CHAPTER 1

1. INTRODUCTION

1.1 ZAP70 Kinase

ZAP70, zeta-associated protein of 70 kDa, belongs to a family of protein tyrosine kinases (PTK) closely related to Src, and is one of the membrane proximal components associated with early cell activation in T and NK-lymphocytes.\(^1\) TCR-mediated activation of T cells is crucial to the immune response. Transplant rejection and diseases, such as allergic and autoimmune disorders, have features of a failure to adequately modulate T cell activation.

The engagement of the T-cell antigen receptor (TCR) with the molecular complex of a peptide antigen bound to a major histocompatibility complex molecule (pMHC) initiates a series of biochemical signals to translate the extracellular binding event into an intracellular response that results in T-cell activation. The signaling cascade that results from TCR stimulation is heavily dependent on protein phosphorylation and is, thus, dynamically regulated by protein tyrosine kinases and phosphatases. Therefore, the protein tyrosine kinases (PTKs) downstream of the TCR have been particularly well studied, including a PTK that directly interacts with the TCR complex, the zeta-associated protein of 70 kDa (ZAP70).
ZAP70 kinase, a 70 kDa member of the Syk family kinase associated with the zeta-subunit of the T cell receptor, is primarily expressed in T and NK cells and plays an important role in initiation of normal TCR signalling, crucial for T cell activation and development. The relative importance of this kinase for lymphocyte activation has been evaluated by disrupting functional ZAP70 which resulted in a marked reduction in protein tyrosine phosphorylation, cytosolic Ca$^{2+}$ level change, and loss of IL-2 production, demonstrating that ZAP70 is absolutely required for IL-2 production by T cells. In addition, human severe combined immunodeficiency patients have been identified that do not express ZAP70 protein. All these reports indicate that ZAP70 is a potentially useful therapeutic target for immune suppression.

Inhibitors of ZAP70 Tyrosine-kinase activity with high affinity and high selectivity can block signaling downstream of the kinase leading to inhibition of T-cell lymphocyte activation. Peptides that block the association of ZAP70 with the ζ subunit and compounds that antagonize ZAP70 tyrosine kinase activity are known to block T cell activation in vitro. Piceatannol blocks Syk and ZAP70 tyrosine kinases involved in immune cell activation and prolongs kidney allograft survival in the stringent ACI-to-Lewis rat model.
The three-dimensional structure of the catalytic tyrosine kinase domain of ZAP70 has been crystallized recently in complex with Staurosporine at 2.3 Å resolution. Staurosporine is a non-selective, ATP inhibitor of many kinases having IC$_{50}$ value of 55.8nM against rhZAP70 tyrosine kinase.$^6$

There is currently no highly specific, small-molecule inhibitor for ZAP70. In situations where overactive T cells are a substantial component of disease, such as in autoimmune diseases or transplantation, targeting ZAP70 could provide a means to target the T cells while avoiding adverse effects on other cells. Thus, a potent and selective ZAP70 tyrosine kinase inhibitor should modulate T cell function in a more targeted fashion than the less specific immunosuppressive agents currently available, such as cyclosporine A and FK-506.

1.1.1 ZAP70 Structure

ZAP70 and Syk are the only two members of the Syk family of PTKs. They share a similar domain organization with two N-terminal SH2 domains and a C-terminal kinase domain (Figure 1.1). The tandem SH2 domains of ZAP70 bind the doubly phosphorylated ITAMs, which serve to dock the PTK at the stimulated TCR complex. The region between the
second SH2 domain and the kinase domain, termed interdomain B, contains three tyrosines, Tyr292, Tyr315, and Tyr319 that are known sites of phosphorylation. Following their phosphorylation, these residues interact with important downstream signaling molecules, including c-Cbl, Vav, CT10 regulator of kinase II (CrkII), Lck, and phospholipase Cc (PLCc), thus making ZAP70 a potentially important adaptor molecule. However, more recent biochemical and structural studies suggest that Tyr315 and Tyr319 are also, in fact, critical for proper regulation of ZAP70 by playing critical roles in an autoinhibitory conformation of the protein.\textsuperscript{10-12}

\textbf{Figure 1.1:} Domain organization of ZAP70 Kinase
Kinase Domain Regulation

