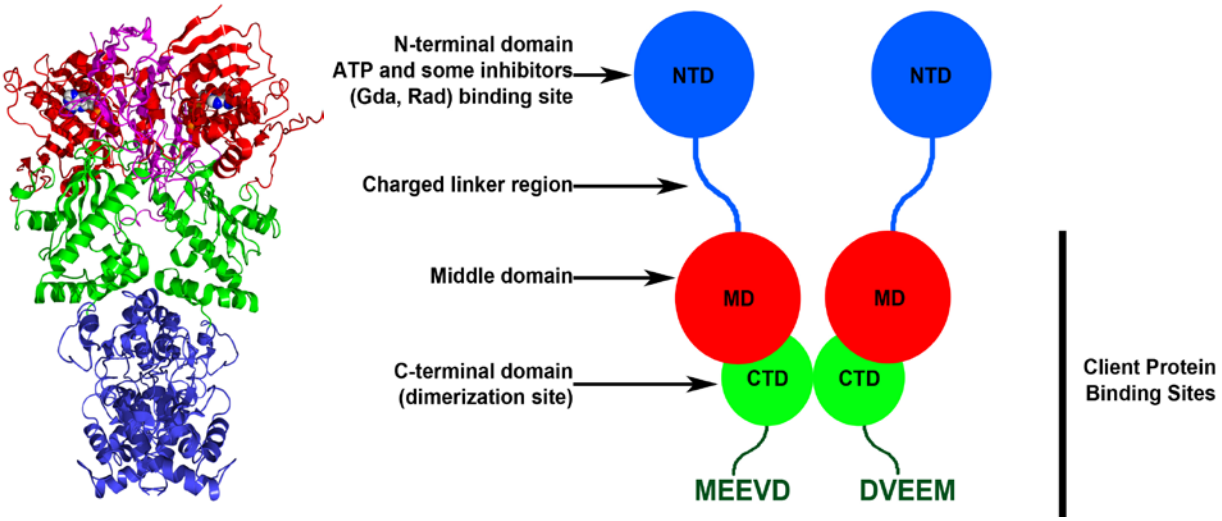
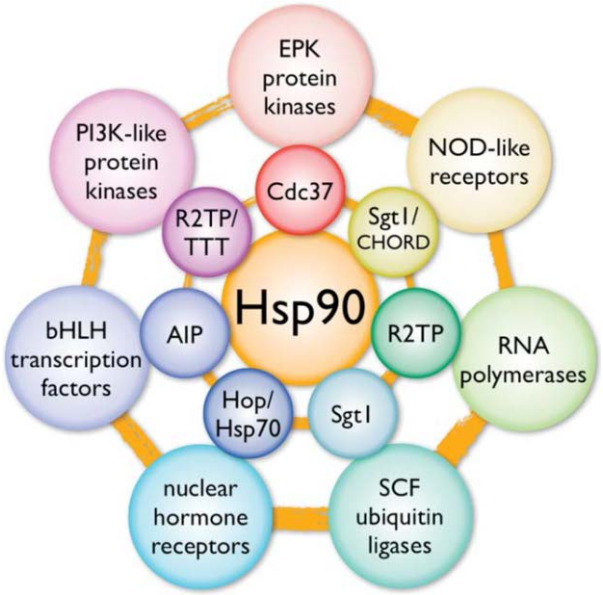


Figure 1: The structure of Hsp90. N-terminal, Nucleotide Binding Domain (NTD) binding site of ATP and small molecule inhibitors such as Geldanamycin (Gda), Radicicol (Rad) as well as co-chaperones and client proteins, MD: intermediate domain, CTD: C-terminal dimerization domain. Adapted from Ref. 58[58].

Co-chaperones are the important interaction partners of heat shock proteins. More than 20 different co-chaperones from Hsp90 are known which involved with the ATP cycle. The co-chaperones of cytosolic Hsp90 include p23, Hop, FKBP51 and 52, CyP40, PP5, Cdc37, SGT-1, CHIP and Aha1. Figure 2 shows an overview of the most famous co-chaperones Interaction network of heat shock protein 90 (Hsp90) and other proteins. The bioinformatics tool, STRING (search tool for the retrieval of interacting genes/proteins) is a database of well-known and predicted protein interactions.

a)



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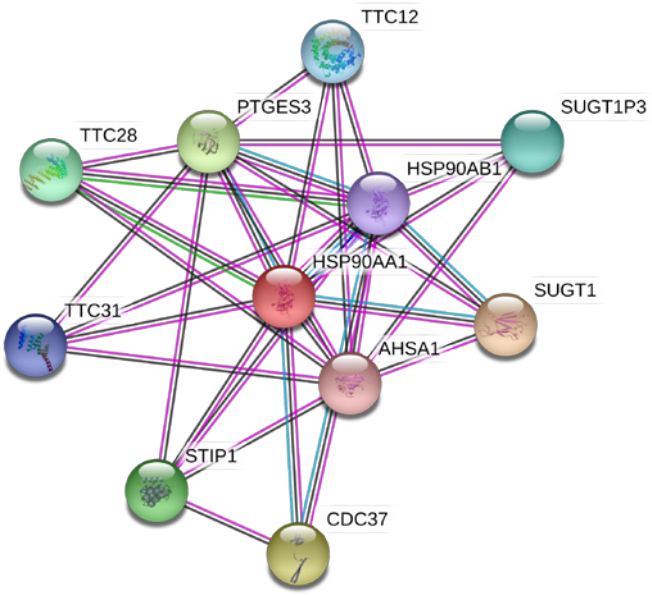


Figure 2: a) Schematic presentation of the Hsp90 and its clients. Hsp90 involves with different client proteins (external circle) has interaction with several co-chaperone proteins or complexes (internal circle. Reprinted from Pearl, L.H., (2016)[12]. **b)** Interaction network of heat shock protein 90 kDa (Hsp90) alpha (cytosolic); SUGT1: suppressor of G2 allele of Skp1 (*S. cerevisiae*), CDC37: Cell division cycle 37 homolog (*S. cerevisiae*), PTGES3: Prostaglandin E synthase 3 (cytosolic), STIP1: Stress-induced phosphoprotein 1, A1: Aha1, activator of heat shock 90 kDa protein ATPase homolog 1 (yeast), TTC28: Tetratricopeptide repeat domain 28, SUGT1P3: Suppressor of G2 allele of Skp1 (*S. cerevisiae*) pseudogene 3, TTC12: Tetratricopeptide repeat domain 12, TTC31: Tetratricopeptide repeat domain 31. Made by STRING software.

Hsp90 is involved with important cellular transformations of intracellular protozoan parasites and human pathogens such as *Plasmodium*, the strain which causes malaria, *Leishmania* (the strain which is responsible for leishmaniasis) and *Trypanosoma* is the strain which triggered Chagas and sleeping disease[48].

Due to the presence and being essential for the most eukaryotic cells and also having different domain and binding sites, Hsp90 grab interest and serve as a therapeutic target. Furthermore, Hsp90 has gained much popularity as a target for treating diverse infectious diseases and cancer as well. It is assessed as a biomarker in cancers (including bladder, breast, prostate, ovarian, etc)[31] and involved in many important cellular processes of intracellular protozoans and other important human pathogens[59]. Since Hsp90 is responsible for the conformational maturation of some enzymes involved in various cancer pathways, it functions as a target for the cancer[60].

Heat shock protein 90 also plays a key role as a therapeutic target in some neurological and pathogenic illnesses including leishmaniasis and malaria[61]. They can be targeted by secondary metabolites known as Hsp inhibitors. The inhibition of Hsp90 results into an incapability to react to the stresses which are usually associated with pathogenic cells especially cancer cells[60].

In these cells, Hsp90 has higher activity as well as ATP-binding affinity comparing to the normal cells. These features could make Hsp90 as a potential therapeutic target[60,62,63].

2.1.1.2. Heat shock protein 70 (Hsp70)

The human genome superfamily of the heat shock protein 70 consists of 13 members. There are four major proteins including constitutively expressed HSC70, endoplasmic reticulum-localized GRP70, mitochondrial mtHsp70 and stress-inducible Hsp70[64]. In principle Hsp70 influences the development of proteins protects the proteome against stress and renature the aggregated proteins. It also influences the transport through membranes and builds the protein complexes.

In the promoter part of heat shock transcription factor 1 (HSF1) the regulation of the expression of heat shock elements (HSEs) is controlled by cellular stress, including the heat shock, oxidative stress, some pathological conditions, exposure to heavy metals, hypoxia and so on. These stresses also cause the proteins to unfold and become inactive[65].

The 70-kDa heat shock proteins (Hsp70s) assist a wide range of folding of newly synthesized, misfolded, aggregated proteins and has a key role in the cancer field, such as induction of resistance to chemotherapy, inhibition of apoptosis and regulation of the stability of oncoproteins[66,67]. It is highly conserved and ATP-dependent chaperones and its structure is similar to Hsp90 which consist of an N-terminal ~44 kDa nucleotide binding domain (NBD) which includes the ATP binding site and hydrolysis, a 15 kDa substrate polypeptide binding domain (SBD) which interacts with the hydrophobic amino acids in peptides and ~10 kDa α-helical C-terminal, a 'lid' domain that closes over the substrate and also mediates the biding of the co-chaperone. In bacterial cells, DnaK is the eukaryotic Hsp70 equivalent[68–71] (Fig 3).

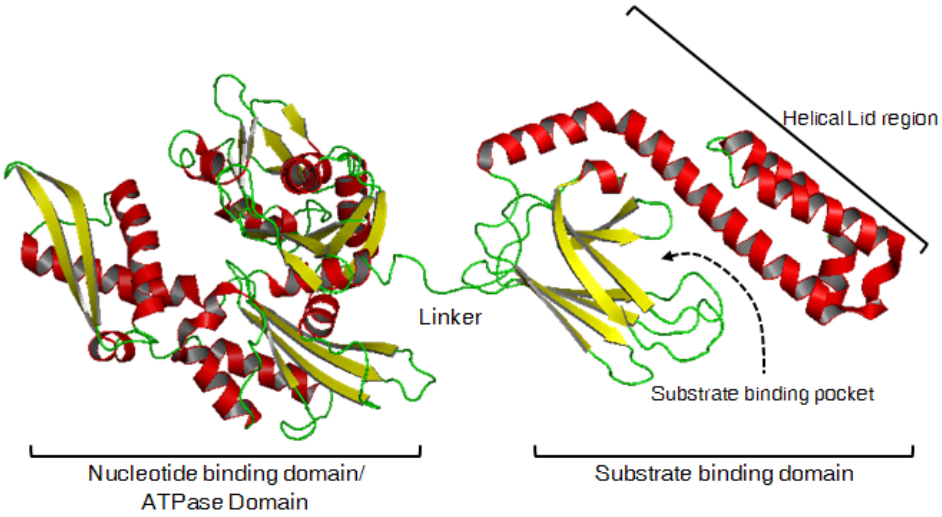


Figure 3: The structure of Hsp70 includes N-terminal (nucleotide binding domain), substrate polypeptide binding domain (SBD) which connect together with a linker. Reprinted from (http://pdslab.biochem.iisc.ernet.in/hspir/hsp70.php).

The ATPase activity of Hsp70 is a center of controlling its structure and function. Completion of Hsp70's ATPase cycle requires the release of ADP, which is a process that is catalyzed by another class of co-chaperones, the nucleotide exchange factors (NEFs). They are essential for the function of the Hsp70[72]. The presence of nucleotide in the N-terminal (NBD) allosterically regulates substrate binding in the C-terminus of DnaK. Hsp70 proteins bind substrates with low affinity whereas the ADP-bound form has a high affinity for substrates. The co-chaperones are important for the function of Hsp70; for example members of the co-chaperone family Hsp40 (J-domain proteins (DnaJ)) which stimulate ATP hydrolysis by Hsp70 and also facilitate the delivery of

some substrates to Hsp70 proteins and a NEF that hasten the nucleotide exchange by Hsp70, in order to promote the substrate release. There is another classification of co-chaperones that bind to the C-terminal domain of Hsp70; like Chip and HOP. They have an important and pivotal role in regulating Hsp70 function[71,72] (Fig. 4).

Genetic investigations have illustrated that there is a strong connection between Hsp70 and its co-chaperones to numerous diseases, including neurodegeneration, microbial pathogenesis and cancer. Therefore the potential of this chaperone as a therapeutic target has remained mainly under investigation[72].

Hsp70 has passed a long way in connection with cancer issues and has different functions in this field. It can protect cells which transformed from oncogenic stress at the initial stages of tumorigenesis. Furthermore, by suppressing the cellular senescence, it provides an important anti-tumor mechanism at the early stages of tumorigenesis and proper response to anti-cancer therapy[64].

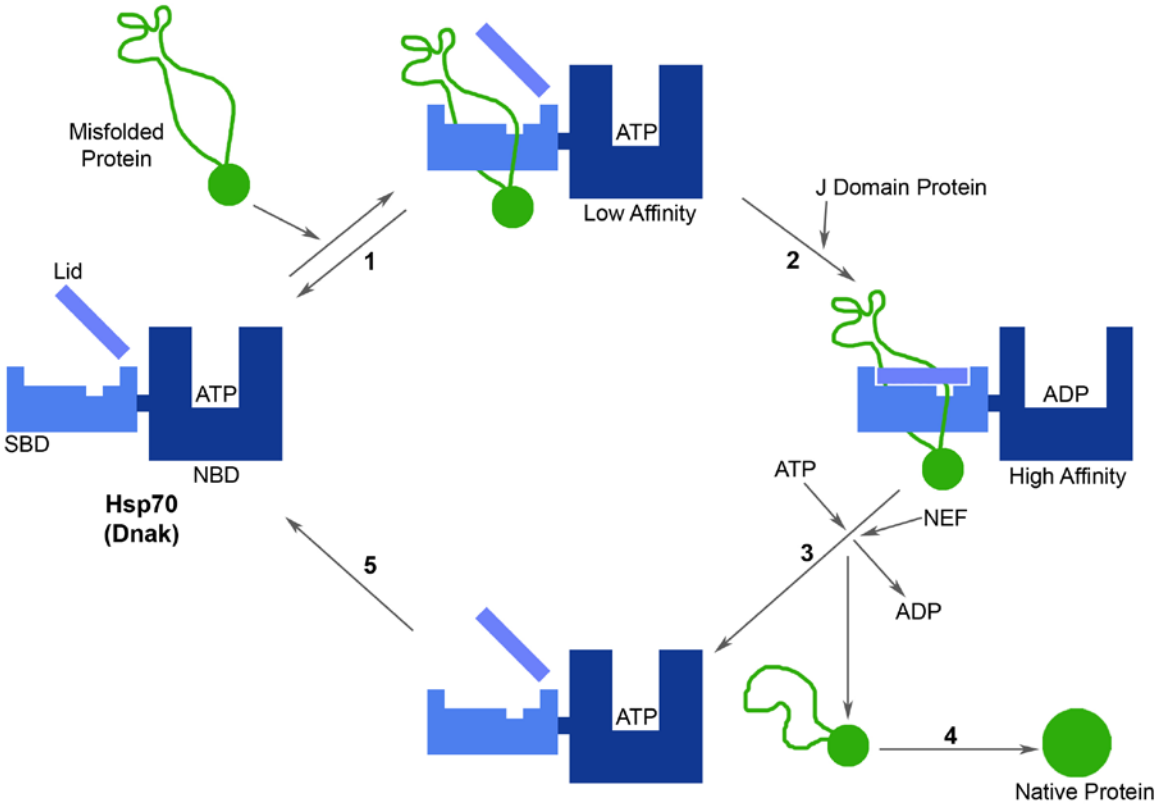


Figure 4: Graphic model for the mechanism of action and the functional cycle of heat shock protein 70 (Hsp70) (DanK in *E. coli*) in interaction with its co-chaperones. (1). DnaK interacts weakly with misfolded proteins 2). The co-chaperone Hsp40 (J-domain proteins (DnaJ)) binds to Hsp70 and induce ATPase activity (3). Nucleotide exchange factor (NEF) catalyzes the release of adenosine diphosphate (ADP) and stimulates nucleotide exchange. It also facilitates the substrate release. The ATP-bound form has a low affinity (4). The released substrate can either refold to the native structure or (5) it binds to Hsp70 thus the cycle is completed and repeated again. Adapted from Ref.72[72].

Additionally, Hsp70 is an essential factor for malignant cells survival and tumor growth. Most tumors show an unusual increased level of Hsp70. This elevating in concentration can act as a tumor marker. An important example of a marker is the mitochondrial Hsp70. It is overexpressed in breast and intestinal tumors. Malignant cells suffer from internal and various environmental stresses such as hypoxia, nutrient deprivation as well as accumulation of mutated and miss/unfolded proteins and the improper activity of deregulated signaling pathways, which can affect their survival. Hence, cancer cells are deeply dependent on Hsp70 as their survival element[64]. So the inhibition of Hsp70 may provide the raise in tumor cell death as well as sensitizing them selectively to chemotherapeutic treatments.

The Hsp selectivity has appeared as an additional key issue on the stage of drug research and Hsp members from other natural sources like bacteria or parasites such as *Leishmania spp.*, *Mycobacterium tuberculosis* and etc need to get more into focus.

2.2. Sources of novel drug compounds

2.2.1. Natural products

Natural products or secondary metabolites are a chemical compound produced by living organisms that are found in nature. Usually, they have pharmacological or biological activities which provide a therapeutic advantage in treating diseases. They present the active components of most traditional and modern medicines. Since the structural diversity of natural products readily achievable by chemical synthesis, natural products are often used as starting points for drug discovery.

During thousands of years, natural products have performed an essential role in health care as well as preventing diseases and ailments[73]. The progress in the genomic mining and engineering of biosynthetic pathways led to the great revolution in the discovery of new natural products[74].

Natural products or secondary metabolites are usually described as small to middle-sized, chemical and therapeutically compounds produced by biological or natural sources like plants, bacteria, fungi and their derivatives that exist in the nature[75,76].

Earlier, the active ingredients were isolated mostly from plants but now there is more attention on investigating the secondary metabolites of different microorganisms[77].

Numerous compounds with pharmacological function derived from natural sources are potent to be used in the drug development as well as biomedical investigations[78]. Several of these compounds apply in the therapy of different diseases such as cancer, protozoan disease[78–80] as well as ailments elicited by bacteria and etc[75,80–83]. Artemisinin[84], quinine, febrifugine[85], etc are some examples of anti-malarial natural products[86].

The antibiotic erythromycin A is one of the most famous examples of natural products which is clinically broadly applied. It is derived from the production of a species of actinomycete bacteria *Streptomyces erythreus*[87].

Since 1941 when antibiotic penicillin was introduced, natural products have performed a considerable role in antibiotic drug discovery and at the moment they have obtained the significant attention and became a great source for drug development[83,88].

Geldanamycin is an important example of natural products which is produced by soil-living actinomycetes *Streptomycetes hygroscopicus*[89]. It has great anticancer potential in cells. Many natural products are able to inhibit heat shock proteins. It is remarkable that these natural products have broadened the insight into the heat shock proteins, which are an important therapeutic target for cancer[90]. Polyketides are one of the main groups of natural products which are biosynthesized by the polyketide synthase (PKS)[32,91]. Polyketide synthases (PKSs) belongs to the family of multi-domain enzymes complexes. In order to produce many clinically important drugs, for instance, the tetracycline, anthracyclines, erythromycin, rapamycin, and lovastatin they use acyl-CoA building blocks as precursors. The three different types of PKS have been recognized in bacteria including the non-iterative type I PKS, the iterative type II PKS and the acyl carrier protein-independent homodimeric type III PKS. Geldanamycin (Gda) is a significant example of natural sources. Due to the hepatotoxic side effects of this compound and its low solubility in aqueous solutions, its clinical application is hindered. Tanespimycin (17-AAG) and Alvespimycin (17-DMAG) and several more new derivatives were presented as a drug candidate instead. There are some of the strategies which generate the Geldanamycin as a lead compound: a) Total and semisyntheses which are the most general and comprehensive ways in natural product chemistry, b) the appliance of the biological tools into synthetic strategies[32,92–96] (Fig. 5).

2.2.2. Chemical synthesis

As mentioned before, natural products are chemical substrates which are produced by the living organisms in nature. It is also possible to prepare them by chemical synthesis. Since the process of isolating the natural products from the living source is not economical and probably time-consuming. By means of the semisynthesis approach, the target biosynthetic intermediate is collected and then converted to the end product by the standard method of chemical synthesis.

2.2.2.1. Total and semisynthetic approaches

Generally, the total synthesis of the natural products is not used as a commercial investigation. However, for the better understanding of the synthesis of the certain natural product and the development of the novel synthesis approaches this method is commonly used.

Total and semisynthesis are a widespread technique in order to access new derivatives of the natural products. Instability of the natural products and finding the highly chemoselective transformations for a given multifunctional is the main challenge from a chemical point of view. Most known geldanamycin derivatives were achieved by semi-synthesis and were modified at C-17 position[32,93,95]. Semisynthetic approaches can be achieved by changing at quinone and hydroquinone part as well as alteration in ansa chain.

2.2.2.2. Combination of biosynthesis and semisynthesis approaches

One of the powerful methods in natural product chemistry is the combination of bio and semisynthesis which can be attained by two different ways through genetic engineering including blocking the selected enzymes or changing the individual enzyme.

Furthermore, there are also several more strategies for developing novel Hsp90 derivatives which can be achieved by using biosynthesis gene clusters, namely combination of biosynthesis and mutasynthesis[32,93]. These methods rely on the genetic engineering and combination of chemical synthesis with the biosynthesis approaches[96]. Using these two strategies led to reach the novel geldanamycin derivative library[32]. Due to the precise chemical biosynthetic production of natural products, the active substance derivatives which interact with the Hsp90 can be generated and their activity becomes visible in the Microarray test system[97]. By the use of modeling the predictions of

reblastatin derivatives in the ATP-binding domain of Hsp90 (N-domain) important positions that have high binding affinity at Hsp90 could be identified.

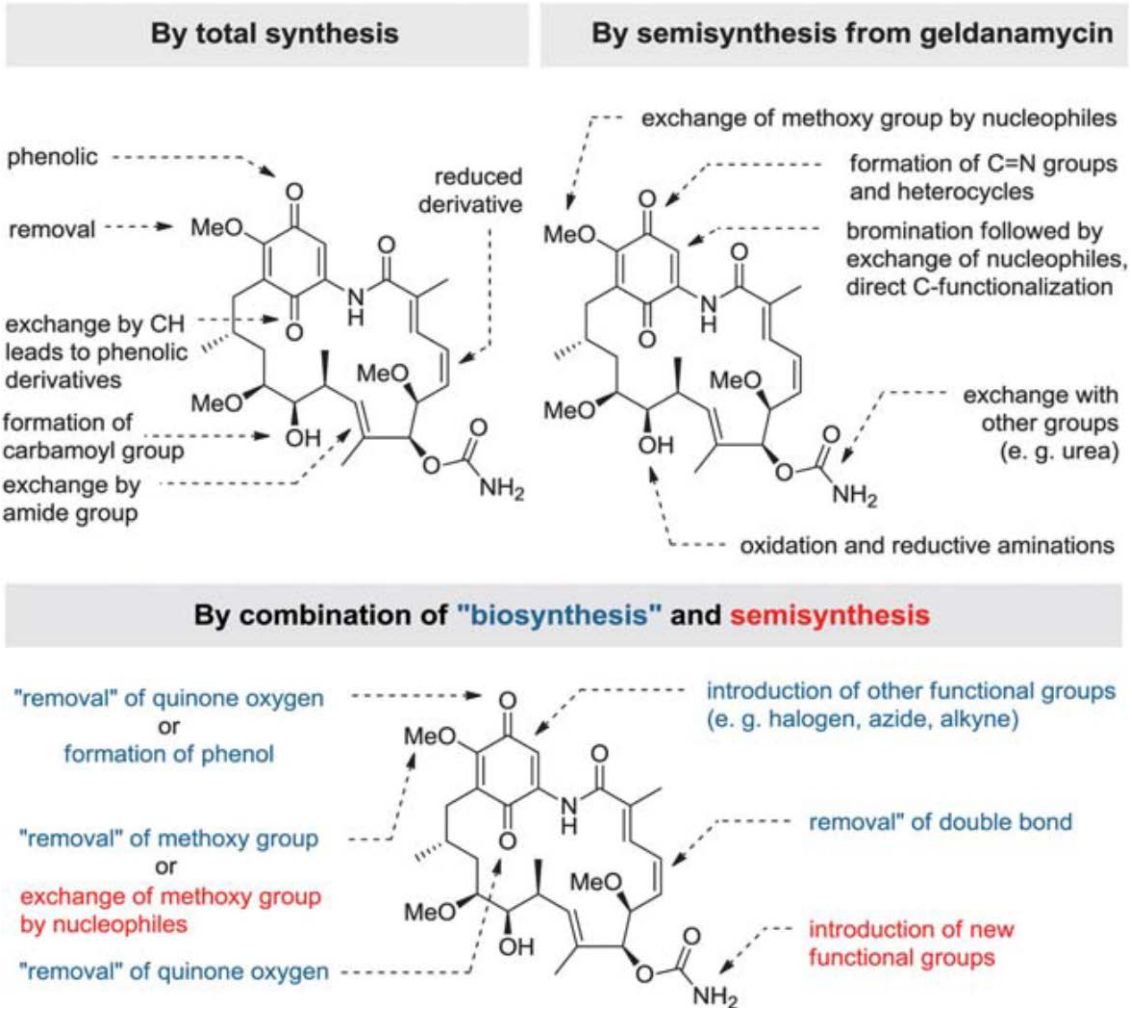


Figure 5: Schematic presentation of the modifications sites obtained from total synthesis, semi-synthesis and the combination of chemical and biosynthesis. Reprinted from Ref. 93[93].

