Aus dem Zentrum für Innere Medizin der Universität zu Köln
Klinik I für Innere Medizin
Direktor: Universitätsprofessor Dr. med. M. Hallek

HSP90 as a novel target therapy for the treatment of Hodgkin's Lymphoma

Inaugural-Dissertation zur Erlangung der Würde eines
doctor rerum medicinalium
der Hohen Medizinischen Fakultät
der Universität zu Köln

vorgelegt von

Farag Ibrahim Eltaib
aus Benghazi/ Libyen

Promoviert am 19. Mai 2010

Dekan:	Universitätsprofessor Dr. med. J. Klosterkötter
1. Berichterstatter:	Universitätsprofessor Dr. med. A. Engert
2. Berichterstatter:	Universitätsprofessor Dr. rer. nat. Dr. h. c. H Pfister

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Die dieser Arbeit zugrunde liegenden Experimente sind von mir mit Unterstützung von Prof. Dr. rer. nat. Elke Pogge-von-Strandmann und Herrn Dr. med. Boris Böll und Frau Dr. Katrin Reiners und dem Tierarzt Herrn Samir Tawadros durchgeführt worden.

Acknowledgements

I would like to graciously thank my supervisor, Prof. Dr. med. Andreas Engert, who accepted and supported me for my PhD study at University Hospital of Cologne.

I am greatly indebted to my thesis co-supervisor Prof. Dr. rer. nat. Elke Pogge-von-Strandmann for her guidance and encouragement. Throughout these years Prof. Dr. rer. nat. Elke Pogge-von-Strandmann has shown me the way of doing quality research.

I would like to especially thank Prof. Dr. Michael Hallek for giving me the opportunity to be a member of the department of internal medicine-I.

I would next like to thank Dr. med. Boris Böll, who guided me through every step of this study. Without his endless patience, this project would not have been possible.

I would also like to thank my thesis committee members for their constructive inputs and encouragement.

I also thank all the members from Prof. Dr. med. Andreas Engert and Prof. Dr. rer. nat. Elke Pogge-von-Strandmann Lab. I owe many thanks to their help, friendship and assistance.

I am grateful to the Libyan Minister of Education which provided me with scholarship and awards to make this possible.

Finally, I would like to express my thanks to my parents Ibrahim and Salma and to my family in Libya for being a source of encouragement and moral support.

This thesis is dedicated to my family

and to the soul of my beloved brother

Youssef (1973-2004)

Rahmat Allah upon him

who regretfully did not live to see this work

List of abbreviations

17-AAG	17-allylamino 17-demethoxygeldanamycin
7-AAD	7-amino-actinomycin
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
c-FLIP	Cellular caspase-8 (FLICE)-like inhibitory protein
C-terminal	Carboxyterminal
DMSO	Dimethylsulfoxide
DOXO	Doxorubicin
EDTA	Ethylenediamintetetracetic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Gemcit	Gemcitabine
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HSP90	Heat shock protein 90
IC50	Half maximal effective concentration
IKK	Inhibitor of NFkB kinase
IκB	Inhibitor of NFkB
kDa	kilo-Dalton
MDR	The multidrug resistance
µM	micro molar
Mg	milligram

min	Minute
mM	Millimolar
MRP	The multidrug resistance-associated protein
NFkB	Nuclear factor kappa-B
NP-40	Nonidet P-40
NQO1	NADPH quinone Oxidoreductase 1
PAGE	Polyacrylamid-Gel electrophoresis
PARP	Poly- (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PMSF	Phenylmethyl-sulfonylfluoride
r.p.m	Rotations per minute
RT	Room temperature
SD	Standard deviations
SDS	Sodiumdodecylsulfate
TEMED	N,N,N,N-tetramethylethylendiamin
Tween-20	Polyoxyethylen-sorbit-monolaurate
v/v	Volume per volume
VPA	Valproic acid
WHO	World Health Organization
XIAP	x-linked inhibitor of apoptosis protein
XTT	Sodium3'-[1-phenylamino-carbonyl]-3,4-tetrazolium]-bis[4-methoxy-6-nitro] benzenesulfonic acid hydrate

1. Introduction

1.1. Hodgkin's lymphoma: classification and epidemiology

Hodgkin's lymphoma (HL) accounts for approximately 11.1 % of all malignant lymphomas worldwide diagnosed in 2008 (www.seer.cancer.gov) (about 62000 annual cases) (Parkin et al., 2005). HL occurs most frequently in two age peaks: the first one is in young adulthood (age 15-35) and the second around the age of 50 years (Diehl et al., 1995). Based on differences in histology, morphology and immunophenotype, the World Health Organization (WHO) classified HL into two different disease entities: the rare nodular lymphocyte predominant HL (NLPHL) and the classical HL (cHL), representing approximately 5% and 95% of cases, respectively (Nogová et al., 2006). Based on histopathological features, classical HL can be further subdivided into: Nodular sclerosis (NS), Mixed cellularity (MC), Lymphocyte rich (LR) and Lymphocyte depleted (LD), and the most common cHL subtypes are NS (40-80%) and MC (20-40%) (Nogová et al., 2006). Hodgkin's lymphoma is characterized by the presence of only a small fraction of malignant Hodgkin and Reed-Sternberg (H-RS) cells. H-RS cells, large mononucleated Hodgkin and multinucleated Reed-Sternberg (H-RS) cells are surrounded by numerous non-malignant reactive cells including B- and T- lymphocytes, eosinophils and fibroblasts (Gruss and Kadin 1996). About 98% of H-RS cells carry monoclonal immunoglobulin (Ig) gene rearrangements and thus derive from germinal center B cells (or rarely, T cells) but have a heterogeneous and largely uncharacterized phenotype (Küppers 2002).

Hodgkin Lymphoma is highly curable, even in advanced stages. To date, approximately 95% of patients with HL in early stages and 85-90% in advanced stages can be cured (Diehl et al., 2007). Current treatments of HL consist of polychemotherapy regimens such as BEACOPP or ABVD. Chemotherapy is commonly combined with involved field radiation in early and intermediate stages

and irradiation of residual masses in advanced stages in the first line treatment. Patients who relapse after chemotherapy can achieve long-term remissions and even cure with high dose chemotherapy (HD-CT) and autologous stem cell support (aSCT). For patients who relapse after HD-CT and aSCT, allogeneic stem cell transplant is considered a potentially curative treatment (Schmitz et al., 2002). Although significant progress has been made in the last decades in the treatment of HL, these treatments cause long-term side effects such as infertility, cardiopulmonary toxicity and secondary malignancies (Diehl et al., 2007). Therefore, new therapeutic agents are needed to enhance the cure rate and to reduce treatment-related toxicity (Franklin et al., 2006; Diehl et al., 2007).

1.2. Hodgkin's lymphoma molecular biology

Recently, two essential features of Hodgkin's lymphoma have been established, germinal centre B cells as a cellular origin of Hodgkin and Reed-Sternberg (H-RS) cells and constitutive NF-kB (p50/p65) activation as a biological base for H-RS cells (Thomas et al., 2004; Müschen et al., 2000). Constitutively active NF-kB protects HL cells from apoptosis and is involved in irregular growth and cytokine gene expression. Several molecules involved in constitutive NF-kB activation in H-RS cells such as Epstein-Barr virus latent membrane protein (LMP-1), mutations in the NF-kB inhibitors IkB-alpha or IkB-epsilon and IL-1β have been identified in the last years (Schmitz et al., 2009; Bräuninger et al., 2005).

In about 40% of cHL cases, the H-RS cells are infected by Epstein-Barr virus (EBV). Interestingly, nearly all cases of HL with destructive Ig gene mutations eliminating BCR expression (e.g. nonsense mutations) are EBV-positive, suggesting that EBV-encoded genes have a particular function to prevent apoptosis of HRS-cell precursors that acquired such crippling mutations (Bräuninger et al., 2005).

H-RS cells are also characterized by overexpression of several TNF family receptors, including CD30, CD40 and receptor activator of NF-kB (RANK) (Zheng

et al., 2004). These receptors frequently share overlapping biologic functions such as activation of NF-kB, regulation of cytokines and chemokines secretion, and regulation of HL cell growth and survival. However, because these receptors have overlapping biologic functions, identifying a shared signaling pathway among these receptors could simplify treatment strategies (Zheng et al., 2003).

Constitutively activated NF-kB in H-RS induces the expression of antiapoptotic genes, such as cFLIP, XIAP and Bcl-xL that antagonize the extrinsic or intrinsic apoptotic pathway in H-RS cells (Thomas et al., 2002; Kashkar et al., 2003; Chiu et al., 2007). NF-kB has also been shown to control the expression of other genes that are linked with tumorigenesis and proliferation (such as c-myc and cyclin D1), invasion (MMP-9) and angiogenesis (VEGF).

Furthermore, the NF-kB and activator protein1 (AP-1) transcription factors are thought to synergistically regulate many genes involved in apoptosis protection and cell proliferation and a large number of cytokines and chemokines produced by the H-RS cells (Re et al., 2005). Thus, constitutive NF-KB activation is likely to be a key mechanism in the pathogenesis of HL (Küppers 2002). In addition several other signaling pathways and transcription factors, including receptor tyrosine kinases and Janus kinase/signal transducer and activator of transcription (JAK/STAT) were also identified to be a pathogenetically important feature of H-RS cells (Schmitz et al., 2009; Bräuninger et al., 2005). A brief summary of the molecular mechanisms of classic Hodgkin's lymphoma listed in (Table 1).

Recently, most of the known key oncogenic proteins involved in HL cell survival have been identified as HSP90 client proteins (Xu et al., 2004; Zhang et al., 2004). Moreover, constitutive IKK/NF-κB activation in HL cells is dependent on HSP90 function and can be inhibited by treatment with the HSP90 inhibitor Geldanamycin (GA) (Broemer et al., 2004). Thus, targeting HSP90 function may be as effective as targeting multiple oncogenic pathways in HL leading to inhibition of all the hallmark traits of malignancy (Broemer et al., 2004; Yang et al., 2006).

Table 1. Summary of the molecular mechanisms of classic Hodgkin's lymphoma (Felberbaum 2005)

Molecular Mechanisms of Classic Hodgkin's Lymphoma
Epstein-Barr Virus-positive cells: Viral LMP2A expression leads to an aberrant transcriptional profile
Epstein-Barr Virus-positive or negative cells: **Constitutive NF-κB activity leads to inappropriate cell growth:** • Arises from mutations in IκB • Arises from mutations in REL • Arises from JunB overexpression leading to CD30 overexpression **Resistance to apoptosis prevents cell death**: • Arises from c-FLIP overexpression • Arises from XIAP overexpression • Arises from inactivation of the tumor suppressor gene RASSF1A

1.3. Heat shock proteins 90 (HSP90)

Heat shock protein 90 (HSP90) is one of the most abundant cellular proteins. HSP90 accounts for 1–2% of total protein in unstressed cells and increases to 2–10% under stress (Ferrarini et al., 1992). HSP90 is a member of the family of heat shock proteins (HSPs) which function as molecular chaperones and play a critical role in protein folding, stabilization, transportation, and degradation (Jaattela 1999; Hartl et al., 2002; Amere and Csermely 2004). These processes are essential for the normal functions of cellular proteins. As their name indicates, HSPs are expressed in response to heat shock and other conditions of stress such as environmental changes, genetic mutations, oxidative stress, heavy metals, hypoxia, acidosis, and metabolic toxins (Lindquist and Craig 1988; Hartl et al., 2002). Heat shock proteins vary in their cellular localization and functions and mammalian HSPs have been classified according to their molecular weights: HSP90, HSP70, HSP60, HSP40 and the small HSPs such as HSP27 (Morimoto et al., 1997; Hartl et al., 2002) (Table 1).

Unlike other HSPs, HSP90 was found to interact specifically with many proteins involved in transcriptional regulation and signal transduction, such as steroid hormone receptors (Cadepond et al., 1991), transcriptional factors (Xu et al., 2004), and protein kinases (Pearl and Prodromou 2000). Recent studies show that HSP90 is abundantly expressed in primary and cultured Hodgkin's lymphoma cells (Broemer et al., 2004; Georgakis et al., 2006). The current study therefore will focus on HSP90 as a target for HL therapy.

1.3.1. Heat shock protein 90 structure

HSP90 is a homodimer containing two to three covalently bound phosphate molecules on each monomer. Each homodimer is made up of monomers that consist of three distinct domains that have important functional interactions: a highly conserved 25 kDa ATP binding N-domain, a 35 kDa M-domain involved in client protein binding and the 12 kDa C-domain, the main region for dimer

interaction with co-chaperones such as the stress induced phosphoprotein 1 (Sti1/Hop). Binding and hydrolysis of ATP at the N-terminal site results in a conformational change in HSP90 which enables conformational changes in the client protein. The presumed site of binding for client proteins, the "client site", is in the V-shaped pocket formed between the C domains, while the main site for the interaction with essential cofactors of HSP90, so called cochaperones and immunophilins, resides between the N and M domains. (Grenert et al., 1997; Pearl et al., 2006) (Figure 1).

In humans, two closely related HSP90 isoforms have been described so far, HSP90α and HSP90β (Csermely et al., 1998). Both isoforms are induced by stress and contain intronic sequences, a rather unique feature of HSP90 compared to the other HSP proteins that lack introns. Although no major differences in the functions of the two isoforms have been determined yet, differences in their respective modes of regulation have been observed: HSP90α is more inducible than HSP90β (Csermely et al., 1998). HSP90α seems to be a fast-response, cytoprotective isoform, while HSP90β seems to be associated with long-term cellular adaptation and facilitated cellular evolution (Amere et al., 2004). Furthermore, HSP90β expression was shown to be associated with the development of drug resistance (Bertram et al., 1996). HSP90β is generally expressed at higher levels than HSP90α, and is therefore considered the major form of HSP90 involved in normal cellular functions, such as maintenance of differentiation and cytoprotection. Additional HSP90 isoforms include HSP90N, which is associated with cellular transformation (Grammatikakis et al., 2002), Grp94 in the endoplasmic reticulum and HSP75/TRAP1 in the mitochondrial matrix (Csermely et al., 1998) were added to the HSP90 family (Table 2). HSP90 isoforms, functions and major expression status are summarized in Table 3. HSP90 isoforms have five highly conserved regions, called ‘signature sequences’, of which three are in the N-terminal domain (amino acids 38-59, 106-114, 130-145 of human HSP90α) and two in the middle domain (amino acids 360-370 and 387-401) (Amere et al., 2004) (Figure 2).