The kinase domain of ZAP70 contains two tyrosine residues, Tyr492 and Tyr493, which are phosphorylated after TCR engagement ([Figure 1.2, Figure 1.3]). These tyrosines can be phosphorylated by Lck or by ZAP70 itself. Mutational analysis of tyrosines 492 and 493 to phenylalanine revealed a negative regulatory role for Tyr492 and a positive regulatory role for Tyr493. A ZAP70 Y492F mutant exhibited increased kinase activity, while kinase activity of the Y493F mutant was impaired. Consistent with these findings, the expression of the ZAP70 Y492F mutant but not the Y493F mutant was able to complement Syk-deficient BCR signaling and induction of IL-2 promoter activity. Phosphorylation of these two tyrosines, located within the activation loop, potentially serves to displace the activation loop from the catalytic site of the kinase. However, crystal structure analysis of the ZAP70 kinase domain in complex with the small molecule inhibitor staurosporine suggests that the activation loop can adopt an active and open conformation, even without phosphorylation of Tyr492 and Tyr493. These results suggest that additional regulatory mechanisms regulate ZAP70 catalytic activity. Indeed, conformational changes in the tandem SH2 domains and Interdomain B are likely to play a role in ZAP70 autoinhibition. Analysis of a ZAP70 mutant that lacks Interdomain B entirely revealed that ZAP70 catalytic activity can be modulated by but is not completely dependent on Interdomain B.
Figure 1.2: Regulation of ZAP70 in TCR signaling

Figure 1.3: T-cell function pathway.
Activated TCRs recruit ZAP70, which phosphorylates proteins such as LAT (linker for the activation of T cells) and SLP-76 (Src homology 2-domain-containing leukocyte protein of ~76 kDa). These proteins then serve as scaffolds to organize signaling complexes essential for onward signal transmission. ZAP70 is further phosphorylated by Lck and/or Fyn and is thus activated. Subsequently, these kinases phosphorylate other specific substrates, resulting in the activation of the various intracellular signaling pathways required for T cell function.

Crystal structures

A comparison of the crystal structures of the tandem SH2 domains of ZAP70 (without the kinase domain) bound to a dually phosphorylated ITAM peptide with the unbound SH2 domains in the autoinhibited conformation indicates that the two SH2 domains are reoriented and move closer together when engaged by ITAM peptide. This large conformational change is directly coupled to a reorientation of helices αL2 and αL3 and a consequent repositioning of Pro147, resulting in a steric clash with Y579/598 (Figure 1.4). For this reason, ITAM binding to the two SH2 domains appears inconsistent with a docking of the tandem SH2 unit onto the catalytic domain.

Therefore, ITAM engagement is likely to dislodge the tandem SH2 unit from the distal surface of the kinase domain, and to destabilize the
linker–kinase sandwich as well as the rigidifying hydrogen bonding network that stabilizes the inactive conformation of the kinase domain.

An initial destabilization of the linker–kinase sandwich would promote access to the regulatory tyrosine phosphorylation sites in the SH2-kinase linker. Once these sites (Tyr315 and Tyr319) become phosphorylated by Lck or by ZAP70 itself in trans, reformation of the autoinhibitory linker–kinase sandwich seems energetically even less favorable. Phosphorylation of the activation loop would then render ZAP70 maximally active by preventing the activation loop from collapsing into the active site. ZAP70 in complex with Staurosporine is also crystallized (1U59) (Figure 1.5)
Figure 1.4: Activation of ZAP70 upon ITAM binding

Figure 1.5: ZAP70 crystal structure (1U59) in complex with Staurosporine
1.1.2 ZAP70 role in different diseases

ZAP70 and the entire TCR pathway must be optimally activated during thymic selection to insure ‘fine-tuning’ of the peripheral T-cell repertoire. A recent study suggested that development and function of regulatory and helper CD4+ T-cell functions are separated based on their different signaling levels. Various functions of CD4+ T cells were dependent on TCR signaling, including forkhead box protein 3 (Foxp3)+ selection, positive and negative selection, autoantibody help, naive CD4+ T-cell numbers, maintenance of memory T cells, and other functions. Therefore, mutations compromising ZAP70 or TCR signaling can have unexpected or even paradoxical outcomes: sometimes immunodeficiency, sometimes autoimmunity, sometimes both.