2.3. Strategies to test the activities for the detection of the ATP binding of the purified Hsps and the binding affinity

In order to test the numerous compounds and their bindings in the fastest possible time, the high-throughput screening (HTS) is necessary. Furthermore, having the access to the

better understanding of cellular systems, need the identification and analysis of the components and their functions[98].

The protein microarray technology provides the investigation of protein function in a productive, fast and affordable possible way. Additionally, it is evolving as a beneficial method for biopharmaceutical research[99].

Nowadays, three types of protein microarrays are available to study the biochemical activities of proteins: analytical microarrays, functional microarrays, and reverse phase microarrays**.** Analytical protein arrays are typically used to monitor protein expression levels, measure binding affinities, biomarker identification and clinical diagnosis. The most common type of analytical microarray is antibody microarrays. In this approach, the arrays are probed with a complex protein solution such as a cell lysate, after a library of antibodies, aptamers are arrayed on the surface of the slide. Then the expression levels of certain proteins in the sample, as well as measurements of binding affinities, are determined by using different detection systems. In this technique, the expression of the proteins can be compared in the different solutions. Also, it makes it possible to identify the response of the cells to the specific factor by comparing the lysates of cells which are treated with specific substances or grown under particular conditions with the control cells. Another application of analytical protein arrays is the identification and profiling of diseased tissues.

The second classification of protein microarrays is the functional protein microarrays which also known as target protein arrays. They are applied in various fields of protein interactions, biochemical activity, and immune responses. In these arrays, which are mainly differed from the analytical arrays, the full-length proteins or protein domains are applied. The usage of functional protein microarrays are: (*i*) investigating different kinds of protein activities, such as interactions between protein-protein, protein-lipid, protein-DNA/RNA, protein-small molecules (drugs), as well as protein-peptide; (*ii*) identifying enzyme substrates and (*iii*) profiling the immune responses[98,100].

Reverse phase protein microarray (RPA) is presented as the third type of protein microarray which is related to analytical microarrays. RPA deal with the complex samples, such as tissue lysates. The presence of altered proteins or other agents that may be the result of the disease can be determined by reverse phase protein microarray. By using this method, the post-translational modifications, which are generally altered as a result of disease, can be detected.

In these arrays, the lysate cells from various tissues of interest are arrayed on the nitrocellulose slide by the means of contact pin microarrayer. The slides are probed with antibodies against the target protein and finally, the antibodies which are against the target proteins are detected by specific dyes[98].

All kinds of microarrays are utilized for two main purposes including:

i) Biomarker and or target detection; to identify the proteins which can be applied for measuring and evaluating the disease development, moreover discover novel therapeutic targets.

ii) Drug screening; to identify the compounds which bind to target proteins and modulate their activities or by performing enzymatic assays. The microarrays have great potential to accelerate drug development[99].

2.3.1. Detection techniques

The detection technology of protein microarray must provide a high signal and a low background. The bindings can be detected by various means. The most commonly used and preferred approach for the detection is fluorescence labeling which is highly sensitive, safe effective and compatible with available microarray laser scanners. Other labels such as affinity, photochemical or radioisotope tags can also be applied[101].

Because the labels which are attached to the probes can interfere with the probe-target protein reaction, several label-free detection methods have been presented. The recent prominent label-free detection technology is surface plasmon resonance (SPR), which optically measure the index of refraction. The other methods include the carbon nanotubes, carbon nanowire sensors. These detection techniques are quite novel and are not yet for high throughput protein interaction detection.

2.3.1.1. Power of miniaturized protein microarray

Protein microarrays, also known as protein chips have emerged as a promising method, are miniaturized and parallel assays that contain small amounts of purified proteins. They can also be applied for clinical diagnostics and observing disease states.[98,100]

It is an outstanding technology for proteomic investigations and diagnostics approaches that facilitate researchers to study interactions between proteins and different molecular partners, such as nucleic acids, peptides, phospholipid, small molecules and so on. The significant advantages of the developed protein microarrays can be defined as:

- The minimum concentration and volume of the proteins are required for the assay. Thousand arrays can be prepared with a tiny protein amount of around 150 µg.
- A variety of proteins and different conditions can be tested simultaneously.
- The cost of each protein chip is comparatively low.
- The remarkable advantage of the protein microarray test system is the possibility to enable flexible adaptation to different experimental questions and extend the application to various conditions and different problem areas.
- It is a fast productive technique in which the duration of each test is approximately less than two days. It could also possible to reduce the duration of the incubation and make the overall time shorter than mentioned.

The developed protein microarray demonstrates a greatly flexible screening platform which enables the researchers to have various levels of the assays in multiple investigations including:

a. Investigating several target proteins which cause different diseases. The assay is performed with the tiny amount of the proteins per spot (800 - 1600 pL) without any limitation in the sensitivity of the proteins. Different proteins can be printed and the interaction with co-chaperones, client proteins and multifunctional natural compounds can be investigated[14].
b. Testing different potential inhibitors in parallel. The power of this miniaturized method is providing for testing many inhibitors at the same time in one assay. Each chip is inserted in the 16 well hybridizations chambers that used to separate the sub-arrays[14].
c. Providing the opportunities to screen the inhibitors of different functional sites within one target protein due to the application of full-length proteins[14,98,102]. The target proteins monitored by an optical label like fluorescence labeled ATP. Consequently, the binding and displacement by potential inhibitors can be determined optically in the microarray format.
d. Protein interactions with labeled proteins or peptides can be detected by this approach as well. Real interaction can be re-enacted on a microarray device and can be used to test protein-protein, protein-ligand and protein-inhibitor affinities. It provides a possibility to screen the client binding site of Hsp for drug development as an additional target site. The Binding affinity between a ligand/binding partner (such as a drug, inhibitor) and a protein is determined by a dissociation constant (K_d)

that evaluate the strengths of bimolecular interactions. The smaller the K_d value, the greater the binding affinity of the ligand for its target. Isothermal titration calorimetry (ITC) is a direct, label-free assay which measures the thermodynamic parameters of interaction and binding affinity between any two biomolecules. It measures the heat that is either released or absorbed during a bimolecular binding and it is the only technique that determines simultaneously all binding parameters in a single experiment and one of the latest techniques to be applied in characterizing binding affinity of ligands for proteins. ITC is widely used in drug discovery and the thermodynamic characterization allows for further optimization of the potential compounds[103,104].

e. Diagnostic applications: The cell lysates can be directly printed on the microarray chip and the increased Hsp levels which originated from the cell lysates can be detected through this method. It is applied for the investigation of tumor marker as a potential diagnostic approach.

2.3.1.1.1. Target-oriented test based on a microarray

The principle of the assay which based on the direct competitive displacement of ATP from the ATP-binding pocket of proteins (Hsp) by potential inhibitors and the fluorescence-labeled ATP/ labeled protein illustrated in Figure 6[14,105].

The heat shock proteins are spotted by means of a nanoplotter, with a nanotip pipette (volume of 800-1600 pL; 8 drops/dot) and become immobilized onto the nitrocellulose membrane. The target proteins can either bind the fluorescence-labeled ATP or the potential inhibitors with high affinity. By the means of fluorescence-labeled ATP (Cy3/Cy5 derivatives) displacement, different interactions or binding can be visualized and evaluated.

When the dye-labeled ligand is bound, this leads to an increased intensity of fluorescence signal whereas an active substance (inhibitor) can displace a fluorescence reduction when they bind to the binding site of the proteins. In the HTS method, the fluorescence signals are evaluated according to the presence or absence of the ligand. The concentration of proteins is 3 mg/ml and they are spotted on the microarray chips in the small volume (~800 pL) by the use of nanotubes.

In order to validate the quality of the microarray tests, the Z-factor should be calculated. For having a powerful and reliable assay the Z-factors for all inhibitors should fit into the range of $0.5 < Z < 1.0$ [14,106].

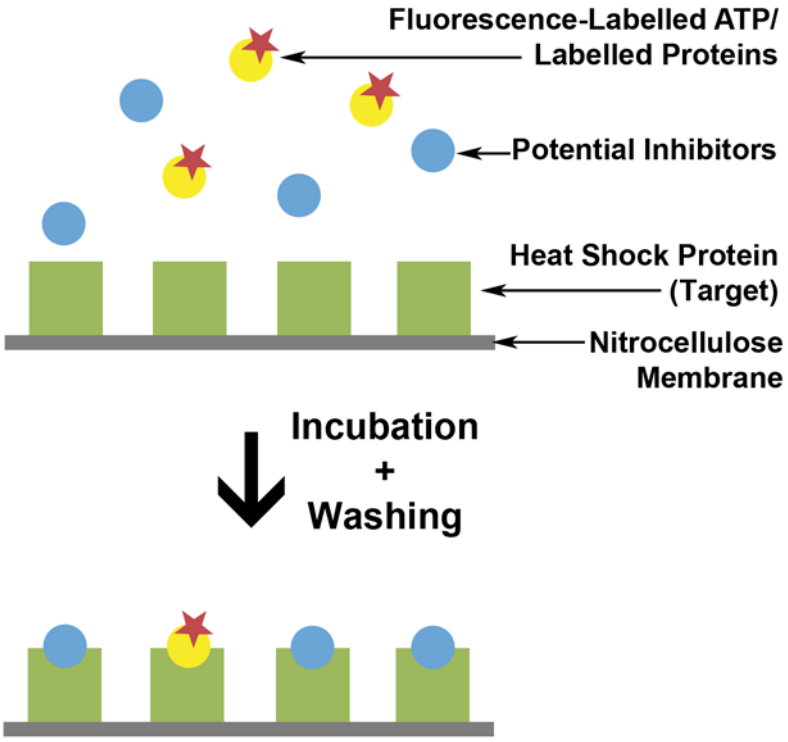


Figure 6: Schematic illustration of the direct competition assay by using the fluorescence-labeled ATP (Cy3-ATP or Cy5-ATP) as a reference. The proteins (Hsps) are immobilized on the nitrocellulose membrane. They can either bind fluorescence dyes or the potential inhibitors with higher affinity. Finally, the binding property of the potential inhibitors is assessed after the incubation and washing steps. Adapted from Ref. 14[14].

2.3.1.1.2. Fluorescence labels

The fluorescent label is a molecule that binds chemically to help in the labeling and detection of a target biomolecule such as a protein, antibody, or amino acid. They are used in a variety of fluorescence detection techniques. In research, the knowledge about fluorescence is utilized to visualize the biological processes at the cellular or molecular level. The most important characteristics to review when choosing an appropriate dye are absorption and emission spectra, size, the fluorescence Stokes shift, stability, as well as the background color. Absorbing light via special molecules like fluorophores, fluorescent dyes or fluorochromes, results to fluorescence. The absorption of light by these molecules increases their energy level to a brief excited state. They emit

fluorescent light when they decay from the excited state. Biotechnology presents a variety of fluorochromes, which differ on their features (Table 1).

Table 1: Examples of some frequently used fluorochromes.

Fluorochrome	**Abbrev.**	**Excitation wavelength (nm)**	**Emission wavelength (nm)**
Pacific Blue		405	455
R-Phycoerythrin (PE)	PE	480/565	578
Fluorescein	FITC	495	520
Alexa 488		495	520
Indocarbocyanine 3	Cy3	550	570
Allophycocyanin	APC	650	660
Indocarbocyanine 5	Cy5	650	670

Cy3 and Cy5 are the most popular fluorochromes from the class of indodicarbocyanines, which are particularly stable. They are used in the detection of two colors. Cy3 fluorescent greenish yellow (~550 nm excitation, ~570 nm emission), while Cy5 is fluorescent in the red region (~650 excitation, 670 nm emission) (Fig.7).

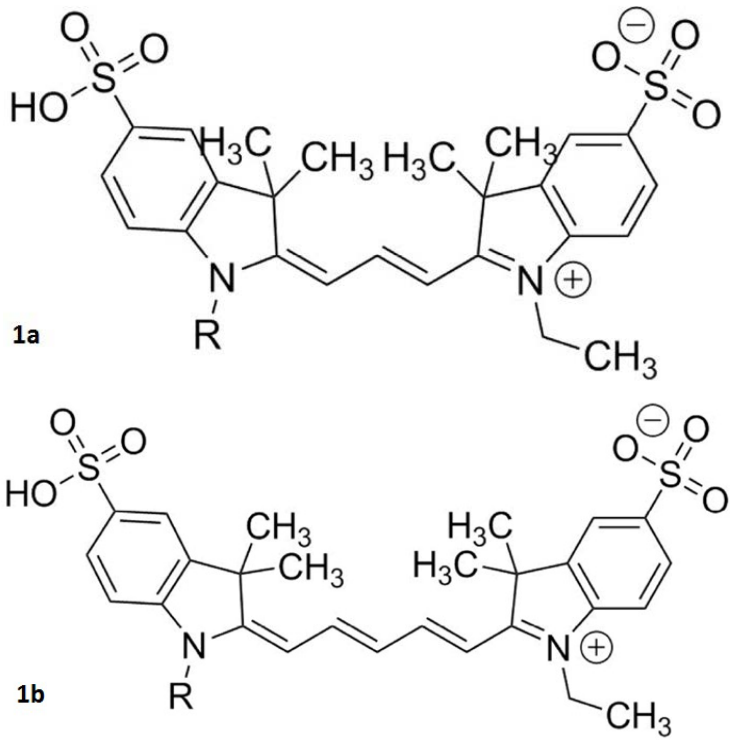


Figure 7: Structures of Indodicarbocyanine, **1a:** Cy3 and **1b:** Cy5

Cyanine dyes are used in proteomics investigations. They are utilized in labeling the proteins, antibodies, nucleic acid probes, and any types of other biomolecules to be used in different fluorescence detection methods such as flow cytometry, microscopy, microplate assays, microarrays, etc. They have become incredibly popular labels in life science research and diagnostics.

The position of the fluorescence labels on adenosine triphosphate (ATP) has a strong influence on the binding of the Hsps, especially in Hsp70 and Hsp90. For instance, the label at the γ-phosphate of ATP can hinder the binding to Hsp70 whereas other positions on the purine ring allow binding, which is opposite in Hsp90.

3. Materials and experimental methods

3.1. Materials

I. Protein purification buffers

Lysis buffer		Tris-HCl, pH:8 KCl β-Mercaptoethanol Imidazole Glycerin Protease inhibitor	20 mM 500 mM 5 mM 2 mM 10 % (v/v) 0,005 % (w/v)
Immobilized Metal Affinity Chromatography (IMAC) buffer	**Buffer A**	Tris-HCl, pH:8 KCl β-Mercaptoethanol Imidazole	20 mM 500 mM 6 mM 2 mM
	Buffer B	Tris-HCl, pH:8 KCl β-Mercaptoethanol Imidazole ATP/Mg^{2+} Tween 20	20 mM 1 M 6 mM 2 mM 1 mM 0.1 % (v/v)
	Elution buffer	Tris-HCl, pH:8 KCl β-Mercaptoethanol Imidazole	20 mM 500 mM 6 mM 250 mM
Dialysis buffer		Tris-HCl, pH:8 KCl β-Mercaptoethanol Glycerin	20 mM 20 mM 6 mM 10 % (v/v)
MonoQ buffer	**Buffer A**	Tris-HCl, pH:8 KCl β-Mercaptoethanol	20 mM 20 mM 6 mM
	Buffer B	Tris-HCl, pH:8 KCl β-Mercaptoethanol	20 mM 500 mM 6 mM
Gel filtration buffer 16/60		Tris-HCl, pH:8 KCl β –Mercaptoethanol	20 mM 500 mM 6 mM
Storage buffer/Hsp buffer		Tris-HCl, pH:7.5 KCl β-Mercaptoethanol Glycerin	20 mM 50 mM 6 mM 10 % (v/v)

II. SDS-Gel

Running puffer	Tris-HCl Glycin SDS H_2O	25 mM 200mM 0.1% (w/v)	0.3 g 14 g 1 g 1000 mL
Stacking gel, 5%	H_2O Acrylamide (37 %) 1.5 M Tris pH:6.8 10 % SDS 10 % APS TEMED		1.7 mL 0.535 mL 0.25 mL 0.02 mL 0.01 mL 0.005 mL
Separating gel, 12%	H_2O Acrylamide (37 %) 1.5 M Tris pH:8.8 10 % SDS 10 % APS TEMED		2.45 mL 3 mL 1,9 mL 0,075 mL 0.075 mL 0.01 mL
2x SDS-sample buffer	Tris-HCl EDTA-Sodium salt SDS Bromophenol blue H_2O		0.315 g 58 mg 5 g 20 mg 80 mL
Laemmli-buffer	2x SDS sample buffer β -Mercaptoethanol Glycerin H_2O	80 % (v/v) 10 % (v/v) 55 % 10 % (v/v)	

III. Buffers used in the Microarray

Storage buffer (Hsp buffer)	Tris-HCl, pH:7.5 KCl β –Mercaptoethanol Glycerin H_2O	20mM 50mM 6mM 10 % (v/v) Add till 1L
Blocking buffer	BSA Storage buffer	1 g 100 mL
FPI 1x buffer	HEPES-KOH pH:7.3 KCl $MgCl_2$ x 6 H_2O Na_2MoO_4 Tween 20 DMSO BSA	20 mM 50 mM 5 mM 20 mM 0,01 % (v/v) 2 % (v/v) 0.1 $mg \cdot mL^{-1}$

IV. Cy5-Labelling Buffer

10x PBS	NaCl pH: 7.4 KCl Na_2HPO_4 x 2H2O KH_2PO_4	1370 mM 27 mM 80 mM 15 mM

3.2. Experimental methods

3.2.1. Molecular biology tools

There were two vectors used in the experiments. The pET15.1 applied for human Hsp90α and the Hsp70 gene was ligated into the pET SUMO vector[15,107]. The constructs carry an N-terminal His-Tag (histidine hexamer) which is essential for binding the protein to the cobalt ion column. His-tag provides a specific binding from the cell lysate among the other proteins of the lysate. The vectors are controlled by the T7 promoter and T7 terminator. Furthermore, the protein expression is regulated by *lac* operon. Whereas the vector pET SUMO with 5643 bases contains a kanamycin resistance site, the pET15.1 vector hold ampicillin antibiotic resistance site which is applied for the selection after the transformation (Figs. 8 and 9).

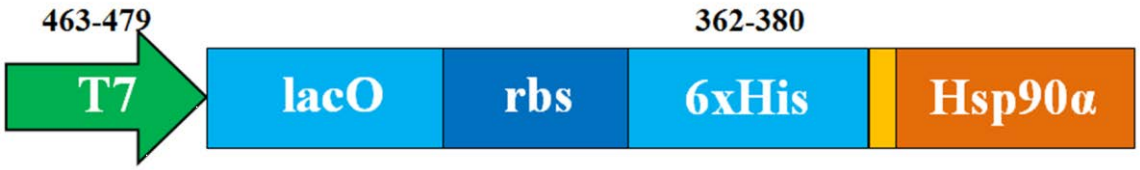


Figure 8: Hsp90α expression vector pET-15b and the sequence traits (more information is available in appendix). Reprinted from Ref. 108[108].

Figure 9: Vector pET SUMO 5643 base pair (Invitrogen Life Technologies) map and the sequence characteristics. The lacOperator is used to regulate the expression and the His-tag is applied for the purification of the protein. The vector has a kanamycin resistance site as a selection marker. Reprinted from Ref. 109[109].

3.2.2. Transformation and synthesis of heat shock proteins

All the steps of the transformation should carry out under the sterile clean bench in order to avoid getting contaminated.

The amount of 3 µl of plasmid DNA (pET SUMO–HsHSP70 and pET15.1–HsHSP90) added to the 40 µl competent *E.coli* cell. The competent cells are stored in -80 ^{o}C freezer so they should be brought out earlier and thaw in ice to get defrosted. The mixture should be incubated for 10-20 min in ice. Then the heat transformation was applied at 42 ^{o}C for 30 s and directly transferred to ice without shaking the tubes and remained there for 3 min. In the following, 400 µl of SOC medium was added to the tubes and incubate for 1h at 37 ^{o}C on the shaker with 200 rpm. After incubation, 50 - 100 µl of the solution of the cell was spread on the agar plates including antibiotics ampicillin and kanamycin which are specific for Hsp90 and Hsp70 respectively. The plates were stored overnight at 37 ^{o}C in order to let the cells grow.

In the next day, one single colony of each plate was taken and added to the individual 100 ml LB medium with 100 µl of a specific antibiotic. The flasks containing LB were stored overnight (16 h) at 37 ^{o}C on the shaker with 200 rpm. After the incubation period, the precultures transferred to two larger flasks for 1 l (50 ml cell culture in the volume of 1 l LB medium including 1 ml antibiotic) and allow them to grow overnight at 37 ^{o}C on the same shaker and condition that mentioned before. In the next day, when the OD reached to approximately 0.6-0.8, 1 ml (1 M/l) of IPTG (Isopropyl β-D-1-thiogalactopyranoside) added to 1 l LB medium and incubated 16 h (overnight) at 16 ^{o}C on the shaker with 200 rpm. IPTG compound triggers transcription of the *lac* operon, therefore it is utilized to induce protein expression where the gene is under the control of the *lac* operon.

The cultures from the flasks transferred to centrifuge vessels and centrifuged at 4 ^{o}C for 15 min with 10,000 xg. Finally, the cells were ready to be harvested. In this step it is possible to store the samples for further testing in -80 ^{o}C freezer.

The cell pellets were suspended in 30 ml Hsp lysis buffer with the addition of protease inhibitor (100 µl for 10 g *E. coli*). The usage of the protease inhibitor is to prevent cell degradation. Then by the means of French press (keeping the pressure at 1,200-1,400 p.s.i.), the cells disruption was done. The lysates were centrifuged at 20,000 xg for 30 min at 4 ^{o}C.

3.2.2.1. Protein purification

After centrifugation, the supernatant was mixed with around 10 ml of cobalt polyhistidine affinity resin (TALON) and incubated for 3-4 hour at 4 ^{o}C to let the protein combined with the ion properly. TALON is an Immobilized Metal Affinity Chromatography (IMAC) resin for the purification of recombinant histidine-tagged (6xHis-tagged) proteins which have a significant specificity for polyhistidine-tagged proteins.

Afterward, the sediments were collected carefully and the purification was carried out by the means of affinity chromatography. IMAC purification procedure is explained below:

i) Three buffers for different purposes (A, B, C) were prepared. The usage of buffer A was to remove the unbound proteins whereas the Buffer B which contained ATP and Magnesium ion ($MgCl_2$) was applied to stabilize the protein structure. Buffer C (elution buffer) including imidazole was responsible for washing the target protein through the column and collected for the next steps.

ii) The column was refilled with the mixture of protein-cobalt ion resin and was washed with 20 ml buffer A

iii) In the next step, the column was washed with 10 ml of buffer B (including 1 mMl ATP 2.5 mM $MgCl_2$) with speed of 2 ml/min.

iv) At last, the column the bound protein was eluted with 3-fold column volume into the elution buffer.

Afterward, the His-tag was removed by addition of TEV protease for pET15 vector (Hsp90) or SUMO protease when the pETSUMO vector (Hsp70) was used. First, the eluted solution was concentrated by Amicon concentrator YM30K to around five mL and transferred into a dialysis tube and dialyzed overnight at 4 ^{o}C against 50-100 fold volume of Dialysis buffer (Tris-HCl, pH:8 20mM, KCl 20mM, β-Mercaptoethanol 6mM, Glycerin 10 % (v/v)).

Next, the dialyzed solution was loaded onto a Mono anion exchanger and a Hsp90 protein fraction eluted with a linear salt gradient from 20 to 500 mM KCl and enriched into around 400 – 450 mM KCl. The eluted samples were concentrated again by

Amicon 30 K concentrators and loaded in a volume of 1 mL onto a SEC16/60 to separate dimeric Hsp90 or enrich Hsp70 proteins. The optical density of the purified protein was measured at 280 nm and calculated the protein concentration according to Gill and von Hippel[110]. The protein concentration adjusted with Hsp storage buffer Amicon 30K concentrator to a final concentration of 3 mg/mL and was frozen in 50 µL aliquots in liquid nitrogen was stored at -80 ^{o}C. In the end, all columns and devices were used should be washed with distilled water and specific buffers.

3.2.2.2. Protein analysis

In order to check the purity and molecular weight of the proteins, SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed. For this purpose, a 5% stacking gel and a 12% separating gel were prepared. First, the separating gel solution was poured between two glass plates in a gel caster, and then by use of isopropanol, it was covered. After polymerization, the isopropanol was removed and the collecting gel was added and a comb was placed in the pockets. When the gel was polymerized, the comb could be removed and the gel was ready for electrophoresis. The prepared samples (5× SDS sample buffer; 1:5) were incubated at 95 ^{o}C for 5 min. Afterward, 10 µl of each protein and 5 µl of the protein marker were applied to the gel. In the beginning, the voltage of the device was adjusted to 100 V and after passing through the stacking gel, the voltage was increased to 180 V.

v) Protein gels have been stained with Coomassie Blue.

