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DIVISION DE NUEVAS CREACIONES
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Re: Expediente No. **07.008.776**
Solicitud de patente a nombre de **NEUTEC PHARMA PLC.**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **NEUTEC PHARMA PLC**, domiciliada en Manchester Gran Bretaña, solicitante de la patente de la referencia, en respuesta al oficio No. 12590 notificado por fijación en lista el 24 de septiembre de 2010 relacionado con el concepto técnico No. 3327, me permito presentar ante ese despacho la siguiente documentación que fue requerida con fundamento en el artículo 46 de la Decisión 486.

- Maloney Alison et al. "HSP90 as a new therapeutic target for cancer therapy: The story unfolds" EXPERT OPINION ON BIOLOGICAL THERAPY; January/2002, vol 2, no. 1 (2002-01), pages **3-24**.

- Schwartz Joseph et al. "Novel Targeted and immunotherapeutic strategies in chronic myeloid leukemia". SEMINARS IN HEMATOLOGY; January/2003, vol 40, no. 1, pages **87-96**.

- Banerji Udai et al. "The clinical applications of heat shock protein inhibitors in cancer-present and future" CURRENT CANCER DRUG TARGETS; October/2003, vol. 3, no. 5 pages **385-390**.

Sobre este particular, nos permitimos manifestar que con la presentación de los anteriores documentos queda total y cabalmente atendido el requerimiento de ese despacho fundamentado en el artículo 46 de la Decisión 486.

Señor Superintendente,

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Cell- & Tissue-based Therapies

HSP90 as a new therapeutic target for cancer therapy: the story unfolds

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Current anticancer drug development strategies involve identifying novel molecular targets which are crucial for tumourigenesis. The molecular chaperone heat shock protein (HSP) 90 is of interest as an anticancer drug target because of its importance in maintaining the conformation, stability and function of key *oncogenic client proteins* involved in signal transduction pathways leading to proliferation, cell cycle progression and apoptosis, as well as other features of the malignant phenotype such as invasion, angiogenesis and metastasis. The natural product HSP90 inhibitors geldanamycin and radicicol exert their antitumour effect by inhibiting the intrinsic ATPase activity of HSP90, resulting in degradation of HSP90 client proteins *via* the ubiquitin proteosome pathway. Anticancer selectivity may derive from the simultaneous combinatorial effects of HSP90 inhibitors on multiple cancer targets and pathways. 17-allylamino,17-demethoxygeldanamycin (17AAG), a geldanamycin derivative, showed good activity and cancer selectivity in preclinical models and has now progressed to Phase I clinical trial in cancer patients with encouraging initial results. Phase II trials including combination studies with cytotoxic agents are now being planned and these should allow the therapeutic activity of 17AAG to be determined. Second generation HSP90 inhibitors may be designed to overcome some of the drawbacks of 17AAG, including limited oral bioavailability and solubility. They could also be engineered to target specific functions of HSP90, which may not only provide greater molecular selectivity and clinical benefit but may also increase understanding of the complex functions of this molecular chaperone. HSP90 inhibitors provide proof of concept for drugs directed at HSP90 and protein folding and this principle may be applicable to other medical conditions involving protein aggregation and stability.

Keywords: apoptosis, cancer, cell cycle, drug development, geldanamycin, heat shock protein, signal transduction

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1. Introduction

Molecular chaperones maintain the appropriate folding and conformation of proteins and are crucial in regulating the balance between protein synthesis and degradation. They have been shown not only to play a vital role in the cellular stress response but also to be important in regulating many important normal cellular functions, such as cell proliferation and apoptosis [1-3].

Exposure of cells to a number of environmental stresses, including heat shock, alcohols, heavy metals and oxidative stress results in the cellular accumulation of a number of chaperones, commonly known as HSPs. This effect is mediated by the transcription factor heat shock factor 1 (HSF1) and is termed the 'heat shock response' [4]. Induction

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of HSPs protects the cell against the initial stress insult, enhances recovery and leads to maintenance of a stress tolerant state. It has become clear, however, that certain HSPs may also play a major molecular chaperone role under normal, stress-free conditions by regulating the correct folding, degradation, localisation and function of a relatively small but growing list of important cellular proteins.

A number of multigene families of HSPs exist, with individual gene products varying in cellular expression, function and localisation. They are classified according to molecular weight, e.g., HSP70, HSP90 and HSP27. Exceptions to this nomenclature rule are a small subset of chaperones that were identified as glucose regulated proteins, e.g., GRP94 and GRP75.

Several diseases in humans can be acquired as a result of protein misfolding [2,5,6]. Hence the development of therapies which modulate the molecular chaperone machinery may prove to be beneficial. In some conditions, e.g., Alzheimer's disease, prion diseases and Huntington's disease, misfolded proteins can cause protein aggregation resulting in neurodegenerative disorders. Also, misfolded proteins may result in loss of wild type protein function, leading to deregulated molecular and physiological functions in the cell. With specific reference to the present review, HSPs have been implicated in cancer. For example, there is evidence of differential expression of HSPs which may relate to the stage of tumour progression [7-12]. As a result of the involvement of HSP90 in various critical oncogenic pathways and the discovery that certain natural products with anticancer activity are targeting this molecular chaperone, the fascinating new concept has been developed that inhibiting HSP function may be useful in the treatment of cancer. This approach has now reached an exciting stage, with the first molecular chaperone inhibitor currently undergoing clinical trials.

The present review will focus on the literature describing the potential use of HSP90 inhibitors as anticancer agents. In recent years a great deal of research has been performed to elucidate the function of HSP90 at both the biochemical and molecular level and this is also summarised. Such fundamental knowledge has enabled researchers to understand more clearly the mode of action of the currently available HSP90 inhibitors and to develop them more rationally. However, these are extremely complex agents, with the potential to deregulate a number of key cellular processes that are collectively responsible for driving the tumour phenotype. Several important questions remain to be answered and these are addressed in this review. The precise details of the molecular functioning of HSP90 are still being elucidated. In particular, although HSP90 inhibitors are known to block the molecular chaperone function of HSP90, the understanding in molecular detail of the downstream consequences of this action and the elucidation of precisely how this may lead to an antitumour effect has only just begun. Selectivity for cancer *versus* normal cells is a crucial requirement for cancer therapies aimed at new molecular targets [13-16]. It is not completely clear how this is achieved with HSP90 inhibitors but possible scenarios are discussed. Many factors may affect tumour sensitivity to these agents and it remains to be seen whether it is possible to dissect out any particular parameter that is responsible for their activity. Alternatively it may be the ability to take out simultaneously the effects of several cancer genes which are critical to multistep oncogenesis that provides HSP90 inhibitors with a unique pharmacological profile. This review discusses how the particular genetic and biological context of the individual tumour may dictate the precise mode of action and biological consequences in any given case but also stresses the potential for broad spectrum anticancer activity in various tumour types, regardless of their genetic make-up. The review will conclude by summarising the status of the current clinical trials with the first-in-class, proof of concept HSP90 inhibitor and by outlining the potential for the discovery and development of future generations of cancer drugs based on blocking the functions of HSP90. The development of new classes of inhibitors, which could for example target specific interactions and functions of HSP90, might not only provide potential clinical benefit in cancer but may also help increase the understanding of the complexity of this molecular chaperone, building on the valuable knowledge already gained with first generation inhibitors. It is noted that HSP90 inhibitors are of broader medical significance, in that they provide general proof of principle for drugs directed at the folding and conformational stability of proteins, an approach that is applicable to pathological conditions as diverse as Alzheimer's and Creutzfeld-Jacob disease.

2. The biology of HSP90

HSP90 is a highly abundant protein which is essential for cell viability and it exhibits dual chaperone functions [17,18]. It plays a key role in the cellular stress response by interacting with many proteins after their native conformation has been altered by various environmental stresses, such as heat shock, ensuring adequate protein folding and preventing non-specific aggregation [2]. In addition, recent results suggest that HSP90 may also play a role in buffering against the effects of mutation, presumably by correcting the inappropriate folding of mutant proteins [19]. However, HSP90 also has an important regulatory role under normal physiological conditions and is responsible for the conformational stability and maturation of a number of specific client proteins, of which about 40 are currently known (see Table 1a and 1b). These can be subdivided into three groups: steroid hormone receptors, serine/threonine or tyrosine kinases and a collection of apparently unrelated proteins, including mutant p53 and the catalytic subunit of telomerase hTERT. All of these proteins play key regulatory roles in many physiological and biochemical processes in the cell.

The highly conserved HSP90 family in humans consists of four genes, namely the cytosolic HSP90α and HSP90β isoforms [58], GRP94 in the endoplasmic reticulum [59] and HSP75/tumour necrosis factor receptor associated protein 1

Table 1a. HSP90 family client proteins.

Client protein	Function	Ref.
Steroid hormone receptors		
Glucocorticoid receptor[a,b]	Ligand mediated gene transcription	[20]
Oestrogen receptor[a,b]	Ligand mediated gene transcription	[21]
Androgen receptor[a,b]	Ligand mediated gene transcription	[22]
Progesterone receptor[a,b]	Ligand mediated gene transcription	[23]
Ser/Thr and Tyr kinases		
c-SRC / v-SRC[a,b]	Signal transduction	[24]
LCK[a,b]	T-cell development and function	[25]
WEE1[a]	Cell cycle regulation (G2)	[26]
MYT1[a]	Cell cycle regulation (G2)	[26]
ERBB2[a,b,c]	Signal transduction	[27]
EGFR (ERBB1)[b,c]	Signal transduction	[27]
FPS/FES[a]	Proliferation	[28]
c-RAF-1 / v-RAF-1[a,b]	MAPK signalling	[29]
MEK[a,b]	MAPK signalling	[30]
Casein kinase 2[a]	Pleiotropic kinase	[31]
CDK4[a,b]	Cell cycle regulation (G1)	[32]
AKT (PKB)[a,b]	PI3 kinase signalling	[33]
Death domain kinase RIP[a,b]	TNF-mediated apoptosis	[34]
BCR-ABL[a,b]	Pathogenesis of myeloid leukaemia	[35]
PIM-1[a,b]	Cytokine mediated proliferation	[36]
MOK[a,b]	MAPK signalling	[37]
Polo-1 kinase (PLK)[a,b]	Cell cycle regulation (G2/M)	[38]
Focal adhesion kinase (FAK)[a,b]	Actin-based cell motility	[39]
c-MET[a,b]	HGF/SF-MET motility signalling	[40]
eIF2 kinase[a,b]	Transcriptional regulation	[41]

HSP90 client proteins can be characterised into three groups: 1. steroid hormone receptors; 2. Ser/Thr or Tyr kinases and 3. proteins with unrelated functions. The type of evidence indicating that these proteins are clients of HSP90 is indicated as follows: a. physical association with HSP90 and b. degradation of client protein by HSP90 inhibitors. Also indicated in the table is whether these client proteins bind to specific HSP90 family members: c. GRP94 and d. TRAP1. Current references describing HSP90-client protein interaction/functions have been cited.

CDK: Cyclin-dependent kinase; EGFR: Epidermal growth factor receptor; HSP: Heat shock protein; LCK: Lymphocyte kinase; PKB: Protein kinase B; RIP: Receptor interacting protein; TRAP1: Tumour necrosis factor receptor associated protein 1.

(TRAP1) in the mitochondrial matrix [60]. It is thought that all the family members have a similar mode of action but bind to different client proteins depending in part on their localisation within the cell. For example, the ERBB2 receptor tyrosine kinase is known to be a specific client protein of GRP94 [59]. Type I tumour necrosis factor receptor (TNFR1) and the retinoblastoma protein (RB) have both been reported to be clients of TRAP1 [50,51].

HSP90 participates in a series of complex interactions with a range of client and regulatory proteins [3,61]. Although the precise molecular details remain to be elucidated, biochemical and x-ray crystallographic studies [62,63] carried out over the last few years have provided increasingly detailed insights into the chaperone function of HSP90.

The monomer of HSP90 consists of conserved 25 kDa N-terminal and 55 kDa C-terminal domains joined together by a charged linker region (not present in TRAP1) [64]. Both the N- and C-termini of HSP90 are reported to bind to substrate polypeptides including client proteins and co-chaperones. The N-terminus contains an unusual ATP-binding site that has structural homology with the Type II topoisomerase gyrase B, an N-terminal fragment of the MutL DNA mis-

Table 1b. HSP90 family client proteins.