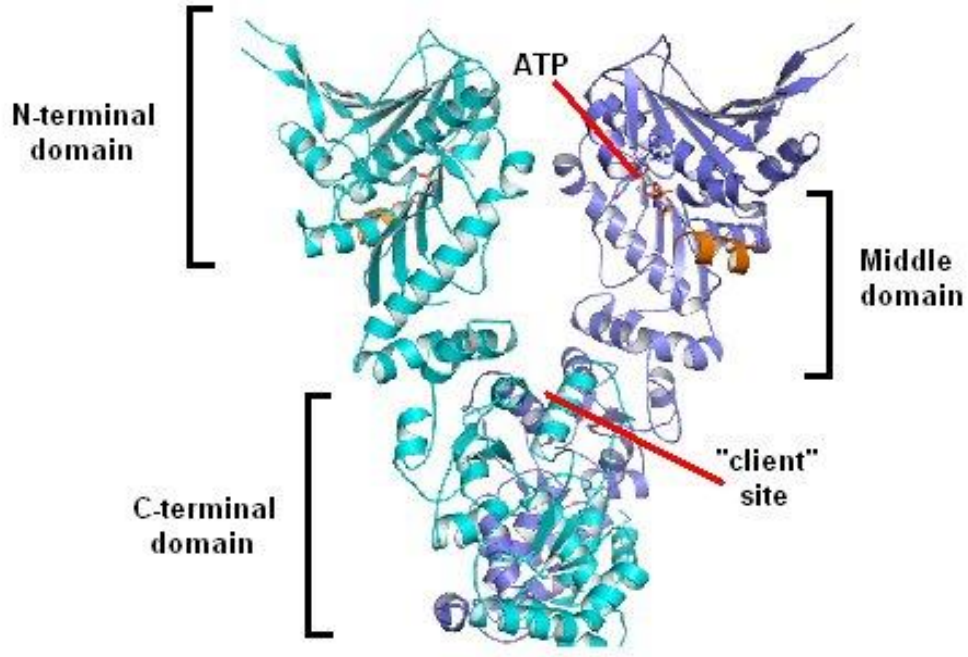

Figure 1. HSP90 structure (Dollins et al., 2007)

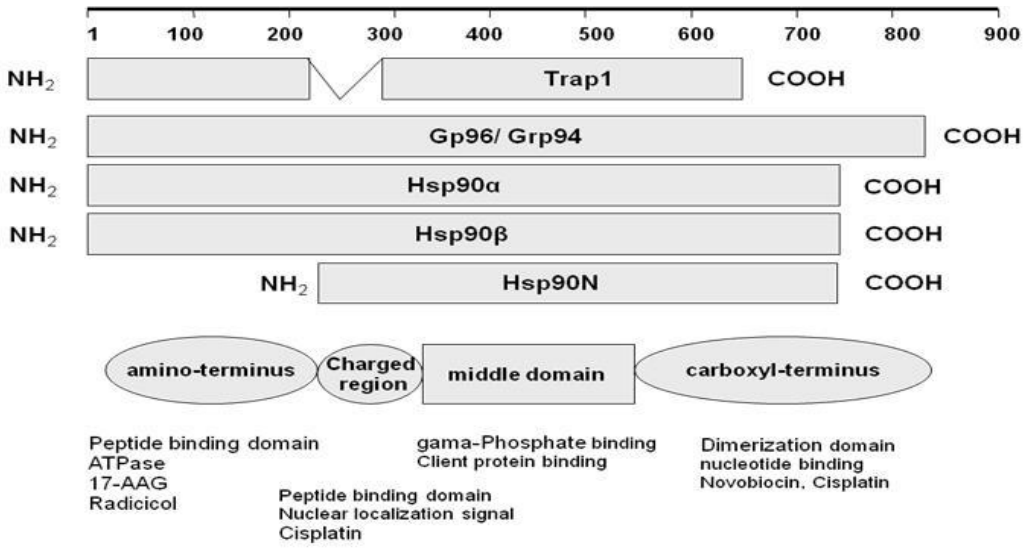

Figure 2. HSP90 isoforms (Dollins et al., 2007)

(The numbering 1-900 refers to the amino acid sequence).

Table 2. HSP90 isoforms, functions and major expression status (Sreedhar et al., 2004)

Isoforms	Specific Function	Major expression status
HSP90α	Growth promotion Cell cycle regulation Stress induced Cytoprotection	Induced
HSP90β	Early embryonic development Germ cell maturation Cytoskeletal stabilization Cellular transformation Long term cell adaption	Constitutive
HSP90N	Cellular transformation	Constitutive
HSP75/ TRAP1	Cell cycle regulation	Constitutive

Table 3. Name, localization and function of heat shock proteins (Goetz et al., 2003)

Name	Localization	Specific Function
HSP104	Cytoplasm	Releases proteins from aggregates
HSP90 α & β	Cytoplasm	Prevents protein aggregation, helps protein stabilization and trafficking, facilitates activation of many regulated proteins
GRP94	Endoplasmic reticulum	Quality control of protein processing in the endoplasmic reticulum
TRAP/ HSP75	Mitochondria	Unknown
HSP70	Cytoplasm	Prevents protein aggregation, helps protein folding
GRP78, BiP	Endoplasmic reticulum	Protein import and folding in the Endoplasmic reticulum
HSP60	Cytoplasm and mitochondria	Prevents protein aggregation, helps protein folding
HSP40/HDJ2	Cytoplasm	Helps protein folding as a co-chaperone of HSP70
HSP27	Cytoplasm	Prevents protein aggregation may have role in cell growth and differentiation

1.3.2. Heat shock protein 90 function

Malignant cells have been shown to express high levels of HSP90 and to be dependent upon HSP90 for the correct folding and function of mutated and overexpressed client proteins (Workman 2002).

HSP90 protects client proteins from degradation and environmental stress, including heat, hypoxia, free radicals, radiation, and chemotherapy. An elevated HSP90 level allows the accumulation of proteins of mutated genes in the cell, which may be evolutionarily advantageous to genetic diversity. In addition, increased HSP90 activity would also permitted the survival of genetically unstable cancer cells (Ma Wen and Adjei 2009). In normal cells, HSP90 exists in an inactive state, whereas in cancer cells HSP90 is present entirely in multiprotein complexes with high ATPase activity (Kamal et al., 2003) (Figure 3).

Since many of HSP90's client proteins are involved in cell signaling, proliferation and survival, HSP90 has received considerable attention as a potential target for cancer therapy. HSP90's client proteins, including several protein kinases, steroid hormone receptors and transcription factors, are involved in signal transduction, cell cycle control and transcriptional regulation and are thus important in controlling cell growth, proliferation, differentiation and survival (Goetz et al., 2003; Pratt and Toft 2003). Interestingly, most of these proteins are of major interest in cancer therapy. These include NF-kB, Akt, Her2, androgen receptor and HIF-1 alpha.

The number of reported HSP90 client proteins now exceeds 100 (See http://www.picard.ch/downloads/Hsp90interactors.pdf for an up to date list of HSP90 client proteins).

HSP90 client proteins are involved in all six features of malignant cells, that have been suggested as “hallmarks of cancer”: self-sufficiency in growth signaling, insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2000) (Figure 4). Specific mutations in the client proteins or the abnormal expression of these

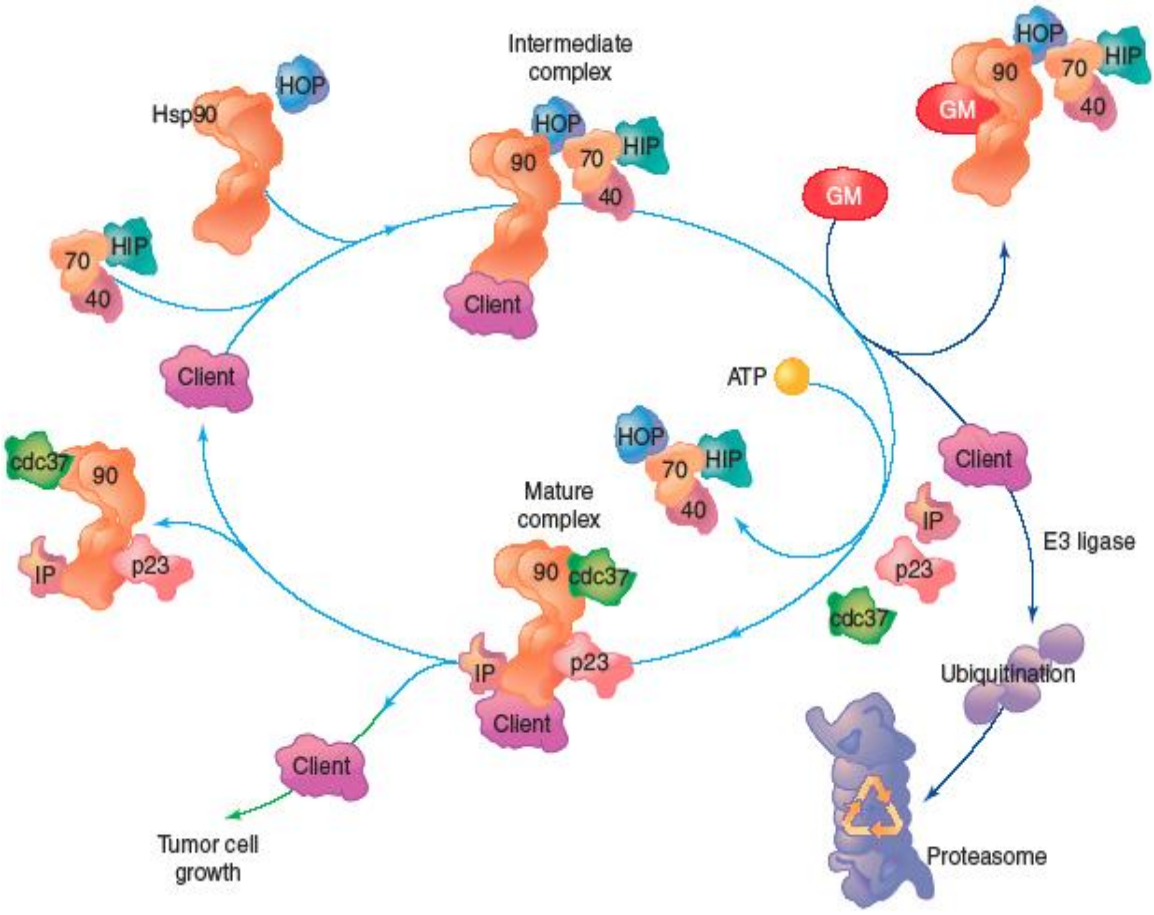

Figure 3. HSP90 Chaperon Complex Cycle (Kamal et al. 2004)

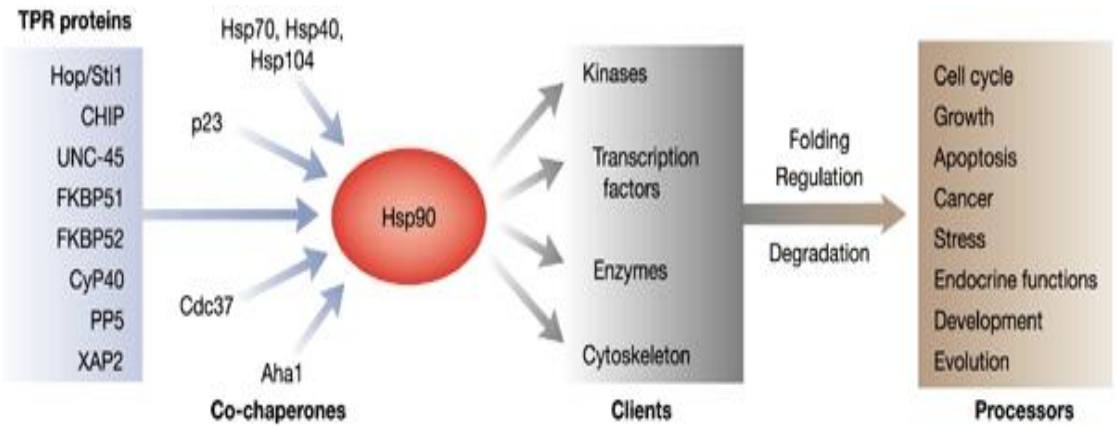

Figure 4. Examples of roles of HSP90 and its co-chaperones in different cellular processes through their interactions with different client proteins (Caplan et al., 2003).

client proteins promote the growth of malignant cells, resulting in tumor and disease progression.

Although there are numerous known client proteins for HSP90, the HSP90 binding process has only been studied with a few of these proteins.

The binding between HSP90 and the progesterone receptor (PR) has provided a useful model for HSP90 clients (Hernandez et al., 2002) (Figure 5). This binding occurs in steps, initial recognition of the PR is performed by HSP40 and HSP70 both binding the PR in a cooperative step that involves ATP hydrolysis (Hernandez et al., 2002). This results in the assembly of a large complex where the proteins HIP and Hop bind to HSP70 and HSP90 is engaged through its interaction with Hop. The last step involves the loss of much of the HSP70, HSP40 and Hop. When HOP leaves the complex, one of the immunophilin proteins can bind to HSP90. Simultaneously, the co-chaperone p23 binds directly to the HSP90 homodimer, the resulting conformational changes results in a PR with an active hormone-binding site (Figure 5). Then the HSP90-receptor complex dissociates, thus allowing the receptor to bind to DNA.

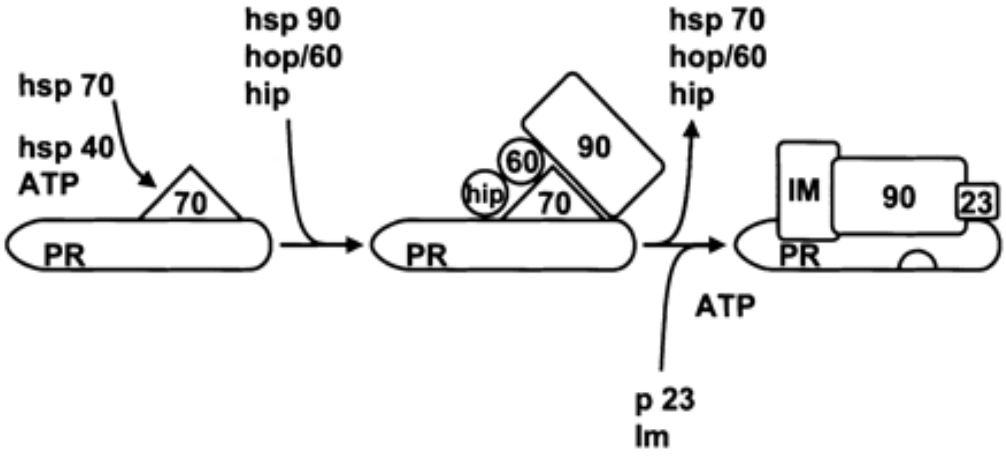

Figure 5. A model for the binding of heat shock protein 90 to the progesterone receptor (PR) (Hernandez et al., 2002).

1.4. HSP90 inhibitors

Since HSP90 client proteins played critical roles in tumor growth and maintenance, inhibition of HSP90 has emerged as a promising approach for the treatment of cancer. Understanding the crystal structure of HSP90 allowed researchers to develop several small molecules, including 17-AAG and others, that bind to the ATP pocket in the N-terminal domain of HSP90 and inhibit the essential ATPase activity of HSP90 leading to inactivation, destabilization, and proteasomal degradation of many HSP90 client proteins by the ubiquitin-dependent proteasome pathway (Whitesell et al., 1994; Solit et al., 2003; Chiosis et al., 2001; Isaacs et al., 2003).

Recent studies suggest that HSP90 found in tumor cells exists in a highly active state that is more susceptible to HSP90 inhibitors compared to HSP90 in nonmalignant cells (Kamal et al., 2003; Zhang and Burrows 2004).

Inhibitors of HSP90 such as 17-AAG exhibited biologic activity against many human tumor cell lines through downregulation of client proteins, such as ErbB2, mutant p53, C-Raf, and Bcr-Abl, and has shown antitumor activity in preclinical experiments (Ramanathan et al., 2005). 17-AAG is currently being tested in various solid and hematological malignancies, either as a single agent or in combination with conventional chemotherapeutics. 17-AAG has completed Phase I clinical trials, and several Phase II trials are in progress for different types of solid and hematological tumors.

Although 17-AAG has entered clinical trials with some promising early data, it demonstrated limitations including poor solubility, stability, and hepatotoxicity (Sharp and Workman 2006). In addition, dose-limiting toxicities of 17-AAG included gastrointestinal disturbances, anemia, thrombocytopenia, dehydration, and hyperglycemia (Ma Wen and Adjei 2009). In addition, expression of P-Glycoprotein (P-GP) or loss or mutation of the NQO1 gene that is required for the bio-reduction of 17-AAG to the more potent hydroquinone 17-AAGH2, have been suggested as mechanisms of de novo or acquired resistance to 17-AAG (Guo et al., 2005; Chandarlapaty et al., 2008). Therefore, second- and third-

generation HSP90 inhibitors that are not substrates for P-glycoprotein and do not require NQO1 metabolism could be preferable (Ma Wen and Adjei 2009). Other HSP90 inhibitors under clinical evaluation include 17-DMAG, IPI-504, and KOS-953, radicicol, novobiocin, LY294002, SNX2112 and BIIB02. Each of the HSP90 inhibitors was active *in vitro*, sometimes leading to more potent disruption and degradation of HSP90 client proteins than 17-AAG (Workman et al., 2007).
Consequently these small molecule inhibitors have demonstrated to be of great value in identifying new HSP90 client proteins and in understanding the biology of HSP90. Furthermore, novel HSP90 inhibitors could have antitumor activity in a variety of tumors (Zhang and Burrows 2004). However, it remains to be elucidated, whether these compounds will offer greater clinical benefit than 17-AAG while maintaining an acceptable toxicity profile (Goetz et al., 2003).

