Technische Universität München
Klinik und Poliklinik für Innere Medizin III
Hämatologie und Onkologie

Identification of point mutation as a mechanism underlying HSP90 inhibitor resistance

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Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Medizin genehmigten Dissertation.

Vorsitzender: Prof. Dr. Ernst J. Rummeny

Prüfer der Dissertation:
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Die Dissertation wurde am 21.03.2019 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 04.12.2019 angenommen.

Selbstständigkeitserklärung

Diese Arbeit wurde vom 05.12.2011 bis zum 22.12.2013 an der

im Forschungslabor für Hämatologie und Onkologie der III. Medizinischen Klinik am Klinikum rechts der Isar

ausgearbeitet.

Diese Arbeit wurde von der deutschen Krebshilfe gefördert.

Betreuer: Prof. Dr. med. Justus G. Duyster
Dr. Rama Krishna Kancha

Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen benutzt habe. Alle Stellen, die wörtlich oder sinngemäß aus Quellen entnommen sind, habe ich als solche gekennzeichnet.

München, ……

Margarete Greither

Dedication

Meinen Eltern.

Ich danke der deutschen Krebshilfe, ohne die dieses Projekt nicht möglich gewesen wäre. Ebenso möchte ich mich bei Dr. Rama Krishna Kancha, Prof. Justus Duyster und Prof. Philipp Jost für die wissenschaftliche Betreuung bedanken.

Index

1. List of Abbreviations

µg	10^{-6} Gram
µl	10^{-6} Litre
µM	10^{-6} Mole
17-AAG	17-N-allylamino-17-demethoxygeldanamycin
17-DMAG	17-Dimethylaminoethylamino-17-demethoxygeldanamycin
bp	base pair
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BCR-ABL	breakpoint cluster region- abelson murine leukemia viral oncogene homolog 1
cDNA	complementary DNA
c-KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue
DNA	Desoxyribonucleic acid
dNTPs	2'-Deoxynucleoside-5'-triphosphate
ERBB2/Her2	v-erb-b2 erythroblastic leukemia viral oncogene homologue 2
EGFR	epidermal growth factor receptor
FCS	fetal calf serum
GA	Geldanamycin
GRB2	Growth factor receptor-bound protein 2
GRP4	G-protein coupled receptor 4
HSF	heat shock factor
HSP90	heat shock protein
IL3	interleukin-3
kDa	kilo Dalton

MAPK	mitogen activated kinase-like protein
MET	mesenchymal-epithelial transition factor
mM	10^{-3} Mole
NMP-ALK	nucleophosmin-anaplastic lymphoma kinase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
RAF	rapidly accelerated fibrosarcoma
RALT	receptor-associated late transducer
RAS	rat sarcoma viral oncogene homolog
SDS	sodium dodecyl sulfate
SRC	sarcoma
TNF	tumor necrosis factor
TRAP	TNF receptor associated protein 1
VEGFR1/2	vascular endothelial growth factor receptor 1/2
WT	wild type

2. Abstract

Background: A significant number of cancers are characterized by an over-expression of wild type or mutated oncoproteins, which are dependent on the HSP90 (Heat shock protein 90) chaperone for their stability. Thus, HSP90 has become an important therapeutic target in a variety of cancers. Initial clinical studies showed promising results with the use of HSP90 inhibitors in malignant melanoma, breast cancer and ovarian carcinoma. Even though the use of kinase inhibitors as part of targeted therapy has shown substantial success in multiple cancers, the development of drug resistance has become a significant clinical challenge. One of the most frequent causes underlying the emergence of drug resistance in patients treated with targeted drugs is the development of secondary mutations, which abrogate the binding of kinase inhibitors to their intended targets. We have thus hypothesized that a similar mechanism of drug resistance may operate upon treatment with HSP90 inhibitors and as a result create major problems in clinical use of these substances.

Methodology/Principal Findings: Using a cell-based chemical mutagenesis screen we were able to identify a novel mutation in the N-terminal domain of the HSP90 that causes resistance to HSP90 inhibitors. In addition to the mutation, we have identified, we researched previously reported HSP90 mutations that were known to cause drug resistance. We then established stable cell-lines that co-expressed both the oncoprotein ERBB2 and HSP90 wildtype or one of the drug-resistant HSP90 mutants. In an immunoprecipitation assay there could be shown that all the HSP90 mutants interact with the client protein ERBB2. In a FACS-controlled competition assay there was no survival benefit in absence of inhibitors for the cells transfected with the HSP90 mutations compared to the cells transfected with the wildtype HSP90 or the parental cellline. The HSP90 inhibitor resistance caused by the F133V mutant was further confirmed by western blotting using ERBB2 as a read-out for interaction.

Conclusion/Significance: We were able to identify and describe a novel drug-resistant mutation F133V in the ATP-binding-pocket of the house-keeping protein HSP90. The disruption of HSP90-F133V and the HSP90 inhibitors Geldanamycin/17-AAG/17-DMAG was confirmed by protein expression analysis, proving the point mutation to be responsible for the inhibitor resistance on a biochemical level. As currently phase-II-trials for HSP90 inhibitors are being investigated and hence the molecular treatment is moving into clinical practice, secondary drug resistance due to

mutations may also develop in patients treated with Geldanamycin-analogues. Thus, there is a major interest to investigate second generation HSP90 inhibitors with different mechanism of action to explore the possibility of overcoming drug resistance in vitro and in vivo. The fact that no significant difference in survival of murine cells carrying exogenous HSP90 wildtype or mutants in the absence of the inhibitors could be shown, poses a number of challenges. Endogenous HSP90, which represents up to 1-2% of protein in functional cells, might interfere with effects of HSP90 overexpression and obliterate growth differences between mutant and wildtype chaperones. This question, the possible impact of HSP90 Mutation F133V on a cellular level and the investigation of second line inhibitors are interesting considerations for investigations to come.

3. Introduction

Despite the tremendous achievements in understanding the pathogenesis and treatment of cancer, the efficacy of standard chemotherapeutics in patients suffering from solid tumors remains unsatisfactory.[90] Since the understanding and knowledge of molecular events has been increasing at a rapid pace, the focus in research has now shifted to targeted therapies.[35] Currently, the development of targeted therapeutics against a variety of malignant neoplasms is intensely pursued. However, the development of secondary drug resistance upon targeted treatment, due to point mutations or protein overexpression, is reported in multiple cancer types. Thus, there is an urgent need to look for alternate strategies that might overcome secondary drug resistance and improve the treatment outcome.

3.1. ERBB2/HER2: An oncogene underlying breast cancer incidence

Breast cancer is a malignant lesion of breast tissue with either a ductal (85-90%) or lobular (10-15%) phenotype. One in every ten females is affected by breast cancer during her lifetime, making it the most common cancer among females. In addition to conventional therapies like surgery, chemotherapy and radiation, there is the option to treat breast cancer using hormonal therapy, if the tumor expresses estrogen and/or androgen receptors. Furthermore, novel strategies like immunotherapy - for instance antibody treatment against specific and overexpressed targets are evolving. One of these targets is the erythroblastic oncogene B 2, ERBB2. Application of ERBB2 antibodies is indicated in 25-30 % of breast malignances, in which ERBB2 overexpression is observed. The increased expression of ERBB2 is associated with high aggressiveness and bad prognosis of breast cancers.[17] In addition, ERBB2 overexpression is associated with gastric cancer, ovarian cancer and endometrial cancer. The ERBB2 proto-oncogene belongs to the family of the Epidermal Growth Factor Receptor (EGFR), therefore also known by HER2/neu or HER2, the human epidermal growth factor receptor 2. [16, 55, 80]

The ERBB family consists of the receptor tyrosine kinases EGFR, ERBB2, ERBB3 and ERBB4. The structure of ERBBs is comprised of an intra cellular kinase domain, a transmembrane domain and an extra cellular ligand-binding domain. It is in the extracellular space where the ERBBs dimerize. As a result of the dimerization, auto- and cross-phosphorylation in the intracellular kinase domain takes place, which promotes various physiological effects in downstream signaling.[38, 62] These effects

include cell cycle control, cell survival, apoptosis, proliferation and angiogenesis.[11] Furthermore, gene expression is altered as a result of ERBB receptor activity.[63] ERBB2 plays an important role in cell proliferation via the Ras-Raf-MAPK pathway and in apoptosis inhibition via PI3K-AKT pathway.[46, 62]

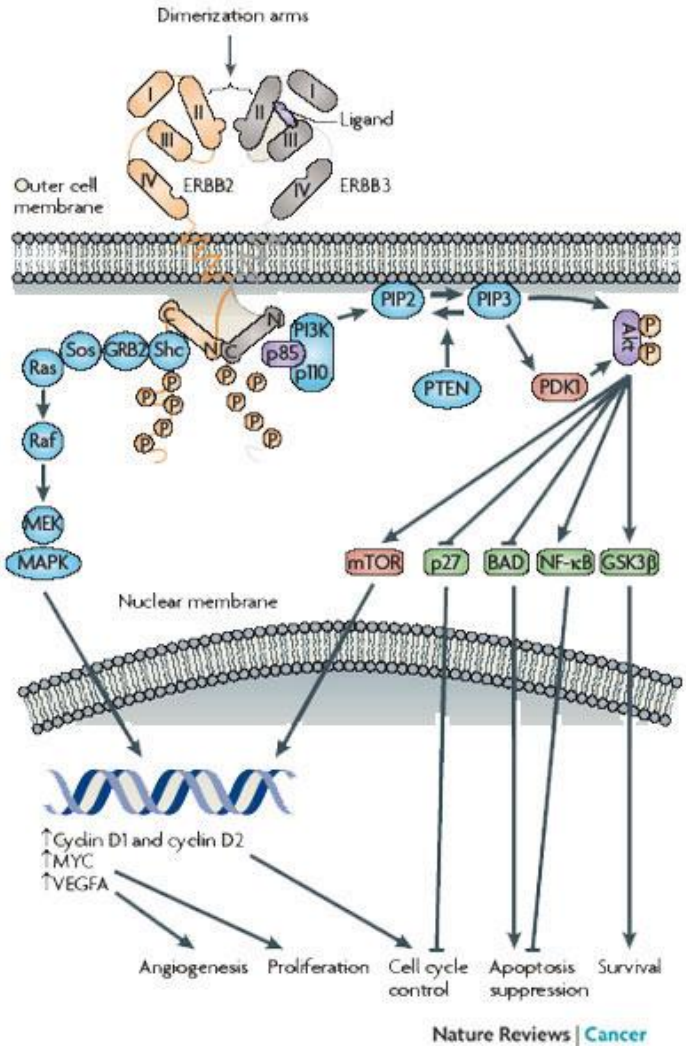


Figure 1: ERBB2 Downstream signaling[11]

3.1.1. Targeted therapeutics against ERBB2 kinase

Targeted therapies have revolutionized the face of modern cancer treatment. As conventional chemotherapy or radiation just strike fast dividing cells in general, personalized drugs are designed to target specific proteins inside and outside of the cell. Targeted agents interfere directly with signaling in the cell (e.g. Inhibitors) or aim for receptors on cells (e.g. Antibodies). Ideally molecular targeted therapy is more effective in cancer treatment and causes fewer side effects because it will - in theory -

damage exclusively tumor cells by mechanisms like interfering with tumor growth, angiogenesis or apoptosis.[20, 39] ERBB2 has been one of the first targets for successful molecular treatment.[27] Chemotherapeutics that specifically target the ERBB2 kinase - for instance Trastuzumab and Lapatinib - are approved and have been successfully used to treat ERBB2 positive breast cancer. [18, 59] Trastuzumab (Herceptin©) is a humanized monoclonal antibody which interacts with the extracellular domain of the ERBB2 receptor. In the case of ERBB2 overexpression in breast cancer patients, treatment with Trastuzumab can not only prolong survival in metastatic breast cancer (33 vs 26 months), but has also approval in Europe to be used as adjuvant treatment after surgery.[52] Studies have shown that patients had no severe side effects from treatment apart from having a higher risk of cardiotoxicity (2.45 times higher in the Trastuzumab group).[92, 94] The interaction of Trastuzumab with an ERBB2 positive cell is shown in Figure 2.