Client protein	Function	Ref.
Proteins with various other functions		
Mutant p53[a,b]	Mutant form of cell cycle checkpoint protein	[42]
Hepatitis B virus reverse transcriptase[a]	Viral transcription	[43]
hTERT (catalytic subunit of telomerase)[a,b]	Cell mortality and senescence	[44]
βγ subunits of heterotrimeric G proteins[a,b]	Signal transduction	[45]
Endothelial nitric oxide synthase (eNOS)[a]	Nitric oxide production	[46]
Calcineurin[a]	Ca^{2+} and calmodulin dependent signalling	[47]
Tubulin[a]	Microtubule formation	[48]
HIF-1α[a]	Hypoxia-induced angiogenesis	[49]
Retinoblastoma protein (RB)[a,d]	Cell cycle regulation (G1/S)	[50]
Tumour necrosis factor receptor type 1 (TNFR1)[a,d]	TNF-mediated apoptosis	[51]
Cystic fibrosis transmembrane conductance regulator (CFTR)[a,b]	Ion channel/cystic fibrosis	[52]
Immunoglobulin chains[a,c]	Immune response	[53]
Fanconi anaemia protein[a,c]	Haematopoiesis	[54]
Apoprotein B[a]	Cardiovascular atherosclerosis	[55]
Aryl hydrocarbon receptor (AhR)[a,b]	Ligand dependent gene transcription	[56]
SV40 T antigen[a]	Viral oncogene	[57]

HSP90 client proteins can be characterised into three groups: 1. steroid hormone receptors; 2. Ser/Thr or Tyr kinases and 3. proteins with unrelated functions. The type of evidence indicating that these proteins are clients of HSP90 is indicated as follows: a. Physical association with HSP90;Degradation of client protein by HSP90 inhibitors. Also indicated in the table is whether these client proteins bind to specific HSP90 family members: c. GPP94 and d. TRAP1. Current references describing HSP90-client protein interaction/functions have been cited.

GRP: Glucose regulated protein; HIF-1: Hypoxia-inducible factor-1; HSP: Heat shock protein; hTERT: Human telomerase reverse transcriptase; SV: Simian virus; TRAP1: Tumour necrosis factor receptor associated protein 1.

match repair protein and a C-terminal fragment of the histidine kinase Che A [64]. Following earlier controversy on this issue, it is now clear that HSP90 is an ATP-dependent molecular chaperone [62], with dimerisation of the nucleotide-binding domains being essential for ATP hydrolysis, which is in turn necessary for chaperone function [65]. Binding of ATP results in the formation of a toroidal dimer structure in which the N-terminal domains are brought into closer contact with each other, resulting in a conformational switch known as the 'clamp mechanism' [64]. The function of HSP90 is regulated by association with a number of co-chaperones that combine in various ways to form a series of multimeric protein complexes. Interactions with these various partners in different heterocomplexes may be restricted by temporal, spatial and biochemical factors. A number of co-chaperones contain a tetracopeptide repeat and binding of these proteins to HSP90 has been localised to the C-terminal MEEVD motif [64].

Previous reviews describe in detail the molecular chaperone role of HSP90 and its importance in the conformational stability and function of known client proteins [3,61]. Current models for client protein interactions with HSP90 are illustrated in Figure 1a and 1b for both steroid hormone receptors, the case in which the chaperone binding and release cycle is best understood, and also for protein kinase clients. Although the general mode of chaperone action is thought to be very similar for all client proteins, the specific complement of co-chaperones present in the complex seems important in determining the particular subset of client proteins that are chaperoned by that complex. For example, the co-chaperone p23 is present in mature HSP90 complexes containing steroid hormone receptors whereas CDC37 is required for targeting the HSP90 complex to certain kinases [32,61,66] (Figure 1a and 1b). Other co-chaperones, such as HIP and HOP, regulate the chaperone-client protein cycle and binding of these proteins often is dictated by the ATP state of HSP90/HSP70 (for more information see legend to Figure 1a and 1b and references [64,67]).

Steroid hormone receptors, including the glucocorticoid, oestrogen and androgen receptors, require association with HSP90 to enable the steroid hormone ligand to bind. This causes dissociation of the mature complex and dimerisation of the receptor, resulting in DNA-binding and gene transcription. In the case of protein kinases, such as c-RAF-1, it seems that association with HSP90 is important for their maturation and in maintaining the correct folding and function of the protein. HSP90 inhibition leads to proteosomal degradation of both steroid hormone receptors and kinase clients, most

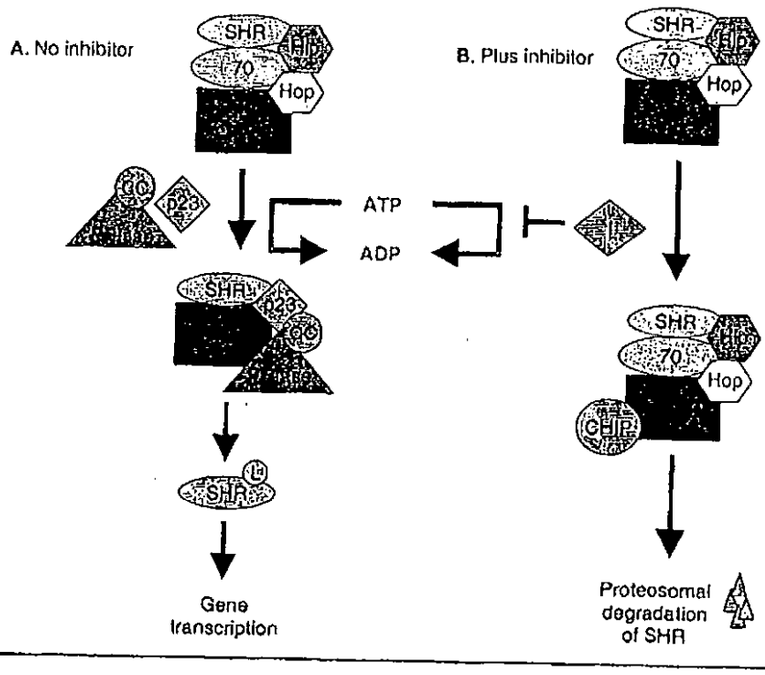

Figure 1a. Proposed mechanism of action of HSP90 inhibitors: the model for steroid hormone receptors (SHRs).
HSP90 function is governed by the dynamic association with client proteins and co-chaperone molecules to form multimeric protein complexes. Spatial and biochemical features, particularly the ATP-binding state of HSP90, dictate which partner can bind at which stage of the chaperone-client protein cycle. The mode of action of HSP90 is best understood for SHRs. A. An intermediate complex is formed involving the chaperones HSP70 and HSP90 and also HIP (HSP70-interacting protein) which stabilises HSP70 in its ADP bound state, enabling it to bind substrate proteins and HOP (HSP70-HSP90 organising protein) which targets HSP90 to this complex. HOP also inhibits the HSP90 ATPase by preventing access of ATP to the nucleotide-binding site and is proposed to be part of a substrate-loading mechanism. When ATP binds to HSP90, HSP70, HIP and HOP are displaced and the co-chaperone p23 and potentially other co-chaperones (CCs) such as the immunophilins (PPIase) are recruited to form a mature complex. Hormone receptors stabilised in this manner are able to bind to their steroid ligands (L) and ATP hydrolysis causes the mature complex to dissociate, resulting in translocation of the receptors to the nucleus where they bind to their cognate DNA response elements causing gene transcription. B. The recruitment of p23 to form a mature complex is ATP-dependent and cannot occur when HSP90 inhibitors (I) interact with the nucleotide-binding site of HSP90. Recent evidence suggests that a ubiquitin ligase, CHIP, associates with the intermediate HSP90 complex, targeting the client protein for degradation via the ubiquitin proteosome pathway. This has only been described with the glucocorticoid receptor but CHIP, or a similar protein, may also provide the same interface between chaperones and the proteosomal machinery with other clients. Based on [61].
CHIP: C-terminus of HSP70-interacting protein; HSP: Heat shock protein.

likely involving recruitment of a ubiquitin ligase to the complex, as demonstrated for the glucocorticoid receptor [68]. It is interesting to speculate why some but not all kinases show a dependence on HSP90. Since binding to HSP90 can be a fundamental requirement for kinase activity, persistence of this dependence during evolution suggests a positive advantage, possibly in terms of regulatory control.

Interestingly, HSP90 may also have an important role in morphological evolution as a result of its buffering effect on genetic mutations [19]. In the fruitfly *Drosophila*, mutations that were previously not evident can be seen to spontaneously produce abnormal phenotypes, in response to the environment, when HSP90 function is compromised. The molecular basis of the buffering effect presumably lies in the ability of HSP90 to encourage the correct folding of mutant proteins.

For example, the activated tyrosine kinase that is encoded by the viral form of the *Src* oncogene (v-*Src*) shows a much greater dependence on HSP90 than the normal SRC protein encoded by the cellular oncogene c-*Src* [24]. These findings reinforce the current view that HSP90 is important in maintaining cellular homeostasis and is vital for the regulation of many biochemical and physiological processes in the cell and in addition support a role for HSP90 in development and evolution. Deletion of HSP90 is lethal in yeast but certain mutations in the *Drosophila* homologue result in defects in eye development due to a lack of the sevenless tyrosine kinase receptor [69]. Mice lacking HSP90β show defects in placental development [70]. Overexpression of HSP90β, but not HSP90α, resulted in impairment of the differentiation of embryonal carcinoma cells into trophoectodermal cells [71].

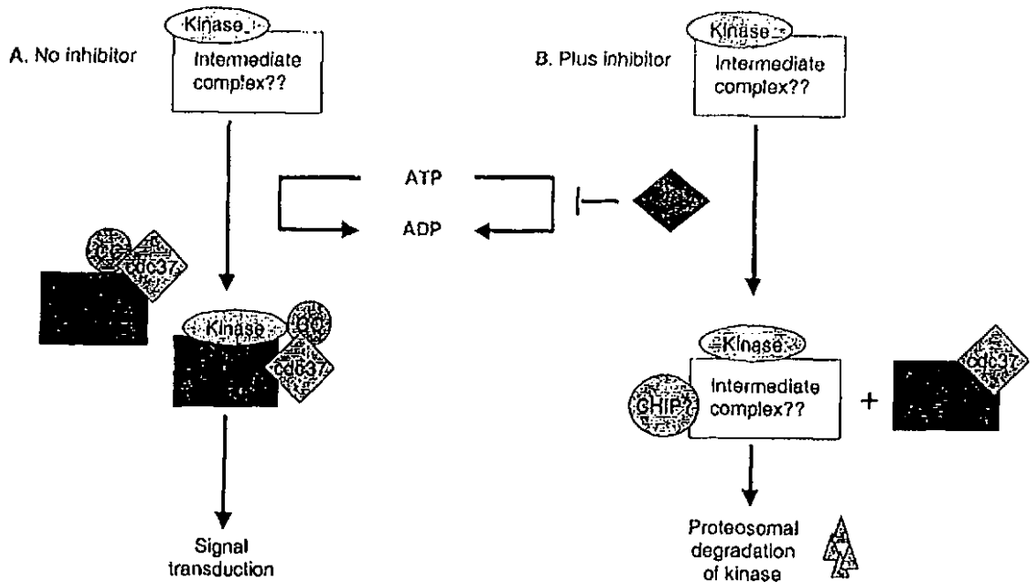

Figure 1b. Proposed mechanism of action of HSP90 inhibitors: the model for protein kinases.
A. It is thought that a similar mode of action occurs with kinases, although the components of these kinase-chaperone pathways have not been as clearly elucidated. However, the major distinguishing feature (in most cases) is that p23 is not recruited to the mature complex. Instead, another co-chaperone CDC37 interacts with HSP90 and targets it to kinase clients. It is not entirely clear from the current literature which other co-chaperones are bound to this HSP90 complex, although immunophilins have been reported to be present in some cases. The co-chaperone HOP cannot bind to HSP90 complexes when CDC37 is present, so it can be predicted that CDC37 is a component of the mature complex. B. Recruitment of CDC37 to HSP90 is not ATP-dependent, in contrast to p23 association, and CDC37-HSP90 complexes are still present following HSP90 inhibitor treatment. However, it is clear that these complexes cannot recruit immature kinases in the presence of geldanamycin. One suggestion is that the ATP state of HSP90 is important for 'high affinity' interactions between HSP90-CDC37 and kinases [67]. It is speculated that in the presence of HSP90 inhibitors, the kinases remain in an intermediate complex, rather like in the case of the steroid hormone receptors, and are targeted for degradation *via* the ubiquitin proteosome pathway. Based on [61].
CC: Co-chaperone; cdc: Cell division cycle protein; CHIP: C-terminus of HSP70-interacting protein; HSP: Heat shock protein.