SCID

ZAP70 deficiency is a rare autosomal recessive form of severe combined immune deficiency (SCID) that is characterized by lack of CD8+ T cells with normal number of circulating CD4+ T cells that however are unresponsive to T-cell-receptor (TCR)-mediated stimulation in vitro. Loss of function or expression of ZAP70 leads to an unusual form of SCID in humans, revealing the critical role of human ZAP70 in both in mature T-cell signaling and differentiation of thymic precursors (Figure 1.6).
Thus far, the ZAP70 mutations occurring in human SCID have resided mostly in the kinase domain, although loss of transcription and one mutation in the N-terminal SH2 domain that resulted in rapid degradation of ZAP70 protein have also been reported.\textsuperscript{3,18} This rare autosomal recessive form of SCID indicates the absolute requirement of ZAP70 for the development of CD8+ T cells.

The importance of ZAP70 in thymocyte development was further demonstrated in an animal model where mice developed rheumatoid arthritis (RA), resembling human RA, caused by a point mutation in ZAP70. As a result of this mutation, an altered signal transduction from the TCR causes a change in thymic selection leading to positive selection of autoimmune T cells that should otherwise be negatively selected against.\textsuperscript{19}

The disease is extremely rare, with thirty or so cases reported so far. During childhood, nearly all victims of ZAP70 deficiency present clinical signs characteristic of SCID: severe pulmonary infection, often sustained by opportunistic pathogens (Pneumocystis carinii), chronic diarrhea, failure to thrive, and persistent candidiasis. Unless treated by bone marrow transplantation, ZAP70 deficiency is ultimately fatal.\textsuperscript{20} Taken together, studies on human ZAP70 immunodeficiency establish
the critical role of ZAP70 in TCR signaling during thymocyte development and in peripheral T-cell function.

**Figure 1.6:** Normal signaling pathway (left) and ZAP70 deficient pathway (right). ZAP70 deficient cells still demonstrate some immune response via Syk, however, Syk does not interact with ζ-chains

**Chronic Lymphocytic Leukemia**

The protein tyrosine kinase ZAP70, normally expressed in T cells and a subset of B cells, is solely expressed in poor prognosis chronic lymphocytic leukaemia and implicated in enhanced B cell receptor signaling (**Figure 1.7**). As a result, the expression of this protein provides an ideal prognostic marker for the disease.
ZAP70 was first recognised as a potential surrogate for IgVH mutational status as a result of gene expression profiling with cDNA microarrays comparing the two subtypes of CLL. Out of the 240 genes found to be differentially expressed between the two types, ZAP70 was identified as the most prominent amongst these genes with expression levels 5-fold higher in patients with unmutated IgVH genes as in those with mutated genes.\textsuperscript{21,22}

\textbf{Figure 1.7:} BCR signaling in CLL
1.1.3 ZAP70 Inhibitors

Breakthrough advances over the past decade have so far resulted in several small-molecule ZAP70 kinase inhibitors. However, broad expression of many of these kinases with much more structural similarity makes them unappealing targets because inhibition of the kinase, when outside of the disease context, can lead to undesirable side effects. There are ~5000 inhibitors reported in the literature with biological activity against ZAP70 (Figure 1.8).
Figure 1.8: Some of the known inhibitors of ZAP70

Conclusion:
The profound immunodeficiency that results from its absence, the
autoimmunity that may develop from its decreased function, and the
poor prognosis associated with its presence in nearly 50% of the cases of
B-CLL, all suggest that ZAP-70 is an attractive therapeutic target for
pharmacologic intervention. After nearly 20 years, since its discovery,
there is still no pharmacologic inhibitor of ZAP-70. Clearly such an
inhibitor would have great therapeutic potential in transplantation,
autoimmunity and possibly in B-CLL. There are so many ZAP70
inhibitors reported in the literature.
1.2 HSP90

The HSP90 super chaperone complex has wide-ranging functions that result from the ability of this sophisticated machinery to assist in the folding and function of a variety of oncogenic ‘client proteins’. In this sense, multiple proteins involved in cell-specific oncogenic processes have been shown to be tightly regulated by the binding of the HSP90 machinery. These include BCR-ABL in the chronic myelogenous leukemia (CML), nucleophosmin-anaplastic lymphoma kinase (NPM–ALK) in lymphomas, mutated FLT3 in acute myeloid leukemia, EGFR harboring kinase mutations in nonsmall cell lung cancer (NSCLC), the zeta-associated protein of 70 kDa (ZAP70) as expressed in patients with aggressive chronic lymphocytic leukemia (CLL), mutant B-Raf in melanoma, human epidermal growth factor receptor 2 (HER2) in HER2-overexpressing breast cancer, mutant c-Kit in gastrointestinal stromal tumors (GIST), and activated Akt in small cell lung carcinoma, to list a few. It is now accepted that at the phenotypic level, the HSP90 machinery serves as a biochemical buffer for the numerous cancer-specific lesions that are characteristic of diverse tumors.