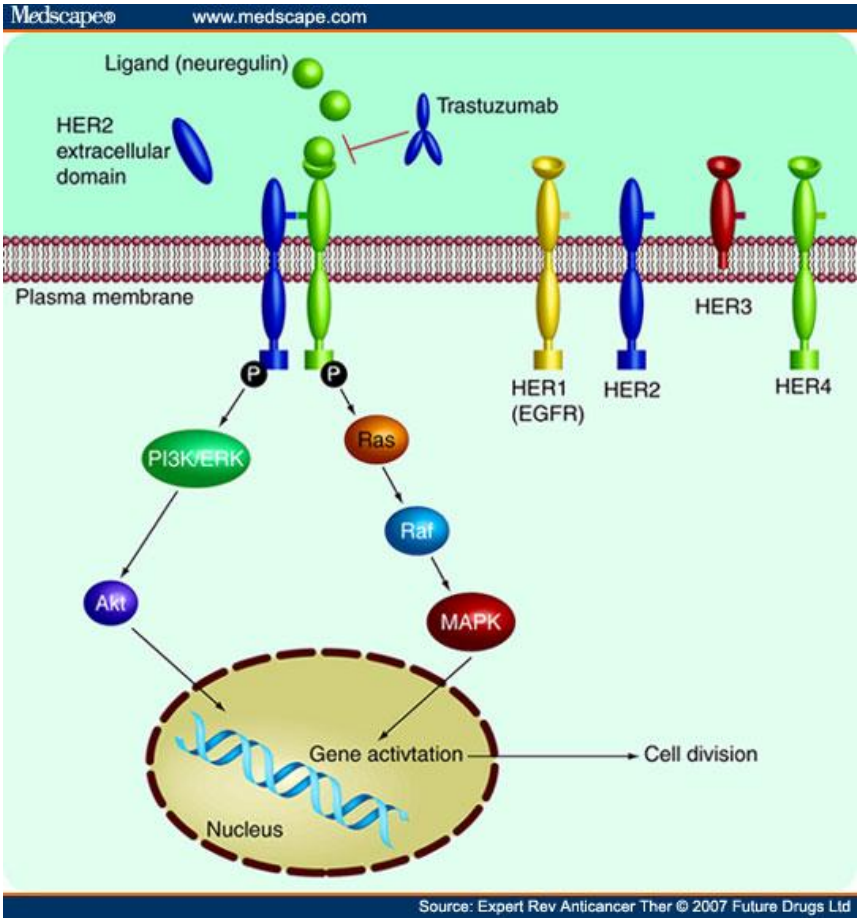


Figure 2: Interaction of Trastuzumab with ERBB2[65]

Trastuzumab blocks the signaling pathway of the ERBB2 receptor, which normally leads to Gene activation and cell division. HER2 as a preferred dimerization agent for the other HERs is shown to activate an intracellular cascade that will trigger proliferation and cell survival.

Lapatinib (Tykerb©) is a dual EGFR/ERBB2 kinase inhibitor that causes cell death in cancer cells which express activated ERBB2.[60] It is approved for the treatment of advanced or metastatic breast cancer either alone or in combination with other chemotherapeutics.[4] It is likely that Lapatinib could also bring benefit to patients with cerebral metastasis as it can cross the blood brain barrier due to its small molecular size. [79] Also a Meta-Analysis implies a benefit of chemotherapy plus dual use of HER2 blockade (Lapatinib plus Trastuzumab) in (neo)adjuvant treatment of breast cancer.[25]

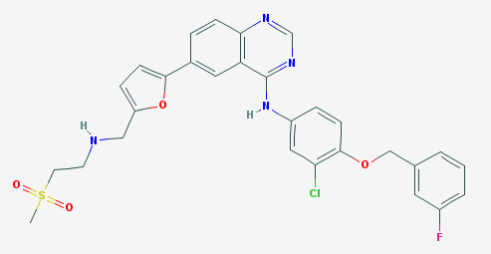


Figure 3 Lapatinib[3]

3.1.2. ERBB2 is a HSP90 client

Xu *et al.* have shown that ERBB2 stability is sensitive to HSP90 inhibition; In their studies treatment with the HSP90 inhibitor Geldanamycin leads to the disruption of the HSP90-ERBB2 interaction and consequently ERBB2 is degraded. To be more specific, Xu *et al.* were able to show, that Geldanamycin treatment only was effective in cells expressing the intracellular HSP90-binding kinase region of ERBB2. This emphasizes the unique connection of HSP90 and his client via stabilization of it through the kinase domain of ERBB2.[104]

3.2. The structure and function of HSP90

Heat shock proteins (HSPs) function as chaperones that help other proteins to fold properly into their mature/native functionally-relevant structure. Thus, HSPs play a very important role both in prevention of the aggregation of misfolded proteins and in directing damaged proteins to degradation. HSPs are able to refold and chaperone proteins, which were denatured by the cellular stress.[71] In addition, HSPs play a significant role in intracellular transport and key signaling pathways.[71] HSPs can be classified into three families based on their molecular mass in kilodalton: HSP60, HSP70 and HSP90.[71] HSP90 is the most remarkable of these proteins, as it constitutes

up to 1-2% of all cellular proteins.[22] HSP90 plays such an essential role in cell physiology that embryonic deletion of it results in a lethal phenotype. Furthermore, there are no known polymorphisms in HSP90 that could be associated with human disease.[10] The role of HSP90 as a chaperone is particularly executed when the cell is exposed to stress, for instance oxygen deprivation or heat shock.[71] In such scenarios, HSP90 is overexpressed to a total percentage of 3-5% of the cellular proteome.[102] Certain transcription factors called Heat Shock Factors (HSFs) activate the overexpression of HSP90.[87] Interestingly, HSFs are also overexpressed upon therapeutic HSP90 inhibition; targeting HSFs may thus enhance the efficacy of HSP90 inhibitors.[77] HSP90 also interacts with a variety of co-chaperones, which have very different functions such as assisting client protein interaction, folding processes or metabolic actions of HSP90.[109] Important co-chaperones of HSP90 are: Aha1[54], p23[7] and Hop[69]. While Aha1 activates ATP Hydrolysis[54], p23 stabilizes the closed HSP90 conformation[7]. Hop is able to bind both HSP70 and HSP90 and link the two chaperones and plays a role in the ATP metabolism of HSP90.[69, 105]

3.2.1. Isoforms

Interestingly, there are several different Isoforms of HSP90. In 2005 Bin Chen *et al.* classified HSP90 those isoforms according to their sub-cellular location: the cytosol, the endoplasmic reticulum and the mitochondria. The following table (Figure 4) depicts five functional genes that code for various HSP90 isoforms. Additionally, there are 12 HSP90 pseudo genes which are not expressed as proteins.[21] The major cytosolic HSP90 isoforms are HSP90α (genetically HSP90AA1 and HSP90AA2), which are inducible by high temperature and HSP90β (genetically HSP90AB1), which is more expressed constitutively.[22] HSP90AA1 and HSP90AA2 constitute of the same amino acid sequence, except for an N-terminal extension of HSP90AA1.[2] GRP-94, also known as Endoplasmin (genetically HSP90B), located in the Endoplasmic reticulum, is induced by lack of glucose to the cell.[22] TRAP1 is the mitochondrial HSP90 homologue, that works as an antiapoptotic protein in correlation to oxidative stress.[9] In addition, another isoform of HSP90 was identified and named as HSP90N.[37] As this observation could not be reproduced, Zurawska *et al.* proposed that HSP90N was created as an artifact of a cDNA synthesis or that it is a chimeric protein.[110] For our studies the constitutively expressed murine HSP90β was used.

Family	Subfamily	Genes	Location	Protein
HSP90A	HSP90AA	HSP90AA1	cytosolic	HSP90α
		HSP90AA2		
	HSP90AB	HSP90AB1		HSP90β
HSP90B		HSP90B1	Endoplasmic reticulum	GRP-94/ Endoplasmin
TRAP		TRAP1	mitochondrial	TNF Receptor Associated Protein 1

Figure 4: Table of HSP90 Isoforms

3.2.2. Structure

As observed with any cytosolic protein, HSP90 is non-polar inside and polar outside. HSP90 has three functional domains: An N-terminal ATP-binding domain (N-domain of about 25 kDa), a C-terminal domain (C-domain of about 10 kDa), that is responsible for dimerization and a linking middle domain (M-domain of about 35 kDa).[8, 88] The amino-terminal domain (N-domain) not only binds ATP, but also binds most HSP90 inhibitors such as Geldanamycin (See 2.2.5). Two layers of α-helices and β-sheets form a wide pocket with a hydrophobic core where Geldanamycin binds. Interestingly, this ATP-binding pocket has two conformations: open and closed. (Figure5)[88] As reported in earlier studies, Geldanamycin binding prevents ATP binding and as a consequence inhibits HSP90 from chaperoning its clients.[68] The carboxyl-terminal domain is responsible for the dimerization of two HSP90 molecules. The middle domain links both the N-terminal domain and the C-terminal domain by a flexible “charged linker”. However, the structure and chaperoning function of HSP90 machinery is not fully understood.[67]

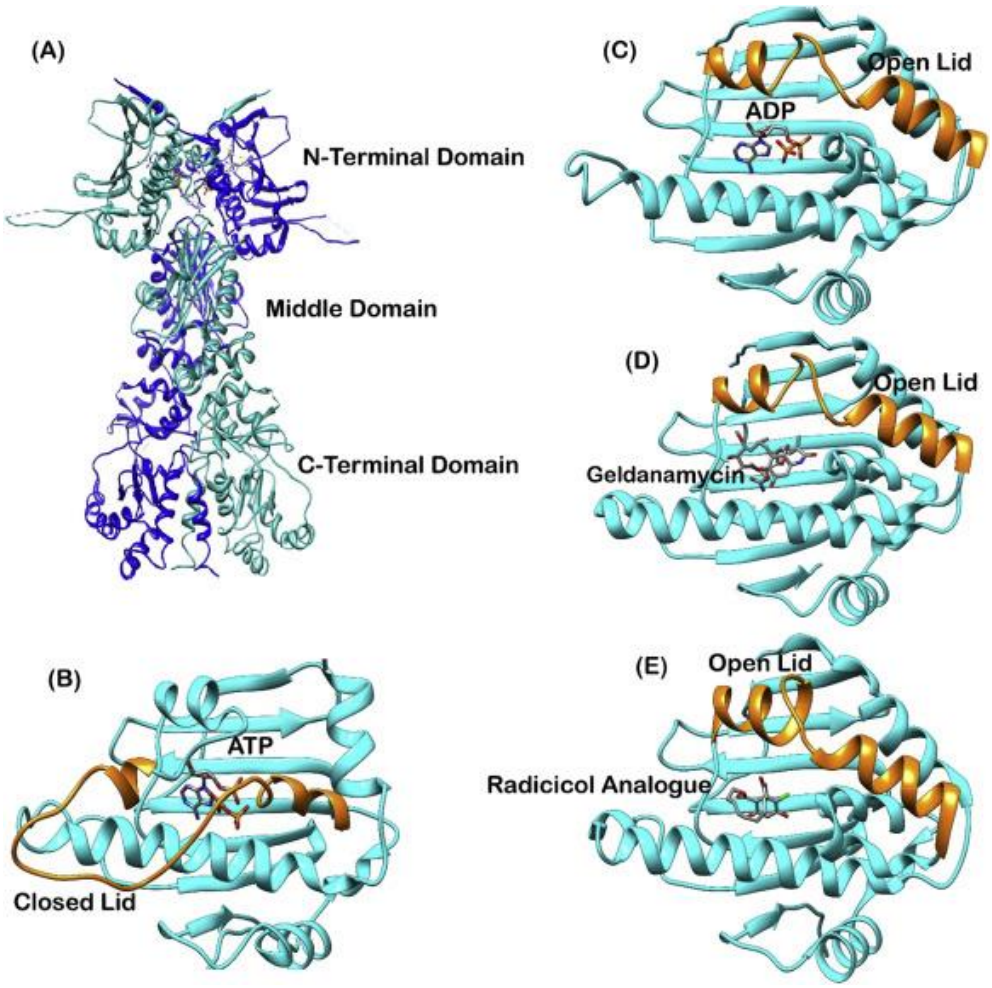


Figure 5: The structure of HSP90[93]