3. HSP90 inhibitors

Due to its involvement in regulating a number of signalling pathways that are crucially important in driving the phenotype of a tumour and in addition the discovery that certain bioactive natural products exert their effects *via* HSP90 activity, this molecular chaperone is currently being assessed as a new target for anticancer drug development [72,73]. Inhibition of HSP90 function has been shown to cause selective degradation of important signalling proteins involved in cell proliferation, cell cycle regulation and apoptosis, processes which are fundamentally important and which are commonly deregulated in cancer (Figure 2). An attractive rationale for developing drugs against this target for use in the clinic is that by simultaneously depleting proteins associated with the transformed phenotype, one may obtain a strong antitumour effect and achieve a therapeutic advantage against cancer *versus* normal cells. Other drugs, which may target only one signalling pathway, or indeed act at just one molecular target within a pathway, do not account for the substantial crosstalk between these signalling networks. This provides the opportunity for the tumour cell to adapt and utilise an alternative signalling pathway and hence circumvent the drug effect, resulting in drug resistance and persistence of the malignant phenotype. Moreover, in addition to blocking proliferative signalling/cell cycle progression and reversing the evasion of apoptosis, HSP90 inhibitors have the potential to target all the other major hallmark traits of cancer (Figure 3) [74]. Thus, insensitivity to growth inhibitory signals, limitless replicative potential, angiogenesis and invasion/metastasis can all be antagonised by HSP90 inhibition *via* effects on key client proteins. Hence such inhibitors simultaneously impact on multiple biological features associated with the disease. Because of this, they would be expected to be much more effective than a drug that hits only one such feature, e.g., proliferation. Conversely, it may be that inhibiting a number of signalling networks and biological features could lead to enhanced toxicity to normal tissue. This issue of therapeutic selectivity can only be resolved by evaluating HSP90 inhibitors in preclinical models and ultimately in clinical trials. The potential mechanisms of

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cancer selectivity will be discussed later in this review and the way in which the various hallmark traits of cancer are affected will also be assessed.

4. Benzoquinone ansamycins

The first class of HSP90 inhibitors to be discovered was the benzoquinone ansamycins, which include the compounds herbimycin A and geldanamycin (Figure 4). These agents are natural products, initially isolated from actinomycete broths in the 1970s [75]. However, it was not until the 1980s that their potential application as antitumour agents was discovered. They were shown to reverse the malignant phenotype of fibroblasts transformed by the v-*Src* oncogene [76] and subsequently to exhibit potent antitumour activity in both *in vitro* [77] and *in vivo* animal models [78]. Initially, the benzoquinone ansamycins were thought to act as tyrosine kinase inhibitors. However, it has since transpired that depletion of oncogenic protein kinases *via* the ubiquitin proteosome pathway, rather than inhibition of their catalytic activity, is predominantly responsible for their antitumour activity. Subsequent immunoprecipitation and affinity matrix studies have shown that the major mechanism of action of geldanamycin involves binding to HSP90 [77,79]. Moreover, x-ray crystallographic and biochemical studies have shown that geldanamycin competes at the ATP-binding site and inhibits the intrinsic ATPase activity of HSP90 [62,80]. This in turn prevents the formation of mature multimeric HSP90 complexes capable of chaperoning client proteins. As a result, the client proteins are targeted for degradation *via* the ubiquitin proteosome pathway. Recent results suggest that this involves the recruitment of other regulatory proteins such as the ubiquitin ligase, C-terminus of HSC70 interacting protein (CHIP) and to the HSP90 complex [68] (Figure 1a and 1b). The particular functions of these HSP90 client proteins and how they may be affected by HSP90 inhibition is discussed in two earlier reviews on HSP90 inhibitors [72,73]. The present review will focus more on the pharmacological consequences of client protein depletion (see Section 5).

Geldanamycin showed activity in human tumour xenograft models but progression of this compound to clinical trial was halted due to unacceptable levels of hepatotoxicity which was seen at doses required for therapeutic activity [78]. However, following screening of a range of geldanamycin analogues at the US National Cancer Institute (NCI), it was discovered that 17AAG (Figure 4) retains the property of HSP90 inhibition, resulting in client protein depletion and antitumour activity in cell culture and xenograft models [77,81], but has significantly less hepatotoxicity than geldanamycin [82]. It remains unclear why 17AAG has a more favourable therapeutic index than geldanamycin, despite the small difference in chemical structure between the two agents and the maintenance of the HSP90 mechanism. It is possible that the hepatotoxicity may be due to some effect of geldanamycin other than HSP90 inhibition. The role of quinone metabolism in the pharmacology and toxicity of benzoquinone ansamycins is not fully understood. Although both geldanamycin and 17AAG are both metabolised by the quinone reductase DT-diaphorase (NQO1), this enzyme potentiates the antitumour activity of the latter but not the former [81,83]. Whether this relates to the therapeutic index of the agents remains to be determined. Nevertheless, 17AAG is currently being evaluated in

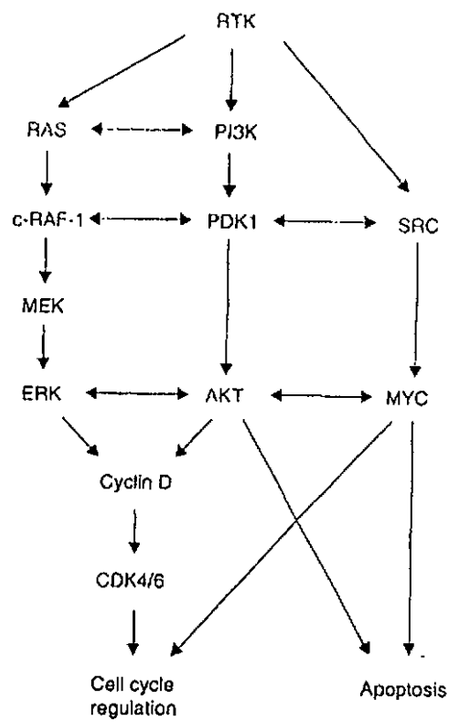

Figure 2. Signalling networks deregulated by HSP90 inhibition. Many signalling cascades exist which provide the interface between external cellular stimuli and control of numerous biological processes within the cell, such as proliferation, cell cycle control and apoptosis. These pathways are commonly deregulated in cancer cells and kinases/phosphatases within these pathways have become important anticancer drug targets. Two of the most well characterised pathways to date are the MAPK and the PI3 kinase pathways, although a number of other cascades exist. It is known that a substantial amount of crosstalk occurs between these pathways. HSP90 inhibitors are particularly attractive anticancer agents as they cause the simultaneous degradation of several key oncogenic HSP90 client proteins that operate within these pathways (as shown in red). Another potential advantage is that HSP90 client proteins are involved at all levels of the signalling pathway, from the membrane to the nucleus. Simultaneous blockade of several key points in these various regulatory pathways that are hijacked by cancer cells leads to cell cycle arrest and apoptosis.
CDK: Cyclin-dependent kinase; ERK: Extracellular signal-regulated kinase; HSP: Heat shock protein; MAPK: Mitogen activated protein kinase; MEK: MAPK kinase; PDK: Phosphoinositide-dependent kinase; PI3K: Phosphatidyl inositol 3-kinase; RTK: Receptor tyrosine kinase.

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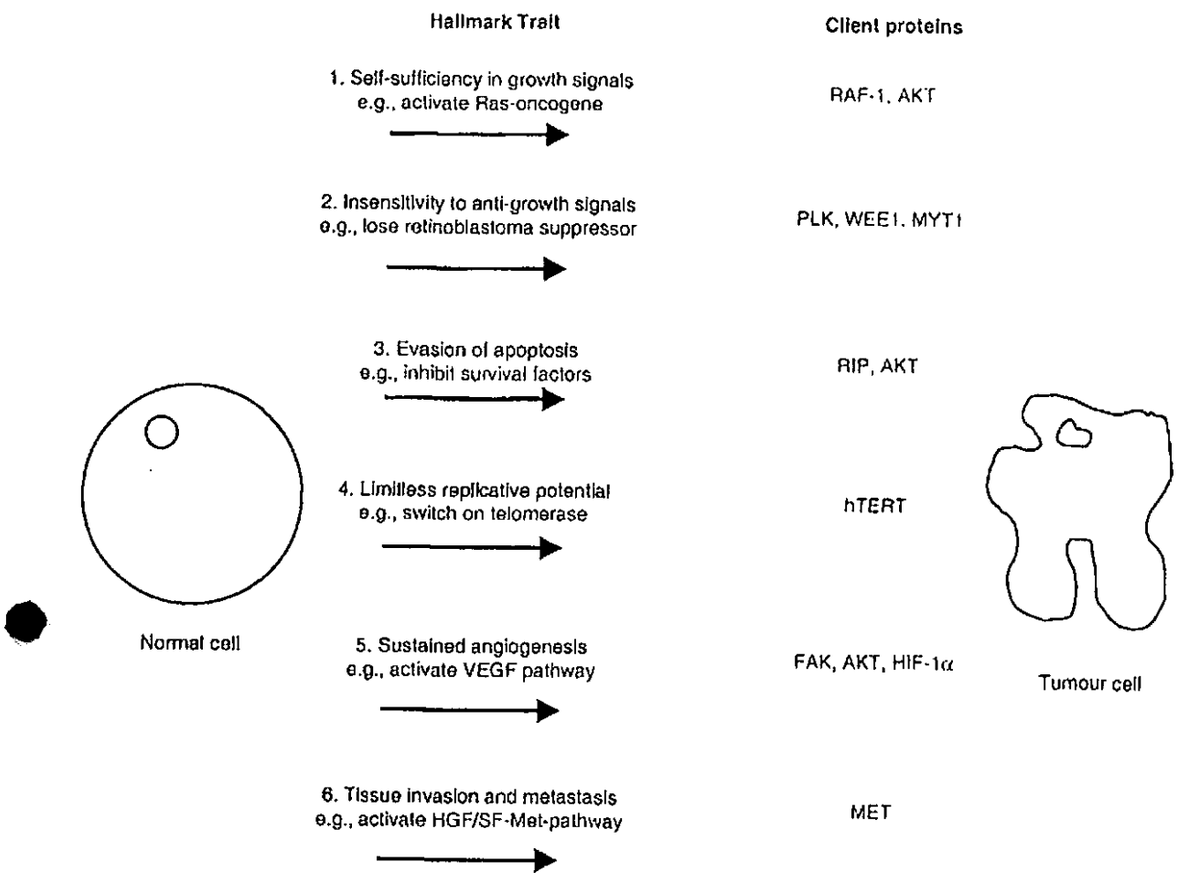

Figure 3. How HSP90 inhibitors may influence the six hallmark traits of cancer. Transformation of normal cells into malignant cells is a multistep process, requiring the accumulation of a number of genetic alterations influencing key regulatory processes in the cell. In a recent review [74], Hanahan and Weinberg described six essential phenotypic traits which are required for development of the full malignant phenotype. We propose that HSP90 inhibitors have the capacity to influence all six of these features. For each hallmark trait, at least one HSP90 client protein has been identified which has the capacity to regulate this process. Examples of client proteins are listed against the relevant hallmark traits. The ability of HSP90 inhibitors to simultaneously block several or all of the key features of malignancy is likely to be a major factor in their effectiveness and selectivity against cancer cells. For more details see text.
FAK: Focal adhesion kinase; HGF/SF: Hepatocyte growth factor/scatter factor; HIF-1: Hypoxia-inducible factor-1; hTERT: Human telomerase reverse transcriptase; PLK: Polo-1 kinase; RIP: Receptor interacting protein; VEGF: Vascular endothelial growth factor.

Phase I clinical trials using a number of different scheduling regimens under the auspices of the NCI and the UK Cancer Research Campaign (CRC) (for interim reports see references [84-87]).