Pharmacologic inhibition of HSP90 by structurally diverse small molecules destabilizes the cancer cell’s aberrant protein subset, leading to protein degradation by the 26S proteasome. Selective depletion of the
cancer cell’s malignancy driving molecules results in growth arrest, apoptosis, and renders cells vulnerable to the actions of chemotherapeutic interventions that otherwise afford limited benefit.\textsuperscript{23,24} Moreover, cancer cells are selectively sensitive to pharmacologic HSP90 inhibitors, and administration of these agents to multiple cancer animal models results in significant antitumor effects associated mostly with little or no target-associated toxicities.\textsuperscript{34,35} In fact, cytotoxic inhibitors of HSP90 are the only cancer chemotherapeutic agents known to impact all six hallmarks of cancer simultaneously.\textsuperscript{36} As defined by Hanahan and Weinberg, this includes 1) self-sufficiency in growth signals, 2) insensitivity to antigrowth signals, 3) evasion of apoptosis, 4) limitless replicative potential, 5) sustained angiogenesis, and 6) tissue invasion/metastasis.\textsuperscript{37}

**Properties of HSP90**

Heat-shock proteins (Hsps) act as molecular chaperones, guiding nascent polypeptides through the process of folding and maturation into three-dimensional structures.\textsuperscript{38,39} Chaperones are also responsible for refolding denatured proteins that result from cellular stresses such as nutrient deprivation, abnormal temperature or pH, malignancy, and exposure to various toxins and drugs.\textsuperscript{40,41}
Heat-shock response is conserved across all species, from prokaryotes to eukaryotes, and provides a mechanism for general upkeep of intercellular processes, including protection against protein aggregation in the cytosol.\textsuperscript{42}

HSP90, the most prominent of the heat-shock proteins, makes up 1–2% of all cytosolic protein and exists in four isoforms: HSP90\textsubscript{α}, HSP90\textsubscript{β}, glucose-regulated protein (GRP94), and Hsp75/tumor necrosis factor receptor associated protein 1 (TRAP-1). HSP90\textsubscript{α} and HSP90\textsubscript{β} can be found in the cytosol, and are the inducible and constitutive forms, respectively. GRP94 resides in the endoplasmic reticulum, while TRAP-1 is located in the mitochondrial matrix.\textsuperscript{43,44}

To date, HSP90 has been found to interact with over 200 client proteins, as well as around 50 co-chaperones, making it a cornerstone in the cellular protein-folding machinery and an emerging target for the treatment of various disease states.

### 1.2.1 HSP90 Structure

HSP90 is a large, homodimeric protein with three main structural domains. The N-terminal domain contains the ATP- and geldanamycin-binding site, and is responsible for the weak intrinsic ATPase activity of
HSP90 (Figure 1.9). The middle domain, which is thought to be the major site of client protein binding, is connected to the N-terminal domain through a highly charged linker region. The C-terminal domain contains the dimerization interface and a conserved C-terminal MEEVD motif, which is responsible for binding TPR-containing cochaperones. C-Terminal crystal structures of bacterial HtpG and eukaryotic HSP90 were solved in 2004 and 2006, respectively.

In 2000 Neckers and co-workers were able to show that inhibition of HSP90 at the C-terminus interrupts activity in a non-ATP competitive fashion. This discovery makes the C-terminus of HSP90 a promising target for drug development, and highlights the importance of utilizing a co-crystal structure to further understand this process. Crystal structures are available for the N-terminal domain of yeast and human HSP90, for complexes of the N-terminus with inhibitors and nucleotides, and for the middle domain of yeast HSP90. Recently structures for full length HSP90 from E. coli (2IOP, 2IOQ), yeast (2CG9, 2CGE), and the dog endoplasmic reticulum (2O1U, 2O1V) were elucidated.
1.2.2 HSP90 role in different cancers

Inhibitors of HSP90 have shown as high as a 200-fold differential selectivity toward malignant versus normal cells. Several mechanisms have been suggested to explain this high selectivity.