5A: Crystal structure of HSP90

5B: N-terminal domain in closed conformation binding ATP

5C, D, E: N-terminal domain in open lid conformation binding ADP (C), Geldanamycin (D) and Radicicol (E)

3.2.3. Mechanisms

As noted above, HSP90 has three different structural domains, each of which contributes to an important feature of its chaperoning function. The function of N-terminal domain is to bind and hydrolyze ATP. Though it is not fully understood how exactly the different domains work together in chaperoning client proteins, it is established that the ATP pocket exists in two conformations: open and closed. The open conformation occurs while HSP90 is nucleotide free or bound to ADP. However, when bound to ATP, the pocket shifts to a “closed” conformation. In addition, further intermediate confirmations were observed, that may have a role in client protein folding and dissociation.[23] The client protein binding region is located mostly in the M-domain of HSP90, but a second substrate binding site may be found on the C-domain.[108] The

middle domain recognizes substrate proteins and contributes to their folding. For this process it requires a number of diverse co-chaperones, which assist with procedures such as the hydrolysis of ATP.[43] Functionally, HSP90 works as a dimer. The dimerization takes place at the C-domains, however the N-domains exist also in a linked and closed conformation within the dimer.[53]

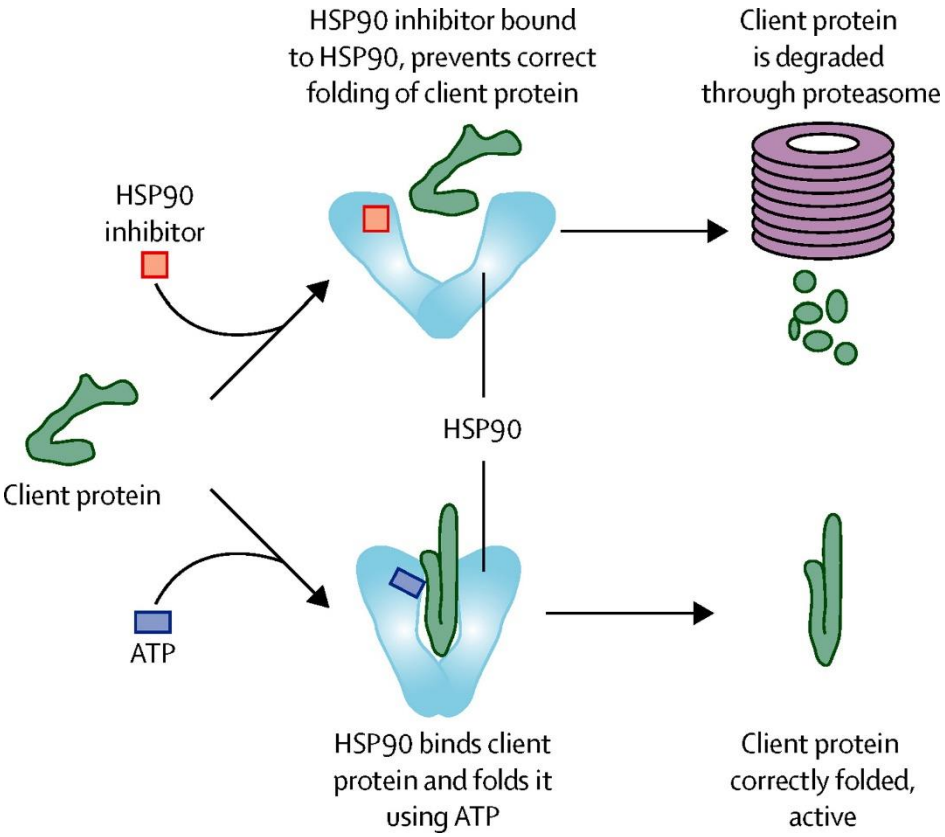


Figure 6: HSP90: The mechanism in presence an absence of inihibitor[33]

3.2.4. Interaction with client proteins

Below (Figure 7) is a list of HSP90 client proteins that have an important role in cancer pathogenesis. In addition, many proteins with diverse functions are HSP90 clients, including signaling oncoproteins, angiogenetic proteins and antiapoptotic proteins. Inhibition of HSP90 can thus lead to apoptosis, interference with angiogenesis and cell death because of high sensitivity of oncoprotein dependent cells.[45] The wide range of functional clients makes inhibition of HSP90 such an interesting target in tumor therapy. A comprehensive list of HSP90 client proteins is available at: *www.picard.ch/downloads/HSP90interactors.pdf*.

Partial Listing of Oncology related clients of HSP90

Signal Transduction Protein Kinases
AKT
BCR-ABL
ERBB2
EGRF
c-KIT
MAPK
MET
NMP-ALK
RAF
SRC
VEGFR1/2
Transcription Factors
Aldosterone receptor
Estrogen receptor
Progesterone receptor
P53

Figure 7: Listing of HSP90 clients with oncogenic relevance
The full listing may be seen on
www.picard.ch/downloads/HSP90interactors.pdf[1]

3.2.5. Clinical significance

HSP90 was shown to be overexpressed in many tumors thus making it an attractive target for cancer therapy. This led to the development of HSP90 inhibitors in a hope to sensitize cancer cells vulnerable to HSP90 activity inhibition. The first HSP90 inhibitor developed and tested is a benzoquinone ansamycin antibiotic named as Geldanamycin (GA). GA binds competitively to the ATP-binding site preventing ATP hydrolysis. Consequently, the house-keeping functions of HSP90 are disrupted resulting in cytotoxicity.[42] GA has shown promising antitumor effects, but unfortunately has a high hepatotoxicity as a side effect.[89] Several HSP90 inhibitors were developed

since then: for example, Geldanamycin analogs 17-AAG and 17-DMAG.[83, 91] 17-AAG, which is also known as Tanespimycin, is found to have promising anticancer effects both *in vitro* and *in vivo* and were tested in phase I and II clinical trials.[75, 78, 81] However, the fact, that 17-AAG is poorly soluble in water[34], could pose problems in regular clinical practice.[44, 82] 17-DMAG (Alvespimycin) is a potent derivate of GA and is more soluble in water.[91] This raises the possibility of oral administration and increased convenience.[29] Alvespimycin showed promising results in phase I trials. In a study of 25 patients with advanced malignancies, one CR could be observed in a patient with prostate cancer, one PR in a patient with metatstatic melanoma and SD >6 months in three patients with chondrosarcoma, prostate cancer and clear cell renal cancer.[64] Combination of HSP90 inhibitors with chemotherapeutics or other targeted drugs could increase effectiveness in further clinical trials. For example, the combination of Tanespimycin and Trastuzumab has led to up to 59% of clinical benefit (CR+PR+SD) in phase II trials in patients who had previously progressed under Trastuzumab.[45, 57] In addition, IPI-504 (retaspimycin) and C-11 are recently developed GA analogs which have been tested in early phase clinical trials.[42,45] A phase III trial for IPI-504 had to be discontinued due to therapy-related toxicity in the study treatment arm.[26] He Wang et al. analyzed 15 phase II trials involving a HSP90 inhibitor (+/-) a second agent in antineoplastic therapy. An ORR of 0,04 to 0,22 percent could be observed. Although there have been promising results in some entities as HER2 overexpressing breast cancer as described above, there are still non satisfying results in others as ovarian cancer (gemcitabine + Tanespimycin), renal cancer, prostate cancer, multiple myeloma or NSCLC.[101] As the reasons for these results might be due to nonsufficient dosage of inhibitor, upregulation of downstream signaling or upregulation of Chaperones like HSP70, other explanations like secondary resistance for drug failure are still to be explored.

3.3. Secondary resistances as a major problem in targeted therapies

Development of secondary drug resistance following treatment with targeted inhibitors is a major challenge in cancer treatment. Our group under the leadership of Prof. Justus Duyster has been engaged in examining secondary resistances due to molecular treatments for a long time.[49, 50, 96, 98, 99] Secondary point mutations in the target protein, overexpression of the target oncoprotein, and drug efflux by membrane proteins are one of the major mechanisms by which drug resistance is developed at

the time of relapse.[97] Some of these resistance mechanisms can be overcome by next-generation targeted inhibitors. For example, in the well-studied case of chronic myeloid leukemia the first line inhibitor Imatinib can be replaced by second line drugs like Nilotinib or Dasatinib, or the third line experimental therapeutics Busotinib.[30, 72, 84] Furthermore Bubnoff *et al*. from the laboratory at TUM (Technische Universität München) have developed an *in vitro* experimental screen that can predict point mutations underlying secondary drug resistance. This method thus speeds up the testing of alternative drugs to overcome drug resistance arising after targeted treatment.[95] In the current project, we have applied this method to identify resistance mechanisms against HSP90 inhibitors.

3.4. Aims and Objectives

1. Study secondary drug resistance mechanisms against HSP90 inhibitors
2. To identify point mutations that cause HSP90 inhibitor resistance
3. Establish cell models to test the efficacy of HSP90 inhibitors

4. Material and Methods

4.1. Material

4.1.1. Chemicals and biogenic Substances

Acetic acid	Roth, Karlsruhe
Acrylamide/Bisacrylamide	Roth, Karlsruhe
Agar	Difco, Detroit, USA
Agarose	Roth, Karlsruhe
Ammonium persulfate (APS)	Sigma-Aldrich Chemie, Taufkirchen
Ampicillin	Sigma-Aldrich Chemie, Taufkirchen
Bactoagar	Difco, Detroit, USA
Bacto-yeast-extract	Difco, Detroit, USA
Bacto-Trypton	Difco, Detroit, USA
Bromphenol blue	Sigma-Aldrich Chemie, Taufkirchen
Chloroform	Sigma-Aldrich Chemie, Taufkirchen
Complete™ Protease Inhibitor tablets	Boehringer, Mannheim
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie, Taufkirchen
Ethanol	Merck, Darmstadt
Ethidium bromide	Roth, Karlsruhe
Ethylendiamide acetic acid	Fluka, Deisenhofen
Glycerol	Fluka, Deisenhofen
Glycerol-2-phosohate	Aldrich Chemical Company, Steinheim
Isopropanol	Merck, Darmstadt
Milk powder	Fluka, Deisenhofen
Methanol	Merck, Darmstadt
N-Ethyl-N-Nitrosourea(ENU)	Sigma-Aldrich Chemie, Taufkirchen

Phosphate buffered saline (PBS)	Biochrom AG, Berlin
Propidium Iodide	Sigma-Aldrich Chemie, Taufkirchen
Sodium azide	Sigma-Aldrich Chemie, Taufkirchen
Sodium chloride	Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe
Sodium dihydrogen phosphate	Merck, Darmstadt
Sodium fluoride	Fluka, Deisenhofen
Sodium hydroxide	Merck, Darmstadt
Sodium orthovanadate	Sigma-Aldrich Chemie, Taufkirchen
Sodium pyrophosphate	Fluka Chemie, Neu-Ulm
Tetramethylethylendiamine (TEMED)	Fluka, Deisenhofen
Tris (hydroxymethyl) aminomethane (TRIS)	Roth, Karlsruhe
Triton X-100	Sigma-Aldrich Chemie, Taufkirchen
TRIzol	Invitrogen GmbH, Karlsruhe
Trypan Blue	Invitrogen GmbH, Karlsruhe
Tween 20	Fluka, Deisenhofen