5. Downstream mode of action of HSP90 inhibitors

Our understanding of how HSP90 inhibitors block the function of HSP90 is becoming clearer but the downstream sequelae of this inhibition and the mechanism by which these agents cause antitumour activity have proved to be very complex. New HSP90 client proteins are continuously being identified. However, the molecular and biological consequences of the degradation of these and the other established client proteins remains to be elucidated. From the authors' studies [88,89] and those from other groups [90,91], it is evident that the precise effects of HSP90 inhibition are often cell line specific and likely to be dependent on the genetic background and biochemical features of individual tumour cells. Despite these differences, broad spectrum antitumour activity is observed across different tumour types. Evidence is emerging that inhibition of signal transduction pathways by HSP90 inhibitors can lead to both cytostasis and apoptosis [89,91]. The pathways that regulate the cell cycle and apoptosis are commonly deregulated in cancer cells, leading to their release from the normal cellular control mechanisms [92]. The impact of HSP90 inhib-

itors on these pathways is described in the following sections, together with effects on invasion, angiogenesis and metastasis.

5.1 Cell cycle effects

Signal transduction pathways provide the interface between external cellular stimuli and the control of the cell cycle. The cell cycle is a highly regulated process involving cyclin-dependent kinases (CDKs), their partner proteins known as cyclins and a number of other regulatory proteins. Different cyclin family members control progression through the cell cycle by regulating the phosphorylation of proteins, particularly RB. Two major checkpoints exist in the cell cycle to prevent cells with damaged DNA from dividing. These are known as the G1 and G2/M checkpoints [93,94]. Key players in cell cycle control are commonly deregulated in cancer cells, so that normal growth controls and checkpoints are evaded [92].

p53, the most commonly mutated tumour suppressor gene identified in cancers, is known as the 'guardian of the genome' because it senses damaged DNA, causing the cell to arrest in the G1 phase of the cell cycle and to implement DNA repair processes [95]. The G1 to S phase transition is dependent on the hyperphosphorylation of RB by CDK4/cyclin D1, causing RB to release the transcription factor E2F-1, thereby allowing gene transcription and S phase entry to occur. Wild type p53 inhibits this process by inducing expression of the CDK inhibitor p21 $^{CIP1/WAF1}$, preventing RB phosphorylation. When p53 is mutated, or otherwise functionally inhibited in cancer, cells can no longer arrest at this checkpoint and commonly arrest at the G2/M checkpoint that is regulated by inactivation of the CDC2/cyclin B complex. This checkpoint can also be influenced by functional p53 but in addition is regulated by a number of checkpoint kinases, e.g., WEE1 and MYT1.

How may HSP90 inhibitors impact on these cell cycle events? The possible cell cycle regulatory mechanisms that could theoretically be modulated by HSP90 inhibition are illustrated in Figure 5. However, in most cases, only limited evidence has been obtained to determine whether these mechanisms are indeed perturbed following treatment with HSP90 inhibitors. Geldanamycin and 17AAG have been shown, in a panel of breast cancer cell lines, to cause a G1 arrest which may be RB-dependent and this may in turn lead to outcomes such as differentiation and apoptosis [90,91]. The G1 block may be caused by downregulation of signalling pathways controlling cyclin D accumulation and or CDK4/cyclin D1 activity [96]. This could be mediated by depletion of a number of HSP90 client proteins. For example, depletion of receptor tyrosine kinases (e.g., ERBB2, EGF receptor) and/or degradation of c-RAF-1 would lead to reduced phosphorylation of extracellular signal-regulated kinase (ERK) downstream of RAS and hence decreased transcription of *cyclin D1*. Effects on cyclin D1 translation could be mediated by depletion of AKT/PKB resulting in reduced phosphorylation of AKT/PKB downstream of phosphatidyl inositol 3-kinase (PI3K) [89,96]. Simultaneous blockade of both pathways by 17AAG

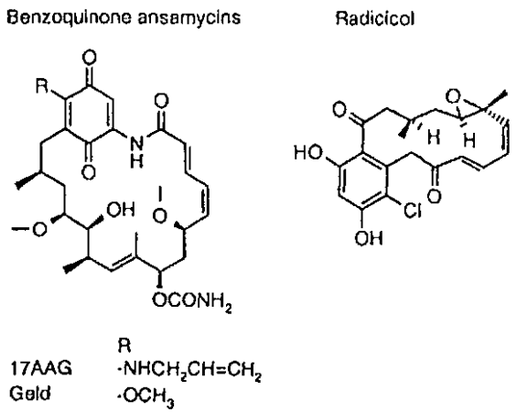

Figure 4. Chemical structures of HSP90 inhibitors.

has been reported in colon cancer cells [89]. In addition, CDK4 has been identified as a HSP90 client protein, which is degraded following HSP90 inhibition [32]. Furthermore, RB has been reported to be chaperoned by TRAP1 during mitosis [50]. The functional consequences of this latter observation have not been elucidated but it may have important implications for RB regulation prior to the G1 phase of the cell cycle.

A G2/M block has been reported to occur following treatment with 17AAG in RB-deficient cells [90,91]. However, the requirement for RB deficiency seems to be cell line dependent, as in other studies with human colon cancer and glioblastoma cell lines, a G2/M block was observed that was independent of RB status [89,97]. The only line in the colon cell study not to undergo an arrest at the G2/M phase was the wild type p53 cell line HCT116 [89]. However, geldanamycin was previously shown to cause a G2/M block in an isogenic cell line model either with or without functional p53 [98]. Therefore, the influence of p53 status on cell cycle arrest and response following treatment with HSP90 inhibitors may not be critical. Another issue to be aware of is that a number of the G2 checkpoint kinases, such as WEE1, MYT1 and Polo-1 kinase (PLK), have been identified as HSP90 client proteins (Table 1a) [26] and degradation of these could clearly influence regulation of the G2 checkpoint. Depletion of PLK would lead to a G2/M block which would be independent of RB status, thereby overcoming loss of sensitivity to growth inhibitory signals (Figure 3 and 5). Conversely, depletion of WEE1/MYT1 would be likely to cause inappropriate mitotic entry and mitotic catastrophe (Figure 5). The cyclin-dependent kinase CDC2 (otherwise known as CDK1) has also been shown to interact with HSP90 in yeast [99] but no evidence has been reported in mammalian cells. However, in vertebrate cells, reduction of HSP90α by disruption of HSF genes caused instability of CDC2 during stress conditions [100].

A mitotic arrest has been observed when cells undergo a G2/M block following 17AAG treatment [89,90] This may be a

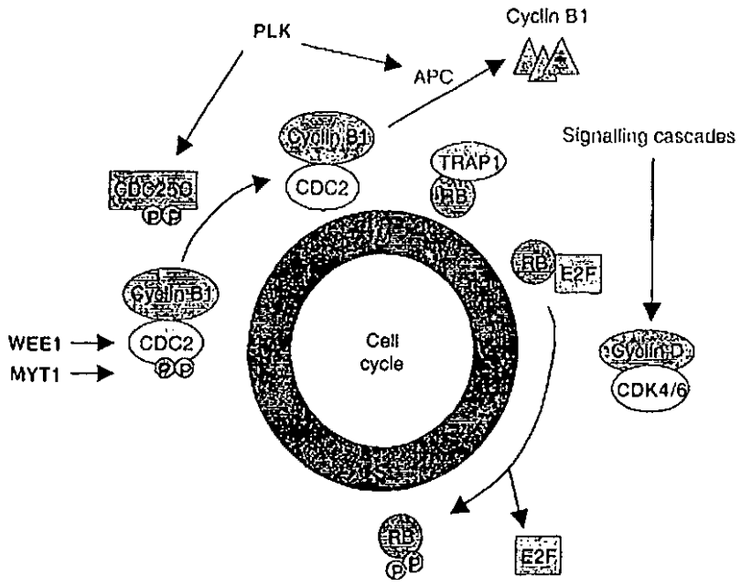

Figure 5. Regulatory cell cycle mechanisms which may be perturbed by HSP90. The cell cycle consists of 4 phases: G1, S, G2 and M. During the cell cycle, a number of checkpoints operate which prevent the production of cells with damaged DNA. The G1/S and G2/M checkpoints are the most well characterised. The G1/S transition requires the phosphorylation of the retinoblastoma protein (RB) by CDK4/cyclin D. The G2/M transition requires the dephosphorylation of CDC2/cyclin B by the phosphatase CDC25C. HSP90 inhibition results in depletion of several kinases that are important in the regulation of both these checkpoints (indicated in red). These include CDK4 which functions during the G1/S transition and WEE1, MYT1 and PLK1 which operate during the G2/M phase. Regulation of the G1/S checkpoint is also governed by upstream signalling cascades (for details of these see Figure 2).
APC: Anaphase promoting complex; cdc: Cell division cycle protein; CDK: Cyclin-dependent kinase; HSP: Heat shock protein; P: Phosphate; PLK: Polo-1 kinase; TRAP1: Tumour necrosis factor receptor associated protein 1.

consequence of inhibition of various upstream signalling cascades following HSP90 inhibition. However, tubulin is a known HSP90 client protein (Table 1b) [48] and therefore it is possible that HSP90 inhibitors may be acting as a novel class of microtubule inhibitors. Interestingly, when the growth inhibitory profile of geldanamycin is compared to other anticancer agents across the NCI panel of 60 human tumour cell lines, for which basal gene expression patterns have been defined, this agent clusters in the same hierarchical node as classical tubulin inhibitors, e.g., vincristine and paclitaxel [89,101]. There are, however, complexities associated with this type of analysis, not least because the geldanamycins may be substrates for the P-glycoprotein (PgP) efflux pump [81]. As is also the case with DT-diaphorase expression [81,102], transport/metabolism factors, such as PgP expression, are likely to interfere with molecular target sensitivity correlations in so-called COMPARE analyses of this type.

HSP90 has been shown to be crucial in centrosome function [103] and this may be attributed to the degradation of the HSP90 client protein PLK which is important for centrosome maturation [38]. As well as influencing the onset of mitosis, PLK degradation could theoretically inhibit exit from mitosis through deregulation of the anaphase promoting complex [104].

Further work will be required to understand whether any common mechanism may be responsible for arresting cells in a particular stage of the cell cycle following HSP90 inhibition. However, it is likely that the mechanisms controlling checkpoint control will be dependent on the genetic background of the cell line examined and may indeed involve a number of regulatory cell cycle mechanisms.

5.2 Apoptosis

Recent work suggests that HSPs play a crucial role in regulating cell survival/death pathways following cellular stress [1] (Figure 6). HSPs are known to function as survival factors and to act at numerous sites in the apoptotic machinery [105]. Both HSP90 and HSP70 have been shown to inhibit apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome [106-108]. Other HSPs, e.g., HSP27, have been shown to suppress apoptosis induced by Fas/Fas L interaction [109]. HSP70 may also intervene at other points along the apoptotic cascade, as it has been shown to suppress activity of JUN-kinase [110] and to impair mitochondrial ATPase function [111,112]. These important findings suggest that the molecular chaperone machinery acts as an interface that co-ordinates the survival or death of cells following cellular stress. Further evidence suggesting that HSP70 acts as a stress sensor comes