3.2.3. Labeling the target proteins (recombinant heat shock proteins)

For this purpose, the target proteins (human heat shock proteins Hsp70 / 90α) were incubated with 1x PBS buffer for 1h in dark room temperature. Then the target proteins were incubated and labeled with Cy5 (Cy5 Mono-Reactive Dye Pack, Amersham, Piscataway, NJ) at 4 ^{o}C for 30 min. The unbound dye label was removed by G25-SEC. In the following, 500 µl of the mixture was filtered and centrifuged by means of the concentrator with a cut-off of 30 kDa / 15000 xg for 1 min. The centrifuge duration was continued till the supernatant of the membrane reached the volume of 100 µl. Afterward, the washing steps were done by 400 µl PBS/ 15000 xg for 1 min. The

washing steps were repeated by the same amount and condition and ended when the flow through became clear. Finally, the supernatant was collected and was covered and stored at 4 ^{o}C. The Cy5 / protein ratio was determined by using nanodrop with the absorption at λ = 280 nm and λ = 650 nm.

3.2.4. Protein microarray method

This method consists of three different steps: 1) spotting 2) incubation 3) analysis that is described below.

3.2.4.1. Protein spotting, incubation and analysis

First of all, the proteins should be diluted to 3 mg/ml with Hsp buffer and poured into a 96- well plate with a volume of 50 µl. Then they were spotted by the means of contactless GeSim Nano-PlotterTM (GeSiM mbH, Großerkmannsdorf, Germany) with a nanotip pipette, on the nitrocellulose-coated glass of a microarray slide (Sartorius Stedim Biotech, Göttingen, Germany). Each ten spots were printed per pad, consists of approximately a volume of 800-1600 pl (8 droplets/dot) protein. The software NPC16 was used to operate the spotting. After the spotting, the slide was dried at room temperature for 30 minutes and then the nonspecific sites were blocked by 1% (w/v) BSA in storage buffer (1 gr BSA in 100 ml HSP buffer) for 45 min at room temperature. This was followed by 3 times of washing steps. Each step lasts 20 minutes with 100 ml of Hsp buffer (storage buffer). Next, the slide was transferred to the 16 well hybridization chambers of Nexterion (Schott Nexterion, Mainz, Germany) in order to subject to the direct competitive displacement assay. All 16 chambers were separated from each other and it provided testing different substances such as proteins, inhibitors and lysates in various conditions simultaneously. Each compartment was filled with 50 µl of a mixture of 100 nM fluorescence-labeled ATP (purchased from JenaBioscience GmbH, Jena, Germany) and geldanamycin derivatives in binding buffer (20 mM HEPES–KOH, pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, 0.01% (v/v) Tween 20, 2% (v/v) DMSO, 0.1 mg ml^{-1} BSA). The concentration of potential inhibitors was varied according to the assay type from 50 µM to 50 pM. A positive and a negative control were applied to evaluate the test. As the positive control, there was a mix of binding buffer, 100 nM fluorescence-labeled ATP and radicicol (typical inhibitor for Hsp90) / ver155008 (typical inhibitor for Hsp70) and as the negative control, the

binding buffer with 100 nM fluorescence-labeled ATP with the lack of the potential inhibitor was applied. Then the slide was sealed with a tape to avoid evaporation of the solution, and the whole chamber covered with aluminum foil and placed in a moist container. It was incubated overnight in the cold room on the shaker (100 rpm) at 4 ^{o}C. After the incubation, the slide was washed three times for 5 min with binding buffer and afterward dried with compressed air.

The protein slide was then scanned and evaluated. Fluorescence originated from fluorescence-labeled ATP was determined by GenePix 4000B Laser Scanner (Molecular Devices, Inc.) with 532/635 nm emission wavelength, laser power 10%, PMT gain 280/380. The data were calculated by using the program GenePix6.1 (Molecular Devices, Inc., Sunnyvale, CA, USA) and ImaGene 5 of BioDiscovery, Inc. (Hawthorne, CA, USA). The half maximal inhibitory concentration (IC_{50}) value was done in order to evaluate the displacement. The dose-response curves were calculated with program OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA) and fitted with the non-linear function logistic, A1 = 0, A2 = 1.

Relative signal intensity was evaluated as the difference of signal means (SM) and background mean (BM)[102,111]. Signal-to-noise ratio (SNR or S/N) is defined as the ratio of signal power to the noise power and was applied in order to check the validity of each data. Thus Signal-to-noise ratio (SNR) was determined by dividing the relative signal intensity by the standard deviation of the background (through the formula)[111–113]:

SNR= (SM-BM) / Background Standard deviation

Background indicates the local spot background intensity which deducts from the hybridization signal of every single spot. It also differentiates the right signal from the noise by measuring each spot[102]. In the present study, the signal intensity for each spot was higher counts than the background counts.

Furthermore, in order to validate the quality of the microarray tests, the Z-factor was determined for each assay. The Z-factor in this work was evaluated according to the Z-factor calculation published by Zhang *et al.*[106].

Since the measured Z-factor for all inhibitors fit into the range of $0.5 < Z < 1.0$, it confirmed the present work as a powerful and trustworthy investigation.

4. Results

4.1. Examination of ATP-binding sites of Hsp chaperone with bioinformatic methods

Hsp90 consists of three conserved domains (as described in detail in the introduction) that each domain has a specific function. The binding pocket for the natural ligand ATP and susceptibility for some inhibitors like geldanamycin is in the N-terminal domain (positions 1-240). It has also reported that there is an allosteric interaction between N- and C-terminal domains of Hsp90. Furthermore, Hsp90 has a second ATP-binding site located in the C-terminal part of the protein (positions 446 –728) of Hsp90[114,115]. Since it is known that Hsp90 is essential for all cells, and is assessed as a biomarker in cancers[31] and involved in many important cellular processes of intracellular protozoans and other important human pathogens[59]. It is known that minor changes in the amino acid composition can influence the affinity for ligands or inhibitors. Therefore this can be analyzed by a bioinformatic predictive strategy by sequence comparison, hydropathy comparison, and interactome comparison[59,116,117]. With sequence alignment the regions of conserved and variable domains are visible and the minor changes which result in larger effects are visualized and later can help in order to interpret experimental results. The hydropathy plot shows the degree of hydrophobicity and hydrophilicity of amino acids along the length of a polypeptide[118,119]. The plot is based on the hydrophobic and hydrophilic properties of the 20 amino acids and illustrates the position and number of the amino acids of a protein on its x-axis whereas y-axis represents the hydrophobicity scores[118,120].

ExPASy presents ProtScale (http://web.expasy.org/protscale/) which make it possible to analyze the hydropathy as a function of the amino acid sequence of the used target protein. The last predictive technique performed was the interactome comparison which exhibits the totality of protein interaction using different databases e.g. experimental, genetic, or cooccurrence. It shows the interactions and the consequences of those interactions among the proteins.

In order to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the amino acid sequences of the Hsps, the N-terminal domain (1-240 amino acids) of human Hsp90α (HsHsp90α) was compared with orthologue binding sites of *Leishmania braziliensis* Hsp83 (LbHsp83),

Streptomyces hygroscopicus HtpG (ShHtpG), and plant Hsp83 *Arabidopsis thaliana* (AtHsp83) (Fig. 10).

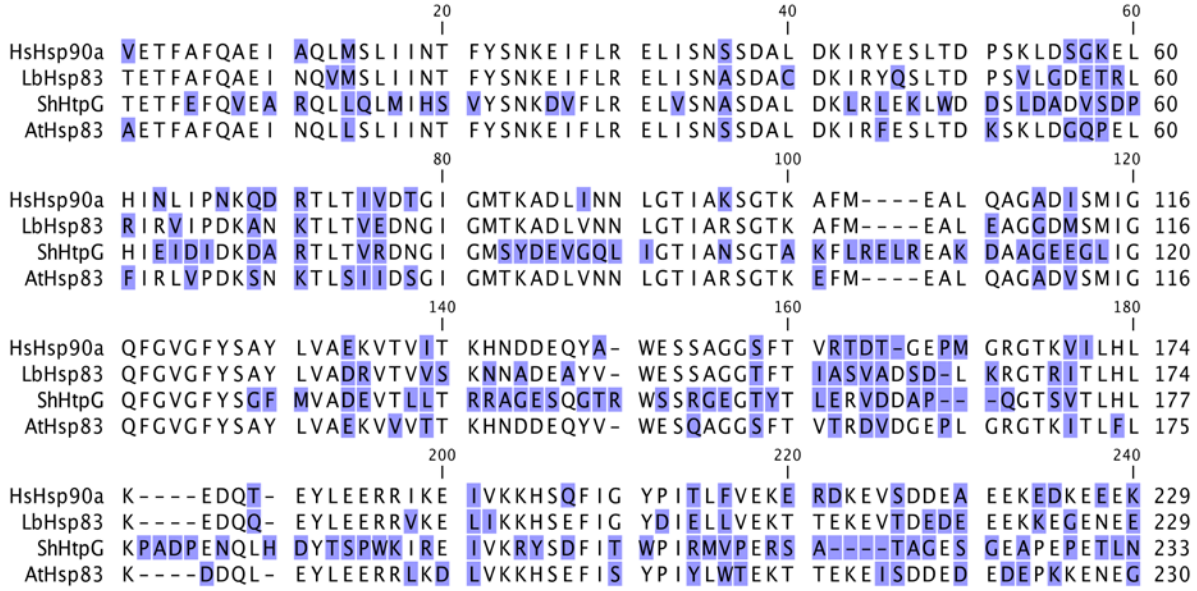


Figure 10: Sequence alignment of different HSP90s; Human HSP90 (HsHSP90α), *Leishmania braziliensis* HSP83 (LbHSP83), *Streptomyces hygroscopicus* HtpG (ShHtpG) and *Arabidopsis thaliana* HSP83 (AtHSP83). The N-domain (1-240 amino acids) was sequenced. The amino acid sequences were taken from the NCBI database and the sequence alignment was done with CLC Sequence Viewer. Deviations are emphasized.

The soil-dwelling actinomycetes *Streptomycetes hygroscopicus* is the producer of the natural product geldanamycin (Gda), a benzoquinone ansamycin antibiotic, which is an antitumor antibiotic that inhibits the function of Hsp90 (Heat shock protein 90) by binding to the ATP-binding pocket of the protein, preventing ATP hydrolysis. Remarkably, the Gda-producer, *S. hygroscopicus* does not bind geldanamycin[32,121,122].

In figure 10, the deviations between the sequences are highlighted in color. These differences may result in diverse drug selectivity of the NBD. As shown recently in Millson *et al.* (2011), small exchanges in the conserved region of the ATP binding site are responsible that *S. hygrsocopicus* has a lower susceptibility for ATP[121].

The sequence differences indicate the different binding behavior of the NBDs thus selective substances can be found which can be helpful in the drug discovery. Sequence alignments of HSP90 have shown that the proteins to have around 40% sequence identity among all homologs, denoting that it is a greatly conserved protein. Because of the high degree of homology between bacterial and human HSPs, the role of HSPs in autoimmunity became a target for investigation[123].

The difference between orthologue proteins of human, plant, pathogenic HSPs and natural product producer *S. hygroscopicus* is determined via a few numbers of amino acids and their hydropathic manner. The hydropathy plot performed by obtaining the amino acid sequence of the *N*-terminal domains from the first homologous 240 amino acids from Human HSP90 (HsHSP90α), *Leishmania braziliensis* HSP90 (LbHSP90), *Arabidopsis thaliana* HSP83 (AtHSP83) and *Streptomyces hygroscopicus* HtpG (ShHtpG) and plotted according to Kyte and Doolittle[118] (Fig. 11).

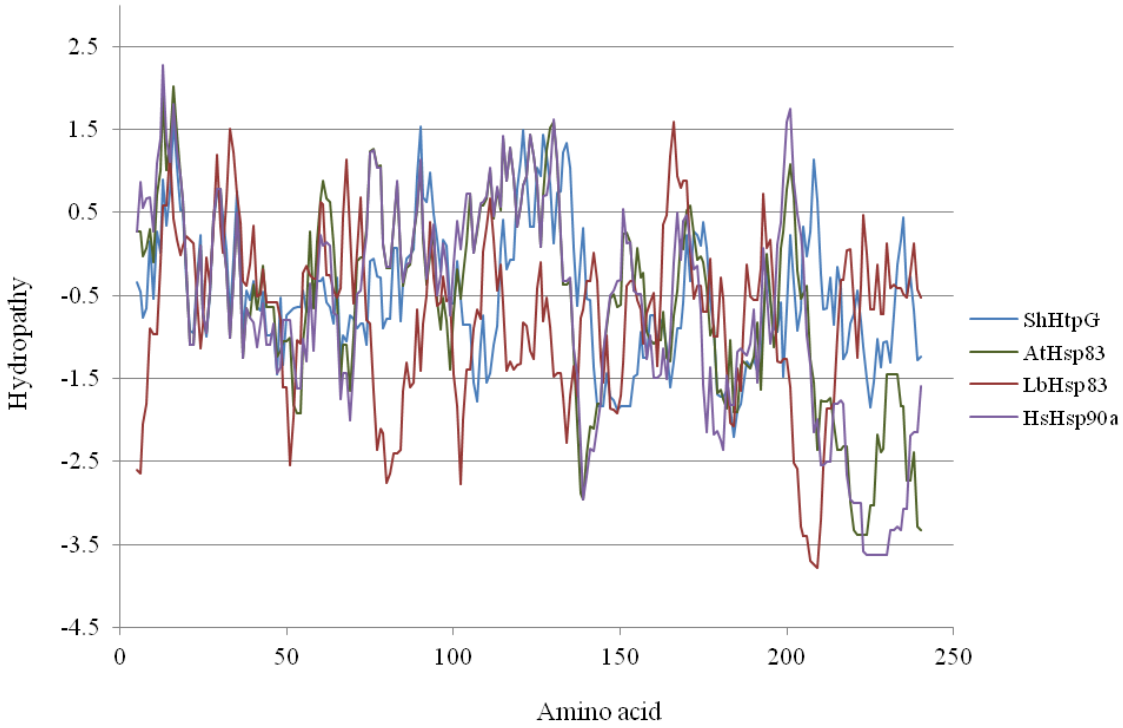
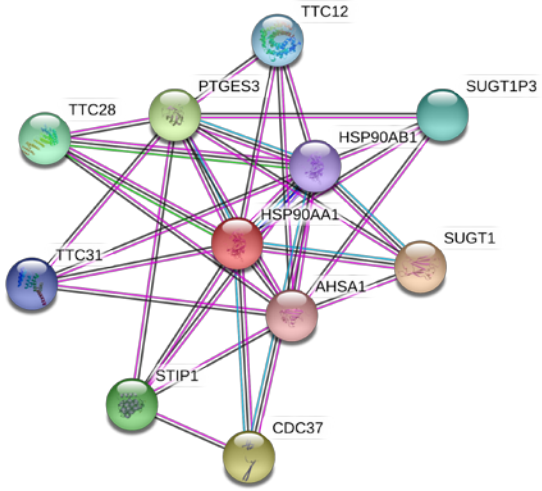


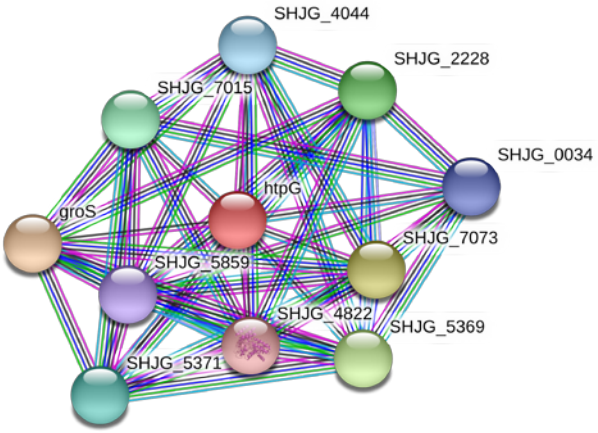
Figure 11: Hydropathy plot of ATP binding domains of four HSP90. The homologous areas of the N-terminal domain of the ATP binding pocket were plotted according to Kyte and Doolittle. LbHSP90 (green), HsHSP90 (purple): HSP90 of Homo sapiens, ShHtpG (blue): HtpG of *Streptomyces hygroscopicus* and *Arabidopsis thaliana* HSP83 (AtHSP83). Hydropathy indices were calculated with a window size of ten in ExPASy ProtScale.

Although a high degree of similarity was shown among the amino acid sequence of HSPs, minor changes may lead to greater effects relating to inhibition, therapeutic access or resistance. The N-terminal domain provides the binding of some inhibitors, for instance, Gda and other small molecules to the ATP-binding pocket. The more hydrophobic amino acid presents the higher score. Figure 12 illustrates the schematic representation of interaction network of four Hsp90s. The detail of the protein-protein interaction of four Hsps is described in following.

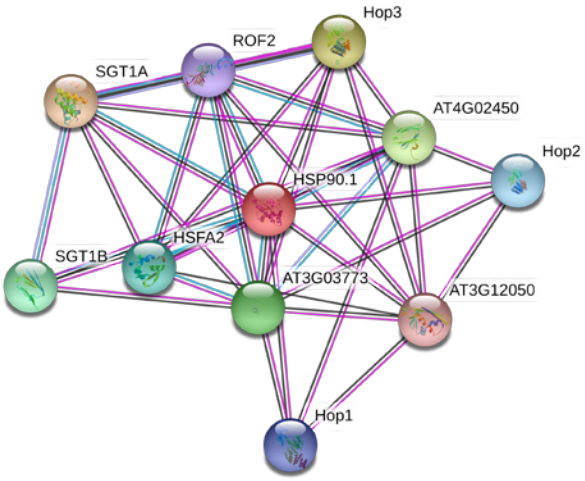
HsHsp90a



ShHtpG



AtHsp83



LbHsp83

Figure 12: Schematic representation of interaction network of four heat shock protein 90 (Hsp90): Human Hsp90 (HsHsp90α), *Arabidopsis thaliana* Hsp83 (AtHsp83), *Streptomyces hygroscopicus* HtpG (ShHtpG) and *Leishmania braziliensis* Hsp83 (LbHsp83). Network nodes represent different proteins. In all 4 images, the colored nodes indicate the query proteins and first shell of interactors. Although the empty nodes show the proteins of the unknown 3D structure, the filled nodes display some known or predicted 3D structure. Made by STRING database.

Human Hsp90α interacts dynamically with various co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function. According to the figure 12, the main predicted functional partners are CDC 37 (Cell division cycle 37 homolog (*S. cerevisiae*), AHSA1 (AHA1)[45,124] and HspA4 (Heat shock 70kDa protein). CDC 37 is a co-chaperone that binds to various kinases and promotes their interaction with the Hsp90 complex[35,124]. AHA1 is also a co-chaperone that is an activator of heat shock 90kDa protein ATPase homolog 1 (yeast) and stimulates Hsp90 ATPase activity[56,59].

AtHsp83 interacts with Hop3 which mediates the association of the molecular chaperones Hsp70 and Hsp90 as well as HSFA2 (Heat shock transcription factor A2). AtHsp83 also interacts with Heat shock protein 70 and Hop2[125]. Hop2 mediates the association of the molecular chaperones Hsp70 and Hsp90 and nuclear encoded chloroplast preproteins binding to Hsp90 prior to chloroplastic sorting[40,126].

Based on ShHtpG interaction network, the putative heat shock protein Hsp70, Dnak and Dnaj which act as molecular chaperone are ShHtpG`s well-known protein interactors. Furthermore, it interacts with chaperonin GroEL[127,128] which hinders misfolding and assist the refolding and proper assembly of unfolded polypeptides produced under the stress conditions.

The LbHsp83 interacts with some proteins that are displayed in figure 12. The most prominent and well-known proteins are sti1 (Stress-induced protein), putative protein kinase and heat shock protein 90 and 70[39,129].

4.1.1. Influence of fluorescence label on the ATP binding site

In earlier studies was shown that FITC-Geldanamycin or Cy3-labeled ATP is able to bind into the pocket of Hsp90[14,94,105]. In order to have a clear sight of the nucleotide binding pockets of Hsp90 and its binding affinity, the potential binding position of cyanine dyes (Cy5/Cy3-labeled) ATP and Hsp90 were studied[16] (Modeled by Prof. Matthias Preller). The schematic presentation of cyanine dye and different label positions on ATP is presented in figure 13. In this experiment, three different positions of the cyanine dye (**1**) on the adenine ring (**2**, **4**) and on the phosphate (**3**) were investigated (Fig. 13).

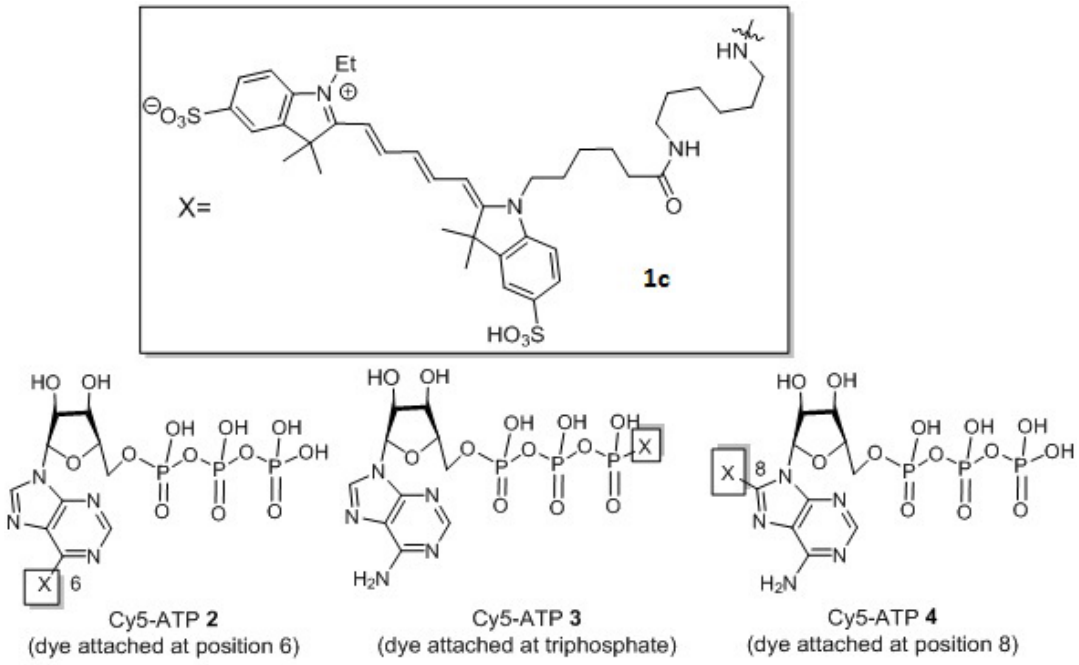


Figure 13: Schematic presentation of cyanine dye (**1c**) and different label positions on ATP (**2-4**)[16]. Adapted from Ref. 15[15].

The question is, can we expect ATP binding on Hsp90 when ATP has an additional dye on the phosphate position? In order to answer this question and predict whether the coupling positions of the dye have an effect on the binding of the ATP in heat shock protein nucleotide binding pockets or not, the molecular simulation exhibit a model (Fig. 14) which is the detailed description of nucleotide binding pockets of Hsp90 (crystal structure of N-terminal domain and the potential binding position of cyanine dye (Cy5/Cy3-labeled ATP). The nucleotide-binding pockets were analyzed with a minimum number of site points per pocket identified of 15 by SiteMap[16,130]. Steered molecular dynamics (SMD) simulations of Cy5-labeled ATP (**2**) or (**3**) bound to human HSP70 were performed in NAMD 2.11[16,131].

The figure shows that ATP can enter the ATP binding pocket of Hsp90 when the label is at the phosphate position, which the phosphate pointing towards the pocket entrance. Therefore we can assume to use labeled Cy5/Cy3 ATP for binding assay. As it is observed from the models, there is a difference between the fitting of cyanine dye Cy3 (Fig. 14a) and Cy5 (Fig. 14b) into the binding site of Hsp90. So the cyanine dye Cy5 does not fit as similar as Cy3 into the binding pocket of the Hsp90.

a) **b)**

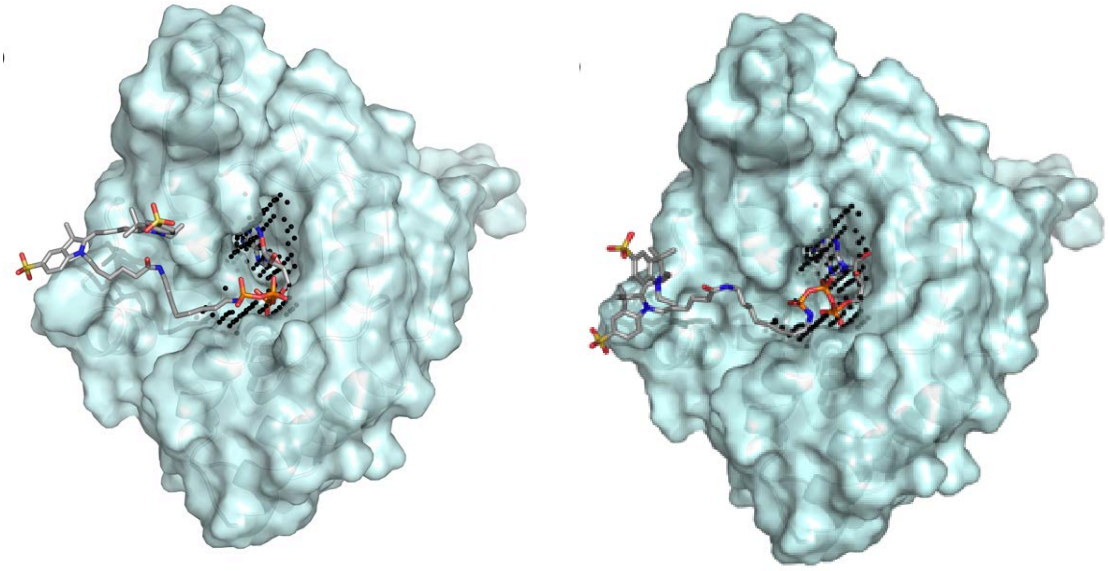


Figure 14: Illustration of the crystal structure of Hsp90 with modeled fluorescently-labeled ATP derivative (3) (gray) analysed with SiteMap. a) Modeled dye-labeled ATP Cy3. b) Modeled dye- labeled ATP Cy5. Reprinted from Ref. 16[16].