4.1.2. Media and Supplements for Cellculture

DMEM-Medium	PAA Laboratories, Cölbe
Fetal Bovine Serum Gold (FCS)	PAA Laboratories, Cölbe
Lipofectamine 2000 Reagent	Invitrogen, Karlsruhe
Mouse Interleukin 3 (IL3)	R&D, Wiesbaden
Neomycin	Invitrogen, Karlsruhe
Opti-Mem® Reduced Serum Medium	Invitrogen, Karlsruhe
Penicillin/Streptomycin Solution	PAA, Pasching
Phosphate buffered saline (PBS),	PAA Laboratories, Cölbe

Sterile	
Polybrene = Hexadimethrine bromide	Sigma-Aldrich Chemie, Taufkirchen
RPMI-Medium	PAA, Pasching
Trypsin-EDTA 10x	PAA Laboratories, Cölbe

4.1.3. Enzymes

Alcalic Phosphatase	Fermentas, St. Leonrod
Pfu DNA-Polymerase	Fermentas, St. Leonrod
Restriction Endonuclease Xh01	Fermentas, St. Leonrod
Reverse Transcriptase	Fermentas, St. Leonrod
T4 DNA-Ligase	Fermentas, St. Leonrod
dNTP Mix	Fermentas, St. Leonrod
Enzyme specific Buffer	Fermentas, St. Leonrod
Master Mix	Fermentas, St. Leonrod

4.1.4. Molecular Marker

GeneRuler^{TM} 1kb plus Ladder	Fermentas, St. Leonrod
PageRuler^{TM} Prestained Protein Ladder	Fermentas, St. Leonrod

4.1.5. Antibodies

Anti-Mouse IgG HRP, conjugated	Amersham, Braunschweig
Anti-Rabbit IgG HRP, conjugated	Amersham, Braunschweig
Anti-Erbb2	Santa Cruz Biotech., Germany
Anti-Flag	Sigma-Aldrich Chemie, Taufkirchen
Anti-HSP90	Sigma-Aldrich Chemie, Taufkirchen
Anti-Actin, mouse	Sigma-Aldrich Chemie, Taufkirchen

4.1.6. Inhibitors

Geldanamycin	Invivogen, San Diego, USA
17AAG	Invivogen, San Diego, USA
17DMAG	Invivogen, San Diego, USA

4.1.7. Bacteria

Escherichia Coli DH5α	Invitrogen, Karlsruhe

4.1.8. Cell lines eukaryotic

Phoenix E (packaging cell line)	Gary P. Nolan, Stanford, USA
Ba/F3	Murine-Pro-B cell line

4.1.9. Kits for molecular and biological approach

GeneJET™ Gelectraction Kit	Fermentas, St. Leonrod
GeneJET™ PCR Purification Kit	Fermentas, St. Leonrod
GeneJET™Plasmid Miniprep Kit	Fermentas, St. Leonrod
NucleoBond Xtra Maxi Kit	Machery-Nagel, Düren
SuperSignal West Pico, Dura, Femto	Perbio Science, Bonn
Whatman®-Paper	Sigma-Aldrich, Taufkirchen
PVDF (Poly vinyliden fluoride) Membrane (Immobilon P)	Millipore, Schwalbach

4.1.10. DNA-Constructs

MigR1 (MSCV-IRES-eGFP)	a kind gift of R.K. Kancha, clinical cancer research
MigR1-Erbb2	a kind gift of Heike Conrad
MSCV(MSCV-IRES-YFP)	a kind gift of Rebekka Dechow
MSCV-HSP90	This work

MSCV-FLAG-HSP90	This work
MSCV-HSP90-FLAG-F133V	This work
MSCV-HSP90-FLAG-G123V	This work
MSCV-HSP90-FLAG-G130V	This work
MSCV-HSP90-FLAG-G127V	This work
MSCV-HSP90-FLAG-I123S	This work
MSCV-HSP90-FLAG-I123T	This work

4.1.11. Primers

MSCV-HSP90-F133V

Forward: 5’ CAGTTTGGTGTCGGA**G**TCTACTCGGCCTATC 3’

Reverse: 5’ GATAGGCCGAGTAGA**C**TCCGACACCAAACTG 3’

MSCV-HSP90-G123V

Forward: 5’ CGGGCAGTTTGGTGTCG**T**ATTCTACTCGGCCTATC 3’

Reverse: 5’ GATAGGCCGAGTAGAAT**A**CGACACCAAACTGCCCG 3’

MSCV-HSP90-G130V

Forward: 5’ CATGATCGGGCAGTTTG**T**TGTCGGATTCTACTCGG 3’

Reverse: 5’ CCGAGTAGAATCCGACA**A**CAAACTGCCCGATCATG 3’

MSCV-HSP90-G127V

Forward: 5’ CATCTCCATGATCG**TT**CAGTTTGGTGTCCG 3’

Reverse: 5’ CGGACACCAAACTG**AA**CGATCATGGAGATG 3’

MSCV-HSP90-I123S

Forward: 5’ CAGGCTGGTGCAGACA**G**CTCCATGATCGGGCAG 3’

Reverse: 5’ CTGCCCGATCATGGAG**C**TGTCTGCACCAGCCTG 3’

MSCV-HSP90-I123T

Forward: 5’ CAGGCTGGTGCAGACA**C**CTCCATGATCGGGCAG 3’

Reverse: 5’ CTGCCCGATCATGGAG**G**TGTCTGCACCAGCCTG 3’

GATC-MSCV-MCS-5-263611

Forward: 5’ CGTTCGACCCCGCCTCGATCC 3’

Reverse: 5’ GGATCGAGGCGGGGTCGAACG 3’

4.1.12. Standard Equipment

Agarose-gel-migration-chamber	Biometra, Göttingen
Analyzing scale BP 221S	Satorius, Göttingen
Centrifuge, Megafuge R 1.0	Thermo Scientific, Karlsruhe
Centrifuge, J2-HS Centrifuge	Beckman, Krefeld
Cooling centrifuge 5417R	Eppendorf, Hamburg
CO2-Incubator SW J 500 TV BB	Nunc, Wiesbaden
Desk centrifuge 5415D	Eppendorf, Hamburg
Digital scale LC 1200 S	Satorius, Göttingen
Developer Optimax	Protec Oberstenfeld
FACS	Beckman Coulter
Heating block, Thermo mixer comfort	Eppendorf, Hamburg
Incubation shaker, Innova 4000	New Brunswick Scientific, Edison, USA
Light microscope Axiovert 40 CFL	Zeiss, Jena
Magnetic shaker CB162	Carl Stuart Limited, Dublin, Ireland
Neubauer counting chamber	Reichert, New York, USA
PCR-Thermo cycler Primus	MWG-Biotech, Ebersberg
PH-Meter inoLab®	TW, Weilheim
Shaker WT 12	Biometra, Göttingen
Spectral photometer, NanoDrop® ND-1000	Thermo Scientific, Karlsruhe

Steam sterilizer, Varioklav	H+P Labortechnik, Oberschleißheim
Sterile work desk, Laminar-Flow 1.8	Holten, Gydewang, Danmark
UV-Lamp TI 2	Biometra, Göttingen
Vertical migration chamber, Mini Protean® Tetra cell	Bio-Rad, München
Vortex Mixer 7-2020	neoLab, Heidelberg
Western Blotting chamber, TE Series Transphor Electrophoresis Unit	Hoefer, Holliston, USA

4.1.13. Standard Fluids and Buffers

Cell culture:

DMEM-Medium:	10% FCS in DMEM-Medium
Freezing Medium 2x:	80%FCS, 20% DMSO
PBS 1x:	10% 10x PBS in Aqua dest.
Trypsin-EDTA 1x:	10% Trypsin EDTA in 1x PBS
RPMI-Medium:	10% FCS and 1% Pen/Strep in RPMI-Medium

Molecular biology/Protein biochemistry:

Ampicillin	50mg/ml in Aqua dest.
Blocking Fluid for Western Blotting:	10% Dry milk powder (degreased) in PBS-Tween-Buffer
DNA loading buffer	60% Glycerol 0.2% Bromophenol blue 0.2 M EDTA in Aqua dest.
Loading Dye 2x:	1M Tris/HCl pH 6.8), 200mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% Glycin in Aqua dest.
Luria-Bertani (LB)-Medium (fluid):	1%Bacto-Trypton, 0,5% Bacto Yeast extract, 1%NaCl in Aqua dest., adjusted with 1M NaOH to pH 7,0, autoclaved

LB-Medium (solid):	1.5% bacto-Agar in LB-Medium (fluid)
Lysis buffer:	10mM Tris/HCl (pH 7.5), 130mM NaCl, 5mM EDTA, 0.5% Triton X-100, 20mM Na_2HPO_4/NaH_2PO_4 (pH 7.5), 10 mM Sodium pyrophosphat (pH 7), 1mM Sodium orthovanadate, 20mM Sodium fluoride, 1mM Glycerol-2-Phosphate, 1Protease-Inhibitor Tablet, 10ml Aqua dest.
Resolving gel buffer (4X):	1.5 M Tris (pH 8.8) 0.4% SDS in A.d
Stacking gel buffer for SDS-Gels (4X):	0.5 M Tris (pH 6.8 < 9) 0.4% SDS in A.d
Stripping Buffer:	0.2M NaOH
SDS running buffer:	25mM Tris, 192mM Glycine, 0.1% SDS in Aqua dest.
TAE Buffer 10x:	0.4M Tris 1.1% acetic acid 2% 0.5M EDTA (pH 8) in Aqua dest.
Transfer Buffer:	25mM Tris, 192mM Glycin, 0.1 %SDS in Aqua dest., 20 % Methanol in Aqua dest.
Washing Buffer for Western-Blotting:	0.1% Tween 20 in 1x PBS

4.1.14. Services

Sequencing	GATC Biotech
Primer	Metabion

4.2. Methods

4.2.1. Methods involving nucleic acids

4.2.2. Transformation and cultivation of bacteria

DNA was transformed into the chemically competent DH5 alpha strain of E. Coli. For transformation, the bacteria were thawed on ice. 100µl of bacteria were taken together with 10µl of the DNA-construct, mixed gently and kept for 30minutes on ice. The mixture was then subjected to heat shock for 42 seconds at 37° C to optimize the effectiveness of transformation. Subsequently the mix was incubated for 2 minutes on ice. The bacteria suspension was then spread on agar plates, containing Ampicillin as the selection marker. The plates were then kept in an incubator overnight and analyzed for colony formation the following day. For amplification, the colonies were inoculated in 5ml (for mini-preps) or 50 ml (for maxi-preps) of LB-Medium containing ampicillin and incubated at 37° C on a 225-rpm shaker overnight.

4.2.3. Isolation of plasmid DNA

Bacterial culture was first centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded. To isolate small amounts of DNA "GeneJET™ Plasmid Preparation Kit" was used as per the manufacturer's instructions. The technique is based on the different qualities of chromosomal and plasmid DNA concerning the denaturation when in contact with sodium hydroxide. Quickly after the pH level is reduced, the chromosomal DNA sticks to proteins and cell fragments, while the plasmids refold and can be salvaged. The non-plasmid component is lost in further centrifugation steps.[14] The eluted DNA was stored at -20° C. Maxi-prep is based on the same principle as Mini-prep, only that it grants large amounts of purified DNA. A "NucleoBond® Xtra Maxi Kit" was used to perform maxi-preps.