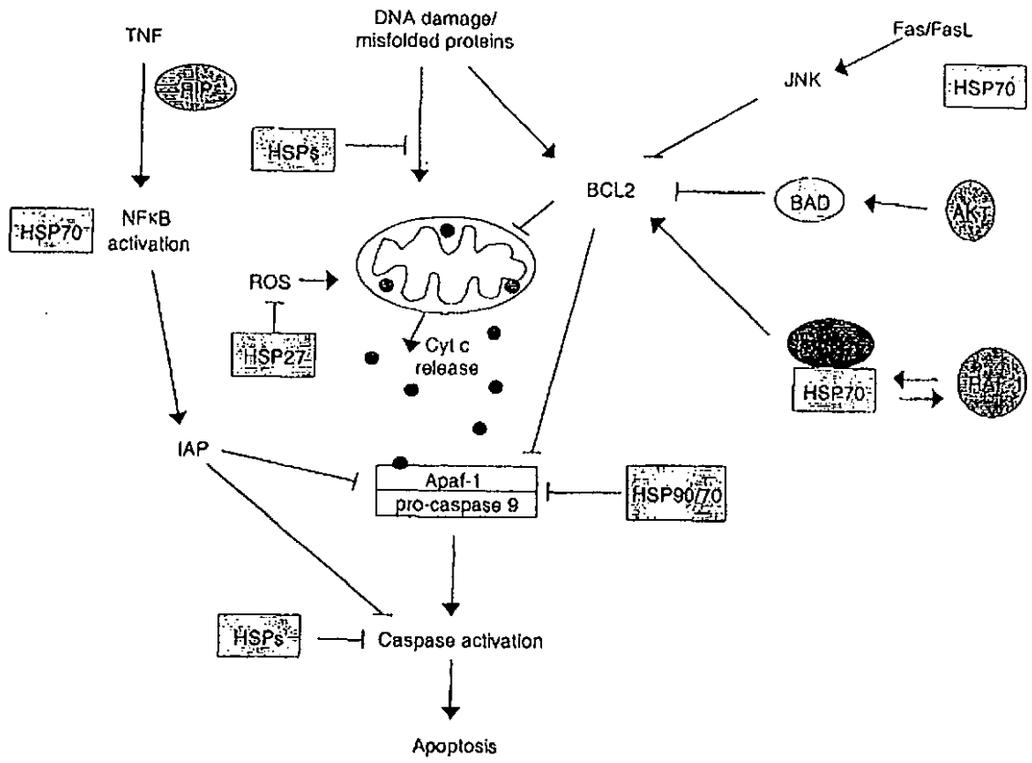

Figure 6. The potential regulation of apoptotic pathways by heat shock proteins (HSPs) and HSP90 inhibitors (based on [105]). HSPs regulate apoptotic pathways at a number of levels and are involved in both the death receptor ligand pathway and cytochrome c (Cyt c) mediated apoptosis. Different HSP family members have been shown to be important in the regulation of the Apaf-1/procaspase-9 apoptosome, caspase activation, the JNK-mediated apoptotic pathway, Fas/Fas ligand mediated apoptosis and mitochondrial ATPase function. HSPs are also required to inhibit the formation of reactive oxygen species (ROS) and to chaperone proteins that have undergone cellular stress caused by heat shock, cytotoxic agents and radiation, thereby enhancing cell survival. A number of HSP90 client proteins have also been shown to influence the apoptotic machinery. These include upstream kinases such as AKT/PKB and c-RAF-1, along with BCR-ABL and receptor interacting protein (RIP) (see text for details). Thus, HSP90 inhibitors have the potential to modulate apoptosis in a number of different ways.
IAP: Inhibitor of apoptosis; JNK: c-Jun N-terminal kinase.

from the finding that this co-chaperone interacts with BAG-1, an anti-apoptotic protein and co-chaperone of HSP70 [113]. c-RAF-1 is also known to bind to BAG-1 but when HSP70 levels are high, BAG-1 preferentially binds to the chaperone resulting in decreased c-RAF-1/ERK activity and cell growth inhibition [113]. Thus, HSP70 and HSP90 may allow the cell to pause and take stock of its ability to repair stress-induced damage before committing to apoptosis, in a fashion that is analogous to a cell cycle checkpoint. Clearly, these recent findings will have to be considered when investigating the mechanism of action of HSP90 inhibitors.

Evidence of an apoptotic response following treatment with ansamycins has been reported in a number of studies. Treatment of human leukaemia cells with geldanamycins causes the degradation of both the BCR-ABL tyrosine kinase and the AKT/PKB Ser/Thr kinase, resulting in apoptosis and differentiation [35]. Depletion of AKT may also cause apoptosis in non-leukaemia cells [89]. It can be hypothesised that depletion of BCR-ABL and/or AKT would lead to reduced BAD phosphorylation, which would in turn increase the activity of proapoptotic proteins such as BAX and reduce the activity of the anti-apoptotic family member BCL2, thereby inducing apoptosis *via* cytochrome c release (Figure 6). However, no change in BAD phosphorylation was seen in colon cancer cells, despite both depletion of AKT and inhibition of AKT phosphorylation by 17AAG [89]. Treatment of human colon cancer cell lines with 17AAG led to various cell line dependent outcomes including either G1 or mitotic arrest, apoptosis, or some combination of these effects [89]. Interestingly, a colon cancer cell line that failed completely to undergo apoptosis in response to 17AAG was one which, in contrast to the other lines, showed no expression of BAX and also exhibited increased expression of BAG-1 [89]. Gene transfection/knock-out studies are required to fully understand the significance of these observations. In human breast cancer cells, apoptosis was noted (together with differentiation) and attributed as a

consequence of RB-dependent G1 arrest following treatment with geldanamycin and 17AAG [90,91]. Interestingly, HSP90 inhibition by geldanamycin treatment has also been shown to cause degradation of the death domain kinase, receptor interacting protein (RIP), resulting in inhibition of TNF-mediated inhibitor κB (IκB) kinase and NFκB activation [34]. This sensitises cells to TNF-induced apoptosis. TNF-R1 has also been shown to be chaperoned by TRAP1 [51] and therefore has the potential to be degraded following inhibition of HSP90 family members by these agents.

5.3 Invasion, angiogenesis and metastasis

Angiogenesis is a multi-step process involving cell motility, invasion and vascularisation and is crucial for the generation of malignant metastatic tumours [114]. HSP90 inhibitors have been implicated as potential anti-angiogenic agents for several reasons. Firstly, many key signalling cascades are important in regulating angiogenesis, for example the PI3-kinase pathway [115], and this review has already described how HSP90 inhibitors can deregulate such cascades at a number of levels. Geldanamycins have also been shown to inhibit the HGF/SF-MET signalling pathway, resulting in decreased cell motility and invasion, as well as reverting the transformed phenotype of c-MET-transformed cells; this occurs by depletion of c-MET which is an HSP90 client protein [40]. In addition, geldanamycin has been shown to affect actin-based cell motility driven by vascular endothelial growth factor (VEGF) [116] as a consequence of degradation of focal adhesion kinases [39]. Furthermore, HSP90 has been implicated in HIF-1α and endothelial nitric oxide synthase (eNOS) activation [49,46], both of which regulate the VEGF pathway amongst others [115].

Precisely how HSP90 inhibition affects angiogenic processes such as motility, invasion and vascularisation *in vivo* remains to be elucidated. However, since invasion of tumour cells and angiogenesis are essential features of the malignant phenotype, leading to metastasis, blockade of these processes by HSP90 inhibitors may contribute in a significant way to their anticancer effects.

6. Factors important in determining cell sensitivity to 17AAG

A number of factors may affect tumour sensitivity to 17AAG and it remains to be seen whether any single parameter which may be responsible for its activity can be dissected out. In the authors' laboratory, gene expression profiling studies were performed using cDNA microarray analysis with the aim of achieving a molecular profile of 17AAG in a panel of colon tumour cell lines [88]. Despite their depletion at the protein level, expression of genes encoding client proteins were generally not altered. Interestingly, induction of *HSP70* gene family members represented the only consistent changes observed in all the tumour cell lines investigated. The authors' group and others have shown that HSP70 is induced at the protein level in a variety of different cultured cell lines [77,88,89] and in xenograft models [117] as part of the heat shock response to HSP90 inhibitors. This induction of HSP70 is mediated by the transcription factor HSF1. Under normal conditions, HSF1 is bound to HSPs but dissociates under stress conditions, trimerises and translocates to the nucleus resulting in increased transcription of *HSP* genes [4]. A recent paper [118] has shown that the degree of growth inhibitory activity of HSP90 inhibitors may also be dependent on the level of cellular stress response caused by these agents. Interestingly, the authors found, from their gene expression profiling studies, that the expression of the gene encoding the constitutive form of HSP70, that is *HSC70*, was induced in some tumour cell lines rather than the inducible form of *HSP70*. The mechanisms involved in this difference are currently unclear. However, since HSP70 has an anti-apoptotic effect (see earlier) it is likely that the increase in its expression may protect the cell against the effects of HSP90 inhibitors.

The gene expression studies demonstrated that some colon tumour cell lines exhibited an increase in *HSP90β* gene expression and also revealed an elevation in HSP90 protein [88]. The results suggested the possibility that cells able to increase the expression of the HSP90 target protein may be less sensitive to 17AAG and may recover more rapidly from its effects. Other genes were found to respond to 17AAG in a cell line dependent manner, including those encoding *keratin 8*, *keratin 18* and *caveolin-1*. The significance of these changes remains to be determined.

As mentioned earlier, it is not clear whether depletion of any particular client protein is essential for the antitumour activity in tumour cells. Indeed, it seems more likely that the importance of specific client proteins to HSP90 inhibition will depend on which particular oncogenic proteins are driving the malignant phenotype in any given cancer cell. In addition, the extent of dependence of the particular client protein on HSP90 for its conformational stability may be important, together with the kinetics of synthesis and degradation of the client. Studies have suggested that ERBB2 may be more sensitive to degradation *via* the ubiquitin proteosome pathway compared to other known clients in breast cancer cell lines [90,91]. Depletion of ERBB2 in tumours that are primarily driven by this oncoprotein, e.g., many breast cancers, may therefore be especially important. However, much more work is required in this area before a detailed understanding can be obtained.

Issues also arise concerning the metabolism of 17AAG in relation to drug sensitivity. As mentioned, 17AAG sensitivity has been shown to be potentiated by DT-diaphorase *in vitro* and to a lesser extent *in vivo* [81]. The drug also undergoes metabolism by cytochrome P450 CYP3A4 to produce the major metabolite, 17-amino,17-demethoxygeldanamycin (17AG), which retains HSP90 inhibitory activity and was shown to have equivalent antitumour activity in cell culture but is not potentiated by NQO1 expression [81]. Both of these metabolic enzymes have potential implications in the clinical application of 17AAG and these are discussed later in this review.

7. Potential for tumour selectivity of HSP90 inhibitors

This review has emphasised that it is not clear why there is therapeutic selectivity for tumour *versus* normal tissue with 17AAG and potentially other HSP90 inhibitors. Nevertheless, preclinical animal models clearly indicate that there is a therapeutic window, as responses are seen in human tumour xenograft models with doses causing no toxicity to the host animals [81].

Although the assessment of therapeutic index can only be fully resolved in the clinic, a number of hypotheses can be put forward as to why therapeutic selectivity can be achieved with 17AAG. It could, for example, be explained simply by the drug's pharmacokinetic properties. 17AAG is retained in tumour tissue at higher levels and for a longer duration compared to normal tissue and plasma in human tumour xenograft models [117,119]. This may in turn be explained by the binding of 17AAG to the higher levels of HSP90 that are expressed in the tumour. However, tumour selectivity is more likely to be a consequence of the molecular mode of action of these agents. One of the most important consequences of HSP90 inhibition is the blockade of multiple signalling pathways, many of which are involved in driving various hallmark features of the malignant phenotype of tumours (Figure 3). Since, as mentioned earlier, HSP90 inhibitors clearly have the potential to deregulate all the six hallmarks of cancer which are involved in crucial steps in tumourigenesis [74], such combinatorial biological effects may be especially damaging to cancers that have been selected to be dependent on these processes.

A universal biological feature of tumour cells is that they contain many mutated proteins that are acquired during tumourigenesis. In an extension of the important theory proposed by Lindquist and colleagues ([19] and see earlier), it is possible that some of these mutations may be buffered by HSP90, thus preventing the manifestation of their potentially damaging biological effects. Such mutations may be synthetically lethal to the tumour cell if unmasked simultaneously by HSP90 inhibition. Another interesting possibility is that the increased number of misfolded proteins that are present in cancer cells as a result of mutation or overexpression may lead to toxic overload of the proteosome when combined with the additional pressure of HSP90 inhibition.

There are numerous reports suggesting overexpression of HSPs in cancer *versus* normal tissues, although the most striking evidence involves HSP27 and HSP70 [9,120,121]. However, a number of papers suggest that HSP90 expression and localisation (along with other molecular chaperones) is deregulated in transformed cells and in human tumours [8,10-12]. HSP90 has been identified by cDNA microarray analysis to be present in a cluster of genes that are overexpressed in breast cancer in a fashion that correlates with clinical stage [7]. Given the above observations and the biological roles of HSP90 discussed earlier, it is not unreasonable to speculate that cancer cells may potentially have greater dependence on HSP90 than normal cells. More extensive studies are required to determine the frequency of overexpression of HSP90 and other HSPs in malignant cells, as well as to elucidate the functional significance of such differences between tumour and normal cells.