1. HSP90 is significantly upregulated in malignant cells to compensate for their dependency on the overexpression of client proteins. The increased concentration of HSP90 in tumor cells inherently results in greater drug accumulation.

2. A second mechanism, introduced by Conforma Therapeutics in 2003, proposes that the HSP90 heteroprotein complex exhibits a higher affinity for N-terminal inhibitors than the inactive homodimer. In cancer cells, HSP90 exists predominantly in a
heteroprotein complex due to the over abundance of mutated, denatured, and naturally expressed proteins. In contrast, the primary form of HSP90 in normal cells is the homodimer, which explains why N-terminal inhibitors accumulate in the high-affinity HSP90 complex found in tumor cells.54,55

3. Finally, a 2006 report by Duvvuri and co-workers56 suggests a physiochemical explanation for selectivity. Under normal physiological conditions, lysosomal pH is around 4–5. HSP90 inhibitors, like many chemotherapeutic agents, often contain basic nitrogens, and in a process known as pH partitioning become protonated as the ammonium salt within the lysosome, trapping them in the organelle and preventing interaction with HSP90, which resides in the cytosol57. Conversely, the lysosomal pH in cancerous cells is essentially neutral, favoring an unprotonated amine and suggesting that the equilibrium drug concentration between lysosome and cytosol favors cytosolic interaction with tumor-derived HSP90 more than in normal cells.58,59.

1.2.3 HSP90 Inhibitors

Natural product inhibitors

Clinical trials have shown that HSP90 inhibitors are not only potent as anti-cancer agents, but are also well tolerated by patients. Two general classes of natural product inhibitors of HSP90 have been discovered
which bind to the N-terminal ATP pocket, and are based on Geldanamycin (GM) and radicicol (RD). These natural products, especially GM, played a vitally important function in the elucidation of the biological role of HSP90 in cancer. They affect HSP90 chaperone function in a similar manner and possess comparable biological activity. In fact, the toxicities and side effects discovered have not been directly linked to HSP90 inhibition, but rather to hepatotoxicity, gastrointestinal irritation, and constitutional symptoms. Given the inherent diversity and vast array of scaffolds that allow for protein interaction, natural products have become a key component in HSP90 research.

Geldanamycin-based HSP90 inhibitors

The first HSP90 inhibitor to enter clinic was the geldanamycin (GM) derivative 17-allylamino-17-desmethoxygeldanamycin (17-AAG) (Figure 1.10). This natural compound, isolated from the broth of Streptomyces hygroscopicus in 1970s, was already known for its antibiotic activities. The antitumor activity of GA was reported near 20 years after, and initially thought to act as a tyrosine kinase inhibitor. Subsequent studies revealed that GA binds to the N-terminal ATP binding site of HSP90, resulting in alteration of its function and the proteolytic degradation of client proteins.
Figure 1.10: Chemical structure of several HSP90 inhibitors currently in clinical evaluation in patients with advanced cancers.

The binding pocket for GA inhibitor is situated in the N-terminal domain of HSP90 (Figure 1.11). Although GA provided very promising antitumor effects, it showed several pharmacologic limitations as poor solubility, limited in vivo stability and high hepatotoxicity in some of the human tumor models. Structure-activity relationship (SAR) studies have shown that structurally and sterically diverse 17-substituents can be introduced without destroying antitumor activity. Therefore, new classes of inhibitors were successfully developed including 17-AAG. Although 17-AAG has shown some encouraging clinical responses, it presents important drawbacks (e.g.; liver toxicity and cumbersome formulation)
that may limit its clinical application. Another noteworthy 17-substituted geldanamycin analogue with increased chemical/metabolic stability is 17-(2-dimethylaminoethyl-amino)-17-demethoxygeldanamycin (17-DMAG), which exhibit significant HSP90 binding activity (Kd < 4µM) and cytotoxicity towards various cancer cells including SKBr3 cells (IC50 < 300 nM). This analogue displayed better pharmacological properties as it was shown to be less toxic, more water soluble and orally bioavailable than 17-AAG, and is being now evaluated in phase II clinical trials. However, the limitation is still the inability to assess quantitatively, as a function of time, the effect of 17-AAG on HSP90 function in patients during phase I trials.63,64 However, the limitation is still the inability to assess quantitatively, as a function of time, the effect of 17-AAG on HSP90 function in patients during phase I trials.
Figure 1.11: Surface view of the HSP90 protein in complex with 17-desmethoxy-17-N,N-Dimethylaminoethylamino-Geldanamycin