4.2.4. RNA-Isolation

RNA isolation was performed using a TRIzol based method in highly sterile conditions. TRIzol, a reagent containing guanidinium thiocyanate and phenol, is an agent that solubilizes biomolecules and denaturizes protein at the same time.[85] $5x10^6$ Ba/F3 cells were pelleted at 1300 rpm for 5 minutes. The cell pellet was then resuspended in 1 ml of TRIzol reagent at room temperature and vortexed briefly. 0.2 ml of chloroform were

then added to the lysate and the samples were vortexed for 15 seconds. Centrifugation of the samples was then performed at 14000 rpm at 4°C for 15 minutes to induce phase separation. The colorless chloroform-containing upper aqueous layer was then collected in a sterile tube without disturbing the lower organic phase. The RNA from the aqueous phase was separated by adding 0.5 ml of isopropanol, incubated for 15 minutes at room temperature for precipitation of the RNA and centrifuged at 14000 rpm at 4°C for 10 minutes. The RNA pellet was then washed with 1 ml of 70% ethanol and air dried for 10 minutes. Nanodrop machine was used to measure the purity and concentration of RNA samples by photometry.

4.2.5. Polymerase Chain Reaction

PCR was performed to clone the HSP90 cDNA by using a one-step reverse-transcription PCR. The following reaction mixture and conditions were used to amplify HSP90 cDNA using RNA as the template.

Reverse Transcriptase PCR

Reaction mixture:

RNA	2 µl
H_2O	19 µl
Master Mix	25 µl
Forward-Primer	1.5 µl
Reverse-Primer	1.5 µl
Reverse Transcriptase	1µl

Program:

Lidght (Lid temperature)	110° C
	45° C for 45 minutes
Initial Denaturation	95° C for 2 minutes
	55° C for 30 seconds
	70° C for 2 minutes
Cycles	35
Denaturation	95° C for 30 seconds

Annealing	55° C for 30 seconds
Elongation	72° C for 2 minutes
End-Elongation	72° C for 20 minutes

DNA sequencing was then performed for the presence of kinase domain mutations in HSP90 using the BLAST program.

4.2.6. FLAG Tagging

A FLAG Tag was inserted to the N-Terminus of HSP90 by PCR using a standard PCR program. This was used to distinguish the exogenously expressed mutants from the endogenous wild type HSP90.

4.2.7. Site-directed-Mutagenesis

Point mutations were introduced into the HSP90 cDNA by site-directed mutagenesis. For the presence of the wanted mutation at the desired nucleotide location primers were designed. Following reaction mixture and conditions were used:

Reaction mixture:

DNA	1.5 µl
dNTPS	1.5 µl
H_2O	20 µl
Pfu Buffer	3 µl
Forward Primer	1.5 µl
Reverse Primer	1.5 µl
Pfu	1 µl

Program:

Lidght (Lid temperature)	110° C
Initial Denaturation	95° C for 30 seconds
Cycles	18
Denaturation	95° C for 30 seconds
Annealing	55° C for 1 minute
Elongation	72° C for 18 minutes

End-Elongation 72° C for 10 minutes

The PCR product contains both mutated and not mutated DNA. To deplete the non-mutated template, a restriction digestion reaction with the DpnI enzyme was performed. Since the original template DNA is derived from bacterial culture, it is methylated. DpnI digests methylated DNA only and so purifies the PCR product to contain only mutant DNA.[19] The gaps left in the circular DNA were closed following the transformation into the bacteria. Following incubation after transformation, bacterial colonies were picked, minipreps were performed and the mutations were confirmed by restriction analysis and DNA sequencing.
All point mutations were introduced into the MSCV-HSP90-WT by primers designed for that purpose, all constructs were confirmed by sequencing.

4.2.8. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to separate and identify the DNA fragments of interest. 1% agarose gels were prepared and used for this purpose. Most essential to the mechanism of this kind of Electrophoresis is the use of Ethidium bromide, a DNA intercalating substance, which will fluoresce when exposed to UV-light.[6] 1 gram of agarose was dissolved in 1x TAE Buffer by slowly and carefully heating it in the microwave. At about 50° C, 2-8µl of Ethidium bromide were added and poured into a clean horizontal gel casting chamber while avoiding the formation of bubbles. A comb was placed into the gel to form wells for loading the DNA samples. Loading Dye was added to each sample and the mixture was then loaded together with a marker (Gene Ruler 1kb). Electrophoresis was performed in a gel chamber under constant voltage until the bands were clearly separated as visualized under UV-light by a UV transilluminator and photographs were taken for records.

4.2.9. PCR-Purification and gel extraction

To purify the DNA by separating reaction mixture components like primers, nucleotides and enzymes, the “GeneJET™ PCR Purification Kit” was used as specified by the supplier. For gel extraction, the bands of interest were cut out of the gel with a sterile scalpel under low UV-light and either frozen for later use or directly analysed. Extraction of DNA from the excised gel pieces was performed using “GeneJET™ Gel

Extraction Kit". The DNA then was in 30µl elution buffer and stored at -20° C for future use.

4.2.10. Restriction digestion

Restriction digestion of target DNA and the cloning vector is the first step of cloning. The enzymes, called restriction endonucleases, recognize specific palindromic base sequences and cut the DNA precisely at these locations thus creating complementary sticky ends (overlapping) or blunt ends.[86] The composition of the reaction mixture for our restriction digestion is as follows:

Insert-DNA	1µl (approx. 1 µg)
10X Buffer	3µl
Enzyme	1µl
H_2O	25µl

The reaction tubes were incubated at 37° C for 15-30 minutes and the samples were further treated with Dephosporylation. HSP90-WT and mutant HSP90 were cloned into the MSCV vector (a kind gift of Dr. Rebekka Dechow) using Xho1.

4.2.11. Dephosphorylation

After restriction digestion, both ends of the plasmid DNA are still compatible and would reunite if not dephosphorylated. The enzyme alkaline phosphatase cuts of the 5'-Phosphate group, thus preventing the religation of the digested vector. 1µl Fast AP (an alkaline phosphatase) was added to the digested vector and incubated for 15 minutes. Following phosphatase reaction, the sample was heated to 75° C for 5 minutes to inactivate the AP. The digested DNA was then seperated by gel electrophoresis, the bands of interest cut out under UV light and extracted by a gel extraction protocol.

4.2.12. Ligation

As the last step on cloning, ligation was performed. Ligation of compatible ends of the vector and the insert was performed using T4 Ligase that links the 3'OH end to the 5'Phosphate end. The following reaction was set up for that purpose:

Vector	2µl

Insert	6µl
5x Rapid Ligation Buffer	4µl
T4 DNA-Ligase	1µl
H_2O	7µl

The reaction was performed for 15 minutes at room temperature and was then frozen or put on ice immediately. The samples were then used for bacterial transformation.

4.3. Mammalian cell culture

4.3.1. Cell cultivation, cell counting and cryopreservation

HEK293 (human embryonic kidney cell line) and Ba/F3 (murine pro-B cell line) cells were cultured in an incubator at 37° C, with relative air moisture of 90% and 5% CO_2. The experiments were performed in sterile workbenches with constant vertical airflow. Centrifugation of cells was performed at room temperature at 1300 rpm and for 3-5 minutes. Ba/F3 cells were cultured in RPMI-1640 and HEK293 cells were cultured in DMEM. Both the media are supplemented with 10% FCS and a Pen-Strep antibiotic mixture. In addition, Ba/F3 cells were cultured in Interleukin-3. However, cells transformed by ERBB2 were cultured in RPMI-1640 medium without IL3.

Cells were stored in liquid nitrogen. For the process of cryopreservation, a freezing medium containing 40 % FCS and 10 % DMSO was used. Cells were then kept in bench-top freezers (containing isopropanol for step-wise cooling) and stored at -80° C overnight and then transferred to liquid nitrogen tank for long-term storage. For thawing, cells were taken out of the freezer into a water bath at 37° C. The cells were then transferred into a 15ml tube containing the culture medium. After centrifugation supernatant was discarded. Th cell pellet was then resuspended in appropriate media and cultured as described above.

Neubauer counting chambers were used for cell counting. The cell suspension in question was mixed at a ratio of 1:1 with 5% Trypan Blue. Then the glass coverslip was put over the Neubauer chamber, which forms little cubes at the exact volume of 0.1µl. The cells were pipetted into the chamber and were counted under a light microscope. Four big quadrates were counted. Then the number of cells per ml was calculated: Concentration (cells/ml) = 2(diluting factor) x number of cells $x10^4$ / number of squares

4.3.2. Transfection and viral transduction

To introduce plasmid DNA in cell lines, the method of lipofection was used. 2 x 10^6 Phoenix cells (a HEK293-based retroviral packaging cell line) in 3 ml DMEM medium were plated in 6 cm plates 12 hours before transfection. The next day, medium was carefully aspirated and replaced with 2ml DMEM medium. The transfection mixture is composed of 1000 µl OptiMem (serum-free medium for transfection), 20 µl Lipofectamine 2000 – a cationic liposome formulation - and 10 µg of DNA. The preparation then was pipetted with caution on to the plate. On day 3, the medium was changed twice before harvesting. The supernatant (containing the retrovirus) was collected and stored at 4° C for later use.

For the infection of the Ba/f3 cells with the virus, 1 x 10^6 cells in 0.5 ml were distributed per well in 12 well-plates. 2ml of retrovirus per well were then added. IL3 and polybrene – a cationic polymer used to neutralize charge repulsions between virions and the cell surface[24] - were added to the mixture and the plates were centrifuged at 32° C for 90minutes at 2400 rpm. Following centrifugation, the plates were kept in the incubator at 37°C for 48 hours and selected with Neomycin at a concentration of 500µg/ml. As neomycin is the resistance marker of the MSCV vector, this was the control that only successfully transduced cells were selected. The cells were stored in liquid nitrogen for further use.

4.3.3. ENU-Screen

N-Ethyl-N-Nitrosourea(ENU) is a very strong mutagen and will induce mutations randomly in a genome. For the ENU-screen we used a cell based screening method established by Bubnoff et al.[95] Ba/F3 cells stably expressing wild type Her2 were treated twice with 100 µg/mL of N-ethyl-N-Nitrosourea (ENU) for 12 hours. The cells were thoroughly washed to remove residual ENU. Treated cells were then cultured in 96-well plates at a density of 4x10^5 cells per well in the presence of 2 µM 17-DMAG, an HSP90 inhibitor. 17-DMAG resistant cell colonies were isolated later and expanded. RNA was isolated, cDNA was amplified and the ATP-pocket of HSP90 was sequenced for mutations.

4.4. Biochemical methods

4.4.1. Cell lysis and Co-Immunoprecipitation

The cell lysis buffer was prepared (refer materials for composition) and stored on ice. 5 x 10^6 Ba/F3 cells were centrifuged, and the supernatant was discarded before placing the pellet on ice. Cell pellets were then resuspended in 1 ml of cell lysis buffer and transferred into a 1.5 ml tube before incubating on ice for 30 minutes. Cell lysates were then subjected to centrifugation at 16000 rpm for 20 minutes at 4° C. Loading dye was added to the lysates and the mix was then heated to 95° C for 5minutes before subjecting it to the SDS-PAGE.

For co-immunoprecipitation of the FLAG-tagged HSP90 protein, Anti-FLAG beads were added to cell lysates and incubated for 2 hours. The lysates were then centrifuged at 10000 rpm for 20 seconds. The pellet was then washed thrice with fresh cell lysis buffer before adding the loading buffer. Samples were then heated to 95° C for 5 minutes and then loaded onto a gel.

4.4.2. SDS-Gel-electrophoresis

Polyacrylamid gels were used to separate protein according to their size. The following composition of stacking gel and resolving gel were used:

Stacking gel:

5% Polyacrylamide, 12.5 mM Tris/HCl (pH 6.8), 0.1% SDS, 0.3% APS and 0.1% TEMED

Separating gel:

8% Polyacrylamide, 375 mM Tris/HCl (pH 8.8), 0.1% SDS, 0.3% APS and 0.1% TEMED

The Separating gel was cast first into the gel casting apparatus. To avoid bubbles, a layer of Isopropanol was poured over the resolving gel. Isopropanol was removed over the polymerized resolving gel with a whatman paper and the stacking gel was cast over it. After polymerization of the stacking gel, the protein samples were loaded to the gel and fractionated under the applied electric field according to their size and further subjected to western blotting.