In view of the complex downstream consequences of HSP90 inhibition, it will take some time to elucidate the precise reasons for the tumour selectivity of HSP90 blockers, particularly since this may well be dependent on biological context, e.g., the particular tumour type concerned and the genetics of individual cancers.

8. Clinical development of 17AAG

Based on the novel mechanism of action and the promising preclinical activity, Phase I clinical trials of 17AAG have been carried out at the Royal Marsden Hospital/Institute of Cancer Research and in four centres in the US. The aim of these studies is not only to identify the maximum tolerated dose (MTD), the recommended dose for Phase II studies, the nature of any side effects and the pharmacokinetic properties of the drug, but also to determine whether the HSP90 target is being modulated in treated patients and to define a biologically effective dose. Pharmacodynamic markers have been developed which provide a molecular signature that is indicative of HSP90 inhibition in tumour biopsies and also in surrogate normal tissue, such as peripheral blood lymphocytes. These molecular end points were validated previously in preclinical tissue culture and human tumour xenograft models [88,89,117]. A number of HSP90 client proteins including lymphocyte kinase (LCK), CDK4 and c-RAF-1, and also the glucose-regulated protein (GRP)-94 client protein ERBB2, were assessed by Western blotting or immunohistochemistry to determine if they are degraded following 17AAG treatment. The most sensitive marker, however, has proved to be the induction of members of the HSP70 family [77,117]. The authors propose that simultaneous induction of HSP70 family members accompanied by depletion of client proteins may be a characteristic and robust molecular signature of HSP90 inhibition.

A number of different scheduling regimes are being assessed in Phase I trials to determine how frequently 17AAG needs to be administered. Two groups in the US are following schedules of 5 days continuous exposure followed by 3 weeks without drug [86,87]. In the authors' own institution and another in the US, a once weekly schedule has been followed [84,85]. In both cases, dose escalation occurs until MTD or a biologically active dose is reached. In human tumour xenograft models, 17AAG was inactive when given as a single dose but produced marked tumour growth inhibition when given as a daily ip. injection at a dose of 80 mg/kg/day for several (e.g., five) days [81]. A cytostatic effect was seen, rather than tumour regression, and tumour growth resumed when the drug was discontinued. Therefore, optimum scheduling for subsequent Phase II trials will need to be determined so as to achieve continuous control of tumour growth. Knowledge

of the pharmacokinetic properties of 17AAG will be extremely useful, as will information on the kinetics of changes in pharmacodynamic markers, in guiding decisions regarding likely optimum treatment schedules. It is also clear from preliminary clinical data that the degree and type of toxicity is schedule-dependent and this also needs to be considered when planning further trials with this agent. Toxic effects seen in the Phase I studies to date include Grade 2 vomiting and diarrhoea, hepatotoxicity (transaminitis) and hypersensitivity [84-87]. In some cases, these effects may be due to the administration vehicle. Also to be taken into account is the likely nature of any future drug combination trials involving 17AAG and the need to adjust the drug schedule in order to optimise any beneficial interactions, e.g., with cytotoxic drugs (see later).

Initial findings from the Phase I trials suggest that plasma concentrations can be obtained that are well above the IC_{50} for tumour cell growth *in vitro* and which are comparable to the plasma exposures seen in responsive human tumour xenograft models [81]. Furthermore, it is clear that a biologically active dose of 17AAG, as determined by pharmacodynamic marker modulation, can be achieved in patients with only limited toxicity being seen. Interestingly, a number of patients have continued to receive the drug for several months, suggesting the possibility of an inhibitory effect on disease progression. Therapeutic efficacy will be assessed in Phase II trials.

Although 17AAG may be useful as an anticancer agent in its own right, it is more likely that it will be combined with traditional therapies for use in the clinic. This may be advantageous given that HSP90 promotes survival in cells following stress [108]. Thus, inhibition of HSP90 may increase sensitivity of tumour cells to cytotoxic agents. Some evidence already exists for this hypothesis. Munster *et al.* [122] showed in a number of breast cancer cell lines that 17AAG treatment was able to enhance the apoptotic response to paclitaxel and doxorubicin (see also commentary by Sausville, [123]). However, it remains to be seen whether this kind of synergy can be achieved in the clinic, particularly as the sequencing of paclitaxel and 17AAG was extremely important. The effects of 17AAG on cell cycle checkpoints may lead to selective potentiation of cytotoxic agents in cancer *versus* normal cells, since the former often have existing checkpoint defects. It has also been suggested that geldanamycins may be useful in treating breast cancers due to their novel ligand independent antihormone action, either in combination with conventional antihormone therapies, e.g., anti-oestrogens such as tamoxifen, or as a single agent in hormone refractory disease [124].

17AAG clearly has activity in experimental cancer models and the early clinical experience appears to be encouraging. The drug has certainly provided proof of principle that inhibition of HSP90 can lead to cytostatic and apoptotic effects in preclinical models. Moreover, the early clinical data suggest that HSP90 can be inhibited in patients without severe side effects. Despite these promising findings, 17AAG has a number of limitations as a clinical drug candidate. It is not readily orally bioavailable [119] and therefore has to be administered as an iv. infusion. The clinical formulation of 17AAG contains DMSO/phospholipid and is not ideal, particularly for chronic dosing. 17AAG is potentially a substrate for PgP [81] and therefore drug uptake problems may be encountered, resulting in drug resistance. 17AAG undergoes metabolism by cytochrome P450 CYP3A4 to produce the major 17-amino metabolite, 17AG [125], which retains HSP90 inhibitory activity [81]. However, CYP3A4 is polymorphic and has the potential to produce variable pharmacokinetic profiles in patients. 17AAG sensitivity has also been shown to be potentiated by NQO1 *in vitro* and to a lesser extent *in vivo* [81]. Polymorphisms in the *NQO1* gene leading to loss of activity arise in approximately 5 - 20% of the population depending on ethnic variation and therefore may have an influence on patient response. Moreover, quinone metabolism may contribute to toxicity to normal tissues, so the potential usefulness of this feature of the 17AAG molecule requires clarification. All of the above issues need to be addressed when designing the next generation HSP90 inhibitors.

9. Development of novel HSP90 inhibitors

9.1 17AAG analogues

A range of geldanamycin analogues have already been described [126,127], of which 17AAG appeared to be the most promising in terms of therapeutic index. The NCI has recently issued a request for submissions for a Collaboration Research and Development Agreement (CRADA) to continue the clinical development of 17AAG and to develop additional analogues that may have improved pharmaceutical properties (e.g., solubility, oral bioavailability) and different pharmacological behaviour [128]. Structure-activity relationships with 17AAG analogues have shed more light on the chemical features required for HSP90 inhibitory activity and also for the NQO1 potentiation effect [126,127,129]. Gene expression profiling of 17AAG analogues as well as HSP90 inhibitors from other chemical classes can be used to dissect out target-specific molecular effects from changes solely due to the chemical backbone of these compounds [130]. Further optimisation of the benzoquinone ansamycin might allow some of the limitations outlined in the previous section to be overcome.

9.2 Radicicol

Radicicol is a macrocyclic antibiotic isolated from *Monosporium bonorden* (Figure 4). It was shown to reverse the malignant phenotype of v-*Src* and v-*Ha-Ras* transformed fibroblasts [131,132]. Like the benzoquinone ansamycins, it was at first thought to act as a tyrosine kinase inhibitor. However, it was shown subsequently to degrade a number of signalling proteins as a consequence of HSP90 inhibition [133]. X-ray crystallographic data confirmed that radicicol also binds to the N-terminal domain of HSP90 and inhibits the intrinsic ATPase

activity [134]. Interestingly, radicicol is more potent at inhibiting HSP90 ATPase activity compared to geldanamycin and 17AAG [134], even though they have similar growth inhibitory effects on tumour cells. This may be a consequence of differences in the cellular uptake or metabolism of these compounds. Radicicol binds to all HSP90 family members, although it has a weaker binding affinity to both GRP94 and TRAP1 than the cytosolic HSP90 isoforms [135].

However, like 17AAG, the structure of radicicol has a number of adverse pharmacological properties that could lead to unfavourable metabolism. These include an epoxide residue, keto group, two phenolic hydroxyl groups and a Michael acceptor (Figure 4). It may be possible to design out these undesirable features, whilst retaining the important interactions required for HSP90 inhibition.

Radicicol lacks antitumour activity *in vivo* due to the unstable chemical nature of the compound. Oxime derivatives of radicicol (KF-25706 and KF-58333) have been synthesised which retain the HSP90 inhibitory activity of radicicol and KF-25706 has been shown to exhibit *in vivo* antitumour activity in human tumour xenograft models [136].

9.3 Coumarins

Coumarin antibiotics are known to bind to bacterial DNA gyrase at an ATP-binding site homologous to that of the HSP90 [137]. The coumarin, novobiocin, was shown to bind to the C-terminus of HSP90, i.e., at a different site to that occupied by the benzoquinone ansamycins and radicicol which bind at the N-terminus [138]. However, this still resulted in inhibition of HSP90 function and degradation of a number of HSP90-chaperoned signalling proteins [139]. Geldanamycin cannot bind HSP90 subsequent to novobiocin; this suggests that some interaction between the N and C-terminal domains must exist and is consistent with the view that both sites are important for HSP90 chaperone properties.

9.4 Purine-based inhibitors

A purine-based HSP90 inhibitor, PU3, has been synthesised based on rationale drug design with the aid of the x-ray crystal structure [140]. This agent was shown to result in the degradation of signalling molecules, including ERBB2, and to cause cell cycle arrest and differentiation in breast cancer cells [140]. Although less potent than 17AAG, it is more soluble and so may be formulated in more conventional vehicles and could potentially have more favourable oral bioavailability.

10. Future HSP90 inhibitors

Analogue development programs are continuing on the known natural product HSP90 inhibitors, i.e., 17AAG/geldanamcyin and radicicol, with the aim of eliminating unfavourable structural features. However, these compounds are difficult to synthesise and this process can ultimately be very time consuming. Another option is to discover completely new chemical classes of HSP90 inhibitors. Such novel chemotypes can be identified by high-throughput screening of diverse compound libraries using both cell-free biochemical assays and also cell-based readouts [141].

It can be speculated that inhibitors may be identified which do not interact with the N-terminal nucleotide-binding site of HSP90 but nevertheless disrupt chaperone activity, as in the case of the coumarins. An intriguing possibility would be to design compounds that introduce a higher degree of molecular selectivity to the HSP90 inhibitory activity in terms of the client proteins that are affected. Geldanamycin dimers have been shown to selectively degrade ERBB2, although the mechanism for this is not understood [142]. An alternative means to bring about degradation of particular subsets of client proteins is by targeting specific co-chaperones such as CDC37. As mentioned earlier, CDC37 is important in directing HSP90 to certain kinases, e.g., c-RAF-1 [66]. Hence such a strategy might be expected to result in selective inhibition of those specific pathways that are dependent only on these kinases. Other client proteins, for example steroid hormone receptors, would be left unaffected. Another strategy for gaining a greater degree of selectivity is to develop agents that inhibit the function of specific members of the HSP90 family. This may result in degradation of client proteins located in particular subcellular compartments. For example, selective degradation of ERBB2 might be achieved by targeting GRP94 which acts as the chaperone for this receptor tyrosine kinase within the endoplasmic reticulum.

11. Other potential therapeutic strategies

Another potential strategy for anticancer treatment involving HSP90 is immunotherapy [143]. HSP90 family members, especially GRP94, have been shown to be important in antigen presentation. Vaccination of animals with tumour derived HSP/peptide complexes provides immunity to the tumour from which the vaccine is manufactured. This results from tumour specific antigens which are processed by antigen-presenting cells and presented to cytotoxic T-cells resulting in a tumour-specific immune response [143].

Immunotherapy using antibodies to tumour specific proteins has been utilised in the clinic and promising results have been seen with the anti-ERBB2 antibody Herceptin™ (trastuzumab, Genetech Inc.). Studies have now shown that when Herceptin is conjugated to geldanamycin, this enhances the antiproliferative activity of the antibody [144]. It also illustrates the potential of targeting geldanamycin, *via* antibodies to other signalling proteins, to tumour cells.