1.5. BIIB021

BIIB021 is a synthetic novel purine-base that binds to the ATP binding site in the N-terminal domain of HSP90. Inhibition of the ATPase activity by BIIB021 results in degradation of various HSP90 client proteins including HER-2, AKT, and Raf-1 (Lundgren et al., 2009). BIIB021 is a promising new oral inhibitor of HSP90 with antitumor activity in preclinical models. Recent studies showed that BIIB021 is more potent than 17AAG and has cytotoxic activity against many cancer cell lines (Lundgren et al., 2009). Furthermore, BIIB021 is not susceptible to tumor cell resistance mediated by MDR, MRP and NQO1 that limit the potency of 17-AAG (Lundgren et al., 2009). BIIB021 was administered to humans for the first time at “MD Anderson Cancer Centre” (USA) for relapsed patients with ZAP-70 positive chronic lymphocytic leukemia (CLL), in October 2005 (Solit and Chiosis 2008). BIIB021 is currently in phase I/ II clinical trials in CLL (NCT00345189) and in gastrointestinal Stromal tumors (GIST) (NCT00618319). Preliminary data obtained from these trials revealed that BIIB021 is a promising novel oral HSP90

inhibitor with improved solubility, bioavailability and cytotoxicity in cancer cells, encouraging us to investigate the inhibitory effect of BIIB021 on Hodgkin's lymphoma cells compared to 17-AAG.

1.6. HSP90 as a novel target in Hodgkin's Lymphoma

Although the treatment of Hodgkin's lymphoma (HL) with a polychemotherapy approach has been very successful, it is often associated with serious complications from therapy, including sterility, cardiovascular disease and high incidence rates of secondary cancers (Diehl et al., 2004).

Therefore, the current goal in HL treatment is to find new specific treatments and therapies that exclusively target the deregulated signaling pathways which cause HL cell proliferation and apoptosis resistance. HSP90 is abundantly expressed by a variety of tumor types and has been recently targeted for HL therapy (Georgakis et al., 2006). Although Hsp90 is highly expressed in most cells, HSP90 inhibitors selectively kill cancer cells.

Constitutive NF-KB activation is likely to be a key mechanism in pathogenesis of HL (Küppers 2002). In addition to NF-kB, several other signaling pathways and transcription factors show deregulated activity in HL cells, including Jak–Stat, PI3K–Akt, Erk, AP-1, notch 1 and receptor tyrosine kinases, were also identified to be features of HL cells (Younes and Carbone 2003; Schmitz et al., 2009; Bräuninger et al., 2005). Members of the inhibitors of apoptosis protein (IAP) family such as XIAP, survivin, cIAP1, and cIAP2 regulate apoptosis by inhibiting caspases activity (Deveraux et al., 1999). The X-linked inhibitor of apoptosis protein (XIAP) has been recently found to be a crucial mediator of apoptosis resistance in Hodgkin's lymphoma cells (Kashkar et al., 2003). It is known that NF-kB mediates apoptosis resistance by upregulation of antiapoptotic genes like XIAP. On the other hand, NF-kB changes its characteristics at selective promoters in presence of DNA damaging agents and so converts from an activator into a repressor of these genes (Campbell et al., 2005).

Interestingly, all of the above mentioned molecular targets have been identified as client proteins of HSP90, i.e. their stability and function depends on HSP90, and inhibition of HSP90 leads to degradation of the proteins via the ubiquitin-proteasome pathway (Wedgwood et al., 2005).

Over expression of HSP90 has been implicated in the protection of various cancer cells against drug-induced apoptosis (Ray et al., 2004; Guo et al., 2005; Schepers et al., 2005; Thomas et al., 2005). HSP90 inhibitors such as GA and 17-AAG inhibit several key signaling pathways including NF-kB and STAT3, leading to a decreased expression of proteins involved in cell proliferation and apoptosis, such as Bcl-xL, Bcl-2, cFLIP, c-IAP1, XIAP, survivin and cyclin D1 (Ramanathan et al., 2005; Bai et al., 2004; Georgakis et al., 2006). Interestingly, 17-AAG caused cell cycle arrest and a significant reduction in HL cell viability. It suppresses the expression of cyclin D1, a component subunit of cyclin-dependent kinases (CDK) 4 (Cdk4) and 6 (Cdk6) (Georgakis et al., 2006; McDonald and El-Deiry 2000).

In addition, cyclin-dependent kinases were extensively studied in relation to the p53, Rb, p16 and p27 expression status because alterations of the p53, Rb/p16 and p27 pathways have been associated with increased expression of G1/S and G2/M cyclins in lymphomas (Bai et al., 2004).

Furthermore, 17-AAG induces apoptosis in HL cells by activating caspase-8, which leads to cleavage of Bid, thus resulting in sequential release of mitochondrial cytochrome C and activation of caspase-9 and caspase-3, poly ADP ribose polymerase (PARP) cleavage (Amere et al., 2004; Georgakis et al., 2006).

Targeting HSP90 could have several benefits in HL therapy. First, HSP90 inhibition increases target-specific damage in tumor tissues. This is due to the increased HSP90 expression in tumors and preferential accumulation of HSP90 inhibitors in malignant cells (Kamal et al., 2003). Second, HSP90 inhibition will result in simultaneous down regulation of multiple pathways because many of the signaling proteins are hsp90 client proteins. Third, because HSP90 inhibition targets multiple pathways, the probability that tumor cells will escape a single-

target therapy is minimized (Kitano 2003; Neckers 2007). Thus, targeting HSP90 represents a promising strategy for the therapy of HL.

2. Objectives

Recent studies demonstrated that HSP90 is highly expressed in primary and cultured HL cells (Georgakis et al., 2006). Thus inhibition of HSP90 is might be a promising approach for the treatment of HL. Inhibition of HSP90 by 17-AAG in HL cell lines induced cell cycle arrest followed by induction of apoptosis (Georgakis et al., 2006). Furthermore, 17-AAG has shown to enhance the effect of chemotherapy and antibodies in HL cells (Georgakis et al., 2006).
17-AAG has limitations as a therapeutic agent such as poor solubility and manageable formulation (Pacey 2006). To date, the effects of BIIB021 on HL cells has not been studied.

The aim of this study was:

1- To assess the effect of BIIB021 on cell viability and apoptosis in Hodgkin's lymphoma cell lines in comparison to 17-AAG as a reference substance.
2- To investigate whether BIIB021 can selectively induce apoptosis and inhibit cell viability in HL cells using donor-derived lymphocytes as a non malignant reference cell population.
3- To test the combination of BIIB021 with gemcitabine, doxorubicin or valproic acid on Hodgkin's lymphoma cell lines.
4- To assess the effect of BIIB021-mediated HSP90 inhibition on X-linked inhibitor of apoptosis protein (XIAP) in Hodgkin's lymphoma cell lines.
5- To assess the effect of BIIB021and 17-AAG on HL cells growth *in vivo*.

3. Materials and Methods

3.1. Materials

3.1.1. Reagents

The HSP90 inhibitor 17-allylamide-17-demethoxygeldanamycin (17-AAG) was purchased from InvivoGen (Toulouse, France), seven novel purine-based HSP90 inhibitors, EC78, EC82, EC137, EC138, EC141, EC144 and EC151 were kindly provided by Biogen (San Diego, CA, USA). All compounds were stored as a 10 mM stock solution in dimethylsulfoxide (DMSO, Sigma) at -80 °C.
Gemcitabine, Doxorubicin and Dexamethasone were obtained from Lilly (Indianapolis, IN), CellPharm (Hannover, Germany) and Jenapharm (Jena, Germany), respectively. Histone deacetylase inhibitor, Valproic acid was purchased from Desitin (Hamburg, Germany).

3.1.2. Antibodies and Molecular Weight Markers

Antibodies	**Manufacturer**	**Dilution**
Rabbit monoclonal anti- PARP	Cell signaling, #9542	1: 1000
Mouse monoclonal anti- XIAP	Becton Dickinson, #610762	1: 1000
Mouse monoclonal anti- HSP90	Stressgen, #830	1: 1000
Mouse monoclonal anti- HSP70	Stressgen, #SPA- 810	1: 1000
Rabbit monoclonal anti- Actin	SantaCruz Biotech, #C1903	1: 1000
Secondary antibodies (HRP) (Goat anti-Mousa IgG)	Dianova, #115-006-071	1: 10 000
Secondary antibodies (HRP) (Goat anti-Rabbit IgG)	Dianova, # 711-036-152	1: 10 000
Magic molecular weight marker	Invitrogen	

3.1.3. Chemicals

All chemicals used were of analytical grade and listed below**.**

Chemical	**Manufacturer**
Annexin-V-FITC Apoptosis detection Kit: Annexin-binding buffer, Annexin-V-FITC, 7-amino-actinomycin (7-AAD)	BD Biosciences
Milk powder	ROTH
TRIS HCL	SIGMA
TRIS Base	SIGMA
Sodiumdodecylsulfate (SDS)	Merck
HEPES	JT. Backer
Acrylamide	ROTH
Ethanol	JT. Backer
Potassium dihydrogen phosphate	Merck
Potassium chloride	Merck
Bovine serum albumin (BSA)	SIGMA
Ammonium persulfate (APS)	SIGMA
Dimethylsulfoxide (DMSO)	SIGMA
Fetal calf serum (FCS)	SIGMA
Formaldehyde solution	ROTH
N,N,N,N-tetramethylethylendiamin (TEMED)	ROTH
Polyoxyethylen-sorbit-monolaurate (TWEEN-20)	SIGMA
RPMI-1640 Medium	GIBCO, BD Biosciences

Phenacin	SIGMA
Trypanblau	SIGMA
EDTA	SIGMA
Sodium Azid	Merck
Propidiumiodid	SIGMA
Di-sodiumhydrogenphosphate dihydrate	ROTH
Sodium Chloride	ROTH
Glycerol	SIGMA
Bromophenol blue	SIGMA
β-mercapitalethanol	SIGMA
Isopropanol	Roth

3.1.4. Buffers and Solutions

NP-40 Lysis Buffer	150mM NaCL, 1% NP-40, 50mM Tris pH 8.0
Transfer buffer	0,01M Di-sodiumhydrogenphosphate dihydrate
5x Laemmli Buffer	250mM Tris-HCl pH 6.8, 5 % SDS, 5 % β-mercaptoethanol, 10 mM EDTA, 35 % glycerol, bromphenolblue
SDS-PAGE running buffer	(10x Tris-Glycin-SDS-Buffer): 0.25 M Tris-HCl, 1.92 M glycine, 0.1% SDS; pH 8.6
10x Phosphate-Buffered Saline (PBS)	1.37mM NaCL, 27mM KCL, 20mM (KH_2PO_4), 100mM (Na_2HPO_4) x 2 H_2O
Secondary antibody solution	5 % dry milk in 1x PBS, 0.01 % TWEEN

Blocking buffer	5 % dry milk in 1x PBS, 0.01 % TWEEN
Washing buffer 1x (PBST)	1x PBS, 0.01 % TWEEN-20
FACS buffer	2g BSA, 2g NaAzid, Add 1L 1 x PBS
FACS binding buffer	0.01 mol/L HEPES/ NaOH, 0.14 mol/L NaCl, 2.5mmol/L $CaCl_2$, pH 7.4

3.1.5. Laboratory equipment and technical devices

Mini Trans-Blot Cell	BioRad, München, Germany
Electrophoresis Power supply:	BioRad, München, Germany
Cell culture: Culture flasks, disposable pipettes, plates, plastic disposable.	Becton Dickinson, Heidelberg, Germany
Cell culture hood (LaminAir HB-2448)	Heraeus, Fellbach, Germany
CO_2 Incubator	HERA CELL, Hanau, Germany,
Biometra Personal Cycler	Biometra, Gottingen, Germany
Microscopes	Zeiss, Jena, Germany
Centrifuges	GR422 (Jouan), J-6B (Beckmann), Eppendorf centrifuge 5415C & 5417C (Eppendorf)
FACS Calibur	BD Biosciences, USA
Imaging Systems: CURIX 60	AGFA, Germany
ECL X ray films	Chemiluminescent Detection Film (GE Healthcare, München, Germany)
ELISA Reader µQuant	BioTech, High Point, USA
Balances	Sartorius, Gottingen, Germany

Sonicator	Bandelin Electronic, Berlin, Germany
Vortex Rotamax 120	Heidolph, Schwabach, Germany
Water baths	GFI, Germany
Software	GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA 92130 USA) Cell Quest software (BD Biosciences, USA) CalcuSyn software (Biosoft, Ferguson, USA)

3.2. Methods

3.2.1. Cell lines and culture conditions

3.2.1.1. Hodgkin's Lymphoma cell lines

The human Hodgkin's lymphoma (HL) cell lines L540, L428, and L1236, L591, DEV and KMH2 were obtained from the German collection of Microorganisms and cell cultures (Brunswick, Germany). L540cy, a subclone of the HL cell line L540 that grows in SCID mice, was kindly provided by Prof. Dr. Engert A. (University Hospital, Cologne, Germany). All cells were cultured in RPMI 1640 (GIBCO, BD Biosciences, Heidelberg, Germany) supplemented with 10% (vol/ vol) heat-inactivated fetal calf serum (FCS), 50µg/mL penicillin, 50µg/ mL streptomycin, and 2mM L-glutamine at 37°C with 5% CO_2 atmosphere. Medium was replaced twice weekly and 24h prior to any experiment.

3.2.1.2. Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated from Buffy coats of healthy donors using Ficoll-density gradient method (Amersham Biosciences GmbH, Freiburg, Germany). Buffy coats were obtained from the Blood Bank of the University Clinic of Cologne, Germany. Separation of PBMCs was done by density centrifugation of 30 ml blood on 15 ml Lymphocyte Separation Medium (LSM) (PAA, Germany), in a 50 ml conical sterile centrifuge tube, at 4000 r.p.m. for 20 min. at 4^{o}C. The cells at the middle layer, constituting the mononuclear cell fraction, were collected, washed twice in serum free RPMI-1640 medium and centrifuged at 1100 r.p.m. for 5 min. After isolation, PBMCs were either lysed for western blotting, or cultured in RPMI-1640 medium under the conditions described above.

3.2.2. XTT cell viability assay

The effect of HSP90 inhibition on cell viability was tested by XTT assay (2-Methoxy-4-nitro-5-sulfophenyl)-2H–tetrazolium- 5-carboxanilide assay) according to the manufacturer's instructions (Roche Molecular Biochemical, Mannheim, Germany). This assay quantitatively measures formazan color development by viable cells.

Briefly, Hodgkin's Lymphoma cells were counted, plated at $2X10^4$ cells per well in triplicates and incubated with the respective compound as indicated in the experiment legend or with vehicle (DMSO; 0.01%) as negative control for 48 hours at 37°C and 5% CO_2. In each experiment, a blank consisting of medium only, no cells, was set up. After 48 hours of culture, 50 µL of fresh plain medium containing 1.49 mM XTT and 0.025 mM phenazinethosulphate were added, and plates were incubated for additional 6-8 hours at 37°C and 5% CO_2. The optical density was measured at 450 nm 690 nm in an ELISA microplate reader (Bio-Tek, Winooski, USA). The half maximal effective concentration (IC50) values were calculated by linear regression using GraphPad Prism analysis software and expressed as mean ±SD of at least three independent experiments.

3.2.3. Flow Cytometric Analysis with Annexin-V/ 7-AAD

The HSP90 inhibitor-mediated induction of apoptosis was assessed by FACS analysis of Annexin V-FITC/ 7-AAD stained Cells. Induction of apoptosis was confirmed in independent experiments by detection of Poly-(ADP-ribose) polymerase (PARP) cleavage in Western blot analysis.