4.4.3. Western Blotting

The transfer of proteins from polyacrylamide gels on to a membrane was performed by western blotting. Both the wet as well as the semi-dry methods were used. A PVDF membrane (Poly vinylidene fluoride) was activated in Methanol first. The gel was carefully put on the membrane and was fixated in the chamber between two whatman papers. As proteins migrate towards the anode, the membrane was faced to the anode and the gel to the cathode. For the wet blot technique, a transfer chamber with nettings is necessary. The transfer happened in transfer buffer at an electric current of 1A for 2.5hours (depending on the molar mass of the proteins in question). The semidry blot was performed for 30 minutes in a Trans-Blot Turbo Transfer System with fewer amounts of buffer.

4.4.4. Assay for activated proteins

After the western transfer the PVDF membranes, carrying the proteins in question were blocked in 5% milk to avoid unspecific antibody binding to the membrane. Then the blocked membranes were incubated overnight in the primary diluted antibody. The primary antibodies were diluted in 5% milk as to the manufacturer´s recommendation. The next day, the PVDF membranes were washed three times for 10 minutes in PBS plus Tween solution before being subjected to the secondary antibody for 30 minutes.

4.4.5. FACS (fluorescence-activated cell scanning)

FACS-analysis was used to determine the percentage of GFP-transfected cells as well as to determine the percentage of living cells in cell death assays.

Based on fluorescing qualities of the cell and light scattering characteristics the Fluorescence-activated-cell-sorting can count and sort individual cells by detecting laser-triggered fluorescence. The level of GFP-transfected cells was assessed as well as the state of cell death. As the GFP-protein is a part of the MIG-vector, successfully transfected cells could be seen green in FACS and the rate of transfection with Her2-MIG could be monitored. Furthermore, the rate of growth of cells carrying the different HSP90 mutations, when transfected with HER2-MIGR1, was analyzed in a competition assay.

5. Results

There is an increasing number of studies reporting drug resistance against targeted kinase inhibitors and antibodies directed against activated receptors[49, 96, 98, 99] Even though a significant fraction of patients were benefited initially from targeted treatment, there is a great number of secondary resistance incidences resulting in remission.[32, 73] However the mechanism of acquired resistance are not yet fully understood. The common mechanisms include factors of the host's environment, rapid drug metabolism, poor absorption, and the lack of drug availability to the tumor. On the other hand, there are acquired mechanisms such as genetic alteration in cancer cells that are exposed to the drug. Under selection pressure, tumor cells mutate the inhibitor's target thus abrogating drug binding and resulting activity. In addition, overexpression of the target protein results in an increase of the drug requirement for inhibiting all the available target protein.[31, 36] As several oncogenic kinases are HSP90 clients, treatment with HSP90 inhibitors were thought to cause specific toxicity to cancer cells. Thus, several HSP90 inhibitors are being increasingly tested for their efficacy against a variety of cancers. However, as seen with kinase inhibitors, it is likely that secondary drug resistance may emerge upon treatment with HSP90 inhibitors.

5.1. A cell-based screen identified drug resistant HSP90 mutation

The primary goal of this work was to investigate the probable occurrence of inhibitor-resistant HSP90 mutations *in vitro*. For this purpose, an ENU-based chemical mutagenesis screen was performed (Figure 8A). N-Ethyl-N-Nitrosourea is a very strong mutagen, which causes point mutations in DNA. Ba/F3 cells stably expressing wild type Her2 were treated twice with 100 µg/mL of N-ethyl-N-Nitrosourea (ENU) for 12 hours. The cells were thoroughly washed to remove residual ENU. Treated cells were then cultured in 96-well plates at a density of $4x10^5$ cells per well in the presence of 2 µM 17-DMAG, an HSP90 inhibitor. Colonies growing in the long-term culture with 17-DMAG were picked and RNA was isolated. PCR was performed using primers specific for the inhibitor-binding pocket of the HSP90 N-terminal domain. Upon sequencing amplicons, a heterozygous point mutation HSP90-F133V was detected in the N-terminal domain of a resistant clone (Figure 8B). This mutation has never before been described in literature. Multiple sequence alignment of various HSP90 isoforms indicated that the F133 is highly conserved across species (Figure 8C). This fact and also its position on the ATP binding site imply that a mutation of such an important

amino acid residue could be of the utmost importance for clinical application. In addition to the mutation we have identified in our screen, we aimed to study the effect of multiple other mutations that were previously reported to be important for the HSP90-inhibitor interaction. (See 5.2.)

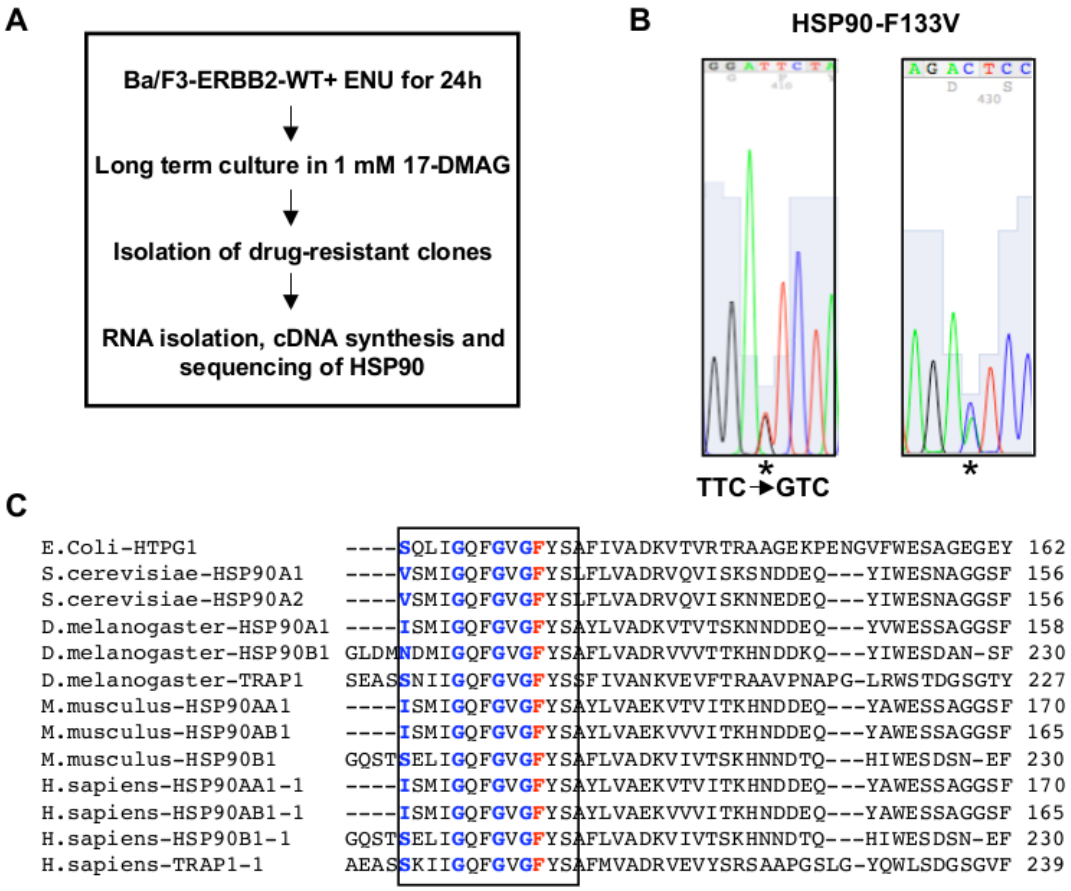


Figure 8: The Mutation F133V

8A: Process of finding the new HSP90 Mutation

We performed an ENU mutagen screen treating Ba/F3 cells transfected with wt ERBB2 with ENU for 24 hours. Cells were then kept in long term culture in 1mM 17DMAG. The growing drug resistant clones were picked, RNA was isolated, cDNA was synthesized, and we sequenced them for HSP90 mutations.

8B: The dark peek indicates a Guanine instead of a Thymine in the position 133

This implicates the successful identification of a never before described mutation in HSP90.

8C: Different sequences of HSP90 in various species[5]

Interestingly the different species all have the Phenylalanine in position 133 in common.

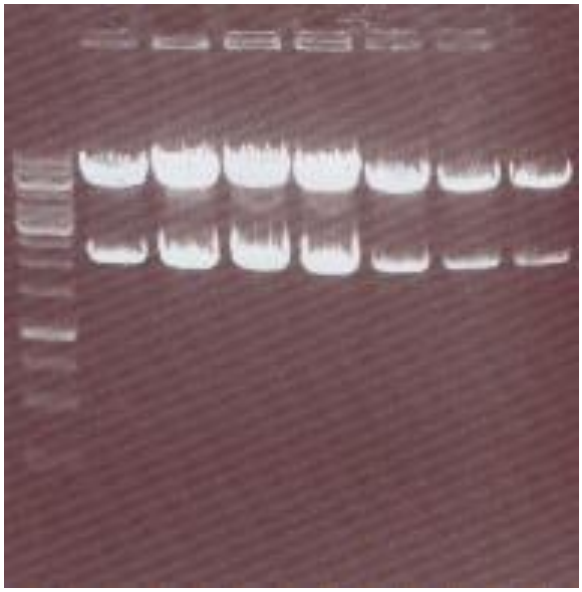
5.2. Cloning of drug-resistant HSP90 mutations

For functional characterization of HSP90 mutants, a FLAG-tagged wild type HSP90 cDNA was cloned into retroviral vectors. Total RNA was prepared from Ba/F3 cells and

FLAG-tagged HSP90 was amplified by PCR and cloned into the MSCV (MSCV-IRES-YFP) vector. Positive clones upon transformation of HSP90-bearing vectors were picked and plasmid DNA was isolated. Cloning of wild type HSP90 was confirmed by test digestion of the isolated plasmid. Positive clones were retransformed and maxipreps were prepared. Site-directed mutagenesis was performed on MSCV-HSP90 and mutant clones were confirmed both by test restriction digestion and DNA sequencing (Figure9).

Apart from the new-found mutation F133V we chose to analyze five other point mutations described in literature. All of them have in common to be located in the ATP-binding region of HSP90. The Glycines G132, G130 and G127 are part of the Bergerat fold, described in 1997 by Bergerat et al. The motif consisting of three Glycines in a row is conserved among species and a component of the helix-sheet-helix orientation.[12, 66] Grenert et al. showed diminished binding of Geldanamycin to the chaperone in HSP90 with Valine instead of Glycine in these positions.[40] The mutation underlying the change of amino acid I123T was described by Zurawsky et al. and found to be resistant to the Geldanamycin analogue 17AAG.[111] I123S is found in the mitochondrial isoform of HSP90, TRAP1 – also a target for molecular antitumor strategies.[5, 9]

A wt F133V G132V G130V G127V I123T I123S



B F133V G132V G130V G127V

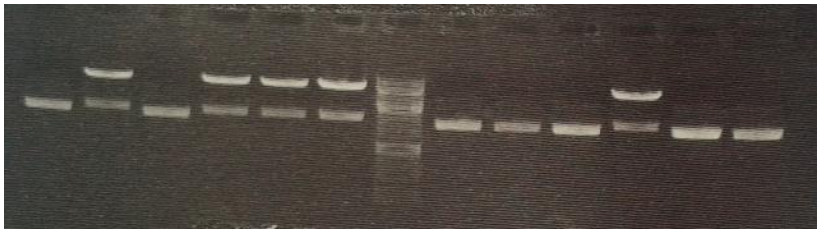


Figure 9: Gel electrophoresis of Digestion from HSP90 constructs in MSCV-Vector
HSP90 wildtype and the 6 Mutations in the MSCV vector, digested with Xho1 in a gel electrophoresis with a ladder of gene ruler 1kb. The lower band is HSP90 at about 50kb and the higher band is MSCV at 7.6 kb)
9A: Test restriction digestion of wt HSP90 and the above described mutations.
9B: Example of cloning of the mutations into the MSCV vector.
The wells with two bands, one upper bright band and one lower dark band were sequenced to confirm successful cloning.