4.2. Application of the ATP binding assay on Hsp90 orthologues

In the next step, the binding of fluorescently-labeled ATP (Cy3-ATP) on purified Hsp90 proteins was performed by the means of a highly miniaturized microarray method. It has emerged as a promising technique, a miniaturized and parallel assay that contains small amounts of purified proteins. The principle of the miniaturized Hsp-microarray is presented in figure 15. The heat shock proteins are spotted by means of a nanoplotter, with a nanotip pipette (volume of 800-1600 pL; 8 drops/dot) and become immobilized onto the nitrocellulose membrane. The target proteins can either bind the fluorescence-labeled ATP or the potential inhibitors with high affinity. By the means of fluorescence-labeled ATP (Cy3/Cy5 derivatives) displacement, different interactions or binding can be visualized and evaluated. When the dye-labeled ligand is bound, this leads to an increased intensity of fluorescence signal whereas an active substance (inhibitor) can displace a fluorescence reduction when they bind to the binding site of the proteins. In the HTS method, the fluorescence signals are evaluated according to the presence or

absence of the ligand. The concentration of proteins is 3 mg/ml and they are spotted on the microarray chips in the small volume (~ 800 pL) by the use of nanotubes.

In order to validate the quality of the microarray tests, the Z-factor should be calculated. For having for a powerful and reliable assay the Z-factors for all inhibitors should fit into the range of $0.5 < Z < 1.0$ [14,106].

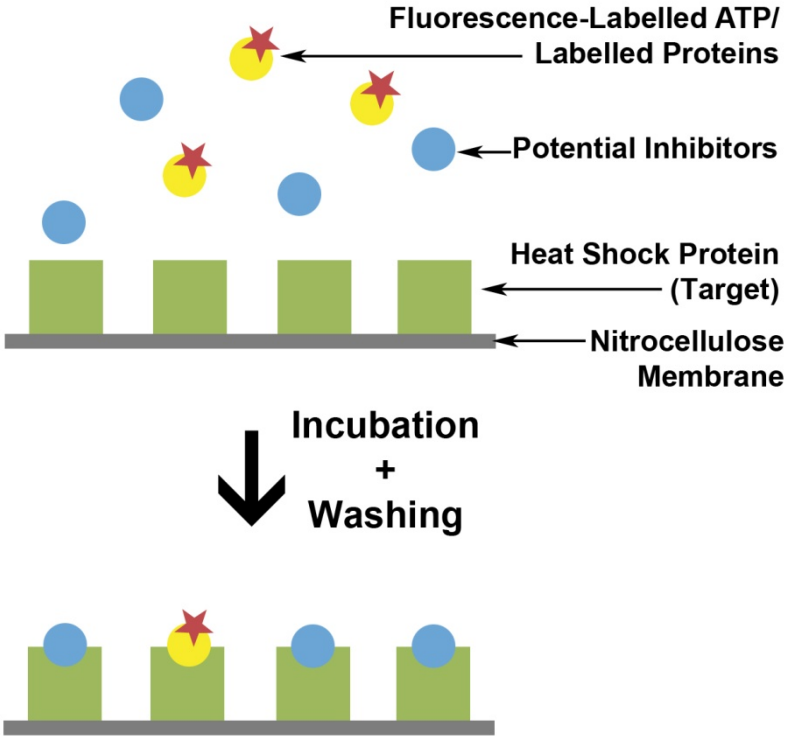


Figure 15: Schematic representation of the principle of the miniaturized Hsp-microarray. (more detail in the theoretical background section). Adapted from Ref. 14[14].

4.2.1. Targeting Hsp90 by microarray-based assay

As shown before on human Hsp90 and bacterial HtpG (HtpG from *Helicobacter pylori*) this assay strategy helps to find novel compounds specific active on Hsp90[14,97]. To understand whether small differences in the ATP binding structure are responsible for the efficiency of compounds on Hsp90 proteins from human as a marker against cancer and *Leishmania braziliensis* as a target against parasites the purified were improved for their 17AAG affinity. The result of displacement experiment is shown in figure 16. Purified human Hsp90 and the orthologue from *Leishmania* (LbHsp90) were spotted

onto the nitrocellulose surface in a row of ten spots in left and right columns of each subarray respectively. After incubation with Cy3 labeled ATP and removing of unbound ATP, bound ATP was visualized as bound fluorescence intensity (Fig. 16a, (first subarray left – negative control (NK)). The presence of 17AAG (17-*N*-allylamino-17-demethoxygeldanamycin) displaced binding of ATP and result in the reduction of the fluorescence signal. In comparison, bound ATP without inhibitor and with a strongly displacing ligand is found first subarray right as a positive control (PK). To estimate the efficient concentration (EC_{50}) where 50% of the signal is displaced from the bound form the following subarrays were arranged from the highest concentration at 50 µM to a very low concentration of 50 pM of 17AAG. The fluorescence intensity was measured as a function of the 17AAG concentration. The result of the displacement experiment is given as a bar histogram in figure 16b.

When the dye-labeled ligand (Cy3-ATP) is bound, this leads to an increased intensity of fluorescence signal whereas inhibitor can displace a fluorescence reduction when they bind to the binding site of the proteins. The fluorescence signals are evaluated according to the presence or absence of the ligand (Fig. 16b).

a) **b)**

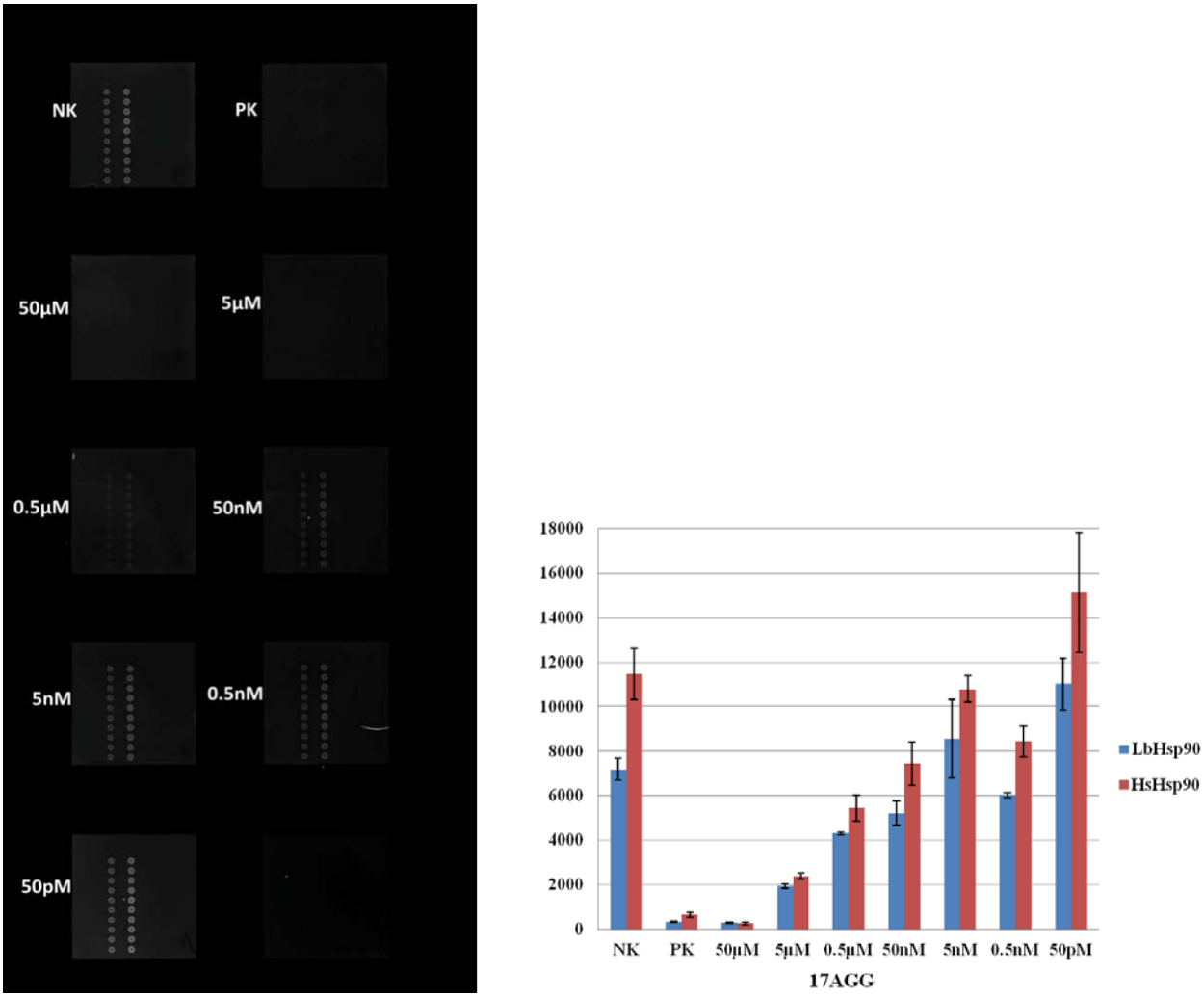


Figure 16: a) Typical protein microarray after incubation with Cy3-ATP, Hsp and inhibitor. LbHsp90 (left columns) and HsHsp90 (right columns) were spotted on the membrane. Bound ATP without inhibitor (the first subarray left – negative control (NK)) and with a strongly displacing ligand (first subarray right – positive control (PK)); following subarrays from high to low concentrations (50 µM- 50 pM) were organized from left to right and from top to bottom. **b)** Bar histogram of microarray competitive displacement test for 17AAG by ATP-Cy3 on LbHsp90 and HsHsp90.

Based on previous findings and our investigations, the Hsp selectivity emerged as the main role in the drug research and Hsp member from other natural resources like bacteria and parasites such as *Leishmania* need to be further studied and more focused.

4.2.2. Identification of novel compounds against Hsp90 from human and *Leishmania braziliensis*

The orthologue proteins of human and pathogenic Hsps vary in a few numbers of amino acids and their hydropathic manner[32,121]. According to the results, in the area of amino acids 50-75, LbHsp90 shows more hydrophobicity comparing to human Hsp90α (Fig. 11). These differences in hydrophobicity may have an effect on the binding of geldanamycin derivatives since this region settle entirely in the N-terminal domain.

With this hypothesis, the recombinant full-length LbHsp90, as well as human HsHsp90 from *E. coli* lysates, were purified. Afterward, the purified proteins were spotted on nitrocellulose membrane coated microarray substrates as described before. In order to identify candidates for inhibiting LbHsp90, some geldanamycin derivatives which were newly prepared as well as previously reported were tested at a 500 nM concentration against HsHsp90 and LbHsp90.

The miniaturized microarray technique was used to test novel synthesized reblastatin derivatives which were synthesized and provided by Prof. Kirschning, Institute of Organic Chemistry, Leibniz University Hannover[94,97]. Several compounds of the in-house compound library were designed by biosemichemosynthetic techniques by the Kirschning group (Institute of Organic Chemistry, Leibniz University Hannover)[95,96]. Numerous inhibitors were tested against Hsp90 from human and *Leishmania braziliensis* which is presented as a heat map in figure 17 and the EC_{50} value of some selected inhibitor is presented in table 2 (detailed in supplementary).

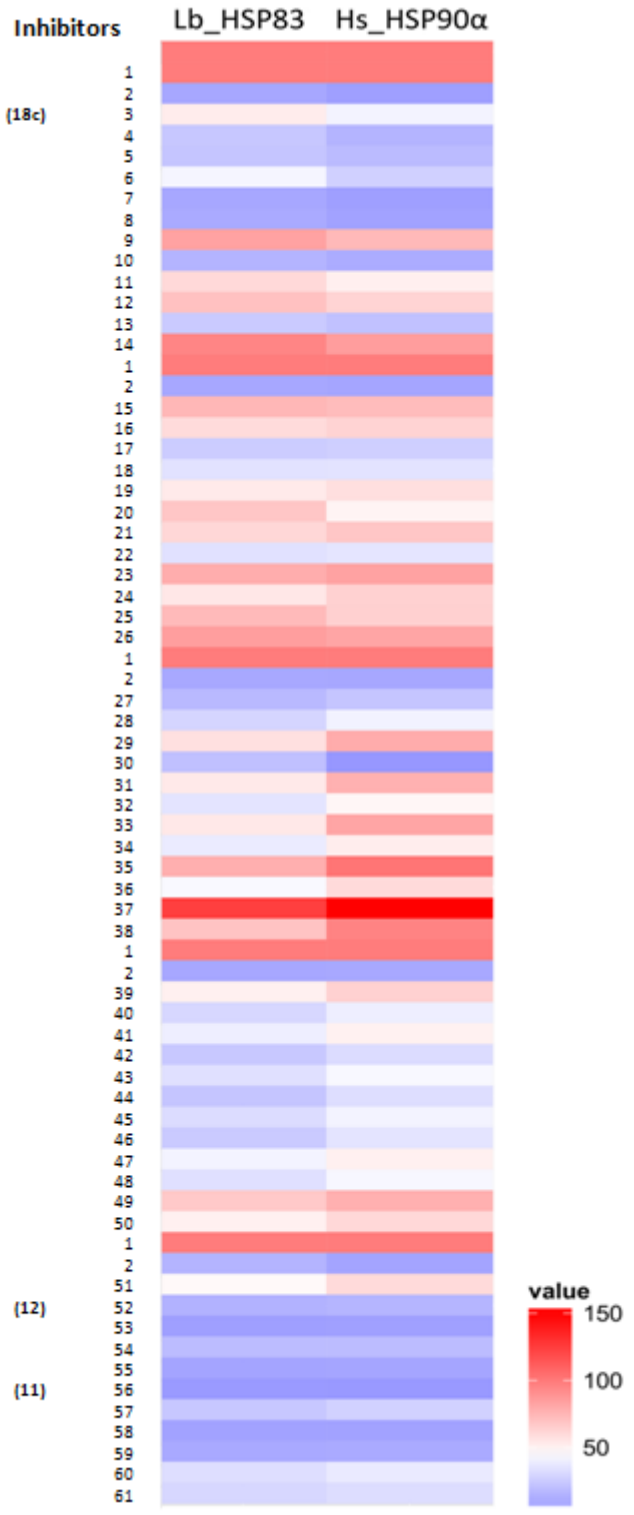


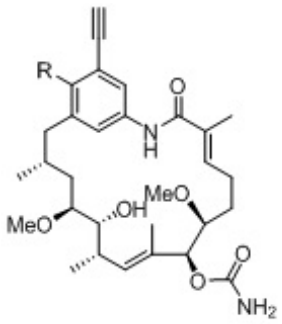
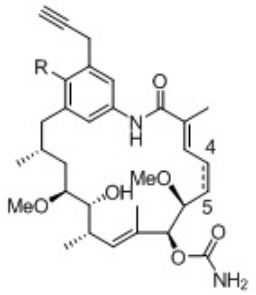
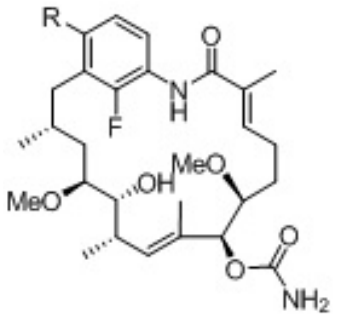
Figure 17: Heat map, a graphical illustration of different tested inhibitors against Hsp90 from human and *Leishmania braziliensis*.

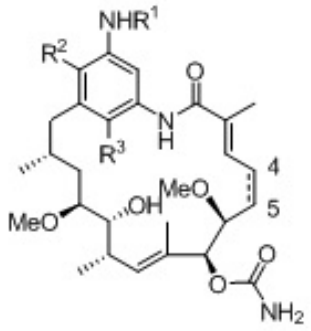
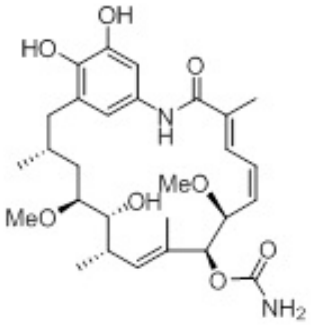
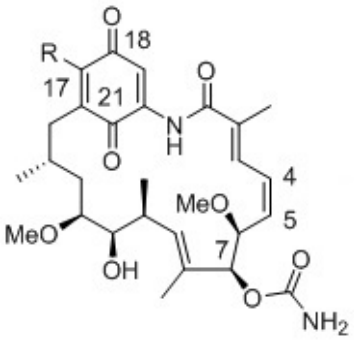
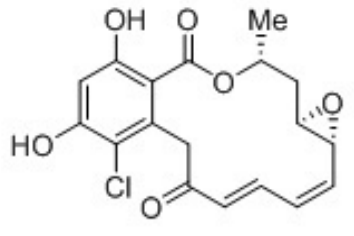
Table 2: List of the EC_{50} value of the inhibitors which were tested against Hsp90 from *Leishmania braziliensis.* The numbering of the inhibitors is according to the heat map in figure17.

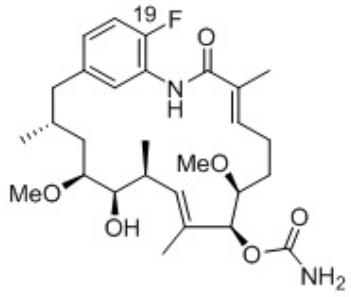
Inhibitor	**LbHsp90 EC_{50} (nM)**
3 (**18c**)	24.95 ± 7.58
5	13.6 ± 1.32
8	28.5 ±1.93
52 (**12**)	25.35 ± 9.29
53	85.5 ± 17.07
54	292.9 ± 33.65
56 (**11**)	5.06 ± 4.21

Several novel nonquinone geldanamycin derivatives were provided and tested against Hsp90 from human and *Leishmania braziliensis* in which the different groups were substituted in C18 or C21 positions (table 3). Although the compounds **5** and **6** have an alkynyl substituent at C18, the compound **7-9** carrying a propargyl substituent at the same position. In the compounds **10** and **11** the fluoro group substituted at C21, whereas an amino or an *N*-acetylamino group substituted at C18 in the compounds **12-16**[15].

Table 3: The structure of new geldanamycin derivatives **5-17** , geldanamycin (quinone) **18**a, (hydroquinone form) **18**b, 17-allylamino geldanamycin (17-AAG) **18**c, radicicol **19** and 19-fluoro geldanamycin derivative **20**, reblastatin **21** [94,97,132]. Adapted from Ref. 15[15].

Position	Structure
5: R=H (0.8mg/L) **6:** R=OH (0.4mg/L)	
7: R=H (0.4mg/L) **8:** R=OH and 4,5 s.b. (0.3mg/L) **9:** R=OH and 4,5 d.b. (0.5mg/L)	
10: R=H **11:** R=OH	

12: R^1=H, R^2=H, R^3 = H, $C_{4,5}$=s.b.(1.7mg/L) **13:** R^1=H, R^2=H, R^3 = H, $C_{4,5}$=d.b.(0.3mg/L) **14:** R^1=H, R^2=OH, R^3 = H, $C_{4,5}$=d.b. HRMS **15:** R^1=Ac, R^2=OH, R^3 = H, $C_{4,5}$=d.b.(1.3mg/L) **16:** R^1=Ac, R^2=OH, R^3 = OH, $C_{4,5}$=s.b.(1.3mg/L)	
17: (0.6 mg/L)	
18a: R = OMe (geldanamycin, quinone form) **18b:** R = OMe (geldanamycin, hydroquinone form, not shown) **18c:** R = $NHCH_2CH=CH_2$ (17-AAG)	
19: Radicicol	

20: R^1 =R^2= H, R^3= F **21:** R^1 = OMe, R^2= OH, R^3=H(reblastatin)	

The inhibitory potency of the new reblastatin derivatives was tested by displacement of fluorescently-labeled ATP from Hsps obtained from *Leishmania braziliensis* Hsp90 (LbHsp90) as well as human Hsp90 (HsHsp90). The dose responsive displacement of the fluorescence signal by the semisynthetically prepared geldanamycin derivative 17-AAG (17-*N*-allylamino-17-demethoxygeldanamycin) is shown in figure 18 (non-linear fit: logistic, fitting parameters: A1 = 0, A2 = 1). The inhibitor concentration is given logarithmically as a function of the normalized fluorescence signal from ATP-Cy3. The calculated EC_{50} for this molecule is 24.95 ± 7.58 nM. The IC_{50} value was obtained from dose-response curve fittings[15].

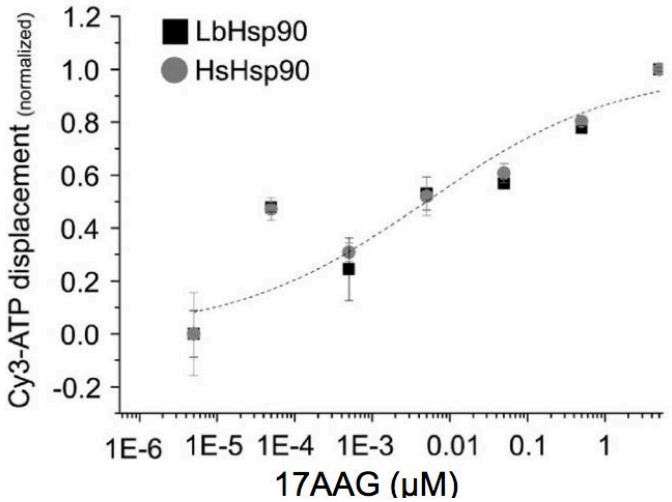


Figure 18: Dose-response curve of the competitive displacement assay for HsHsp90 and LbHsp90 using 17-AAG as displacing ligand (non-linear fit: logistic, fitting parameters: A1= 0, A2= 1). The inhibitor concentration is given logarithmically as a function of the normalized fluorescence signal from ATP-Cy3. The calculated EC_{50} for this molecule is 24.95 ± 7.58 nM. Adapted from Ref. 15[15].

As result, among various tested inhibitors (available in supplementary), six compounds were identified that showed as specific competitors for the both Hsps. The EC_{50} values of 17-Demethoxy-18-dehydroxy-21-fluor-reblastatin (**11**) on LbHsp90 was determined to be 5.1 ± 4.2 nM and for 17-Demethoxy-18-amino-reblastatin (**12**) 25.4 ± 9.3 nM (Fig.19). In comparison, the 17-demethoxy-18-propargyl-reblastatin (**7**) reduced ATP-binding of LbHsp90 with an EC_{50} of 424 nM[15].

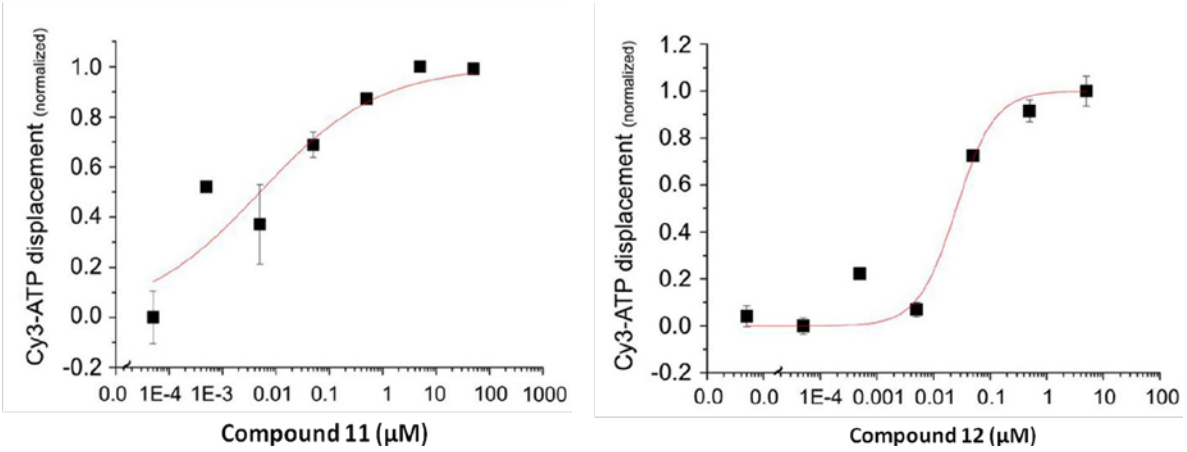


Figure 19: Dose-response curve of the competitive displacement assay for LbHsp90 with **11** and **12**. The inhibitors concentrations were 500 nM. Adapted from Ref. 15[15].