2 $x10^5$ L428, L540cy or PBMC were cultured in 6 well plates for 48 hours at 37°C. Cells were then pelleted by centrifugation at 2000 r.p.m for 5 min. and washed twice with FACS washing buffer and once with 400µl Annexin binding-buffer. Cells were incubated with 2µl Annexin-V-FITC, 2µl 7-AAD or both for 15 min. in the dark at room temperature and resuspended in 300µl Annexin binding-

buffer. As controls, cells were treated with vehicle (0.1% DMSO, negative control), or fixed with 200µL of 37% formaldehyde for 15 min. (positive control).

FACS analysis was performed on a FACSCalibur cytometer (BD Biosciences Hamburg, Germany) equipped with a standard 488 nm laser. FITC and 7-AAD emissions were detected in the FL-1 and FL-3 channels, respectively, after appropriate compensation. A minimum of 10,000 events were counted for each sample. Subsequent analysis was done with Cell Quest software (BD Biosciences).

Apoptosis stages can be measured using a dual parameter staining consisting of Annexin V-FITC and 7-AAD (Cell impermeant dyes). Phosphatidylserine (PS) resides on the inner cell membrane of healthy cells, but will externalize to the outer membrane layer when in early stage apoptosis. Cells with damaged plasma membrane (dead cells) were stained with 7-AAD only. Cells that were positively stained by Annexin-V-FITC but 7-AAD negative (early stages of apoptosis) and positive for both Annexin-V-FITC and 7-AAD (late stages of apoptosis) were quantified, and both subpopulations were considered as the overall cell death. Assays were performed in at least two independent experiments. The following controls were used for compensation and set up:

1- DMSO treated cells: Unstained
2- DMSO treated cells stained with both Annexin-V-FITC + 7-AAD
3- DMSO treated cells fixed with 37% formaldehyde and stained with 7-AAD
4- DMSO treated cells fixed with 37% formaldehyde and stained with Annexin-V-FITC.
5- DMSO treated Cell fixed with 37% formaldehyde and stained with both Annexin–V-FITC + 7-AAD (Positive control).

3.2.4. Combination index Analysis

The human Hodgkin's lymphoma cells (L428 and L540cy) were seeded in triplicates in 96-well tissue culture plates at $2X10^4$ cells per well. After 48 hours of treatment with various concentrations of the compounds, viable cells were assessed by XTT assay as described above. Dose–response interactions between BIIB021 and Gemcitabine or Doxorubicin or Valproic acid at the point of the IC50 were evaluated by the median effect method. The theoretical basis of the median effect method has been described in previous studies by Chou and Talalay (1984). CalcuSyn software (Biosoft, Ferguson, MO, USA) was used to calculate the combination index. Synergy, additivity, and antagonism are defined as combination index < 1, combination index = 1, and combination index > 1, respectively.

For this analysis, each compound were tested alone or in combination at a fixed ratio of concentrations (i.e. the ratio of the IC50 concentrations), and then to create a combination that is 2 to 4 times higher than the IC50, and use serial dilutions of the highest concentration of combination to generate the dose response curve (each single compound in the combination is tested alone in the same manner). The fraction affected was calculated for each concentration and entered into CalcuSyn software. Each experiment provided triplicate data points for each concentration and was repeated at least three times.

3.2.5. Western blot analysis

3.2.5.1. Cell Extracts preparation and protein quantitation

For preparation of whole cell extract for Western blot analysis: Lysates were prepared from $4X10^6$ cells by dissolving cell pellets in 200µl of NP40 lysis buffer. Lysates were collected at 48 hours post-treatment. Lysates were sonicated using Bandelin electronic UW 2070 homogenizer (Germany) on

ice at setting 6 for 5 sec. and pelleted at 14,000 r.p.m. for 20 min. at 4°C, and the resulting supernatants were recovered and stored at -80°C until further use. Protein concentrations were measured by the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Chemical Co., Germany) as described by the manufacturer using BSA as a protein standard.

3.2.5.2. SDS Gel-Electrophoresis and Western blotting

Levels of HSP90, HSP70, XIAP, PARP and Actin after 48 hours incubation with BIIB021 were determined by Western blot analysis of whole cell lysates. Briefly, 20µg of protein were suspended in 5X Laemmle's buffer, boiled for 5 min., and transferred on 10% (w/v) SDS-polyacrylamide gels for 90 min. at 120 V. A 200 kDa Magic molecular weight ladder was used as a size marker (Invitrogen). Gels were electroblotted onto nitrocellulose membranes (Bio-Rad) at 350 mA for 90 min. in Tris-glycine blotting buffer.

The Membranes were blocked for 2 hours with PBS buffer containing 5% non-fat dried milk and 0.01% (v/v) Tween-20 (PBS-T) at room temperature. Blots were washed three times with PBS-T buffer, and incubated overnight at 4^0C in primary antibody diluted in PBS-T with 5% non-fat dried milk. Further, they were washed three times with PBS-T buffer and subsequently incubated with the respective secondary antibodies conjugated to horse-radish peroxidase (anti-mouse HRP or anti-rabbit HRP) in PBS-T buffer for 2 hours at 4°C. Subsequently, membranes were washed three times with PBS-T buffer and subjected to standard technique ECL (Amersham Biosciences) according to the manufacturer’s instructions.

3.2.6. Xenograft model of human HL

All experiments involving animals were conducted following FELASA guidelines and approved by local authorities (authorization number 50.203.2-K 17, 37/05).

Pathogen-free female C.B-17/Icr severe combined immunodeficiency (SCID) mice (Taconic, Lille Skensved, Denmark) were maintained under pathogen-free conditions and fed autoclaved standard chow and water. Briefly, $1x10^7$ exponentially growing L540cy cells were injected into the flank of SCID Mice. When tumors reached a volume of 100 mm^3 animals were randomly assigned to four groups consisting of five animals each.

Mice received an equivalently toxic dose of an HSP90 inhibitor (unpublished data kindly provided by Biogen), or vector as control: (1) 0.1N HCl (vehicle, control) or 120 mg/kg BIIB021 via oral gavage, or (2) PMSE (phospholipon/sucrose emulsion kindly provided by Biogen Idec, vehicle control) or 60mg/kg 17-AAG (1.5 mg/ mouse) per oral gavage every three days. Tumor measurements were taken every 3 days with a calliper, and tumor volume was calculated with the formula [(length x width x height)/ 2]. Mice were killed when tumor volume exceeded 1200 mm^3 or after 30 days. Statistical analysis was performed using the GraphPad-Prism software (San Diego, CA) applying the unpaired t-test.

3.2.7. Statistical Analysis

All data were derived from at least three independent experiments and values were presented as means ± standard deviations. Statistical analyses were conducted using GraphPadPrism software using Student's t-test. Microsoft Excel was also used.

4. Results

4.1. Novel HSP90 inhibitors inhibit cell viability in L428 and L540

To determine the effects of seven novel purine-based HSP90 inhibitors BIIB021, EC82, EC137, EC138, EC141, EC144 and EC151 and 17-AAG on HL cells, L428 and L540 cells were incubated with increasing concentrations of each HSP90 inhibitors (0-10 µM) or vehicle control (DMSO, 0.1% final concentration) for 48 hours and cell viability was determined by XTT assay.

All novel HSP90 inhibitors inhibited cell viability in a dose-dependent manner in both cell lines at low concentrations with IC50 values of 0.04 to 0.71 µM in L428 and 0.05 to 0.9 µM in L540. In contrast, 17-AAG was less effective in inhibiting HL cell viability with an IC50 of 6 µM in L428 and 2.9 µM in L540 (Figure 6).

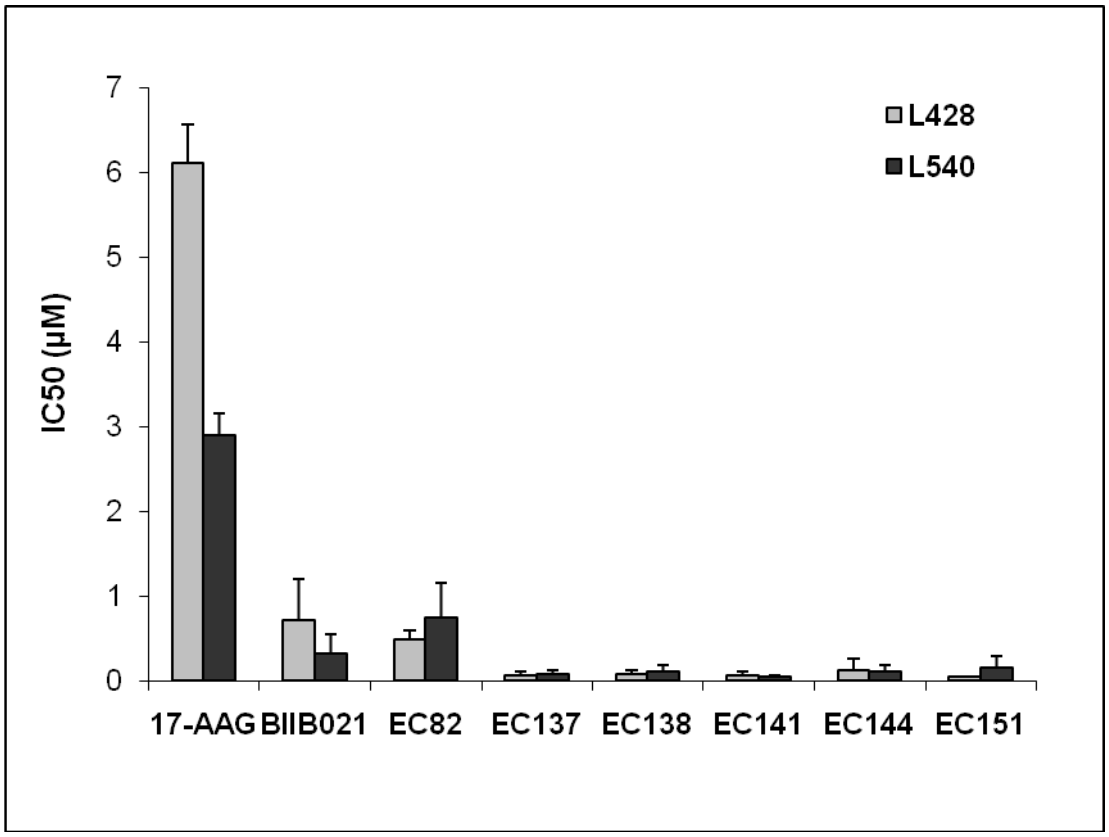

Figure 6. HSP90 inhibitors inhibit cell viability of L428 and L540 cells.

Cells were incubated with vehicle (DMSO, control) or with 0-10 µM of an HSP90 inhibitor (BIIB021, EC82, EC137, EC138, EC141, EC144, EC151 or 17-AAG) for 48 hours in an XTT assay. The half maximal inhibitory concentration of each HSP90 inhibitor was calculated by linear regression as mean and standard deviation of three independent experiments.

4.2. BIIB021 and 17-AAG inhibit cell viability in seven HL cell lines

The effects of BIIB021 and 17-AAG on cell viability were compared in seven different HL cell lines (L540, L540Cy, L428, L1236, L591, DEV and KMH2). HL cells were incubated with increasing doses of BIIB021 or 17-AAG (0-10 µM) or vehicle control (DMSO, 0.1% final concentration) for 48 hours and cell viability was determined by XTT assay.

Both drugs inhibited cell viability in all seven HL cell lines (Figure 7). However, BIIB021 was significantly more effective in reducing cell viability than that of 17-AAG in all cell lines ($P < 0.05$). The IC50 of BIIB021 and 17-AAG were 0.24 to 0.70 µM and 0.56 to 6.10 µM, respectively (Figure 7).

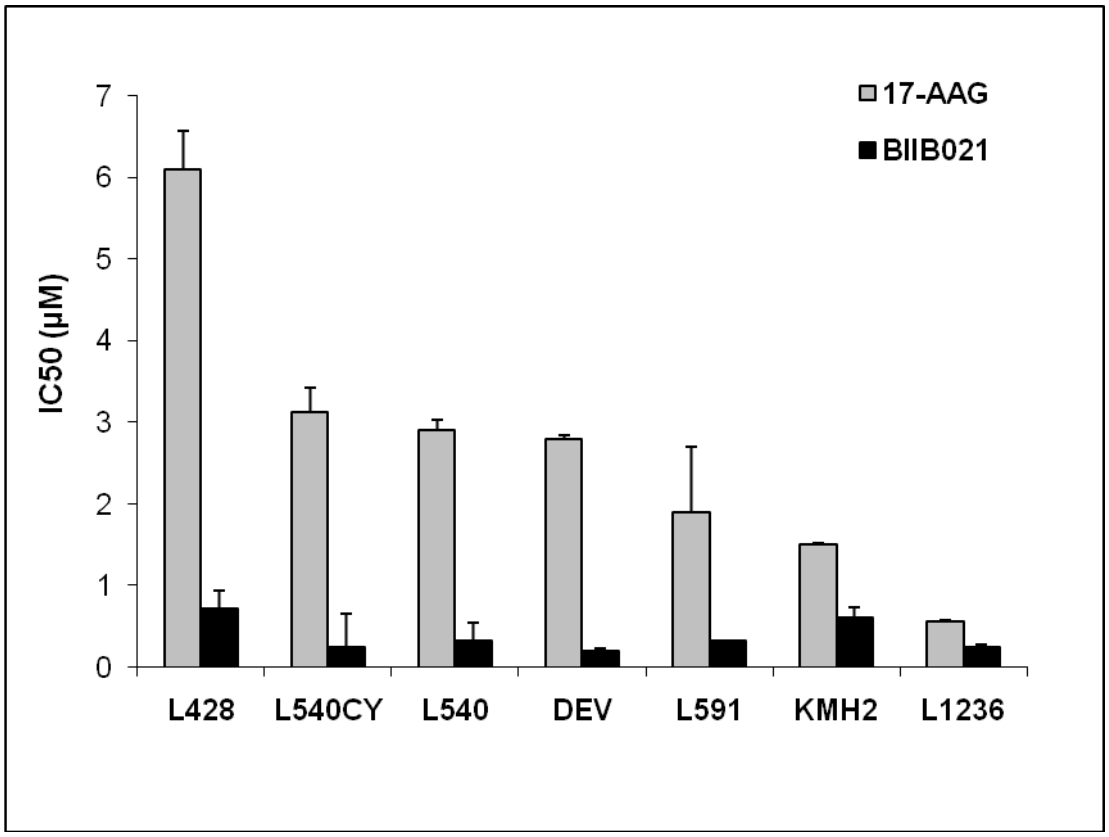

Figure 7. BIIB021inhibits cell viability in seven HL cell lines.

HL cell lines (L428, DEV, KMH2, L591, L1236, L540 and L540cy) were incubated with vehicle (DMSO, control) or with 0-10 µM of BIIB021 or 17-AAG for 48 hours in an XTT assay. The half maximal inhibitory concentration in each cell line was calculated by linear regression as mean and standard deviation of three independent experiments.

4.3. BIIB021 induces Annexin-V/7AAD binding in HL cells

To determine whether HSP90 inhibition induces apoptosis in HL cells, L428 and L540cy cells were incubated with BIIB021 concentrations corresponding to the IC50 in the XTT assay (0.8 µM for L428 and 0.3 µM for L540cy) or vehicle (DMSO, 0.1% final concentration) for up to 72 hours. As a control, cells were incubated with 17-AAG (5 µM) (data not shown). After staining with Annexin-V-FITC/ 7AAD, cells were analyzed by flow cytometry.

Incubation of L428 cells with 0.8 or 5 µM of BIIB021 for 48 hours resulted in an increase from 30% to 38% in Annexin-V/7AAD binding, as an indicator of apoptosis. Moreover, after 72 hours of incubation, the percentage of apoptotic cells increased from 42% to 62% (Figure 8).

Similarly, incubation of L540cy cells with 0.3 or 5 µM of BIIB021 for 48 hours resulted in 19% and 18% in Annexin-V/7AAD binding and at 72 hours the percentage of apoptotic cells increased from 43% to 60% (Figure 8).