5.3. Functional analysis of HSP90 mutants

The mutations that were created by site-directed mutagenesis as described in Figure7A were functionally characterized next.

5.3.1 ERBB2 expression is present respective to exogenous HSP90 mutations

To test if HSP90 mutants retained chaperoning activity, a co-immunoprecipitation assay was performed. Since ERBB2 receptor is a client kinase for the HSP90 chaperone, the interaction between the ERBB2 and HSP90 was chosen as a read-out for the HSP90 activity. For this purpose, Ba/F3 cells that lacked ERBB2 expression were transfected with wild type ERBB2 as well as the FLAG-tagged wild type or mutant HSP90. Analysis of FLAG (and thus ectopically expressed HSP90) immunoprecipitates revealed that all the transfected HSP90 mutants interacted with the ERBB2 receptor thus indicating that the function of HSP90 is not compromised due to the presence of inhibitor-resistant mutations (Figure 10B). The different levels of ERBB2 expression may be caused by different affinity of the exogenous HSP90 mutations to ERBB2, especially HSP90 G127V seems to have a lower efficacy in the interaction with ERBB2. The presence of HER2 in all mutations – even at different expression levels – was an important premise for the following protein degradation assay. (See below)

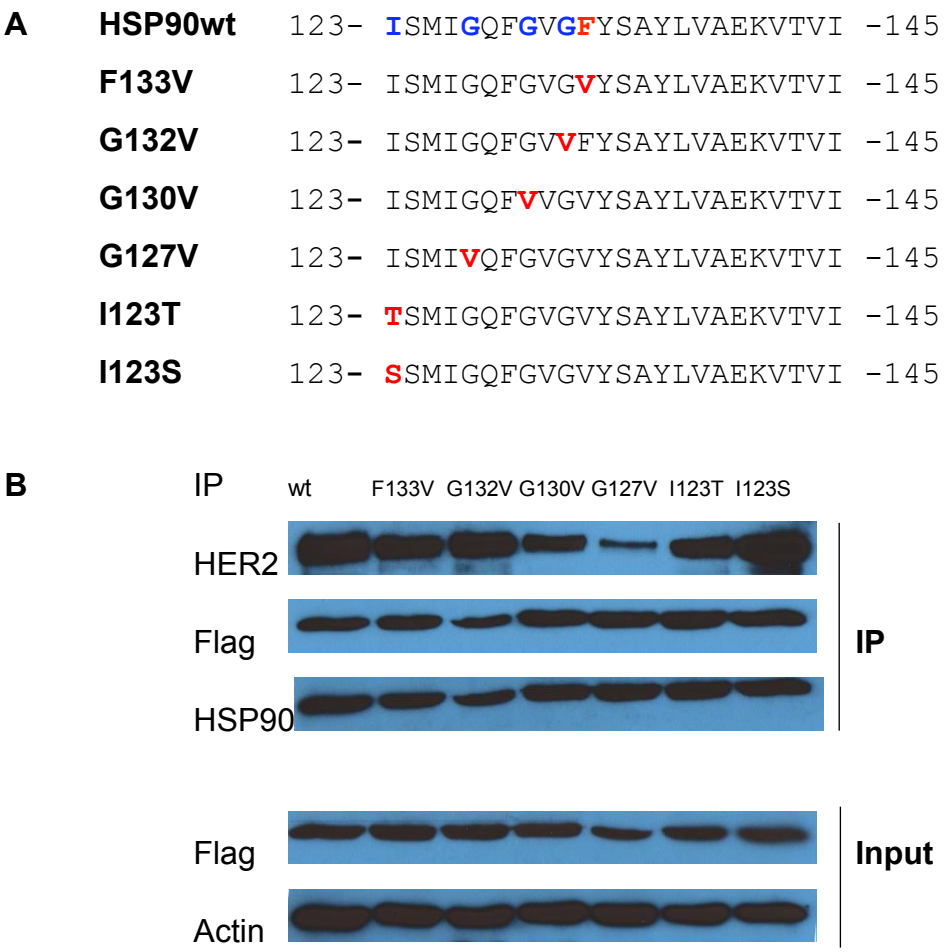


Figure 10: Cloning of the Mutations

10A: Amino acid sequence of Hps90 wt and mutants

10B: Lysis and Flag-IP of Ba/F3 cells transfected with HER2 wt and HSP90 wt and mutants Immunoprecipitation revealed that in the presence of the HSP90 mutations HER2 is expressed at different levels.

5.3.2. High level of endogenous HSP9 lead to interference with oncogenic potential of exogenous HSP90 and ERBB2 in vitro

A GFP-based competition assay was performed to assess the effect of mutations on the chaperoning activity of HSP90. The correct folding, stability, kinase activity and oncogenic potential of ERBB2 are all dependent on the HSP90 activity. We have previously established a competition assay wherein the cells transformed by the ERBB2 oncoprotein outgrow the cells without oncogene.[50] Since the cells that express ERBB2 protein also co-express GFP protein (from an IRES in MiGR1 vector), the competitive outgrowth of IL3-independent Ba/F3 cells over parental Ba/F3 cells in the absence of cytokine can be assayed by FACS analysis. Unselected stable Ba/F3 cells either parental or expressing wild type or mutant HSP90 were infected with MiGR1-

ERBB2 retrovirus (transfection efficiency approx. 20%) were deprived of IL3. The outgrowth of GFP positive cells was measured via FACS analysis over time. FACS analysis showed only a slight difference between the parental cells and the cells that express wild type HSP90 or mutant HSP90 (Figure 11). Ba/F3 parental, Ba/F3 carrying MSCV and Ba/F3 transduced with HSP90 wt showed a similar pattern of growth. Cells transfected with a mutant HSP90, including our new described mutation F133V showed a slower pattern of growth. This could indicate that the mutations have a lower affinity of binding and chaperoning HER2 as shown in the immunoprecipitation assay. As the wild type endogenous HSP90 levels are very high and cells are mostly dependent on these endogenous HSP90, the interpretation of the competitive growth assay remains elaborate. The exogenous HSP90 might not be expressed at the same level as that of the wild type endogenous HSP90 and as a consequence the difference in growth higher in cells without endogenous HSP90. As mentioned above the deletion of endogenous HSP90 leads to a lethal phenotype, ergo the inference of endogenous HSP90 (that constitutes up to 1-2% of total cell protein) is a confounder of in vitro experiments difficult to circumvent.[22]

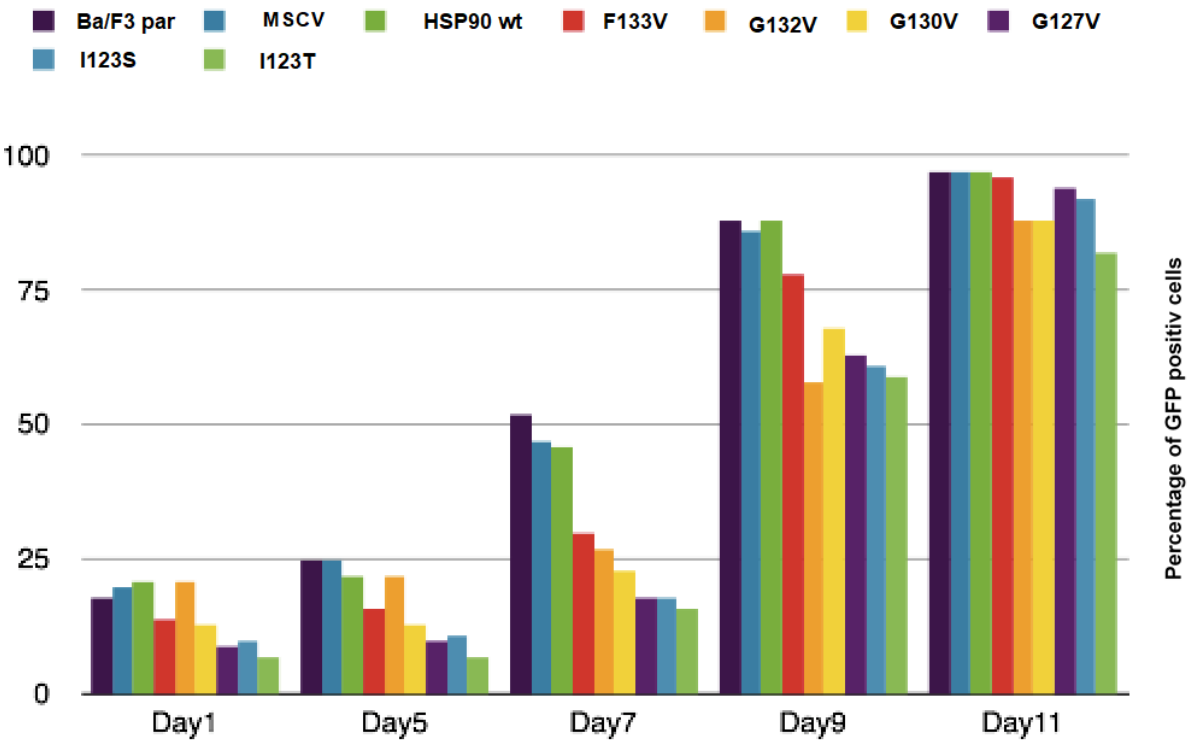


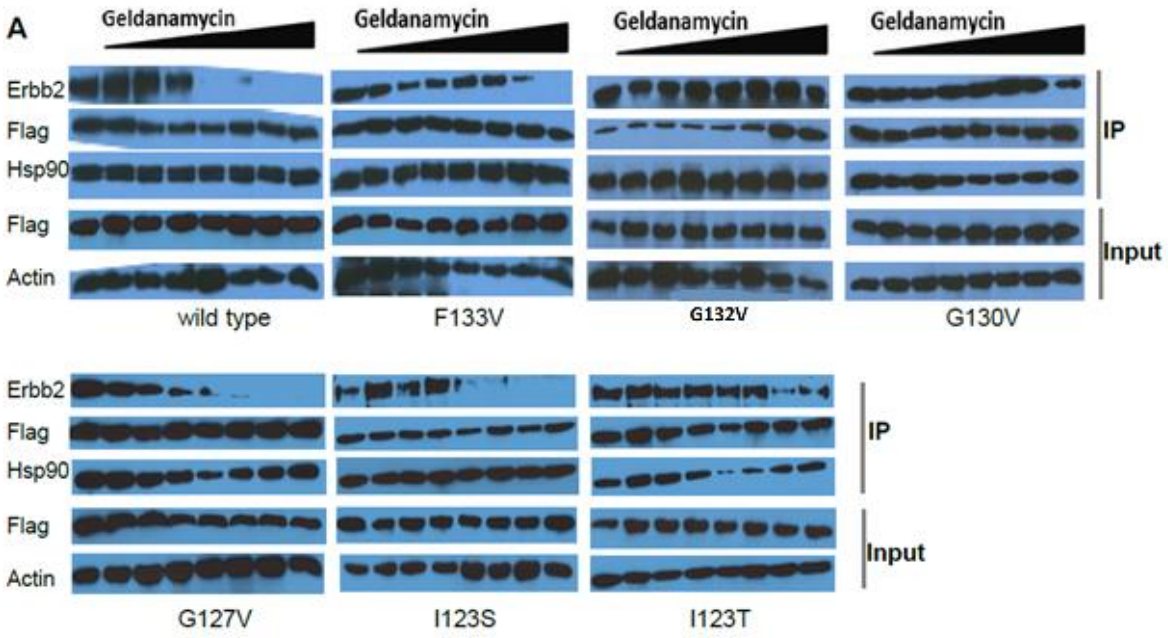
Figure 11: IL3 starving HSP90-Her2-Ba/F3-cells, Comparison

Comparison of Interleukin3 dependency of the cell-lines, transfected with HSP90. Percentage in the y-axis indicates percentage of GFP positive cells, ergo successfully transfected cells with ERBB2.