These compounds are novel nonquinone geldanamycin derivatives that they are different in the groups at C18 or C21 positions. The compounds **5** and **6** carry an alkynyl substituent at C18 whereas the compound **7-9** carrying a propargyl substituent at the same position. Although the fluoro group substituted at C21 in the compounds **10** and **11**, the amino or an *N*-acetylamino group replaced at C18 in the compounds **12-16.**
Since the two derivatives **11** and **12** presented a particular high affinity for LbHsp90, the binding energies were determined individually by ITC (Fig. 20). The ITC data was kindly provided by Dr. Klaus Kock (Institute of Physical Chemistry of Ruhr University of Bochum). The result of ITC experiments for the interaction of two inhibitors 17-demethoxy-18-dehydroxy-21-fluoro-reblastatin (**11,** squares) and 17-demethoxy-18-amino-reblastatin (**12**, triangles) with LbHsp90 is presented in figure 20. After each injection of the inhibitors into the temperature-controlled sample cell including LbHsp90, the alteration in amount of heating power was recorded. Afterwards, the generated heat was gained by integration and plotted against the molar ratio of the

concentrations of compounds **11** or **12** and LbHsp90 after subtraction of linearly decreasing background. The values for stoichiometry (N= 1.4) for compound **12** and (N=0.73) for compound **11**, dissociation constant (Kd)= 830 nM for **12** and 30 nM for **11**, and the change in enthalpy of binding ΔH= -10 kcal/mol for compound **12** and ΔH= -24 kcal/mol for compound **11** were achieved (solid lines) by using a one site binding isotherm to fit the data[15].

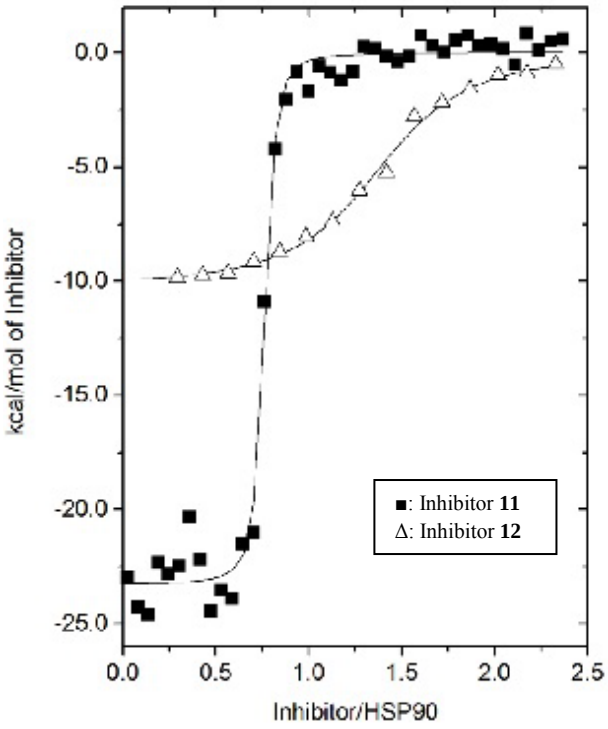


Figure 20: The illustration of ITC results of the interaction of 17-demethoxy-18-dehydroxy-21-fluoro-reblastatin (**11**, squares) and 17-demethoxy-18-amino-reblastatin (**12**, triangles) with LbHsp90. The two compounds were injected into the temperature-controlled sample cell containing LbHsp90 and the change in heating power after each injection was recorded. After subtraction of linearly decreasing background, the generated heat was obtained by integration and plotted against the molar ratio of the concentrations of the two inhibitors and LbHsp90. The values for stoichiometry (N= 1.4) for **12** and (N=0.73) for **11**, dissociation constant (Kd)= 830 nM for 15 and 30 nM for **11**, and the change in enthalpy of binding ΔH= -11 kcal/mol for **12** and ΔH= -24 kcal/mol for **11** were achieved (solid lines) by using a one site binding isotherm to fit the data. Adapted from Ref. 15[15].

By applying the novel geldanamycin derivatives **5-13**, **16** and **17** to cultured human tumor cell lines, the biological aspect as an anticancer agent of them were assessed (Table 4 and 5). The data of applying the inhibitors to tumor call line was kindly

provided by Dr. Florenz Sasse (Department of Chemical Biology, Helmholtz center of Infectious Research Braunschweig (HZI). The compounds **5** and **10** exhibit the strong antitumor activities among all tested compounds overall tested cancer cell lines[15].

Table 4: IC_{50} [nM] value of the antiproliferative activities of compounds 5, 10, 18 and 20 with human cancer cell lines. Adapted from Ref. 15[15].

Cell line	**Origin**	**compounds**			
		5	**10**	**18**	**20**
KB-3-1	*cervix carcinoma*	34	46	53	73
PC-3	*prostate adenocarcinoma*	36	67	18	42
SK-OV-3	*ovary adenocarcinoma*	46	79	125	54
A-431	*epidermoid carcinoma*	17	37	18	62

Replacement at the position C18 had an effect on the binding of Hsp90 in a different way, for instance, alkynylation of **5** and **6** showed low competitive activities on Hsp by the microarray test whereas **6** is a little more active on LbHsp90 than on human Hsp90. By contrast, the tumor cell-based assay showed the completely different result which the compound **5** (17-46 nM) was greatly active. However, compound **6** had no effect. Furthermore, by substitution of propargyl group at C18 in the compound **7**, it showed a weak tumour (295-516 nM) and medium competitive activities (EC_{50}= 424 nM), whereas the compound **10** and **11** revealed the strongest competitive (EC_{50}= 5 nM; Kd= 25 nM) and antitumor activities (37-79 nM)[15].

In the other compound like **12-16**, which bear an amino or an *N*-acetylamino group at C18, the compounds lacked antitumour activity or present medium streaky cell type-dependent of its activity (76-1240 nM). In contrast, the competitive activity in the compound **12**, (EC_{50}= 25 nM; Kd_{ITC}= 826 nM) varies differently[15].

Table 5: The data illustration of IC_{50} and ITC of geldanamycin derivatives **7**, **11** and **12.**

Inhibitor	**IC_{50}, Microarray-based assay nM**	**IC_{50}, Cell based assay anti tumour activity nM**	**(Kd, ITC) Isothermal calorimetry**
7: 17-demethoxy 18-alkynylmethyl-reblastatin	424 ±0.04	295-516	
11: 17-Demethoxy-18-dehydroxy-21-fluoro-reblastatin	5.1 ± 4.2	101-131	Kd = 28 nM ΔHsp = -24 kcal/mol ΔS = -44.3 cal/mol/deg ΔG = -10 kcal/mol stoichiometric factor N = 0.73
12	25.4 ±9.3	76-1240	Kd = 826 nM ΔHsp = -11 kcal/mol ΔS = -7.88 cal/mol/deg ΔG = -8.3 kcal/mol stoichiometric factor N = 1.4

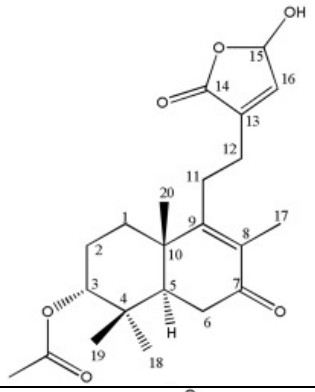
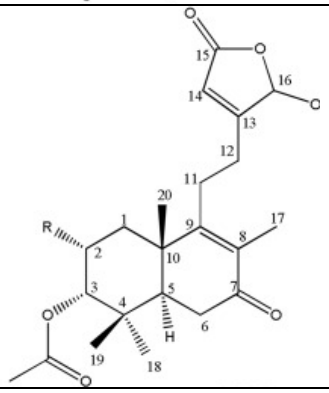
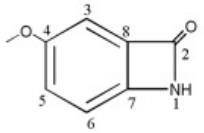
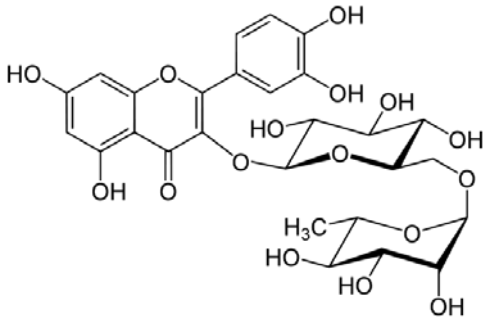
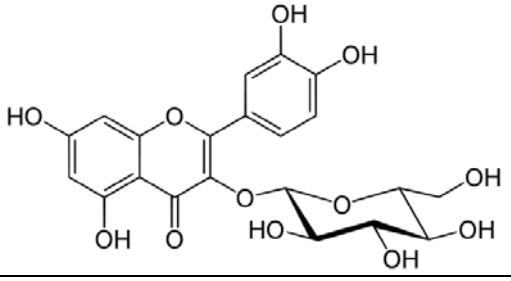
4.2.3. Examination of anti Hsp90 activity of isolated natural compounds from *Roylea cinerea*

Natural products obtained from plant, fungi and bacteria formed during an evolutionary process compounds with strong anti Hsp activity like green tea, or *Garcinia hanburyi* and other[32,95]. Therefore the Hsp-based microarray test system was used to examine the activity of natural compounds isolated recently from the plant *Roylea cinerea* on human Hsp90.

In this assay, the effect of two novel labdane diterpenoids cinereanoid C (**22**), cinereanoid D (**23**), and a new β-lactam cinerealactam E (**24**) from *Roylea cinerea*, as well as six known flavonoid glycosides; rutin (**25**), isoquercetin (**26**) (table 6), nicotiflorin, martynoside, undatuside A and 5´- β -D-glucopyranosyloxyjasmonic acid on the ATP binding site of heat shock proteins Hsp90 were tested[17].

Roylea cinerea is an angiosperm plant belongs to the Lamiaceae family, which is native to the western, Himalayas of Nepal and northern India. Its leaves are traditionally applied in traditional medicine as a febrifuge and a treatment for malaria, diabetes and skin diseases[17,133].

Table 6: Structures of the compounds **22–26**. Two new labdane diterpenoids (**22**, **23**), a new β-lactam **24** from *R. cinerea*, rutin **25** and isoquercetin **26**. Adapted from Ref. 17[17].

Position	**Structure**
22: labdane diterpenoids cinereanoid C	
23: labdane diterpenoids cinereanoid D R=$OCOCH_3$ **23a**: R=H	
24: β-lactam cinerealactam E	
25: rutin	
26: isoquercetin	

A typical fluorescence picture of a microarray with spotted Hsp90 protein is shown in figure 21. The fluorescence is reduced in presence of the Hsp90 inhibitor radicicol. Radicicol is a standard, well-known inhibitor for Hsp90.

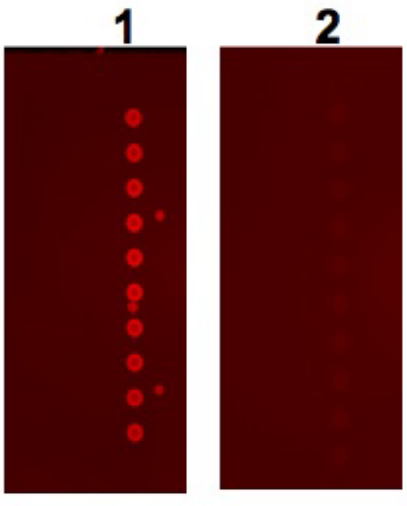


Figure 21: Presentation of a protein microarray after the direct displacement assay with Cy5-ATP labeled protein (1) and displaced by 500 nM radicicol (positive control) (2). Reprinted from Ref. 17[17].

The results illustrated that the compounds including two new labdane diterpenoids, isoquercetin, nicotiflorin and martynoside that are shown by numbers 2, 5, 6 and 7 in the figure 22, had the strongest effect on human Hsp90, whereas the other compounds had weak or no effect on the ATP-binding site of human Hsp90 (Fig. 22)[17].

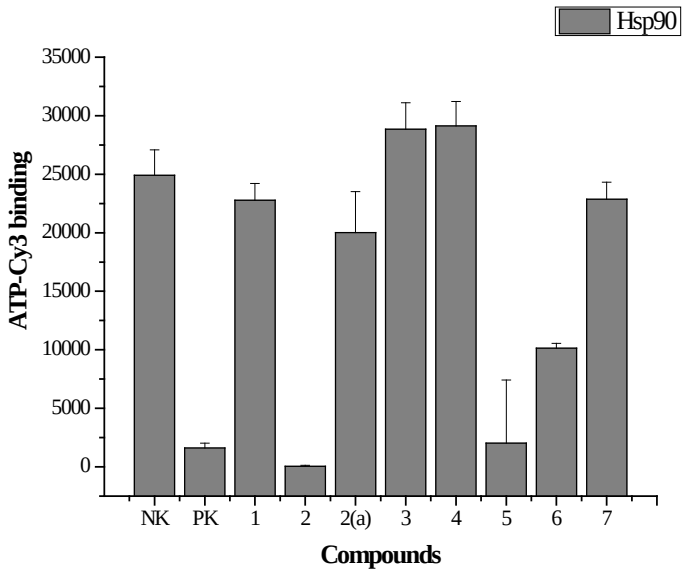


Figure 22: Human Hsp90-Cy5 binding as a function of tested compounds. Compounds were tested at 100 mg mL-1 whereas a standard inhibitor radicicol has 500 nM and a control without inhibitor. Compounds 1, 2: new labdane diterpenoids, 3: new β-lactam, 4: rutin, 5: isoquercetin, 6: nicotiflorin, 7: martynoside. Reprinted from Ref. 17[17].

4.3. Examination of ATP-binding sites of Hsp70 with bioinformatic methods

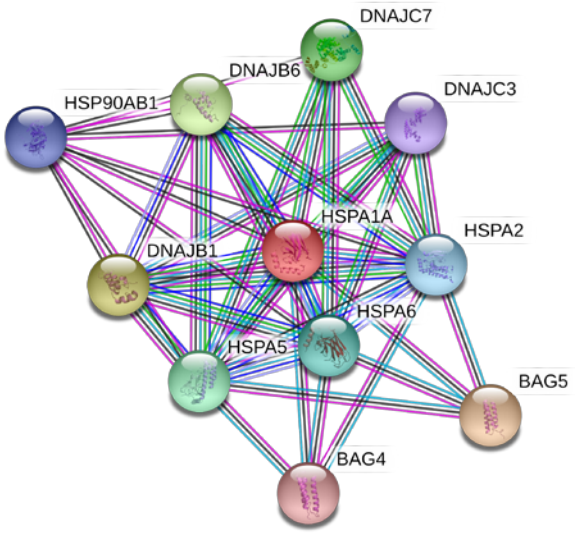
Hsp70 is highly conserved and ATP-dependent chaperones and its structure is similar to Hsp90 which consist of an N-terminal ~44 kDa nucleotide binding domain (NBD) which includes the ATP binding site and hydrolysis, a 15 kDa substrate polypeptide binding domain (SBD) which interacts with the hydrophobic amino acids in peptides and ~10kDa α-helical C-terminal, a 'lid' domain that closes over the substrate and also mediates the biding of the co-chaperone. In bacterial cells, DnaK is the eukaryotic Hsp70 equivalent[68–71]. The binding pocket for the natural ligand ATP and susceptibility for some inhibitors like Ver155008 is in the N-terminal domain (positions 1-388)[134]. Genetic reaserches have illustrated that there is a strong connection between Hsp70 and its co-chaperones to numerous diseases, including neurodegeneration, microbial pathogenesis and cancer. Therefore the potential of this chaperone as a therapeutic target has remained mainly under investigation[72]. The N-terminal domain (1-388 amino acids) of human HSP70 and bacterial HSP70 (MtDnak) from *Mycobacterium tuberculosis* were compared in order to identify regions of similarity between the amino acid sequences of the two HSPs (Fig. 23).

Figure 23: The sequence alignment of the nucleotide binding domains of human HSP70 and bacterial HSP70 from *Mycobacterium tuberculosis* (MtDnak). The differences in the sequences are highlighted in color. The alignment was performed with the CLC Sequence Viewer program and the amino acid sequences were taken from the NCBI database.

In figure 23, the sequence alignment shows the differences within the nucleotide binding domain of HsHSP70 and MtDnak. The differences between the sequences of the amino acids of the proteins may correlate with different binding property of the ligand and consequently has effect on various drug susceptibilities.

Figure 24 presents the schematic image of interaction network of HsHsp70 and MtDnak. The detail of the protein-protein interaction of the two Hsps is described in following.

HsHsp70



MtDnak

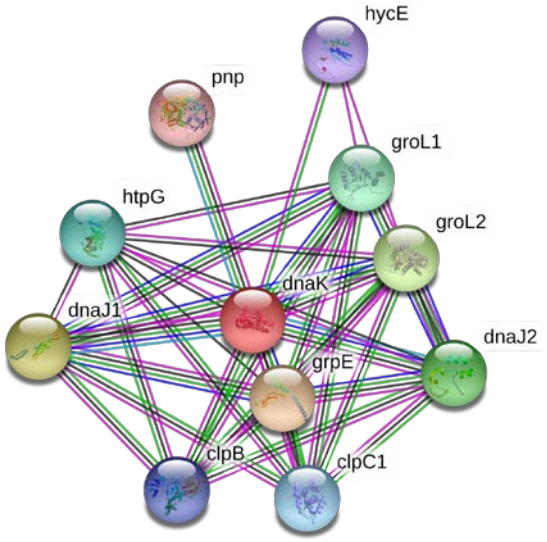


Figure 24: The schematic image of interaction network of HsHsp70 and MtDnak. Network nodes represent different proteins. The colored nodes show the query proteins and first shell of interactors in both images whereas the empty nodes show the proteins of the unknown 3D structure. The filled nodes indicate some known or predicted 3D structure. Made by STRING database.

Heat shock 70kDa protein (HspA1A) is in cooperation with other chaperones. These chaperones attend in all the processes through their ability to recognize nonnative conformations of other proteins. The main predicted functional partners of human which are shown in figure 24 are DnaJ (Hsp40) homolog, Hsp90AB1 (Heat shock protein 90kDa alpha) and HSF (Heat shock transcription factor 1). DnaJ Interacts with Hsp70 and can stimulate its ATPase activity[67,70]. Hsp90AB1 is molecular chaperone (detailed description in the theoretical background chapter) that stabilizes proteins against

stresses, helps in protein degradation as well as raise the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction[135].

According to figure 24, the chaperone protein DnaK interacts with several functional partners such as GrpE, DnaJ and HtpG. The protein GrpE acts as a nucleotide exchange factor (NEF) for DnaK and increase the rate of ATPase activity as well as DnaJ. DnaJ, also known as Hsp40, raise the ATP hydrolysis. Chaperone protein DnaJ participates actively in the response to stresses by preventing the aggregation of stress-denatured proteins and by disaggregating proteins[136]. MtDnak also interacts with HtpG which is the prokaryotic homologue of the Hsp90[137,138].

4.3.1. Influence of fluorescence label on the ATP binding site of Hsp70

The potential binding position of cyanine dye (Cy5-labeled) ATP and Hsp70 were investigated by using the SiteMap in order to predict whether the coupling positions of the dye have an effect on the binding of the ATP in heat shock protein nucleotide binding pockets or not[16] (Modeled by Prof. Matthias Preller). Three different positions of the cyanine dye (**1**) on the adenine ring (**2**, **4**) and on the phosphate (**3**) were tested which is presented in Figure 13. The detailed description of nucleotide binding pockets of Hsp70 (crystal structure of N-terminal domain with modeled fluorescently labeled ATP analogue (Cy5-labeled ATP) is presented in figure 25. The nucleotide-binding pockets were analyzed with a minimum number of site points per pocket identified of 15 by SiteMap[16,130]. Steered molecular dynamics (SMD) simulations of Cy5-labeled ATP (**2**) or (**3**) bound to human Hsp70 were performed in NAMD 2.11[16,131].

The predicted binding mode of (**2**) to Hsp70 is presented in the figure 25a. In this model, the ATP phosphates are laid deeply in the binding pocket whereas the cyanine dye is located outside of the nucleotide binding pocket at the surface of the protein and the amine remains at the entrance of the ATP binding pocket towards the surface direction. Because of the position of the phosphates which lay deeply to the binding pocket, labeling at the γ-phosphate would need to cross the protein with the attached dye to get to the other side of the protein. Base on the modeling, the size of the linker in (**3**) would certainly let crossing the whole length to the protein surface. However, by evaluating the size of the binding pocket, it indicates that the tunnel connecting the

phosphate binding site and this side of the protein is not wide enough to provide a desirable binding of (**3**) (Fig 25 b and c)[16].

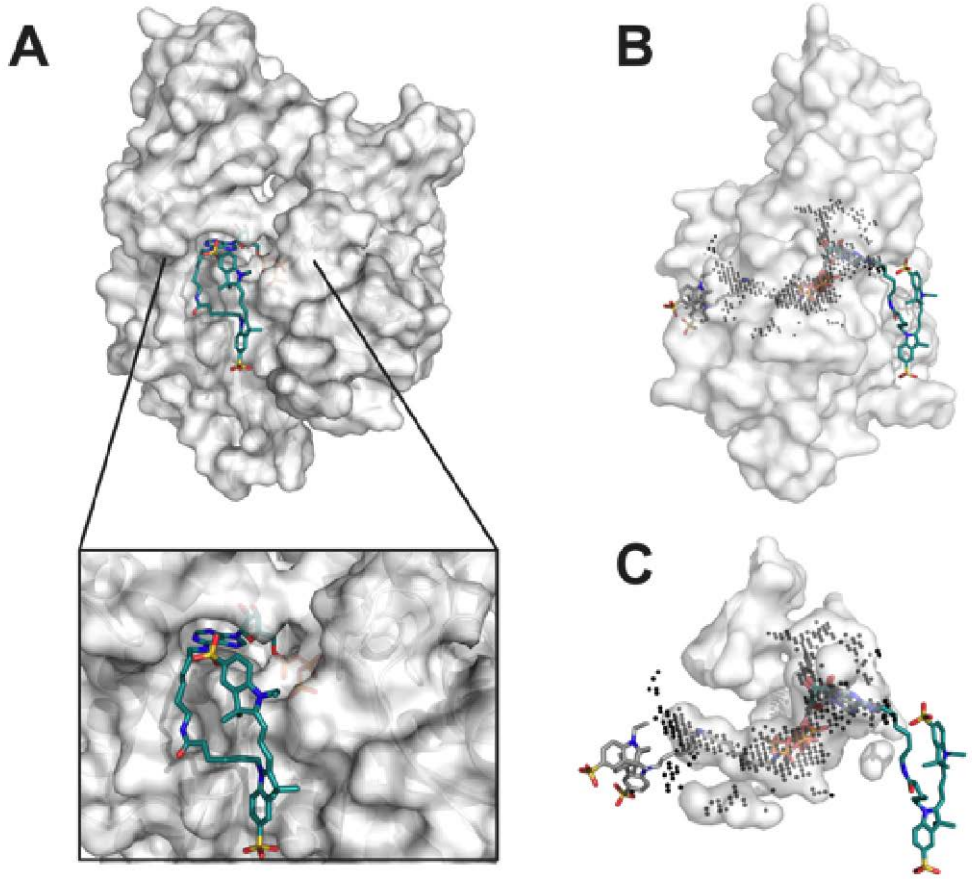


Figure 25: a) Illustration of the crystal structure of Hsp70 with modeled fluorescence-labeled ATP derivative (**2**) analyzed with SiteMap. **b)** Dimensions of the nucleotide-binding pocket and the adjacent tunnels in the proteins (**2**). **c)** Closer view of the binding pocket and the tunnels with the two dye-labeled ATP derivatives (**2**) is shown as sticks in cyan and (**3**) in gray. Reprinted from Ref. 16[16].

The dark sphere in Figure 25c presents the total binding pocket including the tunnels to the surface. Its volume is about 772 $Å^3$ with a tunnel length of around 13 Å and a width of 3 to 5 Å. Whereas ATP with label positions (**3**) and (**2**) feature molecular volumes of 874 $Å^3$ and 859 $Å^3$, respectively, and the dimensions of the cyanine dye (Cy5) is approximately 8 Å by 14 Å. Therefore the size of the dye is too large to fit into the tunnel. Consequently, the binding of (**3**) to Hsp70 is highly improbable[16].

According to the results, only ATP binds to the pocket and the dye remains at the surface of the protein, therefore (**2**) emerge as simply binding to Hsp70[16].

4.3.2. Fluorescence label on ATP influence binding on Hsp70/DnaK in microarray-based compound screening

The effect of three different fluorescence labeled ATP was investigated on the binding of purified human Hsp70 and bacterial Hsp70 (MtDnak) from *Mycobacterium tuberculosis*. The scheme of cyanine dye and different label positions on ATP is mentioned before in figure 13. The results in figure 26, illustrates the binding activities of fluorescence labeled ATP (Cy5-ATP) on purified Hsp70 proteins from human and MtDnaK from *Mycobacterium tuberculosis*. Although the binding activity of the MtDnaK is tentatively higher, both Hsp70 proteins do not bind (**3**) when ATP is labeled at the phosphate group.