It should be noted that given the importance of HSP90 in antigen presentation, HSP90 inhibitors may have the potential for immunosuppression. However, this has not been a side effect reported in the current Phase I clinical trials using 17AAG. Indeed it is possible that stimulation of HSP70 could compensate for or even enhance antigen presentation.

12. Conclusions and expert opinion

There is considerable interest in developing new anticancer drugs that act as novel molecular targets, especially those that are directly involved in the molecular pathology of cancer [13-16]. The current interest in the development of HSP90 molecular chaperone inhibitors for cancer treatment is due to the convergence of two strands of scientific activity, one of them being 'post-genomic' and other 'pre-genomic'. The first was the increasing realisation, based on recent work, that HSP90 plays a crucial role in the control of signal transduction pathways leading to proliferation, cell cycle control and apoptosis. The second was the discovery that two classes of bioactive natural products that have anticancer activity - namely radicicol and the benzoquinone ansamycins such as geldanamycin - are able to exert their cellular effects by binding to and inhibiting the ATP interaction and hydrolysis that occurs at the N-terminal domain of HSP90.

Despite the above arguments, the HSP90 molecular chaperone might not at first sight appear to be the most obvious choice for an anticancer drug target. There is no evidence that it is mutated in cancer (although extensive screening for these may not have been carried out). It is also expressed at high levels in normal cells, where in addition to its classical role in the response to heat shock and other cellular stresses, HSP90 is involved in the control of signal transduction mechanisms, as well as in development and evolution. Surely then, HSP90 inhibitors would cause unacceptable toxicity to normal cells and the therapeutic margin would be minimal?

There are, however, a number of strong arguments that support a therapeutic attack on HSP90 for cancer treatment. HSP90 appears to be overexpressed (or aberrantly localised) in malignant cells. HSP90 inhibition is particularly relevant to the treatment of cancers that are driven by an accumulation of oncogenic events, which is the case for most if not all advanced cancer. HSP90 (together with its endoplasmic reticulum homologue GRP94) appears to be crucial for the correct folding, stability, localisation and function of a subset of proteins that are heavily involved in creating and maintaining the malignant phenotype. These include c-RAF-1, which is a key player in the RAS→ERK signalling pathway, and AKT, a component of the PI3 kinase pathway. Depletion of these oncogenic kinases is readily seen with HSP90 inhibitors and simultaneous blockade of the RAS→ERK and PI3 kinase pathways is demonstrated by inhibition of the phosphorylation of ERK and AKT/PKB. Thus, two of the major mission critical trunk routes leading to stimulation of proliferation and the evasion of apoptosis are both inhibited by HSP90 chaperone drugs. Proliferative signal transduction and cell cycle progression can also be blocked both upstream and downstream of c-RAF-1 and AKT/PKB, for example by depletion of membrane receptor tyrosine kinases such as the EGF receptor and ERBB2 and degradation of CDK4. It is clear that HSP90 inhibitors can overcome three of the hallmark traits of cancer cells, namely:

- self-sufficiency in growth signals
- insensitivity to antigrowth signals
- evasion of apoptosis

The three remaining hallmark traits can also be blocked by HSP90 inhibitors:

- restoration of limited replicative potential by degradation of the catalytic submit of telomerase hTERT
- inhibition of angiogenesis by depletion of focal adhesion kinase (FAK), AKT/PKB and HIF-1α
- blockade of invasion and metastasis by loss of c-MET (Figure 3)

By simultaneously tackling all aspects of the malignant phenotype, an HSP90 inhibitor has the potential to deliver a one-step combinatorial attack on multistep oncogenesis. This provides the capability for a powerful antitumour effect across a broad range of cancers and may make it more difficult for a cancer cell to develop drug resistance.

Although the one-step combinatorial consequences of HSP90 antagonism can be seen as a major and potentially unique selling point of HSP90 drugs, this does not mean that individual cancers may not be sensitive by virtue of effects on a particular locus. For example, a proportion of breast cancers could be especially susceptible to depletion of ERBB2, while chronic myeloid leukaemia patients, even those resistant to the BCR-ABL kinase inhibitor Glivec/Gleevec, could benefit specifically from the degradation of BCR-ABL. It will require much more research to elucidate the principal mechanism of action and downstream consequences of HSP90 blockade and in particular to dissect out the role of context-dependent effects on individual oncogenic players *versus* the combinatorial advantages of chaperone inhibition.

Variations in response to HSP90 inhibitors caused by differences in the genetic make-up of the particular cancer will involve not only differences in the client proteins involved but also the biological outcome of depletion, e.g., in terms of cell cycle arrest, apoptosis and so on. Although such qualitative differences in biological outcome may contribute to differences in the quantitative level of response, it is nevertheless likely that therapeutic activity will be seen across most genetic backgrounds and thus effective HSP90 inhibitors should have broad spectrum anticancer activity.

Researchers should not, however, be complacent about the potentially adverse effects of HSP90 inhibition on normal tissues. The pathways hijacked by oncogenic actors are those which play important roles in normal cell biology, albeit not simultaneously. A major hypothesis for the therapeutic selectivity of HSP90 is that cancers have evolved to be dependent on the mission critical oncogenic gene products that require the attentions of the molecular chaperone. It is also possible that the proteosome machinery of cancer cells is already overworked by having to deal with the upregulated and mutant oncoproteins in cancer cells and that inhibition of HSP90 drives them into toxic overload. Although speculative, extrap-

olation of the mutation buffering/morphological evolution properties of HSP90 to suggest the possibility of unleashing the effects of potentially synthetic lethal mutations in cancer cells is an intriguing notion. The accumulation of 17AAG in tumour tissue, possibly due to increased HSP90 expression, may also contribute to its cancer selectivity.

The development of HSP90 inhibitors has reached an exciting stage with the entry of the geldanamycin analogue 17AAG into Phase I clinical trials in cancer patients. 17AAG has already provided us with proof of concept in animal models that an HSP90 inhibitor can deliver a promising level of antitumour activity at doses that are not toxic to normal tissues. It is not clear why 17AAG has such a therapeutic window, albeit a modest one, whereas the closely related geldanamycin does not. It is also unclear whether the dose-limiting hepatotoxicity of 17AAG and geldanamycin are due to HSP90 inhibition or some other, off-target effect, nor whether the effects of DT-diaphorase/NQO1 are involved in some way. Nevertheless, with the support of the US NCI and the UK CRC, 17AAG has provided us with the first opportunity to test the hypothesis in the clinic that HSP90 inhibition could provide a selective and effective approach to the biological therapy of cancer.

The results emerging from these Phase I trials with 17AAG, though still early, are certainly encouraging. Concentrations significantly above the IC_{50} values for HSP90 inhibition and for anticancer activity in tissue culture are achievable in the plasma of treated patients. Moreover, these exposures are comparable to those required for activity against human tumour xenografts in immunosuppressed mice. Furthermore, using the pharmacodynamic end points developed in preclinical models, in particular the molecular signature of the client protein depletion and HSP70 elevation, there is clear evidence of HSP90 inhibition in treated patients; this is true for peripheral blood lymphocytes and there are early indications that this might also occur in tumour biopsies as well. Another major finding from the ongoing Phase I clinical trial programme is that 17AAG is quite well tolerated. Furthermore, there is anecdotal evidence (no more) of potential therapeutic activity, as indicated by the fact that several patients in the Phase I trials have continued to receive the drug for several months.

Based on the emerging data, it seems likely that 17AAG will enter Phase II efficacy trials. These are essential to allow the therapeutic activity to be determined and will evaluate 17AAG as a single agent and also in combination with cytotoxic agents such as paclitaxel. The design of such trials will be based on hard evidence such as pharmacokinetic behaviour, pharmacodynamic effects and preclinical combination data, together with pragmatic considerations, such as the optimal scheduling of the cytotoxic agent(s) when given alone. Much work remains to be done in the area of optimal drug combinations, preclinically as well as clinically, so that 17AAG is given the best opportunity to fulfil its therapeutic promise.

Despite this promise, it is already clear that 17AAG is not a perfect HSP90 inhibitory drug. It is hoped that this first-in-class agent will provide proof of concept for a therapeutic effect *via* HSP90 inhibition in the clinic and may potentially progress to regulatory approval. Nevertheless it possesses several features that can be improved upon. An optimal 17AAG inhibitor would have better pharmaceutical properties leading to an improved formulation or would be orally bioavailable to support chronic dosing schedules. It would also avoid the attentions of the P-glycoprotein efflux pump and would not be metabolised by the polymorphic cytochrome P450 CYP3A4. In addition, it is not clear whether the effects of DT-diaphorase/NQO1 are advantageous or disadvantageous and this feature could be retained or designed out accordingly.

The promising results with 17AAG are stimulating considerable interest in the search for HSP90 inhibitory drugs. Second generation drugs might overcome some or all of the limitations listed above. Several additional attractive features might be incorporated into future HSP90 drugs. They could be designed to be more selective for individual members of the HSP90 family, e.g., HSP90α/β, GRP94 or TRAP1. This may lead to greater selectivity in terms of depletion of particular client proteins. Alternatively, it may be feasible to target sites on HSP90 other than the ATP-binding site in the N-terminal domain, again with the aim of taking out a more limited subset of clients, e.g., kinases. These approaches have potential advantages and disadvantages that are difficult to predict with any certainty. Approaches to second generation HSP90 inhibitors may be best achieved by analoguing existing inhibitors, by searching for new chemical classes of inhibitors using high-throughput screening with biochemical and cell-based assays, and by exploiting the x-ray crystal structures of HSP90, particularly if these can be obtained for additional regions of HSP90 or for heterochaperone complexes.

In considering the potential of HSP90 inhibitors for cancer therapy, it is important to remember that such drugs not only provide proof of concept for HSP90 as a target but also demonstrate that molecular chaperones and protein folding pathways are feasible targets for small molecule drug discovery programmes. Thus, it is possible to extrapolate beyond HSP90 to other potential chaperone targets in cancer, for example inhibition of HSP70 to modulate apoptosis in malignant cells. A combination of HSP90 inhibition with HSP70 blockade may be of particular interest. As well as being used as anticancer agents, protein folding drugs have the potential to be employed in the treatment of conditions such as Alzheimer's and Creuztfeld-Jacob disease.

It will be fascinating to watch as this story continues to unfold.

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The Clinical Applications of Heat Shock Protein Inhibitors in Cancer – Present and Future

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Abstract: The potential clinical applications of the prototype first-in-class Hsp90 inhibitor 17AAG and other emerging Hsp90 drugs are very exciting. Rigorously planned and executed clinical trials, incorporating measurement of appropriate biomarkers and pharmacodynamic endpoints are critical for selecting the optimal dose and schedule. A detailed understanding of the molecular mode of action of Hsp90 inhibitors alongside the elucidation of the molecular pathology of individual cancers will help us to identify tumour types and individual patients that will benefit most from treatment. Careful *in vitro* and *in vivo* experiments are needed to choose the most potentially advantageous combination studies. It is important to construct a pharmacologic audit trail linking molecular biomarkers and pharmacokinetic and pharmacodynamic parameters to tumour response endpoints. Phase I clinical studies with 17AAG have shown that the drug can be given at does that are well tolerated and that also achieve active pharmacokinetic exposures and elicit molecular signatures of gene and protein expression that are indicative of Hsp90 inhibition. Furthermore, examples of disease stabilisation have been documented, consistent with the generally cytostatic responses that are seen in animal models. Selecting tumour types for Phase II clinical trials must involve balancing 1) our knowledge of molecular response determinants, such as the expression of and dependence upon key client proteins and 2) more pragmatic evidence of antitumour activity in the relevant preclinical models. Examples of likely disease targets include chronic myeloid leukaemia, melanoma, breast, ovarian, brain, thyroid, colorectal and prostate cancer.

INTRODUCTION

The mechanism of action of inhibitors of the heat shock protein 90 (Hsp90) including 17-allylamino, 17-demethoxygeldanamycin (17AAG), has been covered extensively in previous articles in this special issue. The potential clinical applications of 17AAG and other Hsp90 inhibitors are very exciting. However, these have to be carefully thought out. The lessons learnt from rigorously planned and executed clinical trials, incorporating the measurement of appropriate biomarkers and pharmacodynamic endpoints, are crucial to choosing the correct dose and optimal scheduling of novel molecular therapeutic anticancer agents [1, 2]. The detailed understanding of the mode of action of these drugs together with the unravelling of the complex molecular pathology of individual cancers will help us to select the indications where Hsp90 drugs will be most clinically beneficial when used as single agents. Well-designed *in vitro* and *in vivo* experiments will help us to choose the most potentially advantageous drug combination trials. These issues are just as important with Hsp90 inhibitors as they are with other emerging molecular therapeutics.