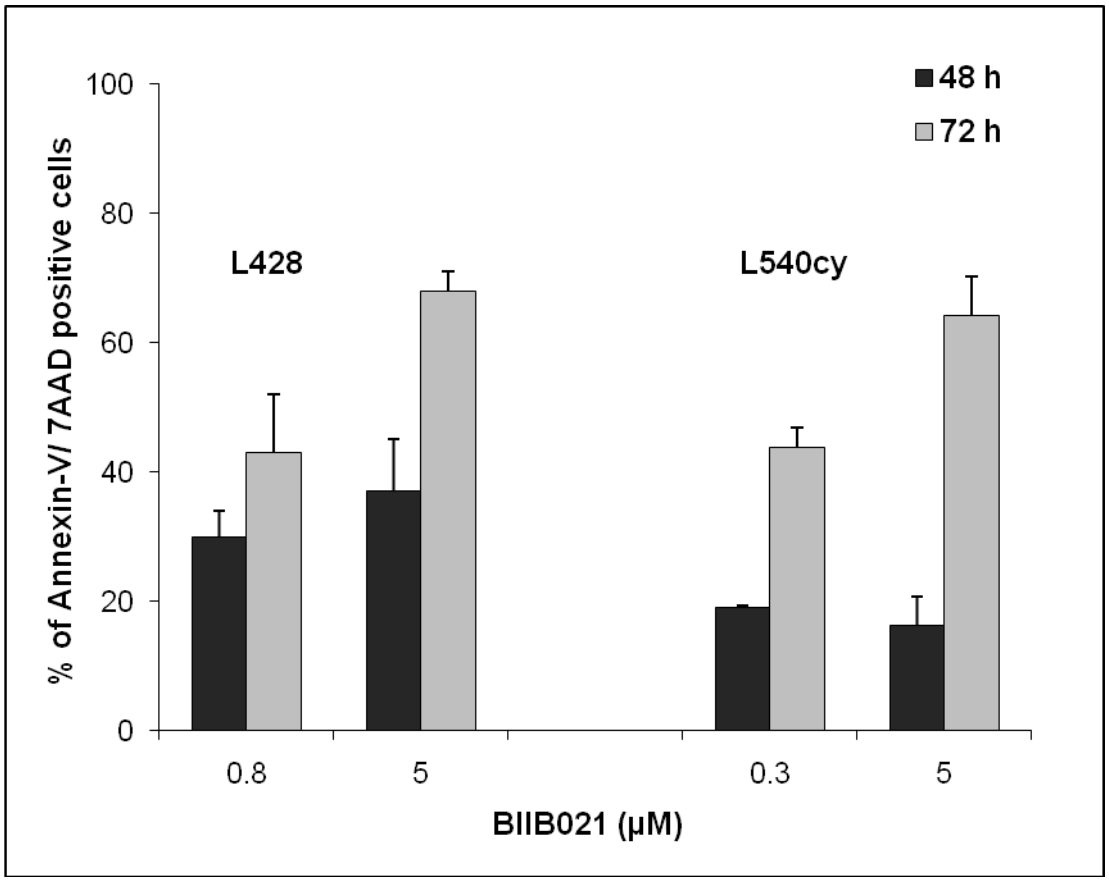

Figure 8. BIIB021 induces Annexin-V/7AAD binding in L428 and L540cy.

L428 and L540cy cells were incubated with vehicle (DMSO, control) or with the indicated concentration of BIIB021 for up to 72 hours. After staining with Annexin-V/ 7AAD, cells were analyzed by flow cytometry. The percentage of Annexin-V/7AAD binding in DMSO treated cells was subtracted as an indicator for background apoptosis, mean percentage and standard deviation of Annexin-V/7AAD positive cells were calculated of three independent experiments.

4.4. BIIB021 selectively induces Annexin-V/7AAD binding in HL cell Lines

Western blot analysis was performed to detect the HSP90 level in HL cells and peripheral blood mononuclear cells (PBMCs) from healthy blood donors. Incubation of L428 and L540cy with BIIB021 (0.8µM for L428 and 0.3 µM for L540cy) or vehicle (DMSO, 0.1% final concentration) for 48 hours resulted in increased HSP90 levels in treated cells compared to untreated cells. In contrast, HSP90 was detectable in a very low level in PBMCs (Figure 9), suggesting an over expression of HSP90 in HL cells.

To determine whether BIIB021 mediated HSP90-inhibition selectively induces apoptosis in HL cells, L428 and PBMCs were incubated with BIIB021 (0.8 µM) or vehicle (DMSO, 0.1% final concentration) for 48 hours. After staining with Annexin-V-FITC and 7AAD, cells were analyzed by flow cytometry. Lymphocytes were gated using light scattering characteristics as non malignant reference cells. BIIB021 induced 35% Annexin-V/7AAD binding in L428 cells; however, no apoptosis was detected in healthy donor derived Lymphocytes (Figure 10).

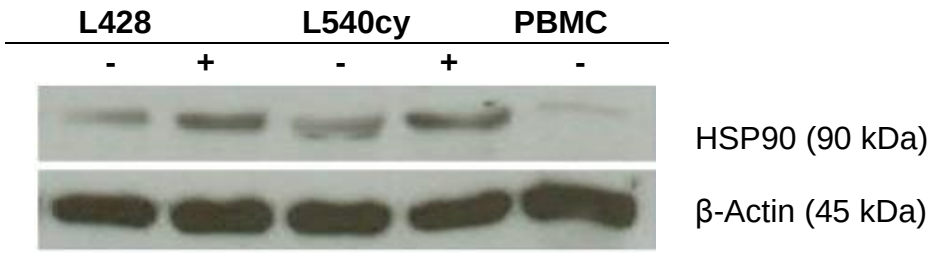

Figure 9. HSP90 is present at low levels in PBMCs.

L428, L540cys and PBMC cells were incubated with vehicle (DMSO, control) or with BIIB021 (0.8 μM for L428 and 0.3 μM for L540cy) for 48 hours. Whole cell lysates were prepared and tested in Western blot analysis using anti-HSP90 and anti-β-Actin antibodies.

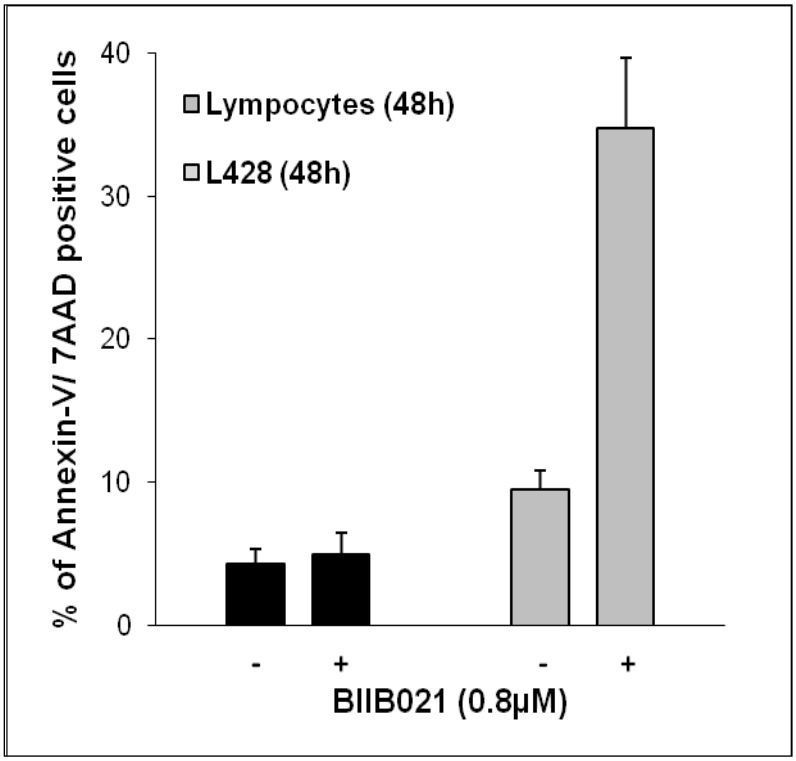

Figure 10. BIIB021 selectively induces apoptosis in L428 cells.

L428 and normal Lymphocytes cells were incubated with vehicle (DMSO, control) or with BIIB021 (0.8 µM) for 48 hours and then stained with Annexin-V/7AAD and analyzed by flow cytometry. Apoptosis was determined by Annexin-V/7AAD binding and expressed as mean percentage of apoptosis in treated (+) and vehicle treated control cells (-). Mean and standard deviation were calculated of three independent experiments.

4.5. BIIB021 induces PARP cleavage in HL cell

The cleavage of poly-ADP-ribose-polymerase (PARP), as an indicator of apoptosis, was detected in western blot analysis. Cells were incubated with BIIB021 (0.8 µM for L428 and 0.3 µM for L540cy) or vehicle (DMSO, 0.1% final concentration) for up to 48 hours. Whole cell lysates were prepared; proteins were separated by SDS-PAGE and analyzed by western blotting. HSP90 inhibition resulted in a marked increase in PARP cleavage in both cell lines (Figure 11). A comparable degree of PARP cleavage as a result of HSP90 inhibition was observed in all other tested HL cell lines after 48 hours (Figure 12). The cleavage of poly-ADP-ribose-polymerase (PARP), as an indicator of apoptosis, was detected in western blot analysis.

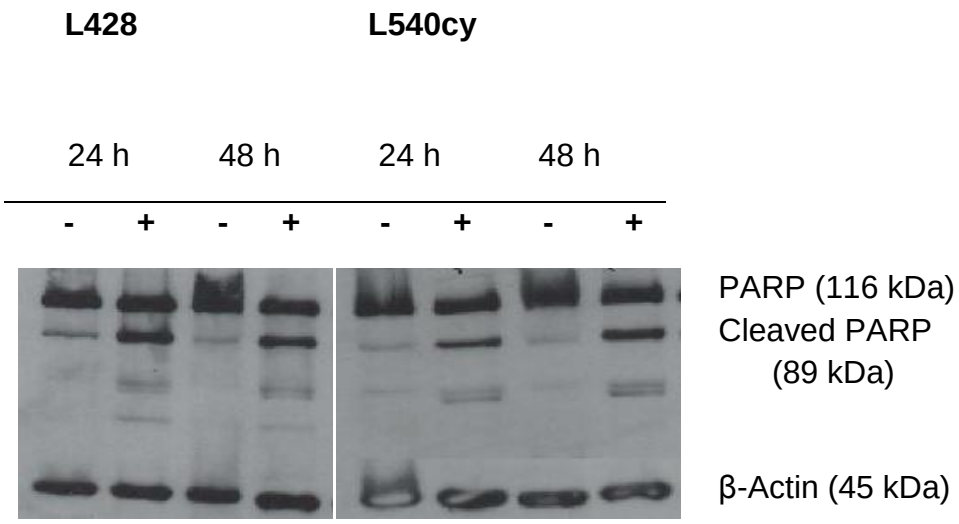

Figure 11. BIIB021 induces PARP cleavage in L428 and L540cy cells.

Cells were incubated with vehicle (DMSO, control) or BIIB021 (0.8 µM for L428 and 0.3 µM for L540cy) for 24 and 48 hours. Whole cell lysates of L428 and L540cy were prepared and tested in Western blot analysis using anti-PARP and anti-β-Actin antibodies.

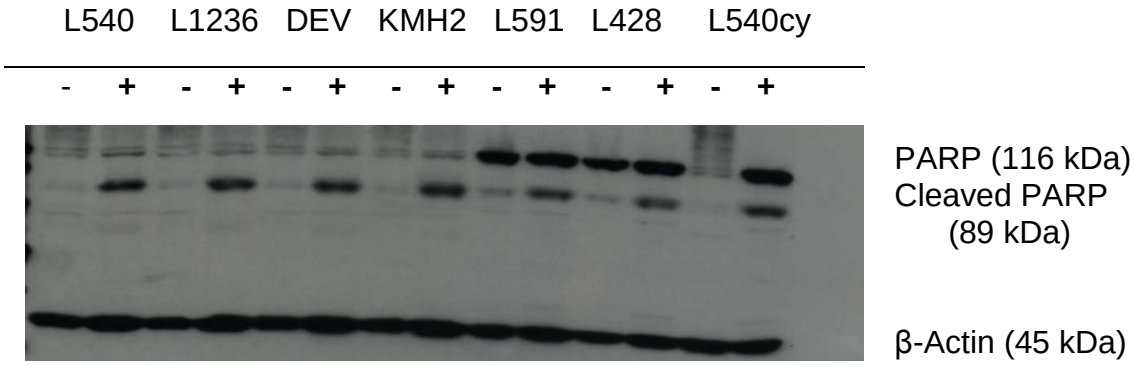

Figure 12. BIIB021 induces PARP cleavage in seven different HL cell lines.

Cell were incubated with vehicle (DMSO, control) or BIIB021 (at concentrations corresponding to the IC50 in a 48 hours XTT assay, section 4.2) for 48 hours. Whole-cell lysates were prepared and tested in Western blot analysis using anti-PARP and anti-β-Actin antibodies.

4.6. BIIB021 reduces cellular levels of XIAP in HL cells

The anti-apoptotic protein X-linked inhibitor of apoptosis (XIAP) has previously been shown to be critically involved in HL cell survival (Kashkar et al., 2003). As XIAP is a potential client protein of HSP90, the level of XIAP in HL cells in response to HSP90 inhibition was analysed. Cells were incubated with BIIB021 (0.8 µM for L428 and 0.3 µM for L540cy) or vehicle (DMSO, 0.1% final concentration) for up to 72 hours. Whole cell lysates were prepared; proteins were separated by SDS-PAGE and analyzed by western blotting.

HSP90 inhibition resulted in a marked reduction in levels of XIAP in both tested cell lines after 48 hours (Figure 13). The decrease was even more pronounced after 72 hours of incubation.

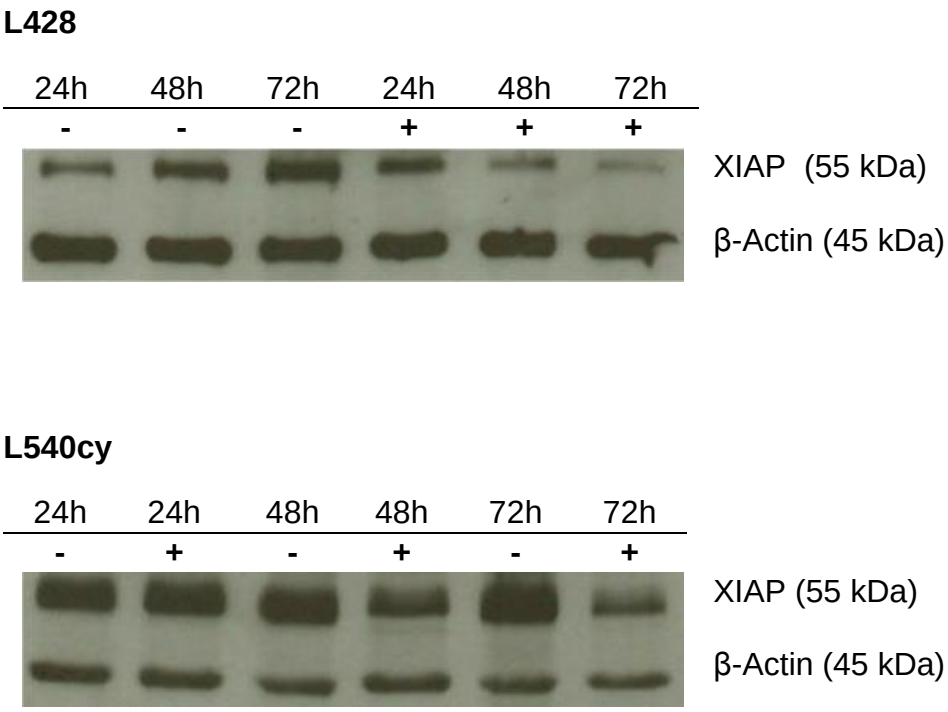

Figure 13. BIIB021 reduces cellular levels of XIAP in L428 and L540cy.