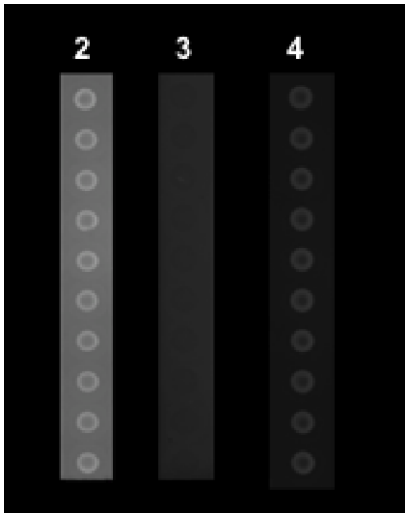


Figure 26: Typical spotted Hsp70 onto nitrocellulose surface and the fluorescence intensity of the proteins. The image shows the signal after incubation with labeled ATP (**2**, **3** and **4**) and the washing steps. Reprinted from Ref. 16[16].

The data clearly shows that the label at the γ-phosphate (**3**) hinder the binding to HsHsp70 as well as on MtDnaK from *M. tuberculosis* whereas other positions on the purine ring (**2**, **4**) allow binding (Fig. 27)[16]. Following the previous experiment, two fluorescence-labeled ATP (**2**) and (**4**) were tested in competition with unlabelled ATP.

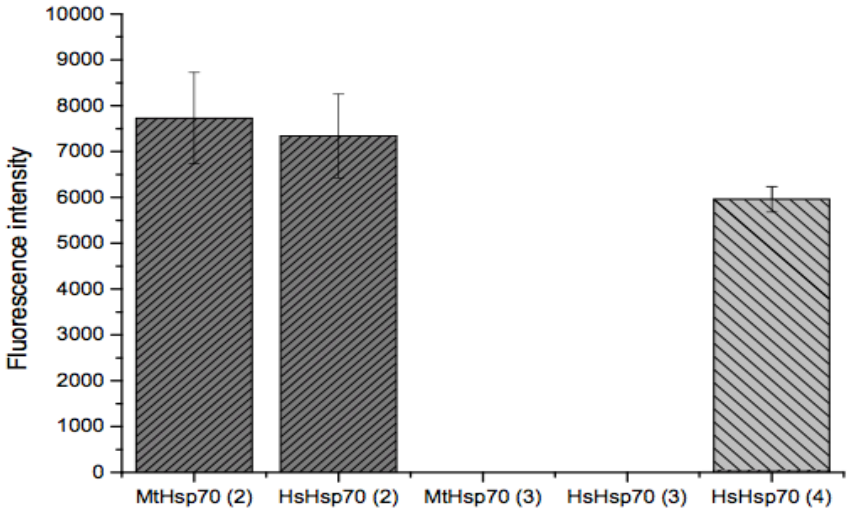


Figure 27: Effect of different label positions on ATP binding of Hsp70 proteins (HsHsp70 and MtHsp70). Adapted from Ref. 16[16].

Figure 28 shows a dose-response curve. The ATP concentration was plotted logarithmically against the displacement of the labeled ATP. The EC_{50} value for MtDnaK and human Hsp70 for the labeling position (**2**) is around 1 nM. The binding affinity of the ATP with the labeling position (**4**) is slightly lower, with an EC_{50} value of 37 nM.

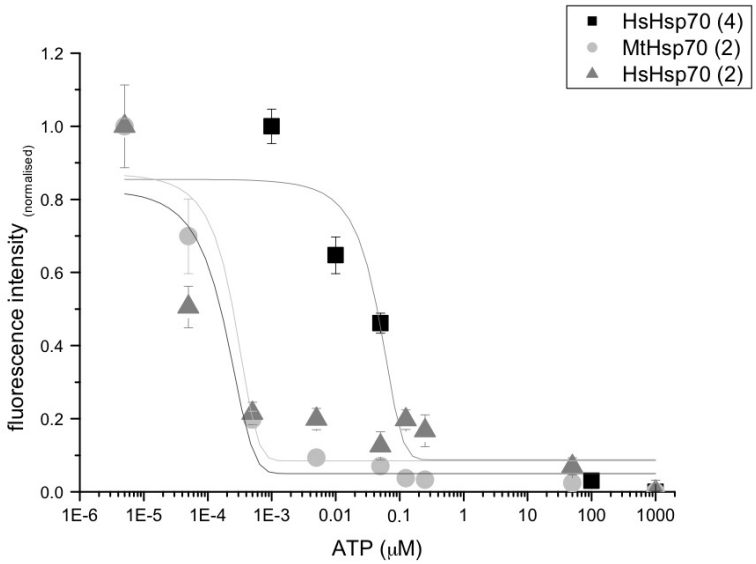


Figure 28: Dose response curve of the competitive displacement test with labeled-ATP and unlabelled ATP. The unlabeled ATP competes with the marked ATP around the binding site. Reprint from Ref. 16[16].

In order to test the assumed drug library on the ATP binding site of Hsp70, VER155008, a well-known, standard inhibitor and the two labeled ATP molecules was applied in the displacement assay (Fig. 29). VER155008 (5'-O-[(4-cyanophenyl) methyl]-8-[[(3,4-dichlorophenyl) methyl] amino]–adenosine) is recognized as an inhibitor, which binds to the N-terminal domain of the Hsp70. Figure 30 illustrates the dose curve response of VER155008 on the binding affinity on HsHsp70. The EC_{50} value for the labeled ATP (**2**) was 484 nM that is approximately close to Kd value 400 nM of ITC measurement. Furthermore, the EC_{50} value of the labeled ATP (**4**) was found as 2.3 µM. whereas the binding of labeled ATP (**2**) on the MtDnaK was assessed to be 30.6 µM[16].

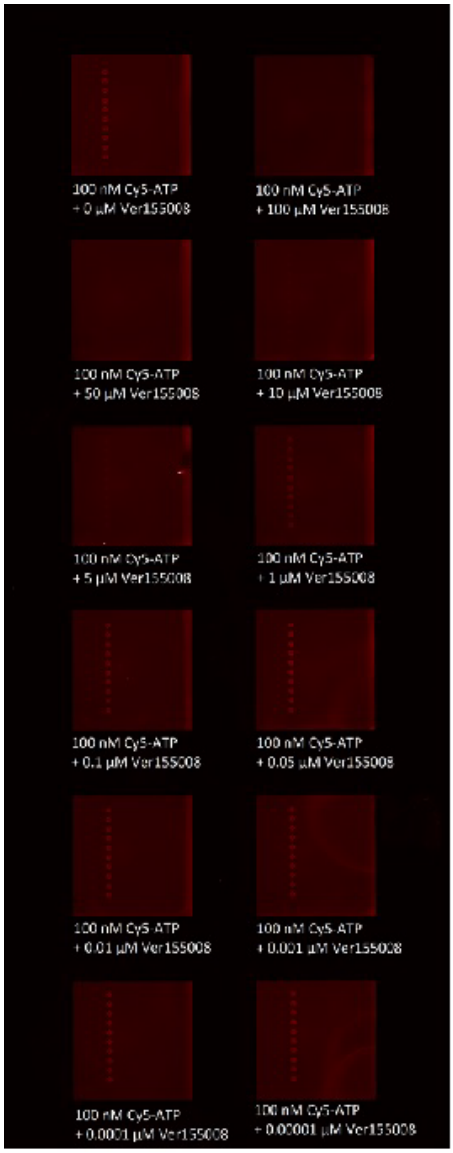


Figure 29: Typical displacement experiment with spotted HsHsp70 on the nitrocellulose surface in a row of ten spots after incubation with Cy5 labeled ATP (**2**) and different inhibitor (VER155008) concentrations on a miniaturized microarray. Reprinted from Ref. 16[16].

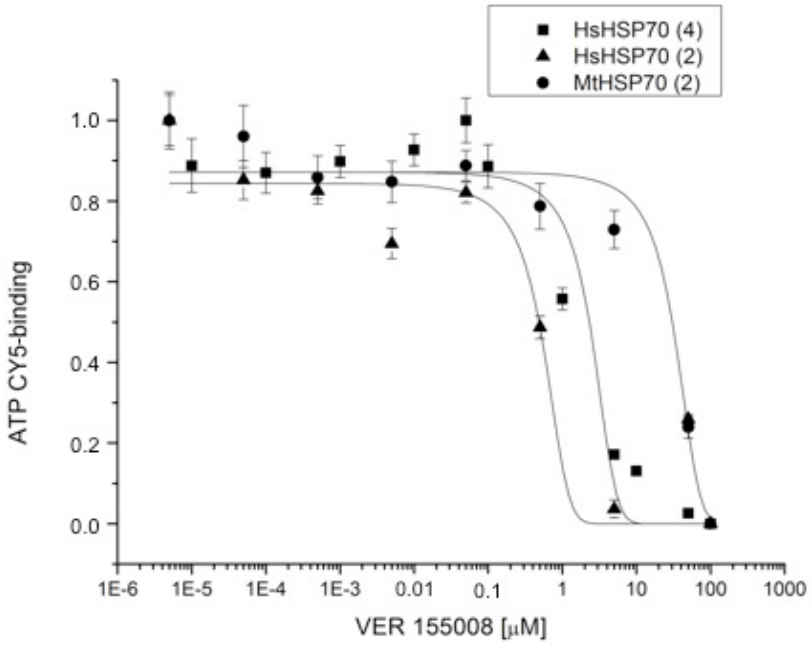


Figure 30: Dose-response curves of the ATP labels and VER155008. The inhibitor competes with the labeled ATP around the binding site. Reprinted from Ref. 16[16].

4.3.3. Examination of anti Hsp70 activity of isolated natural compounds from *Roylea cinerea*

The effect of two novel labdane diterpenoids cinereanoid C, cinereanoid D, and a new β-lactam cinerealactam E from *Roylea cinerea*, as well as six known flavonoid glycosides; rutin, isoquercetin (table 6), nicotiflorin, martynoside, undatuside A and 5´-β-D-glucopyranosyloxyjasmonic acid on the ATP binding site of heat shock proteins Hsp90 were investigated[17](Fig. 31).

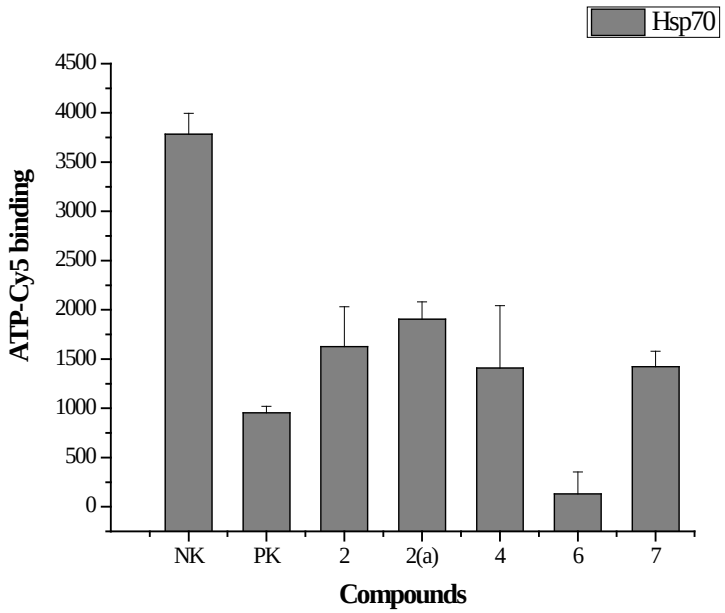


Figure 31: Human Hsp70-Cy5 binding as a function of tested compounds. Inhibitors from *Roylea cinerea* were tested at 1 mg mL^{-1} the standard inhibitor VER155008 was used at 50 mg mL^{-1}. The inhibitors cinereanoid D (2), rutin (4), nicotiflorin (6) martynoside (7) were tested at 1 mg mL^{-1}. Reprinted from Ref. 17[17].

Although the compounds new labdane diterpenoids (cinereanoid D), isoquercetin, nicotiflorin and martynoside compete for ATP from the ATP-binding site of Hsp90, they were not active on Hsp70. The compounds were tested at the concentration of 1 mg/mL and all the values are presented as an average of 10 spots (Fig. 31)[17].

4.4. Detection of the interaction between HsHsp70 and HsHsp90α

The Hsp70 has not only ATP binding property but also has further functionalities. Unknown is which affinity between Hsp90 and Hsp70 exist, although an interaction between both proteins is assumed and shown by different methods[139]. In order to evaluate the binding affinity between the two heat shock proteins 70 and 90, the HsHsp70 was spotted onto the microarray device and then was incubated with Cy5-labeled HsHsp90. The data indicated the binding between labeled HsHsp90 and spotted HsHsp70. According to the dose-responsive curve (Fig. 32), the EC_{50} value was 40 µM and the affinity was sensitive enough to detect HsHsp70 in a very small amount ~240 pg.

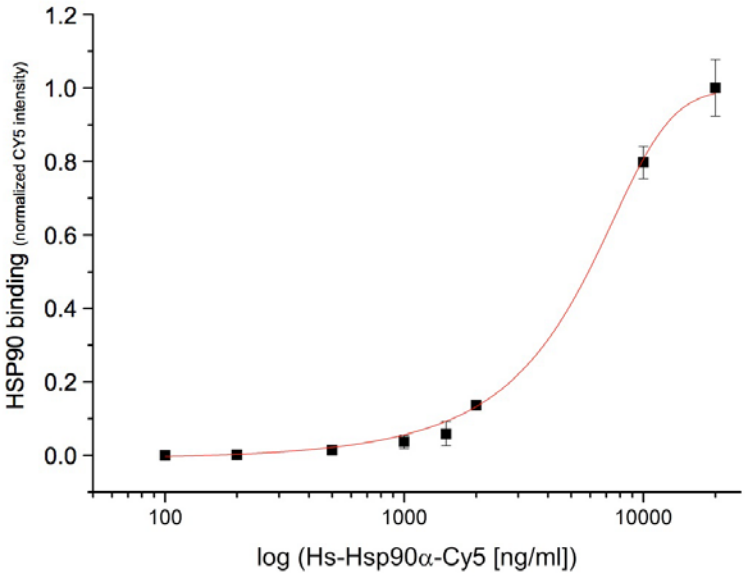


Figure 32: Dose–response curves of the normalized data fitted by a dose response function. Reprinted from Ref. 16[16,140].

4.4.1. Detection the interaction of L1CAM with Hsps and CsgA (Curli)

As shown before Hsp90/Hsp70 interaction and Hsp90 client proteins by Schax *et al.* in 2014, it is possible to visualize protein-protein binding using the microarray technique and to establish this for further strategy to select more specific compounds acting on protein-protein interaction. Following the protein–protein interacting tests, the interaction between some other proteins has been investigated. For this purpose, the five proteins (Human Hsp70, CsgA (Curli), L1CAM, Human Hsp90α, and LbHsp90) were spotted on the nitrocellulose membrane microarray slide. After an overnight incubation with the labeled Cy3-L1CAM, the binding affinity between proteins was determined. Curli are functional amyloid fibers assembled by many gram-negative bacteria as part of an extracellular matrix produced by many enteric bacteria including Escherichia and Salmonella species. Curli have an important role biofilm development in the both mentioned bacterias[141,142].

L1 cell adhesion molecule (L1CAM) is a membrane receptor with an extracellular binding site which is involved in binding with other cells or with the extracellular matrix (ECM) in the cell adhesion process. This 200-220 kDa protein plays an essential role in the development of the nervous system as well as the formation of the myelin and

synapses. It is also involved in the progression of human tumors and treatment-resistant cancers due to its function[143].

The figure 33 illustrates that Cy3-L1CAM interact with HsHsp70, Curli, unlabeled L1CAM and Hsp90 proteins. Interestingly a stronger interaction was found between Hsp70 than with the other proteins.

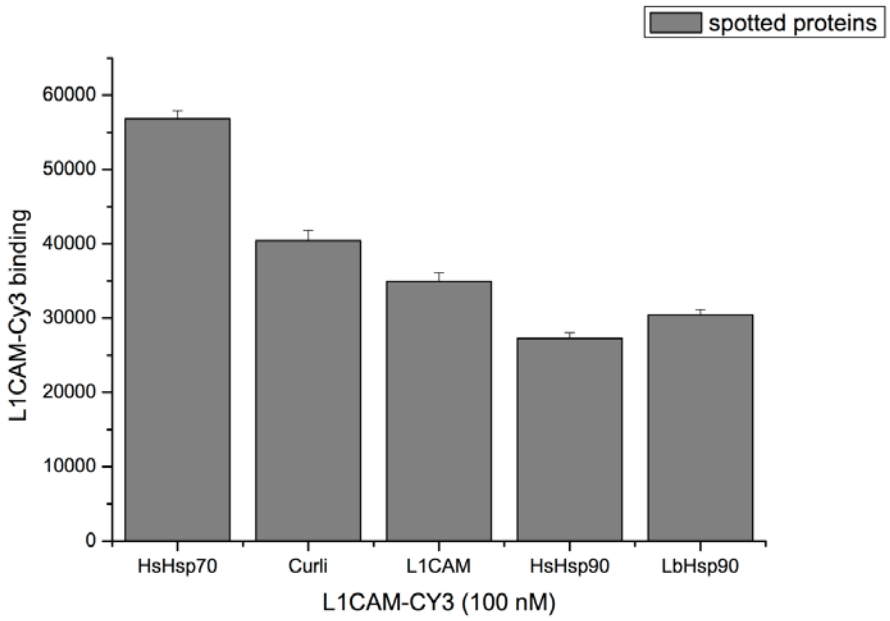


Figure 33: Bar histogram of the interaction of labeled L1CAM with HsHsp70, Curli, L1CAM unlabeled, HsHsp90 and LbHsp90. The proteins were spotted on the nitrocellulose membrane. After the incubation with labeled L1CAM (L1CAM-Cy3) the binding affinity were determined.

5. Discussion

This chapter will begin with the short summarize of the developed microarray technique, and some well-known natural products as well as the labeled ATP, which were used in the present work. Then it continues with the discussion of the results obtained from the effect of the potential compounds through the mentioned method.

The effect of various natural products and chemical compounds to their target is often unidentified. In order to perform an efficient target-oriented drug discovery, target and related tests are needed. These tests are mostly based on a binding or catalytic function, which is usually visualized by optical methods. Subsequently, the drug tests as a cell- or enzyme-based can be done. In many cases, these tests are complex, expensive and time-consuming. To optimize these obstacles, a highly miniaturized microarray-based test for proteins has been developed which can be carried out with very small amounts of material. Purified proteins are the target of the active compounds (substances), while the binding properties of the targets for the ligands and possible drugs and their optimized derivatives are tested. Targets of different proteomes such as heat shock proteins 90 (Hsp90) and Hsp70, a cellular stressed or pathogenic, were studied in term of drug susceptibility or diagnosis.

5.1. Miniaturized Protein Microarray, a flexible, powerful technique

The developed protein microarray-based assay fulfills the demand of screening a wide range of inhibitors and testing the activity of ATP-dependent proteins. As shown by Schax *et al.,* 2014 the microarrays include the immobilized full-length target proteins has a privilege that covers different kinds of fluorescence-labeled inhibitors with different binding sites rather than only ATP-binding site[14,105]. The array present as a remarkably flexible screening method which provides to investigate different target proteins and various potential inhibitors with a tiny amount of them at the same time[14,107].

The protein microarray as a novel technology, compiled data can be afflicted with high background, signal noise, and false signals[102]. In general, the fluorescence signal resulting from unspecific binding of labeled detection molecules and the signals derived from the microarray substrate may cause high background in the microarray

experiments[102,112]. Another critical point is that not absolute values are measured. Actually, all values are relative.

In the present study, the fluorescence signal of cyanine dye displayed the mean (sd) of 10 spots at each concentration. Average values of all replicates were applied for the evaluation.

The question came up, is there a competition signal with a fluorescence dye or do the compounds may influence the label? As a confirmation to this assumption, when the fluorescence dye applied on Hsp70 and Hsp90 label, the inhibitor did not work on Hsp70, but it showed binding on Hsp90. This assay could support the idea that the compounds do not influence the fluorescence labels. Furthermore, a considerable point emerged was due to the interference of label on the binding. To answer this question, the effect of labels on the binding was investigated and reported by Mohammadi *et al.*[16].

Another notable aspect of the protein microarray method that considers as a disadvantage of this technique is masking the binding sites. The proteins usually are not in the same orientation. Therefore, the masking occurs in which all the binding domains are not accessible to all the positions. Comparing to other methods, when the C-terminal is coupled by the antibody, then they all are in the same orientation. The two different regions of the protein should be recognized without masking other binding sites[144]. However one of the advantages of the nitrocellulose is preventing the proteins from the fixation. When the fixation is too strong like when the proteins are spotted on the glass, it can inhibit the protein function in a conformational way[102]. This means in this situation the 3D structure of the protein is ruptured and lose its active configuration. Moreover, by using a three-dimensional matrix, it provides higher binding capacity compared to the glass or other materials. The fixation of the protein on the nitrocellulose which is a net-like system, reveals their suitability for the protein immobilization[102,145].

To be more precise, the diffusion effect happens in the nitrocellulose membrane in the result of drop spread which makes like a doughnut shape. The reason is the diffusion of water is faster than the protein`s, so the water goes out and it concentrates in the outer ring. Thus the concentration in and outside is not the same therefore there is a ring as a doughnut form appears. This ring structure may affect the screening process. This phenomenon becomes more significant when the low concentration of the protein is under investigation. In the low signal area, this problem is inevitable. Thus the binding can not be distinguished.

This limitation may be somehow reduced by some modifications in the setting of the device for example, by shortening the distance between micro spotter tip and microarrays surface or by means of changing the voltage. It is important to keep the concentration of protein at the proper level and use the fresh and active proteins.

Consequently in order to confirm the compound effect as well as the data, comparing the data with other techniques is essential. Supporting the obtained results with the additional methods makes the assay as a sophisticated and reliable test.

Methods for analyzing the proteins and their interactions are significant in the assessment of the cellular functions as long as drug development. All binding methods follow thermodynamic principles having the values Kd and to understand whether the binding is on enthalpy or entropy driven background. For instance, Isothermal titration calorimetry (ITC) a physical method utilizes to determine the thermodynamic interaction factors in the solution which was used in the present study as well as cell-based assay[146,147].

Atomic force microscope (AFM)[148,149], crystallography[150], modeling, nuclear magnetic resonance (NMR)[150,151], fluorescence polarization, SPR[152,153], thermophoresis[154], ATP-hydrolysis test[155], luciferase refolding assay[156,157] can be applied as a further testing in order to make the array as a sophisticated investigation[151,158].

ITC is a functional approach for understanding the thermodynamic image of the binding reactions[146]. Under the isothermal conditions and by means of titrating one reactant into another one, it functions. In order to support the results and prove the data of the present study, ITC measurement, cell-based assay and modeling were applied.

Surface Plasmon Resonance (SPR) is the most powerful biosensing technology in measuring biomolecular interactions. It is an important technique in different fields due to its real-time, label-free and noninvasive feature[159]. The specific information about the interaction such as binding affinity and levels, dissociation and association rate constants as well as thermodynamic parameters can be obtained from SPR method[152]. This approach has its own weaknesses in which it is prohibitively expensive in the commercial devices and need consumable sensor chips. Additionally, the small molecules in the size of <150−180 Da are not simply be detected[159].

Another method is Microscale Thermophoresis (MST) which is a new technology that enables to analyze the interaction of proteins or small molecules in biological liquids at the microliter size. It is on the basis of the thermophoresis of molecules[154]. It is

noteworthy to mention that the thermophoresis is extremely sensitive to alterations of the size, charge along with hydration shell of the molecules[160].

The SPR technique is applied for determining the most antibody affinities and it requires the immobilization of either the antigen or the antibody on a solid surface. Although the surface-based approaches are very sensitive, due to the mass transport effects the high-affinity interactions are usually not attainable for the measurement[154,161].

In contrast, Microscale Thermophoresis is appropriate for the determination of the affinity of protein-protein as well as low molecular weight binders. It is also sensitive to alteration of the size and the charge of molecules. Moreover, it can be carried out in the biological complex solutions[154].

Generally, in order to analyze the interactions in the solution phase, Fluorescence Correlation Spectroscopy (FCS) and Isothermal Titration Calorimetry (ITC) methods are utilized. FCS is extremely sensitive fluorescence microscopy based, however, depends on a significant alteration in the diffusion constant on binding[162]. Because of this reason, it is merely suitable for a little number of interactions. By comparing to ITC method, although ITC is a label-free technique which is an advantage in this method that is not disturbed by a label, it suffers from the limitations such as a low throughput and remarkably high sample consumption[154,163].

Additionally, in ITC measurement, the compounds should be polar or soluble at higher concentrations as well. This method is the only technique can settle the enthalpic and entropic elements of the binding affinity which are correlated to the structural parameters that are utilized in the molecular design and later on drug design[163] (table 7).

Table 7: Methods of estimating the binding affinities and activities.