**Synthetic HSP90 inhibitors**

Novel synthetic HSP90 inhibitors based on diverse chemical scaffolds have been developed, and several are currently undergoing Phase I/II clinical evaluation in cancers (Table 1.1). These are reported to generally have an improved pharmacologic profile when compared to 17-AAG, especially with regard to their availability through synthesis, evasion of multidrug resistance (MDR)-mediated efflux, metabolic stability, water solubility and ease of administration, and retained biological activity over a wider panel of tumors.65,66
The first synthetic HSP90 inhibitor to enter clinic is CNF2024/BIIB021, an HSP90 inhibitor developed initially by Conforma Therapeutics (currently Biogen Idec) based on the purine-scaffold discovered by investigators at Memorial Sloan-Kettering Cancer Center through structure-based design. There are currently several ongoing Phase I trial studies of oral CNF2024/BIIB021 in advanced solid tumors, lymphomas, B cell CLL, and in HER2-advanced breast cancer, alone or in combination with trastuzumab. Results of the Phase I trials were recently released at the ASCO meeting, 2008. CNF2024/BIIB021 appeared to be well tolerated, and a dose of 800 mg twice weekly appeared as maximally tolerated. Biological activity was observed with significant induction of HSP70 and inhibition of the HER2 bio-marker extracellular domain in solid tumors. In CLL, 1 patient at 25 mg had a 39% reduction in lymph nodes, whereas in solid tumors, 11 of 16 patients (68%) reported stable disease. A Phase II evaluation of CNF2024/BIIB021 in patients with GIST was started in March 2008, in which pharmacodynamic assessment of tumor response by 18FDG-Positron Emission Topography has been implemented (http://www.clinicaltrials.gov/).

A second synthetic HSP90 inhibitor to enter clinic is VER-52296/NVP-AUY922 currently in development by Novartis (Figure 1.12). The lead compound that resulted in AUY922 was a pyrazole scaffold derivative identified through a high throughput screening effort by investigators at
the Royal Cancer Institute, and further developed in collaboration with Vernalis.66 A Phase I–II trial of intravenously administered AUY922 is currently open (http://www.clinicaltrials.gov), with the Phase I portion of the trial recruiting patients with several types of cancer, whereas the Phase II portion is limited to patients with either HER2 positive or ER positive locally advanced or metastatic breast cancer.

**Figure 1.12:** HSP90 in complex with VER-52296/NVP-AUY922

SNX-5422 is a third small-molecule HSP90 inhibitor based on the 6,7-dihydro-indazol-4-one scaffold. This agent developed by Serenex, converts to SNX-2122, which is the active HSP90 inhibitor form. A Phase I trial of orally administered SNX-5422 mesylate opened in late May 2007 in patients with refractory solid tumor malignancies. The results of a Phase I dose-escalation study on the safety and pharmacokinetics of SNX-5422 were disclosed at the ASCO 2008 meeting. In March 2008,

STA-9090, developed by Synta Pharmaceuticals is claimed to have a chemical structure unrelated to the ansamycin family of HSP90 inhibitors, such as 17-AAG. STA-9090 is currently enrolling patients in two dose-ranging Phase I clinical trials in solid tumors. The trials are open label studies in patients with solid tumors and are designed to identify the maximum tolerated dose of STA-9090 based on a twice-a-week or once-a-week intravenous dosing schedule (http://www.syntapharma.com/PrdHSP90.aspx).