Cells were incubated with vehicle (DMSO, control) or with BIIB021 (0.8 μM for L428 and 0.3 μM for L540cy) for 24, 48 and 72 hours. Whole-cell lysates of L428 and L540cy were prepared and tested in Western blot analysis using anti-XIAP and anti-β-Actin antibodies.

4.7. BIIB021 increases levels of HSP70 and HSP90 in HL cells

HSP70 and HSP90 are two members of HSP family that were reported to be anti-apoptotic and their elevated expression in cancer cells were detected in several hematological and solid tumors (Jolly et al., 2000). HSP90 and HSP70 protein levels were assessed in seven HL (L428, DEV, KMH2, L591, L1236, L540 and L540cy) by Western blot analysis. Incubation of cells with BIIB021 (at concentrations corresponding to the IC50 in a 48 hours XTT assay, section 4.2) or vehicle (DMSO, 0.1% final concentration) for 48 hours resulted in increased HSP90 and HSP90 levels in treated cells compared to untreated cells (Figure 14). BIIB021 potently inhibits the cell viability of HL cell lines simultaneous with increased levels of HSP70 and HSP90 as hallmarks of HSP90 inhibition.

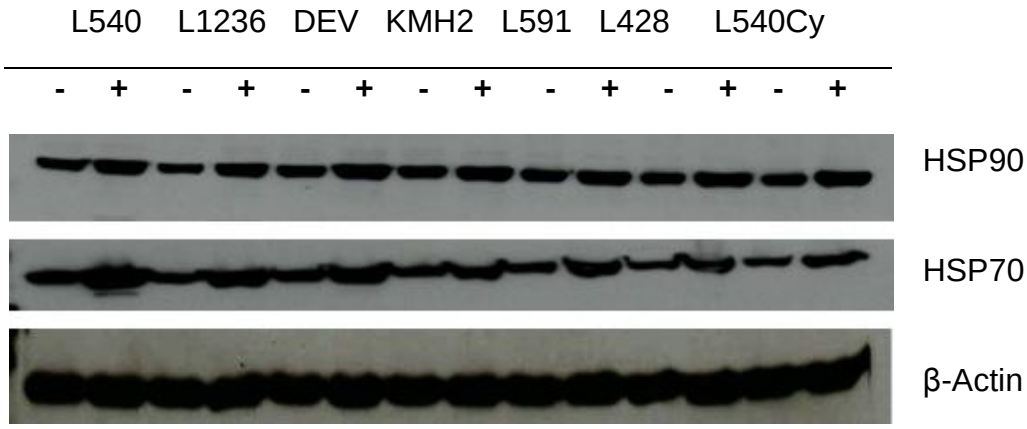

Figure 14. BIIB021 increases levels of HSP70 and HSP90 in HL cells.

Seven different HL cell lines were incubated with vehicle (DMSO, control) or BIIB021 (at concentrations corresponding to the IC50 in a 48 hours XTT assay, section 4.2) for 48 hours. Whole-cell lysates were prepared and tested in Western blot analysis using anti-HSP70, anti-HSP90 and anti-β-Actin antibodies.

4.8. BIIB021 acts in synergy with conventional chemotherapy

4.8.1. BIIB021 in combination with doxorubicin and gemcitabine acts synergistically to inhibit L428 and L540cy cell viability

Doxorubicin and Gemcitabine are chemotherapeutic agents commonly used for treatment of HL in the first-line and relapsed setting, respectively (Canellos et al., 2003; Santoro et al., 2000). In order to evaluate potential combinations for a clinical application of BIIB021, L428 and L540cy were incubated with either BIIBO21 alone or in combination with doxorubicin or gemcitabine for 48 hours in an XTT assay. Combination effects were analyzed using the combination index (CI) method described by Chou and Talalay (1984).

Combination indices were calculated as mean ± standard deviation of three independent experiments. CI<1, CI=1, or CI>1 represent synergism, additivity or antagonism, respectively.

Combination of BIIB021 and doxorubicin synergistically inhibit cell viability of L428 and L540cy cells. In L428 cells, Combination indices were 0.4 ±0.1, 0.6 ±0.11 and 1 ±0.33 (Figure 15). In L540cy cells, combination with doxorubicin resulted in similar effects with combination indices of 0.89 ±0.30, 0.58 ±0.03 and 0.79 ± 0.01 (Figure 15).

Similarly, Combination of BIIB021 and gemcitabine synergistically inhibits cell viability of L428 and L540cy cells. In L428 cells, combination indices were 0.26 ± 0.02, 0.32 ± 0.09 and 0.460 ± 0.22 (Figure 16). In L540cy cells, combination with gemcitabine resulted in similar effects with combination indices of 0.96 ± 0.04, 0.66 ± 0.16 and 0.90 ± 0.42 (Figure 16).

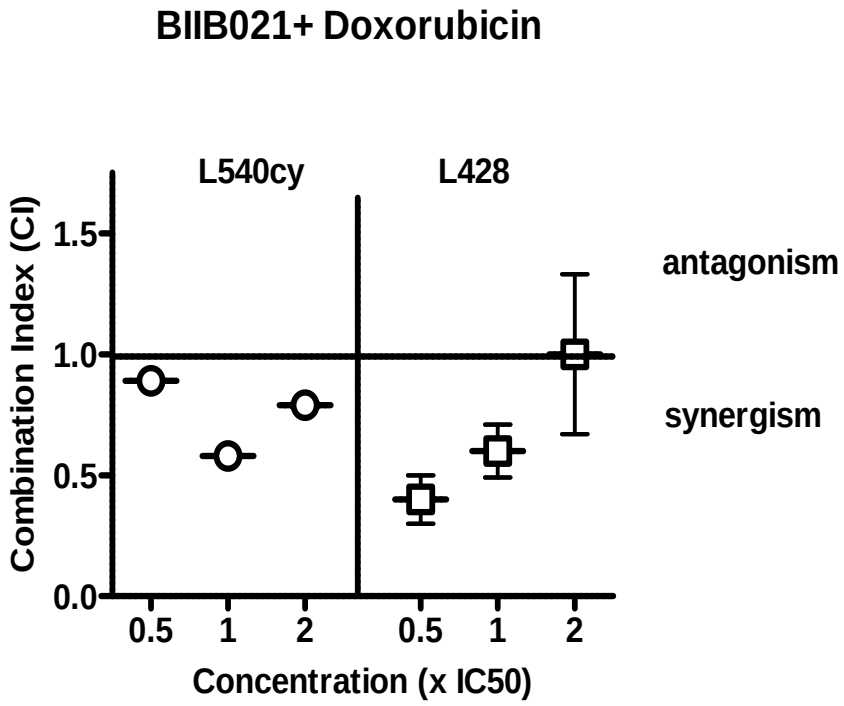

Figure 15. Combination of BIIB021 and doxorubicin synergistically inhibits L428 and L540cy cell viability.

L428 and L540cy cells were incubated for 48 hours XTT assay with varying concentrations of BIIB021 or Doxorubicin alone or in combination at a fixed ratio. Dose-Effect calculations were performed using Calcsyn software (Biosoft, USA) based on the median effect method of Chou and Talalay. CI < 1, CI=1, and CI > 1 represent synergism, additivity and antagonism of the two compounds, respectively. Each combination index (CI) was calculated as mean and standard deviation from three independent experiments.

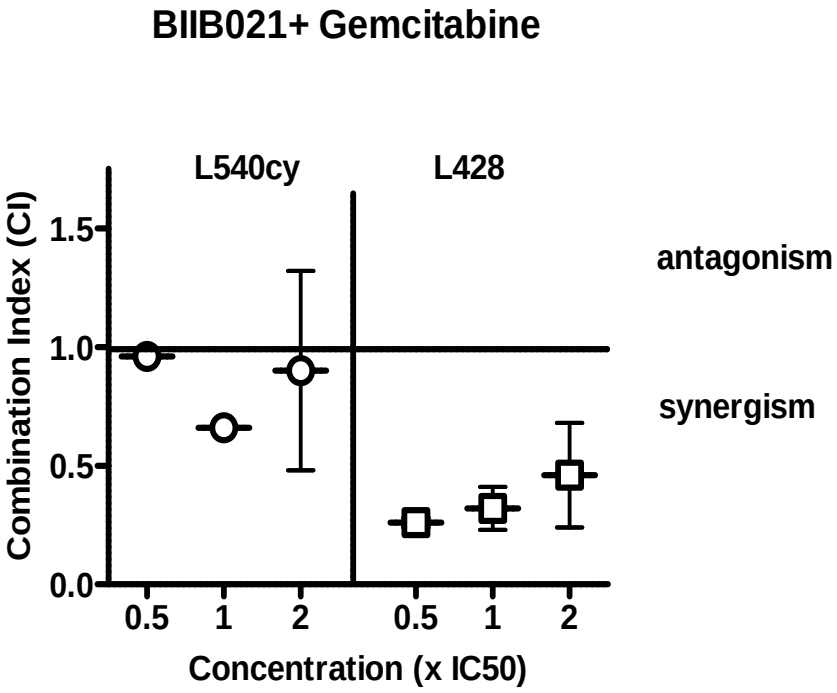

Figure 16. Combination of BIIB021 and gemcitabine synergistically inhibits L428 and L540cy cell viability.

L428 and L540cy cells were incubated for 48 hours XTT assay with varying concentrations of BIIB021 or gemcitabine alone or in combination at a fixed ratio. Dose-Effect calculations were performed using Calcsyn software (Biosoft, USA) based on the median effect method of Chou and Talalay. CI < 1, CI=1, and CI > 1 represent synergism, additivity, and antagonism of the two compounds, respectively. Each combination index (CI) was calculated as mean and standard deviation from three independent experiments.

4.8.2. Combination of BIIB021 and gemcitabine induces apoptosis in L428 cells.

The combination effects of BIIB021 and gemcitabine on Annexin-V-FITC/7AAD binding was investigated in L428. L428 cells were incubated with vehicle or BIIBO21 or gemcitabine alone and in combination for 48 hours. After staining with Annexin-V-FITC/7AAD, cells were analyzed by flow cytometry.

Combined treatment of BIIB021 with gemcitabine resulted in a 2-3 fold increase in Annexin-V-FITC/7AAD binding as an indicator of apoptosis in L428 cells (Figure 17).

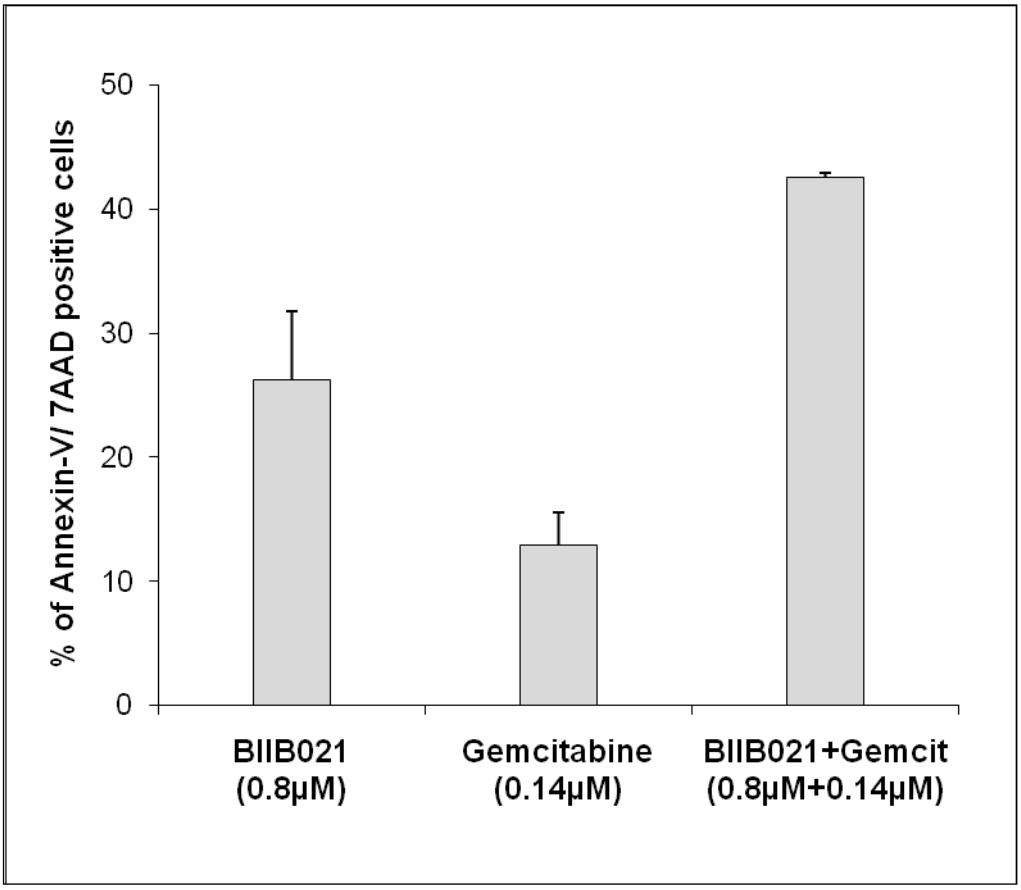

Figure 17. Combination of BIIB021 and gemcitabine induces apoptosis in L428 cells.

L428 cells were incubated with vehicle (DMSO, control) or with the indicated concentrations of BIIB021 and gemcitabine for 48 hours. After staining with Annexin-V/7AAD cells were analyzed by flow cytometry. The percentage of Annexin-V/7AAD binding in DMSO treated cells was subtracted as an indicator for background apoptosis, mean percentage and standard deviation of Annexin-V/7AAD positive cells were calculated of three independent experiments.

4.8.3. BIIB021 in combination with valproic acid acts synergistically to inhibit L428 cell viability

Valproic acid is a potent inhibitor of Histone deacetylase (HDAC). HDAC inhibitors, a new class of chemotherapeutic agents, have been shown to have potent anticancer activities in preclinical studies and are currently being tested in clinical trials in patients with advanced and refractory solid tumors or lymphomas (Rasheed et al., 2007). Interestingly, recent reports suggest significant clinical activity of HDAC inhibitors in refractory HL (Younes et al., 2007).

To assess the effects of BIIB021 in combination with valproic acid in L428 cells, cells were incubated for 48 hours XTT assay with BIIBO2 or valproic acid alone or in combination at a fixed ratio. Dose-Effect calculations were performed using Calcsyn software (Biosoft, USA) based on the median effect method of Chou and Talalay. CI < 1, CI=1, and CI > 1 represent synergism, additivity and antagonism of the two compounds, respectively. Each combination index (CI) was calculated as mean and standard deviation from three independent experiments.

Combination of BIIB021 and valproic acid synergistically inhibit cell viability of L428 cells. Combination indices were 0.56, 0.61 and 0.85 (Figure 18).

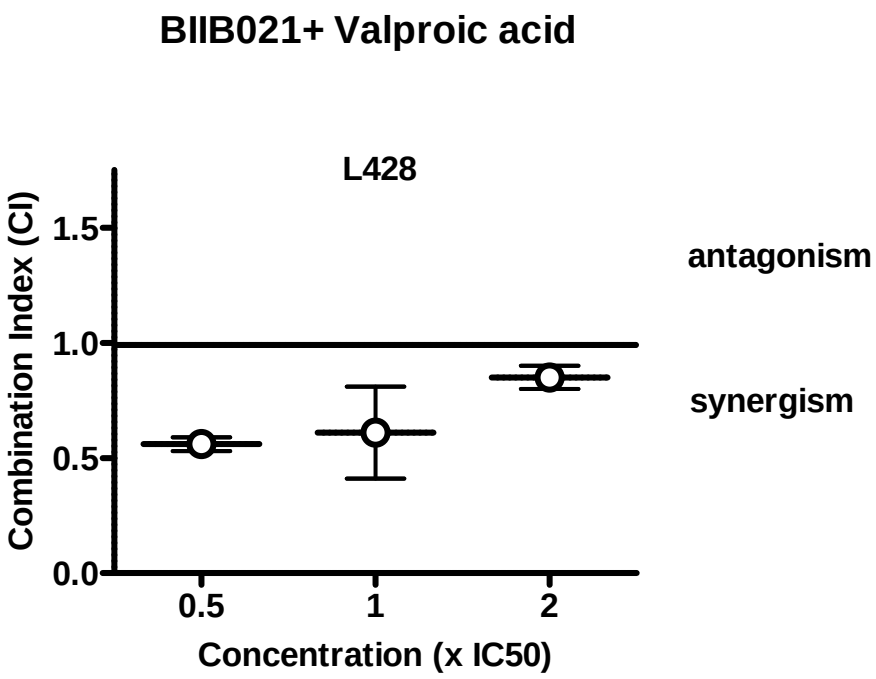

Figure 18. Combinations of BIIB021 and Valproic acid synergistically inhibit L428 cell viability.