	Technique	Advantage	Disadvantage	Description
Binding based assays	(SPR)	• Label-free • Applied to a wide range of molecular systems • Kinetic parameters (k_{on},k_{off}) • Dirty samples possible • Less sample required • High throughput	• Prohibitively expensive, • Not suitable for small molecules in the size of <150−180 Da • Mass transfer limitation • Immobilization artifacts	• Measure binding affinity; binding levels; dissociation and association rate constants and different thermodynamic parameters
	ITC	• Label-free • Thermodynamic parameters (ΔG, ΔH, ΔS) • No immobilization	• More sample required • Lows to medium throughput	• Analyze the interactions in the solution phase
	FCS	• A little number of interactions	• Need label	• Analyze the interactions in the solution phase
	Luciferase refolding assay	• Enzyme emits light • Has a very low noise-to-signal ratio • Label-free • Protected by high levels of molecular chaperones		• Measure the enzymatic activity of molecular chaperones • Enzyme activity is thermolabile and easily measured by a luminometer • Luciferase can be targeted either to the cytoplasm or nucleus
	ATP hydrolysis test	• Measure phosphate release using colorimetric, fluorescent, or radioactive substrates	• Need label	• Different techniques for quantitating *in vitro* ATPase activity
	MST	• Detect a wide range of binding • Low sample consumption	• Need label	• Measures the interactions with high affinity as well low-affinity interaction sensitive to alteration in the size and charge of molecules
	X-ray Crystallography	• Whole 3D structure can be obtained	• Solutions and behavior of molecules in solution can not be examined.	• Structure-based ligand binding assay • Measures the changes of the diffracted X-ray beams to produce a 3D image of electron density within the molecule of interest
	NMR	• Analyze the structure of proteins and the molecular details of protein-ligand interactions • Apply for structure-based drug design	• Expensive and time consuming, • Resolving power of NMR is lower than X-ray crystallography	• Structure-based ligand binding assay • Apply for almost all membrane proteins, soluble proteins, as well as natively unstructured proteins or

5.2. The concept of choosing the natural product, a potential source of drug discovery

Natural products or secondary metabolites are a chemical and therapeutically compounds produced by different living organisms like plants, fungi, and bacteria and their derivatives that exist in the nature[75]. They present diverse structures and serve an important role as anticancer agents as well as the therapy of various illnesses[164,165].

The main group of natural products is polyketides which are biosynthesized by polyketide synthase (PKS). It is found in various microorganisms and is responsible for the production of the complex natural products[32,166,167].

This part of my thesis is allocated to how the targets were found and how the structures were selected as well as the description of their pathway.

By blocking the biosynthesis of AHBA[168] (the starting building block of ansamycin antibiotics), a knock out the mutant strain of the *Streptomycetes hygroscopicus* is available, that let to feed derivatives of AHBA. This process is termed mutasynthesis which provides the new geldanamycin derivatives[96]. In mutasynthesis method, the generation of mutants of a producer organism that are blocked in the formation of a biosynthetic building block of the end product is needed[96]. By means of mutasynthesis strategy, there would be a considerable potential for obtaining a large library of the natural products.

Franke *et al.* pointed out that by having the detailed genetic background and by means of several strategies for using biosynthetic gene clusters, novel derivatives of the Hsp inhibitors can be created[169]. These strategies include biosynthesis that relies on genetic engineering and mutasynthesis which is the combination of chemical and bio synthesis[96].

Heat shock proteins play a great role as a target in the therapy of several pathogenic illnesses namely malaria, leishmania, and neurological diseases. Although geldanamycin is the main and practical inhibitor candidate for Hsp90, the hepatotoxic side effects and low solubility in aqueous solutions, hinder the clinical application of geldanamycin[169]. Therefore in the present study new analogue such as 17-AAG which has fewer side effects and gained the clinical trials, as well as novel derivatives of Geldanamycin, were tested.

Geldanamycin (quinone and hyroquinone form) and radicicol are natural products that inhibit Hsp90 function by binding to the N terminal pocket. The hepatotoxic side effects of the geldanamycin are linked with the benzoquinone moiety which the reaction of thiol nucleophiles at the position of C19 is responsible for the toxicity of the compound. Actually, the quinone group is not necessary for binding to the ATP binding pocket of Hsp90[32,170].

By comparing reblastatin with geldanamycin, reblastatin reveals less hepatotoxic side effects. Interestingly it is only varied by saturation at C4-C5 and by benzenic chromophore instead of quinon moiety.

17-aminoallyl geldanamycin (17-AAG) which achieved the phase 2 of a clinical trial, obtained by semisynthesis from geldanamycin (quinine form). Semisynthesis is a common technique to achieve the new derivatives of complex natural products.

Hermane *et al*. reported in 2015 that cytotoxic non-quinone geldanamycin derivative **20**[97] binds to human Hsp90 with improved affinity compared to 17-AAG[14]. The fluoro derivative (table 3) presented the same inhibitory effect towards human full-length Hsp90 as well as the analogous bacterial HtpG.

Interestingly, 17-AAG does not exhibit a strong binding affinity to HtpG of *Helicobacter pylori*[14]. The reason probably based on the similarity between the HtpG of *H. pylori* and the bacterial HtpG of *Streptomyces hygroscopicus*, the geldanamycin producer which is not inhibited by its own metabolite[32].

Based on these findings, it is possible to develop selective inhibitors for different Hsps exist in diverse organisms with various therapeutic goals. Moreover, the non-quinone derivatives which carry the structural resemblance to reblastatin, are well suited for future drug developments[15].

The interesting effects of several plants on humans were reported, in which the green tea is an outstanding example among others.

Tea is a popular drink worldwide which produced from leaves of a plant called *Camellia sinensis*. In the matter of catechins the tea polyphenols, it showed cancer chemoprevention in humans[171]. Moreover, among tea catechins, “green tea” produces epigallocatechin-3-gallate (EGCG) (Fig. 34), a polyphenolic natural product which is the most abundant and active constituent of the green tea, has a marvelous effect on humans[165,172]. It targets the Hsp90 function and exhibits anti-cancer properties as Palermo *et al.*, reported[173].

Gambogic acid (GA) is another example of the natural products which isolated from gamboge a brownish to the orange dry resin of south Asian tree called *Garcinia hanburyi*[164] (Fig. 34). Gambogic acid carries out various intra and extracellular actions such as apoptosis, autophagy as well as anti-inflammatory activities[174]. Many investigations performed on the Gambogic acid by different research groups[175]. According to Kashyap *et al.* and Zhao *et al.* studies, GA can be applied as a remedial factor for dreadful diseases and it hindered early wounds and lung metastasis of *in vivo* orthotopic model of A549 cells respectively[174,176].

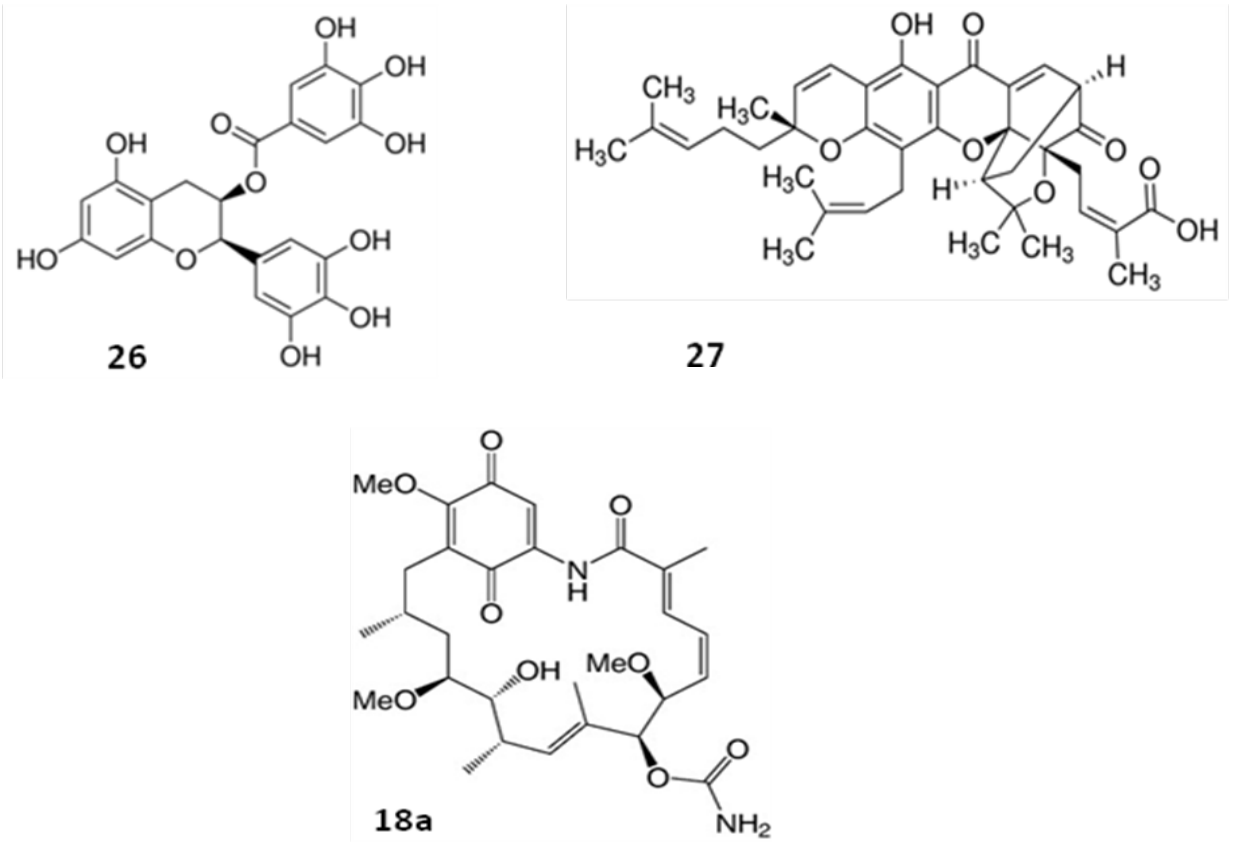


Figure 34: Structure of some natural products; **26**: epigallocatechin-3-gallate (EGCG), **27**: Gambogic acid and **18a**: Geldanamycin.

Roylea cinerea (D.Don) Baill is a genus of flowering plant belongs to the Lamiaceae family. It is indigenous to the western Himalayas of Nepal and north part of Indea[177].

The plant leaves are traditionally utilized in India order to treat diabetes, malaria and skin disorders[178] as well as febifuge[133,177]. Despite holding good potential therapeutic properties, it is not well studied and considered as drug component by the pharmaceutical companies.

Sharma *et al.* indicated in their publication that due to their phytochemical investigations, they could isolate labdane diterpenoids from the aerial parts of *R. cinerea*[179]. Furthermore Bhatt *et al.* reported in 2017 that they discovered two anti-diabetic compounds which were isolated from the aerial part of *Roylea cinerea*[177]. Following Sharma *et al.* published their new founding of the isolation and structural study of two novel labdane diterpenoids (**22, 23**) and a new β-lactam (**24**) from the aerial parts of the *Roylea cinerea*, as well as six known flavonoid glycosides. Additionally, they investigated the effects of the compounds on the displacement of the ATP binding pocket of Hsp90 as well as Hsp70[17](table 8).

The most important and well known natural product among numerous examples is geldanamycin which exhibits potential anti-cancer effects by inhibiting the Hsp90 functions through binding to the ATP binding pocket of the chaperone[180]. It is benzoquinone ansamycin which originally has been discovered in the culture of soil-living actinomycetes *Streptomycetes hygroscopicus* var. geldanus[89].

Although geldanamycin targets and inhibits the function of Hsp90, because of its toxicity side effects and poor stability it is not clinically applied[181,182]. Therefore, based upon the geldanamycin structure, many semisynthetic derivatives have been developed in order to inhibit Hsp90 activity[181].

Due to the strong side effects owing to cell toxicity in quinone based geldanamycin and its derivatives, the demand and necessity of improved Hsp90 inhibitors have been increased[169,183,184]. Kim *et al.* reported that the screening of abundant inhibitors for Hsp90 binding is the feasible way of identifying the novel compounds against this chaperone[185].

Table 8: Some well known plants and bacteria species utilized in different diseases.

Scientific name	**Chemical compound**	**Plant part used**	**Medicinal uses**
Artemisia roxburghiana		Plant extract	Skin allergy
Camellia sinensis		leaves	
Garcinia hanburyi	Gambogic acid	Fruits	
Roylea cinerea		Leaves decoction	Febrifuge
Rosa sericea Lindl.		Flower juice & Fruits	Headaches, Liver & bowel pain
Streptomycetes hygroscopicus	Geldanamycin		Anti cancer
Tripterygium regelii	Celastrol	Plant extract	Anti-diabetic,anti-inflammatory, anti cancer

There are several novel screening methods for testing the binding of the inhibitors, but the high-throughput screening (HTS) is the fastest technique among them which enable the testing of plenty of compounds in the short period of time[186].

As Hall *et al.* showed a comparison between the conventional method and high-throughput approach, he reported that the traditional method is time and cost consuming as well as labor intensive[98]. Another application of protein microarray has been already studied and reported by several research groups. Protein microarrays keep a considerable pledge to hastening the development of remedial and diagnostic biomarkers[101,187]. Recently Huang *et al.* in 2017 also indicated the role of protein microarray in identifying the biomarkers, protein expression profiling, and clinical diagnosis/prognosis, as well as environmental and food safety analysis[188].

5.3. Screening the natural product by the miniaturized protein microarray method

During an evolutionary process, numerous numbers of natural products which obtained from plant, bacteria, fungi etc were found. Strong effect of anti Hsp activity of Green tea, *Garcinia hanburyi* and other were proved. Here the effect of some other natural products against Hsp function is discussed.

5.3.1. Anti Hsp90 activity of novel natural compounds derived from aerial parts of *Roylea cinerea*

The Hsp90/Hsp70 inhibitory activity of the isolation of two new labdane diterpenoids, cinereanoid C **22**, cinereanoid D **23** and one new β-lactam compound cinerealactam E **24** along with six known compounds [rutin **25**, isoquercetin **26**, nicotiflorin, martynoside, undatuside A and 50-b-D-glucopyranosyloxyjasmonic acid] from the aerial part of *Roylea cinerea* were tested (table 6). *Roylea cinerea* is an evergreen, aromatic shrub belongs to the Lamiaceae family. The leaves of this plant were collected from Yol region (Dharamshala district of Himachal Pradesh of Western Himalayas in India)[17] (Fig. 35).

Sharma *et al.* pointed out that the structure of the nine compounds was found out by MS, IR and NMR spectroscopy. Furthermore, by single crystal X-ray diffraction, the structure of cinereanoid D (**23**) was confirmed.

According to the results, the compounds **23**, **26**, nicotiflorin and martynoside showed a significant effect against Hsp90. Since heat shock proteins are overexpressed in a wide range of human diseases such as cancers and are involved in tumor cell proliferation, metastasis etc, they considered as an important target for the novel drugs[189]. Inhibition of the Hsp can hamper Hsp-induced protein regeneration. Kirschning *et al.* reported in 2015 that they have identified some Hsp inhibitors by testing natural products as well as bio semisynthetic appoarches[32].

Shrama *et al.* pointed out in their previous phytochemical investigation in 2015 that they isolated the two new labdane diterpenoids from the extract of the aerial part of *Roylea cinerea*[179]. In the same year, Bahuguna *et al.* announced the anti-diabetic potential of

this plant in the traditional medicine, which is used in the treatment of diabetes[190]. Later on Bhatt *et al.* found out the two compounds isolated from the aerial part of *Roylea cinerea* present remarkable anti-diabetic activity in *in vitro* as well as *in vivo* models[177].

Figure 35: *Roylea cinerea* (D.Don) Baill. The picture downloaded from the website (http://globalscitechocean.com).

In this assay, the natural products isolated from the plant *Roylea cinerea* were tested by applying a target oriented screening with recombinant full-length Hsp70 and Hsp90[17]. The heat proteins were printed on the nitrocellulose membrane slide and the competitive binding of the fluorescence-labeled ATP and mentioned inhibitors to the ATP-binding site of Hsp were determined. The significant power of the miniaturized protein microarray is using low amount of proteins and inhibitors for the screening. In this assay, the concentration ranges of (0.1–1 mg/mL) in 45 µl of binding buffer were applied[17].

Due to the hard and time consuming of the isolating and producing processes of natural products, using low amount of natural products as inhibitors is highly considerable.

Although the compounds **23**, **26**, nicotiflorin and martynoside with a concentration of 1 mg/mL revealed the strongest effect on Hsp90 among other compounds, no effect could be measured at the same concentration on the ATP binding of human Hsp70.

By testing different extracts of aerial part of *R. cinerea*, Sharma *et al.* reported in their recent finding that the extraction of this plant has a potential anti-diabetic property and can decelerate the progression of diabetes in rats and also it has an strong antioxidant effects[191].

5.3.2. Fluorescence-labeled ATP and its effects on ATP binding site of Hsp

Earlier studies showed that FITC-Geldanamycin is able to bind into the pocket of Hsp90[14,94,105,192]. But fluorescence dye labeled-ATP proved that it is more stable and sensitive by comparing to FITC-Geldanamycin. Thus, based on Schax`s investigations (Schax *et al.,* 2014), all the experiment in the present study, were carried out by applying Cy3/Cy5-ATP as a label[14,94,105].

According to the previous investigations, heat shock protein 70 does not bind the ATP dye labeled at the phosphate position. By contrast, this label is applied for testing heat shock protein 90 in the microarray assays[97].

In this work, in order to have a clear sight of the nucleotide binding pockets of Hsp70/Hsp90 and its binding affinity, the potential binding position of cyanine dyes (Cy5/Cy3-labeled) ATP and the two Hsps were studied[16]. The results illustrate the fact that the position of the ATP label has a remarkable influence on the binding of Hsp70 and Hsp90.

The position of the fluorescence label ATP has a strong effect on the binding of Hsp70 and bacterial Hsp70 (MtDnak). The Hsp70 proteins do not bind fluorescence labelled ATP which the ATP is labelled on the phosphate group (**3**) whereas, Hsp90 showed the significant binding with the same label[16].

In order to test whether the coupling positions of the three Cy5 derivatives influence the binding of ATP at the Hsp nucleotide binding site, the modeling of the potential binding position of the Cy5-ATP labeled derivatives (**2**) and (**3**) on Hsp70 and 90 by use of molecular mechanics and analyzing with the SiteMap program, a program for identifying and analyzing for binding sites and for predicting target drugability, could prove the results as well[130]. The crystallographic data indicated that any managing in the phosphate position, make problem for the ATP binding of the Hsp70 pocket.

According to programme SiteMap of the predicted binding mode of ATP derivative (**2**) to Hsp70, the cyanine dye is placed outside of the nucleotide-binding pocket beside the protein surface when it attached to the amino group of the adenine ring. While the amino group is laid at the entrance of the ATP pocket pointing to the protein surface and the ATP phosphate is intensely hidden in the Hsp binding pocket. Thus, due to the situation of the buried binding site of the phosphate group labeling at the ɣ-phosphate

would need to pass the protein to the other side of it with the attached dye. The size and structure of the linker in ATP derivative 3 would let crossing the whole way to the protein surface. By analysing the size of the binding pocket showed that the tunnel connecting the phosphate binding site and this side of the protein is too narrow to let the suitable binding of the ATP-labeled dye (**3**)[16].

According to the molecular volume of the ATP with the label positions of (**3**) and (**2**) as well as the dimension of the dye Cy5, the dye is too large to fit through the tunnel. Hence it is very improbable that the ATP-labeled dye (**3**) binds to Hsp70. By comparison, the ATP-labeled dye (**2**) easily binds Hsp70, because only ATP binds to the pocket while the dye stand near the surface of the protein[16].

The result from displacement assay and data from all-atom steered molecular dynamics (SMD) simulations in water presented here confirm the suggestion that the fluorescence ATP label on the adenine (purine) ring (**2, 4**) does not hamper the ATP binding on Hsp70 and Dnak while it hinder the binding to Hsp90. The fluorescence labeled-ATP with the position on the phosphate (**3**) is used for testing Hsp90 in microarray system. The results are confirmed by the molecular modeling and SMD stimulation, which is described in the result part[16].

Based on the result and analyzing the competition of the displacement of dye labelled ATPs (**2, 4**) with label-free ATP, it is indicated that the label influence the binding force. However, the Hsp70 proteins have a higher affinity for ATP comparing to Hsp90. The value which was identified by ITC measurement on Hsp70 with ADP, indicating that the label itself affect the affinity[16].

Moreover, by comparing EC_{50} values of the displacement of dye labeled ATP by VER155008 and the ITC measurements on the binding activities on HsHsp70 and MtHsp70, the data clearly showed that VER155008 is more potent against human HsHsp70 rather than Hsp70 from *Mycobacterium tuberculosis*[193,194]. Also the label position influences the binding affinity.

5.3.3. Direct competitive assay with novel compounds against Hsp90 from human and *Leishmania braziliensis*

The obtained results of the present investigations is focused on patho-targetomics which is an approach for the identification of new targets specific in pathogenic cells such as leishmaniasis and cancer. To target Hsps from different host cells, new geldanamycin derivatives were collected by means of a chemo-biosynthetic synthesis. Inhibitory potency was tested by binding of fluorescent-labelled ATP to Hsps, obtained from *Leishmania braziliensis* Hsp90 (LbHsp90) as well as human Hsp90α and by displacement of the inhibitors subjected to the assay. Microarray assays combined with ITC measurements revealed that the new geldanamycin derivative 17-desmethyl-18-deshydroxy-21-fluoro-reblastatin exerted the highest potency towards LbHsp90 and HsHsp90α.

Based on the previous investigations of different research groups, Hsp90 known as a target for drug development and the treatment of protozoa diseases[195]. It has been studied as a possible target for the therapy of the protozoa ailment caused by Plasmodium falciparum, Trypanosoma cruzi as well as Leishmania donovani[45,61,196–198].

The reason and the importance of using Leishmania Hsp90 among the different chaperones identified, is owing to its high gene copy numbers which result in the increased parasite protein levels up to 2-3% of the entire cell protein content[44,199].

Human heat shock protein90 (HsHsp90) and *Leishmania braziliensis* heat shock protein90 (LbHsp90) are orthologous proteins and they are varied in a few amino acid residues. Silva *et al.* analyzed the amino acid sequence of LbHSP90 and compared it with the *Leishmania major*, *Leishmania donovani* and human. They pointed out that LbHSP90 showed 63% of identity with HsHSP90. Moreover it is approximately 93% identical with the HSP90 proteins of *L. major* and *L. donovani*[195]. Their finding supports the result of the present study.

As a consequence, it was assumed that LbHsp90 should also be a target for heat shock protein inhibitors related to geldanamycin as well as reblastatin[15].

In this study, the ATP displacement assay was used as a screening tool and in some selected cases complemented the assay data with isothermal titration calorimetry (ITC) measurements[15].

First the competitive binding between Cy3-ATP and unlabeled ATP to the human Hsp90 (HsHsp90) and LbHsp90 from *Leishmania braziliensis* was investigated. This competition for the ATP binding pocket was observed and measured by the intensity of the fluorescence signal. When the dye-labeled ATP is bound, this leads a high intensity of fluorescence signal whereas when unlabeled ATP binds to the binding site of the proteins, the reduction in the fluorescence signal is exhibited. By means of miniaturized protein microarray technique, the inhibitory activities of novel reblastatin derivatives and conventional inhibitors against HsHsp90 and LbHsp90 were tested. Schax *et* al., and Hermane *et al.,* reported that they assayed Hsp90 inhibitory properties of some compounds with the same approach[14,97,107].

The ATP binding pocket of Hsp was strobed by the huge inhibitory library against Hsp90. Due to this concept, the binding activities of the human Hsp90 (HsHsp90) and its orthologous protein LbHsp90 from *Leishmania braziliensis* were tested by means of the developed microarray system[14,97]. According to the result (Fig. 11), these two proteins differ in a few amino acids and in their hydropathic manner. LbHsp90 shows more hydrophobicity comparing with human Hsp90α in the region of amino acids 50 - 75; the mentioned region is embedded in N-terminal domain profoundly. For this reason, the idea rose that LbHsp90 should also be a target of geldanamycin and reblastatin which are heat shock protein inhibitors and even small differences in hydrophobicity can affect the binding of geldanamycin derivatives[32].

Although the present results confirmed that the 17-AAG, the benzoquinone geldanamycin derivative hinder LbHsp90 as well as human Hsp90, Eichner *et al.* and Schax *et al.* demonstrated that the compound barely inhibit the bacterial HtpG in the same manner[14,94].

In this study as mentioned before, the inhibitory activities of large library of natural products including new reblastatin derivatives with substitution of alkyno, amino and fluoro against HsHsp90 and LbHsp90 were investigated. Among these compounds, the fluorinated compound (**11**) inhibits the two Hsps strongly[15]. Also due the low hepatotoxic side effects of this non-benzoquinone fluorogeldanamycin, it can be considered as appropriate example for clinical trials, which was reported by Hermane *et al.* in 2015[97].

Various experiments were carried out in the present study, for the purpose of detecting the competitive activities by EC_{50} values and all the results are supported by previous reports[94,132,183,184,186,200–202]. The novel geldanamycin derivatives were tested showed the

inhibitory properties towards HsHsp90 and LbHsp90 and their dose-response curves of normalized data fitted by a dose response function were illustrated in the graphs obviously present a higher affinity to the ATP binding pocket comparing to ATP itself. The Z-factor was determined for each assay in order to validate the quality of the microarray tests. The Z-factor in this work was evaluated according to the Z-factor calculation published by Zhang *et al.*[106]. Since the measured Z-factor for all inhibitors fit into the range of $0.5 < Z < 1.0$, it confirmed the present assay as a powerful and trustworthy investigation.