5.4. The mutant F133V is resistant to inhibitor treatment

We performed a protein degradation analysis to investigate the sensitivity of HSP90 wildtype and the mutations F133V, G123V, G130V, G127V, I123S, and I123T towards three HSP90 inhibitors. For the three inhibitors Geldanamycin, Tanespimycin and Alvespimycin the same conditions and concentrations of inhibitor were used at 8 different concentrations: untreated, 50nm, 100nm, 250nm, 500nm, 750nm, 1000nm and 2000nm. The cells were incubated with HSP90 inhibitor for 30 minutes at different concentrations and western blotting was performed on HSP90 immunoprecipitates (FLAG IPs).

We were able to demonstrate that for Geldanamycin and Alvespimycin all the mutations were more resistant to the inhibitor than that of the wildtype HSP90. This is shown by the intact mutant HSP90-ERBB2 interaction at higher HSP90 inhibitor concentrations compared to that of the mutant HSP90-ERBB2 interaction. Interestingly, the novel mutation F133V seems to be highly resistant to all inhibitors, it's interaction with the ERBB2 client is lost only at 2000nm Geldanamycin. Mutation G130V seem to be sensitive to only Tanespimycin but not Geldanamycin or Alvespimycin. In all the experiments, we ran HSP90 and FLAG as a control, being expressed stable.



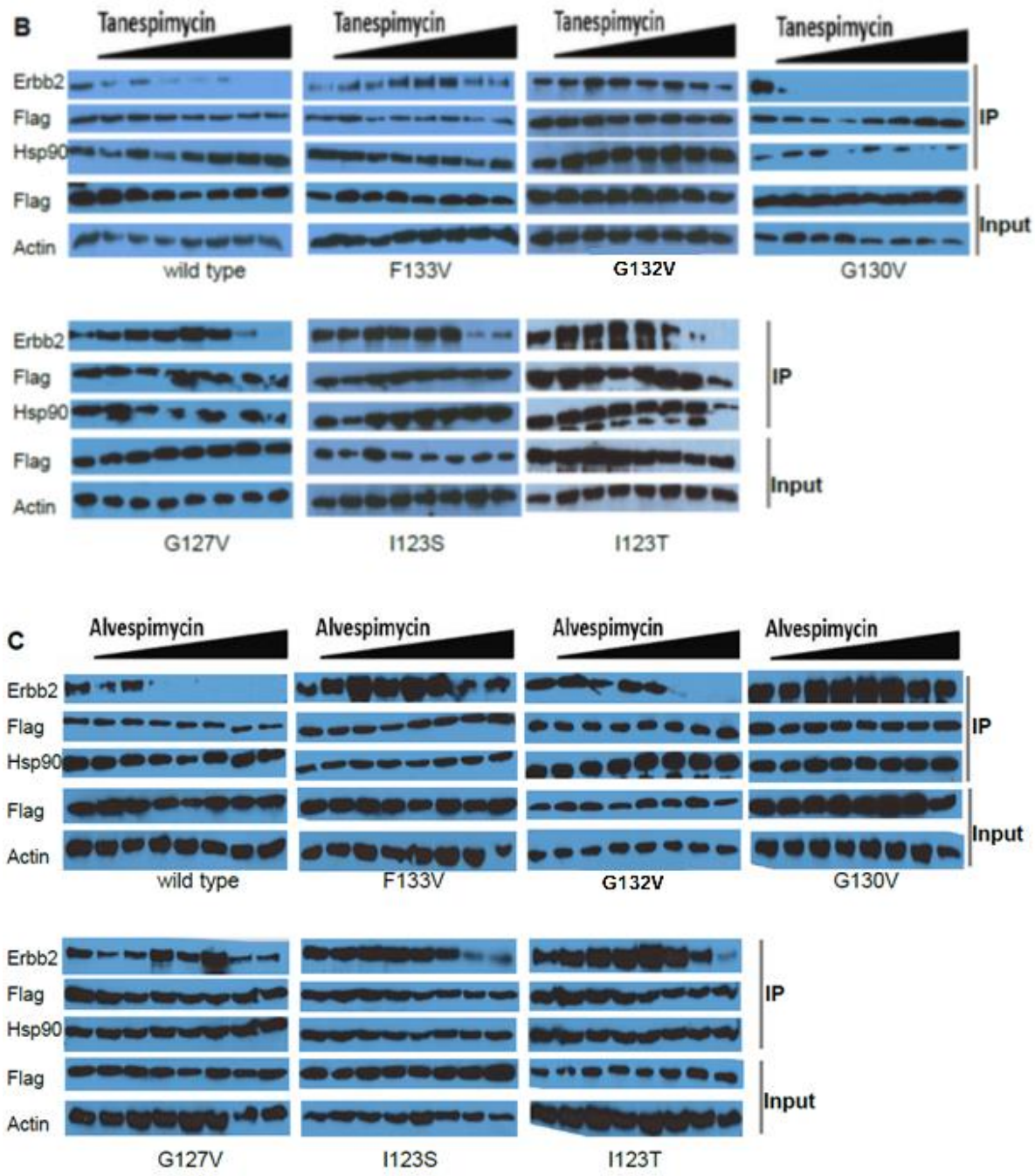


Figure 12: Protein degradation analysis

12A: Geldanamycin

12B: Tanespimycin

12C: Alvespimycin

6. Discussion

Targeted therapy of cancer has shown significant success in the last decade. However, the emergence of secondary drug resistance due to multiple mechanisms prompted researchers to look for alternate treatment strategies. Targeting molecular chaperones offers one such opportunity to treat cancer. HSP90 is a molecular chaperone whose role in cancer pathogenesis has been well documented. As it chaperones multiple oncogenes, a therapeutic approach of the ubiquitary expressed protein would be very convenient in the strategy of molecular treatment.[51, 56] HSP90 is an ATPase and chemical inhibitors that target its enzymatic activity have been developed and tested in pre-clinical studies, as a molecular target alone as well as in combination with other cytotoxic substances. [13, 58, 70, 100, 106] Moreover, several clinical trials have run and are underway to test the utility of HSP90 inhibitors as potential cancer chemotherapeutics.[47, 56, 61, 74, 103, 107] As emergence of drug resistance is observed with almost every targeted treatment, we tested if drug resistance occurs upon HSP90 treatment also. For this purpose, we have employed an in vitro drug resistance screen which was previously shown to successfully predict the drug resistance mechanisms upon targeted kinase inhibitor treatment.[95]

A point mutation in the HSP90 N-terminal domain was found to cause secondary drug resistance against ansamycin inhibitors. This observation is important given that HSP90 inhibitors are currently tested in the clinic. This further indicates that the patients who don't respond to HSP90 inhibitors need to be tested for the presence of HSP90 mutations. However, additional molecular mechanisms have been previously predicted that might cause HSP90 inhibitor resistance including the overexpression of HSP90 protein and drug efflux.[76] To test if the observed in vitro drug resistance was a result of HSP90 mutation, biochemical analysis was performed which indicated that the point mutation caused the resistance. This was done by cloning both the wild type and the mutant HSP90 cDNA followed by their expression in a mammalian system.

Before conforming the resistance of F133V, analysis of ectopically expressed HSP90 immunoprecipitates revealed that all the transfected HSP90 mutants interacted with the ERBB2 receptor. As we saw different levels of ERBB2 expression, we hypothesized a different affinity of the exogenous HSP90 mutations to ERBB2 or a lower ATPase activity due to the mutation. Especially HSP90 G127V – part of the three Glycines described by Bergerat, a crucial position of the ATP binding site – seems to interact

less with ERBB2.[12, 40] Even at different expression levels the presence of HER2 in all mutations was important for the following confirmation and comparability of resistance.

In a competitive growth assay with a murine cell line a difference in growth could be observed after transduction with ERBB2. While Ba/F3 parental, Ba/F3 carrying MSCV and Ba/F3 transduced with HSP90 wt showed a similar pattern of growth, Cells transfected with a mutant HSP90 were impaired in growth. This could indicate a lower affinity of binding and chaperoning HER2 also consistent with our data from the immunoprecipitation assay. As to why we could not observe a higher impairment, there is to state that exogenous HSP90 might not be expressed at the same level as that of the wild type endogenous HSP90 and might interfere with the expected results. Deletion of endogenous HSP90 after transduction with a functional exogenous chaperone would be an interesting way of avoiding this confounder.[22]

A murine cell line that also expresses the HSP90 client protein ERBB2 kinase was chosen to test the effect of HSP90 mutation on its interaction with the ERBB2 kinase upon inhibitor treatment. Disruption of interaction between the HSP90 chaperone and the ERBB2 client kinase after inhibitor treatment was chosen as a read-out to study the effects of point mutations in drug resistance. As documented before, the dependence of ERBB2 stability and its interaction with wild type HSP90 on dose-dependent inhibitor treatment proved to be a good biochemical assay system.[48] Importantly, the point mutation F133V identified in this study, when introduced into the HSP90 cDNA followed by its overexpression and immunoprecipitation analysis indicated the need for higher HSP90 inhibitor concentrations than observed for the wild type HSP90 to disrupt its interaction with the ERBB2 client kinase. In addition, three glycines in the same region (in the Bergerat fold), when mutated displayed similar effects indicating its important role in inhibitor binding. In this case regarding the Glycines in the position 127, 130 and 132.[12] Furthermore an amino acid change in I123 seems to have the same effect of inhibitor disruption. Interestingly this was the case not only the point mutation leading to I123T, that was described to be resistant to 17AAG, but also for the mitochondrial HSP90, TRAP1.

Previous studies have reported the importance of F133 in ATP binding as well as the binding of HSP90 inhibitor geldanamycin.[68] It was shown that F133 forms an H-bond with the ATP and several HSP90 inhibitors. Based on crystal structure data, it is likely that the mutation identified in this study could weaken the HSP90 interaction with

ansamycin inhibitors due to steric hindrance thus leading to drug resistance that emerged in the cell-based screen.[88] This is indeed proven from the western blot analysis of HSP90 immunoprecipitates after HSP90 inhibitor treatment. Thus, this study predicts the emergence of such point mutations in the N-terminal domain of HSP90 chaperone, that might cause secondary drug resistance in cancer patients when treated with HSP90 inhibitors. As mentioned above in multiple sequence alignment of various HSP90 isoforms F133 was found to be highly conserved across species,[5] which highlights the importance of the position this specific amino acid.

In addition to cancer therapy the development of mutations in Hsp90 may have an effect on various other entities, for instance it has been shown that also in Alzheimers disease the Hsps play an important role in degrading of the Tau protein.[15] This stresses the importance of research in this particular field of molecular medicine. Furthermore the toxicity seen in Hsp90 inhibitor studies could implicate the need of further exploration of inhibition of more specific inhibiting structures such as isoform dependent drugs,[28, 51] which could also overcome point mutation resistance shown in this work. New therapeutic goals could involve targeting the C-domain like Novobiocin, a recent discovery in the ally of Hsp90 inhibition, evaluating the co-chaperone activity and finding ways of blocking the interaction of Hsp90 with its clients.[41]

As HSP90 inhibitors are increasingly tested in various clinical trials, it is important to consider testing the HSP90 sequence as well as gene expression in non-responders to identify potential factors that determine the success of HSP90 inhibitor treatment. Therefore, further treatment strategies that involve HSP90 inhibitor in combination with other chemotherapeutics need to be tested to prevent the emergence of drug resistance against HSP90 inhibitor treatment. Moreover, additional HSP90 inhibitors as mentioned above that may prevent drug resistance may be developed and tested in pre-clinical settings. In conclusion, this study predicts possible factors that may determine the efficacy of HSP90 inhibitor treatment.

7. List of Figures

8. List of References

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