L428 cells were incubated for 48 hours in an XTT assay with varying concentrations of BIIB021 or valproic acid alone or in combination at a fixed ratio. Dose-Effect calculations were performed using Calcsyn software (Biosoft, USA) based on the median effect method of Chou and Talalay. CI < 1, CI=1, and CI > 1 represent synergism, additivity, and antagonism of the two compounds, respectively. Each combination index (CI) was calculated as mean and standard deviation from three independent experiments.

4.9. Combination of 17-AAG and Cyclosporine A (CsA) in L428 cells

Resistance to 17-AAG has been previously linked to expression of P-glycoprotein (P-gp) and the multidrug resistant (MDR) phenotype. However, the novel HSP90 inhibitor BIIB021 is not a P-gp dependent (McCollum et al., 2008).

To test, whether the partial resistance of HL cells to 17-AAG- mediated HSP90 inhibition could be caused by P-gp activity, 17-AAG was combined with Cyclosporine A (CsA), an inhibitor of multidrug resistance protein P-gp (P-glycoprotein). After 48 hours incubation, the effects on L428 cell viability were analyzed using an XTT assay.

Both 17-AAG and CsA caused only minor cytotoxicity alone. Combination of 17-AAG (1, 2.5 and 5 µM) with CsA (0.08 or 0.42 µM) for 48 hours resulted in no significant increase of toxicity ($p=0.34$; 10-15% reduction in cell viability) (Figure 19).

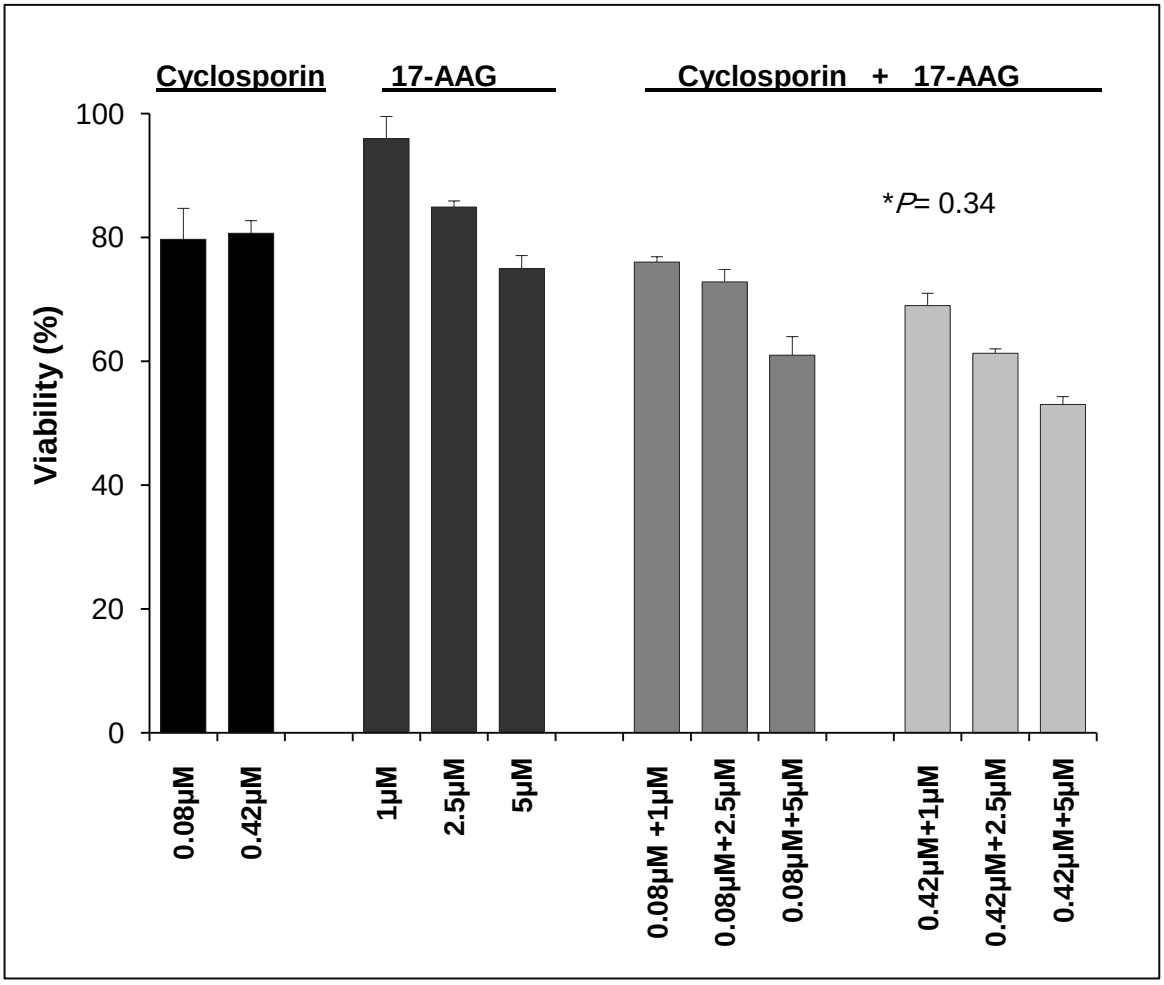

Figure 19. Combination of 17-AAG and Cyclosporine shows a minor effect in L428 cells.

The L428 cells were treated with the indicated concentrations of 17-AAG and Cyclosporine for 48 hours. Cell viability was assessed by the XTT method. Viability was calculated as mean percentage of control-viability and standard deviation from two independent experiments and p values derived from Student's t-test was 0.34.

4.10. HSP90 inhibition inhibits HL growth *in vivo*

L540cy cells were injected subcutaneously into the flanks of SCID Mice to give rise to exponentially growing tumors. When tumor volume reached a volume of approximately 100 mm ^{3} Mice (randomly divided into four treatment groups of five animals) were treated once every three days with an equivalently toxic dose of an HSP90 inhibitor (17-AAG or BIIB021, 1/5 MTD communication by Biogen) or vehicle as control (PMSF or 0.1 N HCl, respectively).

Treatment of tumor-bearing mice with 17-AAG (60 mg/kg per day, intraperitoneally) for 20 days effectively reduced tumor growth compared to the control group (Figure 20A) indicating *in vivo* antitumor efficacy in HL. The differences in tumor growth between the 17AAG-treated and the PMSF-treated control mice were not statistically significant (p=0.10, unpaired t-test), due to the small number of animals per treatment group.

Furthermore, treatment of tumor-bearing mice with BIIB021 every three days (120 mg/kg per day, orally) for 28 days, effectively reduced tumor growth of subcutaneous L540cy (Figure 20B). Notably, the differences in tumor growth was statistically significant (p=0.029, unpaired t-test) (Figure 20C) and no dose-limiting toxicity was observed in either group.

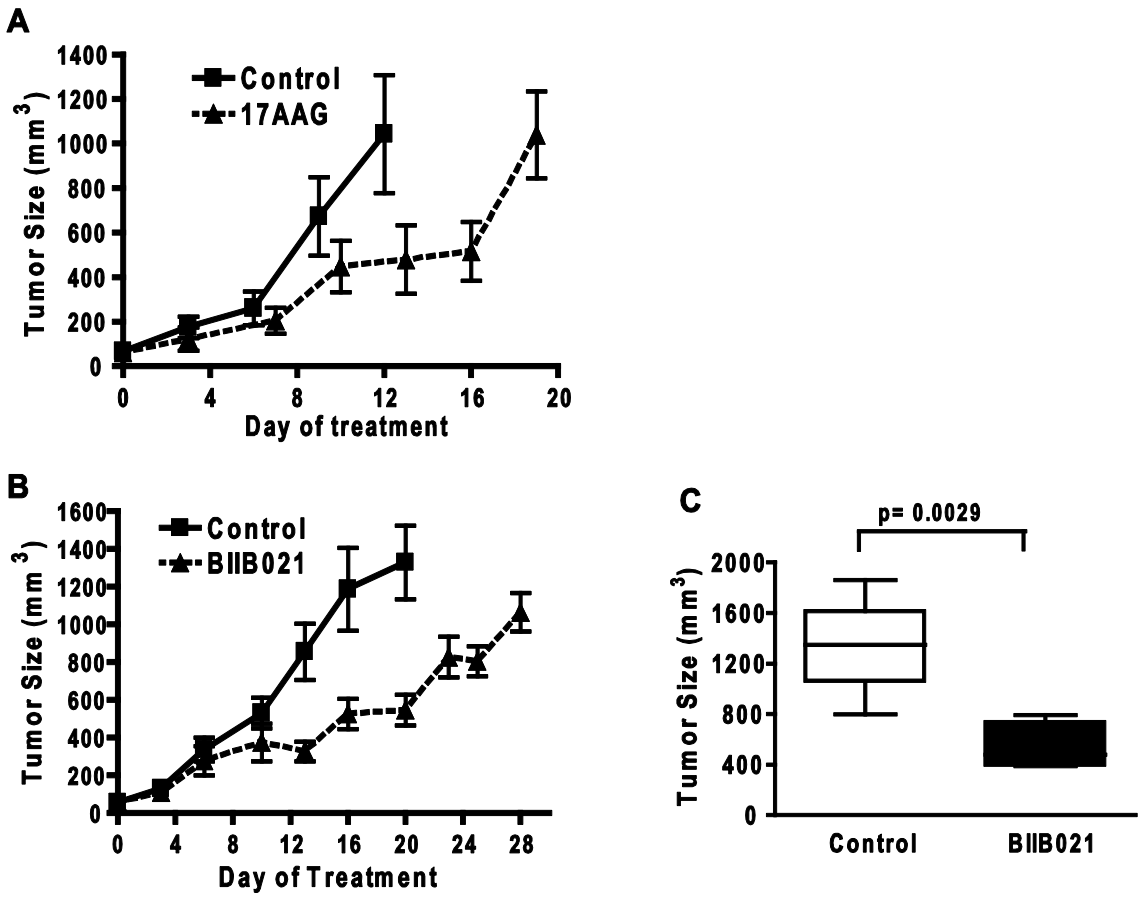

Figure 20. HSP90 inhibition inhibits HL tumor growth *in vivo*

SCID mice with L540cy xenograft flank tumors were treated every three days with 17AAG or BIIB021 or the respective vehicle (control). Data shown are mean relative tumor volume of five mice. The experiment was stopped and mice were killed after 30 days or when the median tumor diameter exceeded 1200 mm^3.
(A) 17-AAG was administered at a 2x60 mg/kg in PMSF (1.5mg/ mouse) or PMSE only (control) per intraperitoneal injection every three days. The mean tumor volume and standard error were calculated. **(B)** BIIB021 was administered at a 120 mg/kg in 0.1N HCl (3 mg/mouse) or 0.1N HCl only (control) per oral gavage every three days. The mean tumor volume and standard error were calculated. **(C)** Statistical significance in mean tumor size on day 23 between the BIIB021-treated and the respective control mice were analyzed using the unpaired t-test on a GraphPad Prism software program (San Diego, USA).

5. Discussion

HSP90 is essential for the maintenance of appropriate folding and conformation of many cell signaling proteins, which are involved in cell proliferation and survival and already several HSP90 inhibitors are under clinical evaluation (Workman 2004). A major attraction of HSP90 inhibitors is their potential to inhibit a range of oncogenic client proteins and cancer pathways, thereby blocking all of the 'hallmark traits' of cancer and exhibiting broad-spectrum antitumor activity (Hanhan and Weinberg 2000). Many studies have suggested that targeting of the HSP90 molecular chaperone has great potential for cancer therapy (Workman 2004). The effect of HSP90 inhibition by geldanamycin and 17-AAG have been thoroughly investigated and clarified in terms of altered association of the chaperone to its client proteins, which are thus degraded (Isaacs et al., 2003; Sharp and Workman 2006).

Importantly, inhibition of HSP90 is required for the stability of multiple pathways that drive signaling, proliferation and survival of Hodgkin's lymphoma cells. Previous work from Georgakis and colleagues has demonstrated that 17-AAG induces cell cycle arrest and apoptosis in HL by degradation of extracellular signal-regulated kinase (ERK), and Akt. However, 17-AAG has limitations as a therapeutic agent such as poor solubility, hepatotoxicity and manageable formulation (Sharp and Workman 2006).

Therefore, the aim of this study is the preclinical evaluation of a panel of orally active purine based HSP90 inhibitors for the targeted therapy of Hodgkin's Lymphoma.

5.1. Novel HSP90 inhibitors inhibit cell viability in HL cell lines

In this part of study, the cytotoxicity of 17-AAG and a panel of seven novel small molecules (BIIB021, EC82, EC137, EC138, EC141, EC144 and EC151) were assessed in two HL cell lines L428 and L540 represent B- and T- cell origin,

respectively, by means of XTT-assays. XTT detects mitochondrial dehydrogenase activity as a measure of cell viability. The results showed that both HL cell lines are susceptible to HSP90-inhibition. In equimolar concentrations, the new compounds were significantly more potent than 17-AAG reducing the cell viability of all seven cell lines examined. However, the orally bioavailable BIIB021 was then selected for further study because it is already in phase I/ II clinical trials, and showed antitumor activity against many cancer types (Lundgren et al., 2009). The effects of BIIB021 on the cell viability of HL cell lines in comparison with 17-AAG, as a reference substance, was assessed using an XTT assay. Six of HL cell lines used were of B-cell origin (L428, L540cy, L1236, DEV, KMH2, L540 and L591), one of T-cell (L540) origin and the EBV-positive L591 cell line.

The results showed that all tested HL cell lines were susceptible to HSP90-inhibition. Both BIIB021 and 17-AAG effectively inhibited cell viability of all tested HL cell lines in a dose-dependent manner. The IC50 values obtained from the XTT assay showed that BIIB021 was up to 7.5 fold more potent than 17-AAG in all HL cell lines, irrespective of B- or T- cell origin. The basis for this appears to be the weak activity of 17-AAG against hematological tumor lines compared with solid tumor lines, although these lines are not reported to carry the NQO1 mutation shown to confer 17-AAG resistance (Okawa et al., 2009). Furthermore, 17-AAG has significant limitations as a drug, including poor solubility, low oral bioavailability and sensitivity to MDR efflux pumps. In contrast, BIIB021 is not susceptible to tumor cell resistance mediated by MDR, MRP, NQO1 that limit the potency of 17-AAG (Lundgren et al., 2009). Thus, such resistance can be overcome with this novel HSP90 inhibitor. A current study by Lundgren et al. (2009) documented that BIIB021 effectively inhibits cell growth in various types of tumors.

5.2. BIIB021 inhibits HL cell viability and acts synergistically with conventional chemotherapy

Hodgkin's lymphoma treatment is based on combination chemotherapy and radiation therapy. Although this treatment strategy produces a high cure rate, short and long term toxic effects continue to be challenging for young cured patients.

The cytotoxicity of BIIB021 in combination with standard chemotherapy commonly used in the treatment of HL such as doxorubicin and gemcitabine was evaluated using the combination index (CI) method described by Chou and Talalay (1984).