By evaluating the ATP binding pocket the HsHsp90, Hermane *et al.* presented the location of K112 (lysine) in HsHsp90α, as a key factor in stabilization of the benzoquinon ring. The equivalent position in LbHsp90 is substituted by R112 (arginine). This exchange may affect the binding of the potential inhibitors as well as new geldanamycin derivatives[97].

It has reported that several geldanamycins derivatives exhibit antiproliferative activity against different human tumor cell lines[94,132]. Due to the structure of the novel reblastatin derivatives, they showed different range antiproliferative effects on cancer cell lines tested. For example, aminoreblastatin derivative 12 showed potency against the epidermoid carcinoma (A-431). Because of the existence of the free amino group in the derivatives 12 and 13 which is prone to form ammonium salts, the transmembrane transport can be hindered hence the activity is reduced. The non-quinone macrolactams like reblastatin such as derivatives 5, 10 and 16, the hydroxyl group at C17 results in some or even complete loss of cytotoxicity effects comparing to other derivatives 6, 7, 8 and 11. By comparing reblastatin 21 with compound 17, the methoxy group at C17 caused improved activity. It demonstrates that a hydrogen donor group at C17 as well as a nitrogen function at C18 greatly decreases the antiproliferative activity[15].

5.3.4. The protein activity in the form of protein-protein interaction

One of the outstanding advantages of the protein microarray test system is having the potential to let the flexible adaptation to various experimental challenges.

By means of this technology, it is feasible to test nucleotide binding sites with dye labelled ATP. Furthermore, the full-length protein makes it possible to test the interaction of proteins. Here in this work, the binding affinity between the two heat

shock proteins 70 and 90 was investigated.

Schax *et al.* in 2014 and Hermane *et al.* in 2015 reported that identifying the novel compounds which affect the specific Hsp90/Hsp70, Hsp90/client interaction by predictive design and other methods will be achievable in the future[97,105].

The present study has been performed for the first time for the reconstitution of Hsp90/Hsp70 complex on the protein microarray test system in order to get the affinity values[15]. According to earlier assays based on mass spectrometry which announced by Ebong *et al.,* and later on with Schmidt *et al.*, the complexes of co-chaperone Hop, Hsp70 and Hsp90 are heterogen[203,204]. Their report is in contrast to the present study that shows an affinity between Hsp90/Hsp70.

By use of different methods such as structural cryo/EM studies as well as spectrometric analysis, the complex of Hsp90/Hsp70 and together with some co-chaperones and clients were determined[26,204,205]. Moreover, Genest *et al.* pointed out in 2015 that Hsp90-DnaK formed a complex with an increased ATP hydrolysis[206].

6. Summary and conclusion

The aim of my thesis was to use the microarray technology for a broader application in systematically screening of compound derivatives and natural products against ATP-binding sites of Hsps. Although the ATP-binding sites are highly homologs, the small differences are sufficient for differences in compound affinities. Therefore several Hsp proteins were tested on the microarray platform simultaneously.

In the context of the present work, the application of miniaturized microarray-based screening method for the identification of novel drugs was investigated. Hundreds of potential drug candidates were tested whereas highly specific compounds were identified from biosemi-synthetically synthesized reblastatin derivatives acting different Hsp proteins from human, bacteria and protozoa.

It was also shown that this technique is useful to identify novel compounds from a natural compound producer like fungi and plants.

Although the significant advantage of the miniaturized protein microarray test is by using a low amount of protein numerous arrays can be prepared, it also enables flexible adaptation to various experimental challenges.

In the first project (section 4.2.2.), inhibitory potency of the new generations of reblastatin derivatives, the mutaproducts, as well as many natural products (~150 inhibitors) and derivatives was tested on the purified heat shock protein obtained from *Leishmania braziliensis* (LbHsp90) as well as HsHsp90. Results clearly indicate that the Fluoro-reblastatin derivative **11** has a significant antiproliferative activity against various cancer cell lines. In addition, it showed strong binding affinity to the ATP-binding site of the HsHsp90 and LbHsp90 respectively. By analyzing the ATP binding pocket of HsHsp90 and LbHsp90, the structural similarity between the two heat shock proteins was confirmed. They have differences in only a few amino acids. In contrast, the bacterial and human Hsp90 show more pronounced structural differences and consequently variations in the affinity to ATP and selected inhibitors.

According to the second project (section 4.3.1.), ATP binding to human Hsp70 and DnaK from *Mycobacterium tuberculosis* was tested. The method was based on the use of Cy5 dye-labeled ATPs (**2–4)** (figure 13). The results demonstrate that the position of the fluorescence label on ATP had a strong influence on the binding to Hsp70/DnaK. Moreover, when the Cy5-label is attached to the γ -phosphate group (**3)** it hampers the binding to human Hsp70 (HsHsp70) as well as on MtDnaK from *M. tuberculosis*

whereas other positions on the purine ring (**2, 4**) allow binding. This result is supported by the molecular modeling and steered molecular dynamics (SMD) simulations (figure 25). The structure of the labeled ATP which includes the length of the linker connects to cyanine dye as well as the position of the connection to the nucleobase could affect the binding.

These two projects exhibit that the parasitic heat shock proteins, as well as MtDnaK from *Mycobacterium tuberculosis*, can serve as pharmaceutical targets. This might represent a turning point for the development of specific inhibitors with broad application range towards infectious diseases and pathogens. Currently, regarding the side effects of known antibiotics as well as rapid emerge of antibiotic resistance which is the biggest threats to the global health, the urgent need of new and effective antibiotics is demanded[207,208].

In the third project of the present study (sections 4.2.3 and 4.3.3.), the effect of two new labdane diterpenoids and one new β-lactam from the aerial parts of *Roylea cinerea* on the ATP-binding of Hsp90 and Hsp70 were investigated. Some of the compounds showed strong effects on the ATP-binding of human Hsp90, while they have no effect on the Hsp70 at the certain concentration.

Another novel application of the screening system is to study protein-protein (Hsp70/90) or protein-ligand interactions (section 4.4.). This application will also be used as a screening method for finding novel inhibitors in the future.

As a conclusion, all the assays developed and performed in the present work prove the fact that miniaturized microarray systems are effective tools to investigate different proteins and various inhibitors functions in a quick, efficient and affordable way[14].

7. References

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8. Appendix

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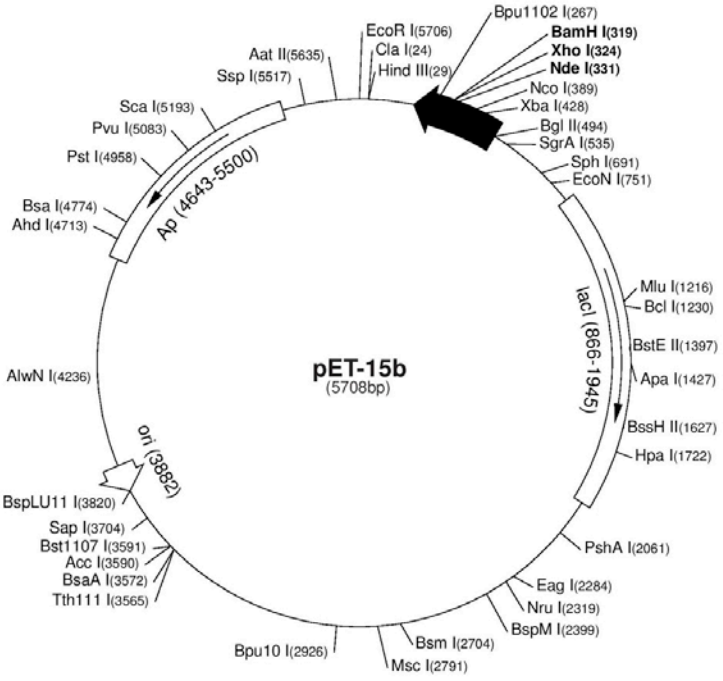
- Many thanks go to **Christen Ahlbrecht** for preparing the data of interaction test (figure 32) during her master thesis.

Hsp90α expression vector pET-15b and the sequence traits (Novagen)[108]

Vector sequence traits:
T7: T7 Promoter (463-479)
T7: Terminator (213-259)
lacO: lac Operator
RBS: Ribosome binding site
6xHis: His-Tag (362-380)
TEV: TEV-Protease



Vector pET SUMO 5643 base pair (Invitrogen Life Technologies) map and the sequence characteristics[109]

Vector sequence traits:

T7: T7 Promoter (bases 209-225)

lacO: lac Operator (bases 228-252)

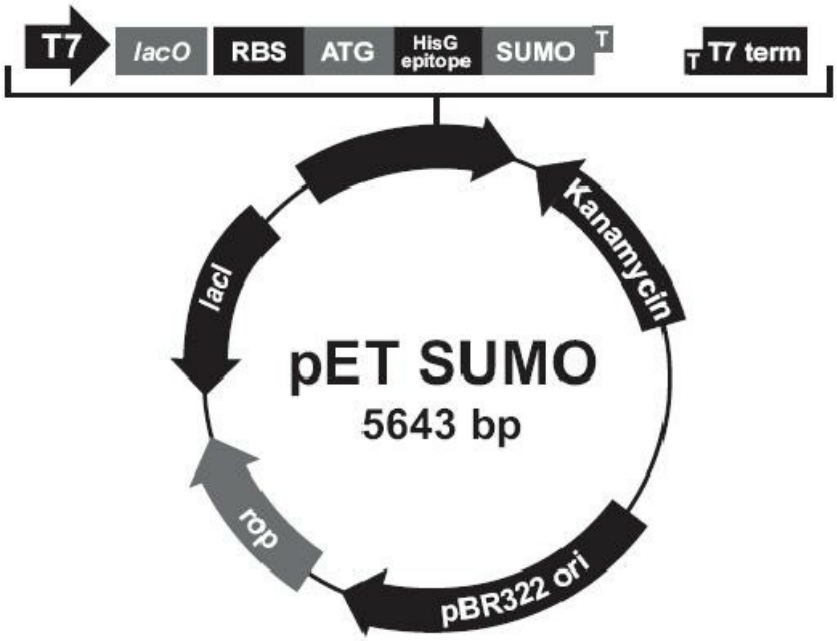
RBS: Ribosome binding site (bases 282-288)

ATG: Initiation ATG (bases 297-299)

His G epitope: His-Tag (bases 309-329)

SUMO: SUMO-Protease

T7: T7 Terminator (bases 744-872)



***Homo sapiens* heat shock protein 90 alpha family class A member 1 (HSP90AA1), transcript variant X1, mRNA**
NCBI Reference Sequence: XM_011536718.2

"MPPCSGGDGSTPPGPSLRDRDCPAQSAEYPRDRLDPRPGSPSEASSPPFLRRAPV
NWYQEKAQVFLWHLMVSGSTTLLCLWKQPFHVSAFPVTASLAFRQSQGAGQH
LYKDLQPFILLRLLMPEETQTQDQPMEEEEVETFAFQAEIAQLMSLIINTFYSNKE
IFLRELISNSSDALDKIRYESLTDPSKLDSGKELHINLIPNKQDRTLTIVDTGIGMT
KADLINNLGTIAKSGTKAFMEALQAGADISMIGQFGVGFYSAYLVAEKVTVITK
HNDDEQYAWESSAGGSFTVRTDTGEPMGRGTKVILHLKEDQTEYLEERRIKEIV
KKHSQFIGYPITLFVEKERDKEVSDDEAEEKEDKEEEKEKEEKESEDKPEIEDVG
SDEEEEKKDGDKKKKKKIKEKYIDQEELNKTKPIWTRNPDDITNEEYGEFYKSL
TNDWEDHLAVKHFSVEGQLEFRALLFVPRRAPFDLFENRKKKNNIKLYVRRVFI
MDNCEELIPEYLNFIRGVVDSEDLPLNISREMLQQSKILKVIRKNLVKKCLELFTE
LAEDKENYKKFYEQFSKNIKLGIHEDSQNRKKLSELLRYYTSASGDEMVSLKDY
CTRMKENQKHIYYITGETKDQVANSAFVERLRKHGLEVIYMIEPIDEYCVQQLK
EFEGKTLVSVTKEGLELPEDEEEKKKQEEKKTKFENLCKIMKDILEKKVEKVVV
SNRLVTSPCCIVTSTYGWTANMERIMKAQALRDNSTMGYMAAKKHLEINPDHS
IIETLRQKAEADKNDKSVKDLVILLYETALLSSGFSLEDPQTHANRIYRMIKLGL
GIDEDDPTADDTSAAVTEEMPPLEGDDDTSRMEEVD"

***Leishmania braziliensis* heat shock protein 83-1 (HSP83), partial mRNA**

"TETFAFQAEINQVMSLIINTFYSNKEIFLRELISNASDACDKIRYQSLTDPSVLGD
ETRLRIRVIPDKANKTLTVEDNGIGMTKADLVNNLGTIARSGTKAFMEALEAGG
DMSMIGQFGVGFYSAYLVADRVTVVSKNNADEAYVWESSAGGTFTIASVADS
DLKRGTRITLHLKEDQQEYLEERRVKELIKKHSEFIGYDIELLVEKTTEKEVTDE
DEEEKKEGENEEEPKVEEVKDGEEDKKKTKKVKEVTKEYEIQNKHKPLWTRDP
KDVTKEEYAAFYKAISNDWEDPAATKHFSVEGQLEFRSILFVPKRAPFDMFEPN
KKRNNIKLYVRRVFIMDNCEDLCPDWLGFVKGVVDSEDLPLNISRENLQQNKIL
KVIRKNIVKKCLDLFDELAENKEDYKQFYEQFGKNIKLGIH"

***Arabidopsis thaliana* heat shock protein (HSP90.1), partial mRNA**
NCBI Reference Sequence: NM_124642.4

"MADAETFAFQAEINQLLSLIINTFYSNKEIFLRELISNSSDALDKIRFESLTDKSK
LDGQPELFIRLVPDKSNKTLSIIDSGIGMTKADLVNNLGTIARSGTKEFMEALQA
GADVSMIGQFGVGFYSAYLVAEKVVVTTKHNDDEQYVWESQAGGSFTVTRDV
DGEPLGRGTKITLFLKDDQLEYLEERRLKDLVKKHSEFISYPIYLWTEKTTEKEIS
DDEDEDEPKKENEGEVEEVDEEKEKDGKKKKKIKEVSHEWELINKQKPIWLRK
PEEITKEEYAAFYKSLTNDWEDHLAVKHFSVEGQLEFKAILFVPKRAPFDLFDT
RKKLNNIKLYVRRVFIMDNCEELIPEYLSFVKGVVDSDDLPLNISRETLQQNKIL
KVIRKNLVKKCIEMFNEIAENKEDYTKFYEAFSKNLKLGIHEDSQNRGKIADLL
RYHSTKSGDEMTSFKDYVTRMKEGQKDIFYITGESKKAVENSPFLERLKKRGYE
VLYMVDAIDEYAVGQLKEYDGKKLVSATKEGLKLEDETEEEKKKREEKKKSFE

NLCKTIKEILGDKVEKVVVSDRIVDSPCCLVTGEYGWTANMERIMKAQALRDS
SMSGYMSSKKTMEINPDNGIMEELRKRAEADKNDKSVKDLVMLLYETALLTSG
FSLDEPNTFAARIHRMLKLGLSIDEDENVEEDGDMPELEEDAAEESKMEEVD"

***Streptomyces hygroscopicus* (Chaperone protein HtpG) (ShHtpG)**

"MPTETFEFQVEARQLLQLMIHSVYSNKDVFLRELVSNASDALDKLRLEKLWD
DSLDADVSDPHIEIDIDKDARTLTVRDNGIGMSYDEVGQLIGTIANSGTAKFLRE
LREAKDAAGEEGLIGQFGVGFYSGFMVADEVTLLTRRAGESQGTRWSSRGEGT
YTLERVDDAPQGTSVTLHLKPADPENQLHDYTSPWKIREIVKRYSDFITWPIRM
VPERSATAGESGEAPEPETLNSMKALWARPRDEVSDDEYHELYKHIAHDWRDP
LETIRLQAEGTFEYQALLFLPEHAPHDLFTRDFKRGVQLYVKRVFIMDDCEALL
PPYLRFVKGVVDAADLSLNVSREILQQDRHIEMMRRRLTKKVLSTVKEMMAK
DQERYATFWREFGTVLKEGLVTDSENRDAILAVASFASTHHDTEPTTLKSYVER
MKDGQEDIYYLTGESRQSIENSPHMEAFRDRGIEVLLLTDPVDEVWADAVGEY
EGKKLRSVAKGQIDLDAKDEDTADDEREKQTEEYAGLLGWMKEQLDEDIKEV
RLSSRLTVSPACVVSDAHDLTPALENMYRAMGQEVPRAKRILELNPDHQLVKG
LNQAYKEREDRSELAETAEVLHGLAVLAEGGQPKEPARFVKLMADRLERSL"

HSPA1A heat shock protein family A (Hsp70) member 1A [*Homo sapiens* (human)] Gene ID: 3303

"MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGD
AAKNQVALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPFQVINDGDKPKVQV
SYKGETKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATK
DAGVIAGLNVLRIINEPTAAAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDD
GIFEVKATAGDTHLGGEDFDNRLVNHFVEEFKRKHKKDISQNKRAVRRLRTAC
ERAKRTLSSSTQASLEIDSLFEGIDFYTSITRARFEELCSDLFRSTLEPVEKALRDA
KLDKAQIHDLVLVGGSTRIPKVQKLLQDFFNGRDLNKSINPDEAVAYGAAVQA
AILMGDKSENVQDLLLLDVAPLSLGLETAGGVMTALIKRNSTIPTKQTQIFTTYS
DNQPGVLIQVYEGERAMTKDNNLLGRFELSGIPPAPRGVPQIEVTFDIDANGILN
VTATDKSTGKANKITITNDKGRLSKEEIERMVQEAEKYKAEDEVQRERVSAKN
ALESYAFNMKSAVEDEGLKGKISEADKKKVLDKCQEVISWLDANTLAEKDEFE
HKRKELEQVCNPIISGLYQGAGGPGPGGFGAQGPKGGSGSGPTIEEVD"

Chaperone protein DnaK [*Mycobacterium tuberculosis* H37Rv] sequence:
NCBI Reference Sequence: NP_214864.1

"MARAVGIDLGTTNSVVSVLEGGDPVVVANSEGSRTTPSIVAFARNGEVLVGQP
AKNQAVTNVDRTVRSVKRHMGSDWSIEIDGKKYTAPEISARILMKLKRDAEAY
LGEDITDAVITTPAYFNDAQRQATKDAGQIAGLNVLRIVNEPTAAALAYGLDKG
EKEQRILVFDLGGGTFDVSLLEIGEGVVEVRATSGDNHLGGDDWDQRVVDWL
VDKFKGTSGIDLTKDKMAMQRLREAAEKAKIELSSSQSTSINLPYITVDADKNP
LFLDEQLTRAEFQRITQDLLDRTRKPFQSVIADTGISVSEIDHVVLVGGSTRMPA
VTDLVKELTGGKEPNKGVNPDEVVAVGAALQAGVLKGEVKDVLLLDVTPLSL

GIETKGGVMTRLIERNTTIPTKRSETFTTADDNQPSVQIQVYQGEREIAAHNKLL
GSFELTGIPPAPRGIPQIEVTFDIDANGIVHVTAKDKGTGKENTIRIQEGSGLSKED
IDRMIKDAEAHAEEDRKRREEADVRNQAETLVYQTEKFVKEQREAEGGSKVPE
DTLNKVDAAVAEAKAALGGSDISAIKSAMEKLGQESQALGQAIYEAAQAASQA
TGAAHPGGEPGGAHPGSADDVVDAEVVDDGREAK"

Table S1: List of fluorescence labeled ATP used in the experiment. Purchased from Jena Bioscience GmbH, Jena, Germany)

Catalogue Nr.		**concentration**	**Volume**
1	NU-805-CY3	1mM	40µl
2	NU-805-CY5	1mM	160 µl
3	a) NU-807-CY5	1mM	40 µl
	b) NU-807-CY5	1mM	40 µl
4	NU-814-CY5	1mM	120 µl
5	NU-833-CY3	1mM	40 µl

Table S2: List of some inhibitors used in the present thesis.

position	inhibitor	position	inhibitor	position	inhibitor
1	17AAG	26	Novobiocin	51	HK571
2	SEB44	27	Radicicol	52	HK571d
3	SEB13	28	Celastrol	53	HK568F1b
4	SEB14	29	Proansamitocin	54	HK592
5	SEB01	30	Pyrronazol	55	HK556b
6	SEB07	31	Vioprolid A	56	HK556a
7	SEC17	32	Socein		
8	SE44	33	Meltepolid A		
9	SE238	34	Kulkenon		
10	SeD51	35	Noricumazol A		
11	SED17	36	Argyrin		
12	SEB27	37	Haprolid A		
13	SEB43	38	Crocapeptin A		
14	SEB45	39	Carolaction A		
15	SEE17	40	Antalid A		
16	SEC42	41	Antalan A		
17	SEB32	42	Ambraticin VS3		
18	SEC43	43	PentacaronsSure		
19	SEB51	44	Sulfangolid C		
20	SEF17	45	Leupyrin A		
21	SEI17	46	Tichunal		
22	SED42	47	Noricumazol C		
23	SED239	48	Atropisomer A (107a)		
24	SEJ17	49	HK568j2		
25	SEB23	50	HK581F10		

Table S3: List of inhibitors were used in the experiments

Compound	**Concentration (stock)**
RS 337	2mg/ml
RS IQ	2mg/ml
RS 218	2mg/ml
RS 234	2mg/ml
RS RU	2mg/ml
RS - 5-EtOAc	2mg/ml
RS - 160	2mg/ml
RS 49	2mg/ml
RS -75L	2mg/ml
RS B4-110	2mg/ml
Amlexanox	50 µM
ND1	4,5 µM
ND2	23,67 µM
ND3	21, 932 µM
ND4	13,148 µM
ND5	12,396 µM
ND6	8,582 µM
ND7	9,645 µM
ND8	7,807 µM
ND9	5,511 µM
ND10	7,992 µM
ND11	19,536 µM
ND12	17,760 µM
Hri001 (Rickiol A)	2,34 mM
Hri003 (Rickenyl A)	2,62 mM
Hri004 (Oxodehydrodihydrobotrydial)	4.35
Hri011 (Rickitin A)	3 mM
Hri013 (3-Hydroxy-10-oxodehydrodihydrobotrydial)	4,1 mM
Hri017 (10-Oxodihydrobotry-1(9),4(5)-diendial)	4,3 mM
Hri018 (Hypoxylan A)	4,4 mM
Hri020 (Rickenyl C)	2,62 mM
Hri021 (13-hydroxysilphiperfol-6-en)	4,4 mM

Publications

1. Sharma, R., **Mohammadi-Ostad-Kalayeh, S**., Stahl, F., Zeilinger, C., Dräger, G., Kirschning, A., Ravikumar, P. C., (2017) Two new labdane diterpenoids and one new β-lactam from the aerial parts of *Roylea cinerea*. Phytochem. Lett. 19, 101–107. (DOI:10.1016/j.phytol.2016.12.013)

2. **Mohammadi-Ostad-Kalayeh, S**., Hrupins, V., Helmsen, S., Ahlbrecht, C., Stahl, F., Scheper, T., Preller, M., Surup, F., Stadler, M., Kirschning, A., Zeilinger, C. (2017) Development of a microarray-based assay for efficient testing of new HSP70/DnaK inhibitors. Biorg. Med. Chem. 25, 6345-6352. (DOI: 10.1016/j.bmc.2017.10.003)

3. **Mohammadi-Ostad-Kalayeh, S.**, Stahl, F., Scheper, T., Kock, K., Herrmann, C., Batista, F. A. H., Borges, J. C., Sasse, F., Eichner, S., Ongouta, J., Zeilinger, C., Kirschning, A. (2018) Heat shock proteins revisited using a mutasynthetically generated reblastatin library for comparing the inhibition of human with Leishmania Hsp90s. ChemBioChem. 19, 1–14. (DOI:10.1002/cbic.201700616)

4. Yue, Q., Bothe, S., **Mohammadi-Ostad-Kalayeh, S**., Warnecke, A., Scheper, T., Zeilinger, C. (2018) Adhesiomics - testing protein-protein interaction of cell adhesion proteins on microarrays. Adv Biotech & Micro 10(3). (DOI: 10.19080/AIBM.2018.10.555790)

5. **Mohamadi-Ostad-Kalayeh, S**., Stahl, F., Walter, J., Scheper, T., Kirschning, A., Zeilinger, C. Identification of novel drug compound targeting AtHsp83 on protein microarray. (under preparation)

Curriculum vitae

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Name: Sona Mohammadi Ostad Kalayeh
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Education

2014 – 2018	Ph.D. study at Institute of Biophysics and Center of Biomolecular Drug Research (BMWZ), Leibniz University of Hannover
2012 - 2014	Research Assistant, Faculty of Natural Sciences, Leibniz University of Hannover
2006 - 2009	M. Sc. in Agricultural Engineering, Azad University of Tehran (Science and Research Branch), Iran
2001 - 2006	B.Sc. of Agricultural Engineering, Gorgan University of Agricultural Sciences and Natural Resources, Iran

Internship

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