**Conclusion**

The ability of HSP90 inhibitors to affect, simultaneously, multiple transforming molecules and pathways is a unique and therapeutically attractive feature of targeting this chaperone. These findings suggest that HSP90 inhibitors might provide a broader, more effective anti-cancer, anti-neurodegenerative, and anti-infectious therapy than molecules targeting single, activated, but dispensable signaling molecules that are the focus of most current drug discovery efforts. Moreover, the apparent increased requirement for HSP90 activity in at least cancer and neurodegenerative diseases suggests the real possibility of an exploitable therapeutic index for this approach.
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**Table 1.1**: HSP90 inhibitors in the clinical trials.
1.3 **ZAP70 degradation via inhibition of HSP90 in CLL**

Chronic lymphocytic leukemia (CLL) is a disease caused by the accumulation of monoclonal CD5+ B cell lymphocytes with a characteristic immunophenotype in bone marrow, peripheral blood, and lymphoid tissues. CLL predominates in the elderly and has an extremely variable clinical course. At least 2 subtypes of CLL can be differentiated by clinical presentation, mutational status of the immunoglobulin heavy-chain variable-region \( (IgVH) \) gene, and more recently also by gene expression profiling using DNA microarray technology.\(^{69}\)

1.3.1 **Prognostic biomarkers of CLL**

An important advance in CLL, not only at biological level but also in its prognostication, was the demonstration that about 50% of patients present in their leukemic cells somatic hypermutations in the rearranged variable regions of the immunoglobulin heavy chains \( (IgVH) \). In 1999, the Hamblin and Stevenson and Chiorazzi groups independently reported on the prognostic importance of \( IgVH \) genes in CLL, showing that \( IgVH \) mutational status separates CLL into two different forms of the disease.\(^{70,71}\) Patients with \( IgVH \) unmutated genes (unmutated-CLL) have a more malignant condition, including evidence of advanced, progressive disease, atypical peripheral blood cell morphology, adverse cytogenetic
features, clonal evolution, and resistance to therapy than those with mutated $IgVH$ genes (mutated-CLL).\textsuperscript{72}

Several prognostic factors correlate with the clinical progression of patients with CLL, and among those the level of expression of the zeta-associated protein of 70 kDa ($\text{ZAP70}$) appears the strongest indicator of the need for early treatment.\textsuperscript{73}

In addition to clinical prognostic parameters, several biologic characteristics associated with patients’ outcome have been identified (Table 1.2). Moreover, response to therapy, particularly the achievement of negative minimal residual disease (MRD) status, has also been correlated with a better outcome.\textsuperscript{74-76}
Table 1.2: Prognostic biomarkers of CLL

Zeta-associated protein of 70 kDa (ZAP70), a cytoplasmic tyrosine kinase essential for T-cell–receptor signal transduction, is preferentially expressed in CLL B cells whose immunoglobulin genes have not undergone somatic hypermutation (Ig-unmutated CLL), while a second CLL subtype with mutated immunoglobulin genes most often lacks ZAP70 expression (Figure 1.13). Expression of ZAP70 in CLL B cells is associated with more rapid disease progression and shorter survival and may be a better predictor of clinical course than Ig-mutation status (Table 1.3, Table 1.4).
**Figure 1.13**: Role of ZAP70, oncogenic client of HSP90, in cell proliferation

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<td>Rassenti et al.,</td>
<td>307</td>
<td>Flow cytometry, Becton</td>
<td></td>
<td>4%(^a)</td>
<td>28%(^a)</td>
<td>57%(^a)</td>
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<td>Dickinson clone 1E7.2</td>
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<td>Krober et al., 2006</td>
<td>133</td>
<td>Flow cytometry, Upstate,</td>
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<td>17%(^a)</td>
<td>41%</td>
<td>29%(^a)</td>
<td>11%(^a)</td>
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<td>(27)</td>
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<td>Wiestner et al.,</td>
<td>107</td>
<td>DNA microarray</td>
<td></td>
<td>14%(^a)</td>
<td>46%</td>
<td>70%(^a)</td>
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<tr>
<td>Laurenti et al.,</td>
<td>92</td>
<td>PCR (ZAP7/Syk ratio)</td>
<td></td>
<td>23%(^a)</td>
<td>3%</td>
<td>36%(^a)</td>
<td>28%(^a)</td>
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<td>Catherwood et al.,</td>
<td>42</td>
<td>RQ-PCR</td>
<td></td>
<td>4%(^a)</td>
<td>32%</td>
<td>55%(^a)</td>
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<td>2%(^a)</td>
<td>38%(^a)</td>
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**Table 1.3**: Correlation Between IgVH Mutational Status and ZAP70 Expression in CLL