Doxorubicin is commonly used as first-line chemotherapy in patients with HL, while gemcitabine is commonly used as second-line chemotherapy in relapsed patients with HL. They exert their antitumor effects by interference with the synthesis and function of DNA (Cappella et al., 2001; Frederick et al., 1990). Combinations of BIIB021 and doxorubicin or gemcitabine synergistically inhibit cell viability of L428 and L540cy cells, suggesting that combining these agents with BIIB021 may enhance the effect of the drug by sensitizing tumor cells or potentiating their anticancer activity.

Recent studies suggest that acetylation of HSP90 effects ATP binding, which could affect HSP90 activity (Qian et al., 2006). ATP binding and hydrolysis cause conformational changes in HSP90 that are required for its function. Several HDAC inhibitors induce HSP90 acetylation, inhibit its binding to ATP, and stimulated enhanced degradation of many HSP90 client proteins. HDAC inhibitors have also been reported to inhibit angiogenesis. 17-AAG has been reported to act synergistically with HDAC inhibitor, SAHA, to induce mitochondrial damage, caspase activity and apoptosis in leukemia and lymphoma cell lines (Rahmani et al., 2003). Interestingly, a recent study suggests significant clinical activity of HDAC inhibitors in refractory HL (Younes et al., 2007). Thus, BIIB021 in combination with the HDAC inhibitor, valproic acid,

was tested in L428 and L540cy cells. Similarly, BIIB021 showed synergy with valproic acid, causing greater cell viability inhibition than either drug alone.
This study demonstrated that BIIB021-mediated inhibition of HL cell viability at low concentrations as a single agent or in combination with doxorubicin, gemcitabine or valproic acid, can provide synergistic activity. Thus, combination of BIIB021 with such agents may represent a novel anti-Hodgkin's lymphoma strategy.

5.3. BIIB021 increases levels of HSP70 and HSP90 in HL cells

Levels of HSP90 and HSP70 in HL cell lines in response to BIIB021 were examined as well as in healthy donor-derived lymphocytes using Western blot. Both, HSP90 and HSP70 were abundantly expressed in all tested HL cell lines (L428, l540cy, L1236, DEV, KMH2, L540 and L591, L540). In contrast, healthy donor-derived lymphocytes only weakly expressed HSP90 and HSP70. BIIB021-mediated HSP90 inhibition in HL was associated with an increase in HSP90 and HSP70, possibly as cellular response to stress, but these elevated levels did not prevent the antiproliferative effect of BIIB021. These results were in agreement with that reported by Georgakis et al. for 17-AAG in HL cells.
Treatment with BIIB021 disrupts the association between HSP90 and heat shock factor 1 (HSF-1), thereby resulting in induction of HSP70, so that HSP90 and HSP70 levels are regulated through the transcriptional activity of HSF-1 (Jolly and Morimoto, 2000). HSF1 transcription is activated upon heat shock and exists as an inactive monomer in a complex with HSP70 and HSP90 and involved in regulating the transcription of the heat shock proteins involved in the cochlear stress response.

5.4. BIIB021 selectively induces Annexin-V/ 7AAD binding in HL cell lines and reduces levels of XIAP and PARP in HL cell lines

The effect of BIIB021 on inducing apoptosis in HL cells applying Annexin V-binding assay and Western blot analysis was evaluated. Treatment of HL cells with 0.8 µM BIIB021 for up to 72 hours resulted in a massive induction of apoptosis as the number of Annexin V-FITC/7AAD positive cells was significantly increased.

Furthermore, the cleavage of poly-ADP-ribose-polymerase (PARP), as an indicator of apoptosis, was also detected in Western blot analysis. Treatment of HL cells with 0.8 µM BIIB021 for up to 48 hours resulted in a marked increase in PARP cleavage in all cell lines as a result of HSP90 inhibition. Cleavage of PARP more specifically points to the involvement of caspases in the cascade that mediates BIIB021-induced apoptosis.

In contrast, no cytotoxicity in PBMCs from healthy blood donors was observed, suggesting selective cytotoxicity against tumor cells; however, our data indicated that the expression of HSP90 in HL cells is much higher than in normal cells.

Recently, Kashkar et al. have reported that XIAP is a key mediator of apoptosis resistance in HL cells (Kashkar et al., 2003). XIAP seems to be an attractive target for HL, as it influences cell-cycle progression and supports NF-kB activity. Therefore, the level of XIAP in HL cells in response to HSP90 inhibition was analyzed using Western blot analysis. The results showed that XIAP was highly expressed in all HL cell lines. Treatment of HL cells with 0.8 µM BIIB021 for up to 72 hours resulted in downregulation of XIAP levels as compared with control DMSO treated cells. The decrease was even more pronounced after 72 hours of incubation. However, Georgakis et al. reported that 17-AAG has no effect on XIAP levels in HL cell lines and this could be due to inhibitors of apoptosis family members (XIAP, c-IAP1, c-IAP2, and survivin) bind to caspases-3, -7, and -9 and prevent the onset of the caspases activation cascade, thereby inhibiting apoptosis (Reed 1999). In contrast to 17-AAG, BIIB021 may activate caspases and involve a mitochondrial pathway to induce apoptosis in HL cells. Recent

results from our laboratory demonstrated that BIIB021 could also induce cell cycle arrest in L428 cells as an early effect of HSP90 inhibition (Böll et al. 2009).
So far, various molecular mechanisms have been proposed to play a role in the pathogenesis of classic Hodgkin's lymphoma (Felberbaum 2005). Targeting these could lead to the development of novel therapies for this disease. Thus, new therapeutic targets are identified that could give rise to a new generation of rationally designed therapies that may be even more effective and less toxic than current treatments.

5.5. HSP90 inhibition inhibits HL growth *in vivo*

To date, the effects of HSP90 inhibitors on Hodgkin's lymphoma cell growth *in vivo* have not been described. Therefore the effect of 17-AAG and BIIB021 on HL tumor growth in a xenograft model of subcutaneous L540cy HL tumors was tested. Either 17-AAG or BIIB021 showed significant inhibition of tumor growth in HL xenograft model compared to mice treated with control vehicle, suggesting *in vivo* activity of HSP90 inhibition. These results although HL xenograft model is artificial and neither reflect the biology of HL in humans nor the potential BIIB021-mediated human effector mechanisms, underlining the importance of clinical research trials.

In conclusion, as BIIB021 was shown to develop high antiproliferative effects on several types of tumors (Lundgren et al., 2009), the effect of BIIB021 on seven different HL cell lines was tested. Furthermore the effect of BIIB021 on the apoptosis rate and XIAP level in HL cells was investigated. All tested HL cell lines were susceptible to HSP90-inhibition regardless of EBV status, histological subtype and origin (T- or B- cell derived). The results of this study showed that the cytotoxic effect of BIIB021 was associated with the induction of apoptosis and associated increase of intracellular HSP90 and HSP70. Induction of apoptosis in HL cells by BIIB021 was also associated with cleavage of PARP and XIAP which

seemed to be mediated through an inhibition of the NF-kB signaling pathway (Böll et al., 2009). The ability of BIIB021 to disrupt multiple cellular functions makes it a potentially attractive drug for combination therapy. Combination of BIIB021 and doxorubicin, gemcitabine or valproic acid resulted in synergistic effect in two HL cell lines (L428 and L540cy). Importantly, the effect of BIIB021 alone or in combination with gemcitabine was selectively toxic for malignant HL cells since no cytotoxic effect was observed in healthy donor-derived lymphocytes. Thus, this combination represents a novel strategy to increase the efficacy of BIIB021.

The results of this study clearly indicate that HSP90 is associated with the growth and survival of HL cells and that inhibition of HSP90 may have therapeutic potential in the treatment of HL. Furthermore, BIIB021 is capable of inducing cell growth inhibition and apoptosis in HL cells *in vitro* and *in vivo* and should therefore be further investigated in clinical trials.

6. Summary

The molecular chaperone heat shock protein 90 (HSP90) is required for the stability and function of various proteins which play critical roles in tumor cell growth and survival. HSP90 has been recently targeted for cancer therapy with HSP90 inhibitors, such as 17-AAG. Although 17-AAG has entered clinical trials with some promising early data, it demonstrated limitations including poor solubility, stability, and hepatotoxicity. The objective of this study was to assess the cytotoxicity effect of the novel HSP90 inhibitor BIIB021 on Hodgkin's lymphoma cells *in vitro* and *in vivo* in comparison to 17-AAG as a reference substance. Furthermore, to test the combination of BIIB021 with gemcitabine, doxorubicin or valproic acid and to assess the effect of BIIB021-mediated HSP90 inhibition on the X-linked inhibitor of apoptosis protein (XIAP) in Hodgkin's lymphoma cell lines.

Immunoblotting experiments showed that HSP90 is highly expressed in HL cells, while healthy donor-derived PBMCs express low levels of HSP90. In XTT assays, both BIIB021 and 17-AAG inhibited HL cell viability effectively. However, BIIB021 was about 8 times more potent, with an IC50 below 0.8 µM in all tested HL cell lines. Treatment with BIIB021 resulted in a marked decrease in cell viability and induction of apoptosis in HL cells but not in healthy donor-derived lymphocytes. Induction of apoptosis was associated with degradation of XIAP, cleavage of PARP and increased HSP70 and HSP90 expression levels. Combination of BIIB021 and Gemcitabine, Doxorubicin or Valproic acid exerted synergistic effects in HL cell lines. Furthermore, combination of BIIB021 and Gemcitabine induced more apoptosis in L428 cells than treatment with either agent alone.

In conclusion, these results suggest that HSP90 is required for cell viability and signaling in HL. Thus, BIIB021 mediated inhibition of HSP90 could simultaneously disrupt multiple pathways involved in cancer development and might be a valuable therapeutic target in HL as a single agent or in combination.

To date, the effects of HSP90 inhibitors on Hodgkin's lymphoma cell growth *in vivo* have not been described. When 17-AAG or BIIB021 was tested in a xenograft HL model, a significant inhibition of tumor growth compared to mice treated with control vehicle was observed suggesting *in vivo* activity of HSP90 inhibition in HL. The results of this study show that HSP90-inhibition has direct anti-tumor activity in HL *in vitro* and *in vivo.* Iinhibition of HSP90 may have therapeutic potential in the treatment of HL and should therefore be investigated in clinical trials.

7. Zusammenfassung

Das molekulare Chaperone Protein “Heat Shock Protein 90“ (HSP90) wird für die Stabilität und Funktion zahlreicher Proteine benötigt, die eine kritische Rolle im Wachstum und Überleben von Tumorzellen spielen. HSP90 wird seit kurzer Zeit als Zielstruktur der Krebstherapie mit HSP90-Inhibitoren wie 17-AAG genutzt. Obwohl 17-AAG mit vielversprechenden ersten Ergebnissen in klinischen Studien getestet wird, zeigten sich bereits klinische Grenzen des Einsatzes, wie eine schlechte Wasserlöslichkeit, Stabilität und Hepatotoxizität.

Das Ziel der vorliegenden Arbeit war es, den zytotoxischen Effekt des neuen HSP90 Inhibitors BIIB021 in Vergleich zur Referenzsubstanz 17-AAG auf Hodgkin Lymphomzellen *in vitro* und *in vivo* zu testen. Außerdem sollte die Kombination von BIIB021 mit Gemcitabin, Doxorubicin oder Valproat, und der Effekt auf das X-linked inhibitor of apoptosis protein (XIAP) in Hodgkin Lymphomzellen (HL Zellen) getestet werden.

Immunoblot Experimente zeigten, dass HSP90 in HL Zellen hochgradig exprimiert ist, während gesunde Spenderleukozyten niedrige Spiegel von HSP90 exprimieren. In XTT-Assays inhibierten sowohl BIIB021 und 17-AAG die HL-Zellviabilität effektiv, jedoch hatte BIIB021 eine etwa achtfach höhere Effektivität mit einer IC50 unter 0.8 µM in allen getesteten HL-Zelllinien. Die Inkubation mit BIIB021 bewirkte eine deutliche Reduktion der Zellviabilität und eine Induktion der Apoptose in HL Zellen, jedoch nicht in gesunden Spenderlymphozyten. Die Induktion der Apoptose war assoziiert mit einer Degradation von XIAP, Spaltung von PARP und einer erhöhte Expression von HSP70 und HSP90. Die Kombination von BIIB021 mit Gemcitabin, Doxorubicin oder Valproat hatte synergistische Effekte in HL Zelllinien. Darüber hinaus induzierte die Kombination von BIIB021 mit Gemcitabin mehr Apoptose als die Einzelsubstanzen.

Zusammenfassend deuten diese Ergebnisse darauf hin, dass HSP90 für die Viabilität bei HL Zellen benötigt wird. BIIB021-induzierte Inhibition von HSP90 könnte simultan mehrere zur Kanzerogenese essentielle Signalkaskaden

nterbrechen und könnte als Einzelsubstanz oder in Kombinationstherapien eine wertvolle Zielstruktur bei der Therapie des HL sein.

Bisher ist der Effekt von HSP90 Inhibition *in vivo* beim HL nicht beschrieben worden. In einem HL Xenograft Modell des HL bewirkten 17-AAG und BIIB021 eine signifikante Inhibition des Tumorwachstums im Vergleich zum Tumorwachstum bei Vehikel-behandelten Kontrolltieren.

Die Ergebnisse der vorliegenden Arbeit zeigen eine direkte *in vitro* und *in vivo* anti-Tumorwirksamkeit von HSP90 Inhibitoren beim HL. HSP90 Inhibition könnte therapeutisches Potential in der Behandlung des HL haben und sollte in klinischen Studien evaluiert werden.

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9. Publications

1. Boris Böll, Farag Eltaib, Katrin S. Reiners, Bastian von Tresckow, Samir Tawadros, Venkateswara R. Simhadri, Francis J. Burrows, Karen Lundgren, Hinrich P. Hansen, Andreas Engert and Elke Pogge von Strandmann (2009). Heat Shock Protein 90 inhibitor BIIB021 (CNF2024) depletes Nuclear Factor kappa B and sensitizes Hodgkin Lymphoma cells for Natural Killer cell-mediated cytotoxicity. Clin Cancer Res. Aug 15;15(16):5108–5116.

Posters

1. B. Böll, F. Eltaib, K. Reiners, P. Borchmann, F. Burrows, A. Engert, E. Pogge von Strandmann. Inhibition of HSP90 for the targeted therapy of Hodgkin`s Lymphoma. Onkologie 2006; 29 (suppl 3): 1–236 (P726).

2. B. Böll, V.R. Simhadri, K.S. Reiners, H.P. Hansen, F. Eltaib, A. Engert, E. Pogge von Strandmann. Release of the BAT3 (HLA-B-associated transcript 3) from Hodgkin lymphoma cells in response to the histone deacetylase inhibitor sodium valproate.
   Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaften für Hämatologie und Onkologie Basel, Schweiz, 5-9 October 2007

3. B. Böll, F. Eltaib, K. Lundgren, K. Reiners, S. Tawadros, B. von Tresckow, F. Burrows, A. Engert, E. P. von Strandmann. Novel hsp90 inhibitor CNF2024 selectively kills Hodgkin lymphoma cells by depleting NF-kB and inhibits HL tumor growth in vivo.
   7th International Symposium on Hodgkin Lymphoma, Köln, Germany,
   3-7 November 2007.

10. Lebenslauf

Name:	Eltaib, Farag Ibrahim Mohamed
Geburtsdatum:	09. 08.1966
Geburtsort:	Benghazi, Libya
Staatsangehörigkeit:	Libyen
Eltern:	Ibrahim Mohamed Eltaib, Rentner
	Salma Khalil Erig, Hausfrau
1981-1984	Gymanasium (High School), Omar Al-mukhtar, Benghazi, Libya
1985-1989	Bachelor of Science (B.Sc) in Medical Laboratory, Higher Institute of Technology- Brack, Universität zu Sebha, Libya
1996-1999	Master of Science (M.Sc) in Biochemistry, Universität zu McGill, Kanada
2006-2009	Doktorarbeit (Dr. rer. medic.) by Prof. Engert A. und Prof. Dr. Elke Pogge von Strandmann- Labor für Immunotherapie, Haematology and Oncology, Klinik I für Innere Medizin der Universität zu Köln