<table>
<thead>
<tr>
<th>Study</th>
<th>N</th>
<th>Survival</th>
<th>ZAP-70 &lt;20% (yr)</th>
<th>ZAP-70 &gt;20% (yr)</th>
<th>P</th>
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<tbody>
<tr>
<td>Bosch et al., 2004</td>
<td>222</td>
<td>TTP(^a)</td>
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<td>&lt;0.001</td>
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<td>OS</td>
<td>16.3</td>
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<td>&lt;0.001</td>
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<td>Orchard et al.,</td>
<td>167</td>
<td>OS</td>
<td>24.4</td>
<td>9.3</td>
<td>&lt;0.0001</td>
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<td>2004 (25)</td>
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<tr>
<td>Rassenti et al.,</td>
<td>307</td>
<td>TTT</td>
<td>9.2</td>
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<td>OS</td>
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<td>0.005</td>
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<td></td>
<td></td>
<td>Not reached</td>
<td>8.5</td>
<td></td>
<td>0.004</td>
</tr>
</tbody>
</table>

\(^a\)TTP, time to progression; OS, overall survival; TTT, time to therapy; TFS, therapy-free survival.
\(^b\)Refers only to Binet's stage A patients (N = 187).

**Table 1.4**: ZAP70 expression and prognosis in CLL
1.3.2 ZAP70 and HSP90 Relation

Heat-shock protein 90 (HSP90) is a molecular chaperone that catalyses the conformational maturation of a large range of signalling proteins in cancer that are collectively known as ‘clients’. In advanced tumours, it exists in an activated form complexed with other molecular chaperones and with high ATPase activity, whereas in normal tissue it is in a latent, uncomplexed state.\textsuperscript{54} 17-allyl-amino-demethoxy-geldanamycin and 17-dimethylaminoethylamino-17-demethoxygeldanamycin are HSP90 inhibitors that bind to the activated form, competing with ATP and locking the complex in an intermediate, nonproductive state, resulting in release and degradation in the proteasome of the client protein.\textsuperscript{78}

Using HSP90 inhibitors, Castro et al.\textsuperscript{29} have demonstrated that ZAP70 is an HSP90 client protein in tumour cells, but not in T cells, from patients with ZAP70 positive CLL (Figure 1.14). In ZAP70-negative CLL cells that have been transduced with an adenovirus vector coding for ZAP70, the ZAP70 is likewise an HSP90 client protein. The inhibitors blocked BCR signalling in ZAP70-positive cells, caused the degradation of ZAP70 and induced apoptosis in the CLL cells.
HSP-90 inhibitors selectively induce degradation of ZAP-70 expressed in CLL B cells (a). ZAP-70 positive CLL-B cells were treated \textit{in vitro} with increasing concentrations of 17-AAG (△) and the analogue EC116 (■) for 48 hours. Cells incubated in media with DMSO 1% final concentration were used as a control (◇). ZAP-70 expression was evaluated by intracellular staining.

Consistent with the notion, it is demonstrated that ZAP70 +ve CLL cells expressed HSP90 in multichaperone complexes with high binding affinity for 17-AAG. Conversely, ZAP70 -ve CLL cells as well as normal T and B lymphocytes expressed the inactive, uncomplexed form of HSP90. Thus it can be said that the molecular basis for the selective antitumor activity of 17-AAG and other ansamycins is related to the presence of an increased binding affinity to these compounds in tumor tissues compared with normal cells. The increased affinity appears to be due to cochaperone-induced changes in the ATP binding site of HSP90, because tumor HSP90 is present entirely in multichaperone complexes with highATPase activity, whereas HSP90 from normal tissues is in a latent, apparently uncomplexed state.\textsuperscript{54}
**Conclusion**

Above data suggest that HSP90 is necessary for ZAP70 expression and activity in CLL; that ZAP70 is unique among HSP90 clients, in that its chaperone-dependency appears conditional on the cell type in which it is expressed; and that ZAP70 is required for cell survival and signaling in CLL. Additionally, ZAP70 expression in CLL cells confers markedly heightened sensitivity to 17-AAG or 17-DMAG, suggesting that these or other HSP90 inhibitors could be effective in the therapy of patients with aggressive CLL.