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TAKING STOCK OF THE PRESENT STATE OF KNOWLEDGE – 17AAG

In the course of evaluating a new molecular cancer therapeutic drug in modern phase I clinical trials, the critical questions to be asked are:

1) Is it possible to achieve drug levels in plasma and tumour that are likely to inhibit the target?

2) Is it possible to actually demonstrate inhibition of the target in the tumour?

3) Is it possible to achieve the above with no side-effects or with tolerable toxicity?

To address the first question, various schedules of administering 17AAG have been investigated. In once a week dosing studies that have administered 320 – 450 mg/m^2, peak plasma concentrations have been 10-15 μM [3][1,2,3,4] The mean IC_{50} of 17AAG for the US National

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Cancer Institute 60 human tumour cell panel is around 120 nM[5]. Thus it has been possible to achieve peak drug levels that exceed concentrations that are needed to inhibit Hsp90 in cancer cells *in vitro*. In regimens exploring q5/7 once every 3 weeks, the maximum tolerated dose (MTD) was reported as 40mg/m^2 with peak plasma concentrations in the range of 1μM.[6,7] However, the length of time for which relevant drug levels are maintained may not be optimal. At the doses of 450mg/m^2 /week, the time above 120 nM was approximately 24 hrs.[1] Of course, careful attention has to be paid to the tissue concentrations as well as plasma concentrations, especially because 17AAG is accumulated extensively in cancer cells and tumour tissue, against a concentration gradient [2, 3]. This explains the greater cellular potency of 17AAG than would be expected on the basis of its modest potency as an Hsp90 ATPase inhibitor.

The second question, as to whether pharmacodynamic changes reflecting target inhibition have been achieved, has been addressed in normal as well as tumour tissue. A well-validated molecular signature has been defined that appears to be highly specific for Hsp90 inhibition [2]. Normal tissues studied include peripheral blood leukocytes (PBLs)[1,2,6] and buccal smears. Reproducible pharmacodynamic changes have been seen in PBLs at the 450 mg/m^2/wk dose, with c-RAF-1, CDK4 and LCK depletion being clearly demonstrable while Hsp70 induction is maintained for up to 96 hrs[1]. In some cases it has been possible to obtain tumour biopsies before and after treatment. Reproducible c-RAF-1 and CDK4 depletion along with Hsp70 induction has been demonstrated 24 hr after treatment[1,2]. Present efforts at our institution are focussing on defining the duration of these pharmacodynamic changes.

As regards the third question of patient tolerance, the toxicity predicted from preclinical models has been hepatotoxicity.[8,9] In both once a week schedules and q5/7 every 3 weeks regimens, elevation of liver enzymes has been reported[1,2,7]. Gastrointestinal toxicities including nausea, vomiting and diarrhoea have also been observed[1,2] and prophylactic anti-emetics are necessary at doses above 320 mg/m^2.[1] Varying degrees of fatigue and anaemia have been reported.[1,6] Of concern, although not a dose limiting toxicity, is the strong odour of DMSO excreted by the patients as the present parenteral formulation of 17AAG incorporates 0.4% DMSO and at doses of 450mg/m^2 this amounts to 20-40 ml of intravenous DMSO per infusion. The extent to which DMSO contributes to the non-hepatotoxic side effects of the drug is not fully known. Improved formulations for 17AAG would be advantageous. Alternatively the development of new Hsp90 inhibitors with better pharmaceutical properties would be desirable.

HSP90 INHIBITORS USED AS SINGLE AGENTS

One of the drawbacks of drugs targeted to specific components of complex signal transduction pathways is that there is often redundancy and cross talk between pathways, leading to the activation of multiple downstream transcription factors. Furthermore, cancers are commonly driven by multiple oncogenic abnormalities and they hijack many pathways that sustain the malignant phenotype. For these reasons, incomplete responses are likely to occur when only a single molecular target is modulated pharmacologically. Hsp90 inhibitors deplete multiple proteins important to signal transduction, cell cycle regulation, apoptosis, invasion, angiogenesis, metastasis and immortalisation: thus combinatorial attack on these multiple oncogenic targets should potentially result in a powerful anticancer effect when Hsp90 inhibitors are administered as single agents [4]. Furthermore, blockade of multiple targets should also provide Hsp90 inhibitors with a broad spectrum of activity against many tumour types, regardless of the particular combinations of cancer genes that drive those cancers. Some of the key targets, their biological functions and examples of links to certain cancers are illustrated in Table 1.

Table 1. Client proteins of the Hsp90 Chaperone Complex that are Known to be Deregulated in Malignant Disease

Client Protein	Function	Example of malignancy
Oestrogen Receptor	Ligand mediated transcription	Breast [5]
Androgen Receptor	Ligand mediated transcription	Prostate [6]
ERB-B2	Signal Transduction	Breast [7]
RAF	Signal transduction	Melanoma [8,9]
BCR-ABL	Fusion protein causing proliferation	CML [10]
AKT	PI3 kinase signalling	Ovarian [11], Cervical [12]
CDK4	Cell cycle regulation (G1/S checkpoint)	Ovary [13]
SRC	Signal transduction	Colon [14], Breast [15]
h-TERT	Cell senescence	Breast, Lung [16]

Conventionally, phase I trials establish the optimal dosage and scheduling of novel agents and these are then examined in phase II trials in a wide range of malignancies. This broad approach has pitfalls, as evidenced by a phase II trial in breast cancer of trastuzumab (Herceptin; a monoclonal antibody to ERBB2/HER2), where it was found that while the overall response rate was 26%, the response rate for tumours that were scored as more strongly ERBB2 positive by immunohistochemistry was 35% as compared to

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0% in those with lower expression of ERBB2 [17]. This underlines the importance of choosing a patient population with a clearly defined molecular pathology when planning a phase II study. The selection of a disease setting based on an anatomical site of origin together with a defined molecular abnormality, or even entirely selected on molecular abnormalities, will be appropriate with many molecular therapeutic agents targeting highly specific molecular abnormalities.

How does this analysis fit with Hsp90 inhibitors, which we have agued above will hit multiple molecular abnormalities and tumour types? The answer to this question is not clear. 17AAG has shown a broad spectrum of activity against various different types of cancer cells in tissue culture [18][5]. Differences in sensitivity have been seen, but these are probably attributable to factors such as the expression of multidrug resistance pump proteins and the quinone-metabolising enzyme DT-diaphorase/NQO1 [18]. Nevertheless, it is also possible that other molecular factors, including the status of and dependence on key oncogenic abnormalities, may also affect differential sensitivity to Hsp90 inhibitors. A detailed discussion of this issue is provided in reference [4] (see also article by Maloney *et al*, this issue). Examples of key oncogenic proteins that have been considered in regard to response include RB, ERBB2 and AKT/PKB. Other factors include the ability of cancer cells to alter the expression of potentially protective gene products in response to drug treatment, including the anti-apoptotic Hsp70, the molecular target Hsp90 itself and also the Hsp90-activating protein AHA1 [19, 20]. The cellular status of various cell cycle control proteins and apoptotic regulators such as BAX and BAG-1 may also be critical, particularly in terms of whether cancer cells respond to 17AAG by undergoing G1 arrest, G2M blockade or apoptosis [21]. It has been proposed that simultaneous inhibition of both the RAS → RAF → ERK1/2 MAP kinase pathway and the PI3 kinase → PDK1 → AKT/PKB pathway may be especially important, as shown for example in colon cancer cells [21]. Published human xenograft studies have demonstrated that colon, prostate, breast and melanoma tumours are responsive to 17AAG (e.g. ref. [18]; see also ref. [4] for review).

All of the above studies are helpful but do not directly point to a definitive set of phase II studies for 17AAG or other Hsp90 inhibitors. A pragmatic solution, which nevertheless takes into account current mechanistic knowledge, is to prioritise those cancers that are established to express and be at least partially driven by, or dependent upon, the gene products and pathways that are established to be affected by 17AAG, while at the same time ensuring that there is evidence for antitumour activity in laboratory models corresponding to those cancers. Looking at Table 1 for example, an obvious candidate disease would be chronic myeloid leukaemia (CML) as it is almost solely driven by BCR-ABL, which is a client protein for Hsp90. Other clear possibilities could include: HER-2 positive breast cancer; ovarian cancer, glioblastoma and other tumours in which the PI3 kinase pathway is activated, for example by loss of PTEN (based on depletion of AKT/PKB and inhibition of its phosphorylation); melanomas, thyroid cancer and colorectal cancers harbouring BRAF mutations (based on the speculation that BRAF as well as c-RAF-1 may be a client protein); and also hormone resistant prostate cancer where oncogenic kinases are likely to be activated. On the other hand, one may argue that the list of client proteins of Hsp90 is still incomplete and that until such time as we fully understand the biology of cancer, it may be premature to restrict the phase II trials of Hsp90 inhibitors to narrow molecularly defined options.

Hsp90 Inhibitors in Combination with Conventional Cytotoxics

Combinations of heat shock protein inhibitors and conventional cytotoxic agents have been studied *in vitro* and *in vivo*. Drugs investigated have included paclitaxel, doxorubicin, cisplatin and topotecan.

17AAG has shown enhancement of paclitaxel-mediated apoptosis in SKBr-3 breast cancer cells and antiproliferative synergy with paclitaxel has been demonstrated in SKBr-3 and also BT-474 breast cancer cells [22]. The scheduling of the 17AAG in relation to paclitaxel is of vital importance. It has been shown that if 17AAG was administered before paclitaxel in SKBr-3 cells, there was a reduction of paclitaxel mediated apoptosis; however, if administered after paclitaxel, 17AAG enhanced paclitaxel mediated apoptosis [22]. A possible explanation put forth was that 17AAG caused an RB-dependent G1 arrest, thus limiting the cells that proceeded to the G2M checkpoint at which antitubulins such as paclitaxel exert their effects [22]. Non-small cell lung xenografts (H358) treated with 17AAG and paclitaxel showed additive growth inhibition *in vivo* and also enhanced apoptosis and reduced the microvasculature in the treated tumours [23].

The possibility of combining 17AAG with cisplatin has been explored in A2780 ovarian cancer and in HT29 and HCT116 colon cancer cell lines[10] [23, 24]. The data from these *in vitro* studies have shown either additivity or antagonism. As with paclitaxel, the key may lie in exploring the relative scheduling of the two drugs.

When 17AAG was added after the anthracycline doxorubicin *in vitro*, enhanced apoptosis was observed in SKBr and BT-549 breast cancer cells [22]. The combination of 17AAG with the topoisomerase I inhibitor topotecan was found to be antagonistic in A2780 ovarian cancer cells[11,12].

HSP90 INHIBITORS IN COMBINATION WITH OTHER NOVEL AGENTS

As highlighted earlier in this article, Hsp90 inhibitors have the advantage of depleting a variety of oncogenic client proteins crucial to highjacked signal transduction and deregulated cell cycle control. It may then seem to be

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perverse to suggest combining Hsp90 inhibitors with other drugs that intervene with specific molecular targets in these areas. On the other hand, the activities of these latter agents may well be incomplete and suboptimal. Therapeutic modulation of the same target or pathway by two different approaches may ensure a more complete blockade. Combination of an Hsp90 inhibitor with a signal transduction antagonist or cell cycle blocker could provide a 'belt and braces' effect. It could also help prevent resistance that might otherwise arise via a single molecular mechanism.

Obvious candidates of this type that might be combined with 17AAG to beneficial effect include the BCR-ABL inhibitor imatinib (Gleevec) [24] and the anti-ERBB2 monoclonal antibody trastuzumab (Herceptin) [17]. Both these agents are currently approved and their targets are both client proteins of the Hsp90 chaperone complex.

The receptor tyrosine kinase → RAS → RAF → MEK → ERK1/2 MAP kinase pathway has been found to be deregulated very frequently and in diverse ways in multiple forms of cancer. Inhibitors of individual components of this pathway have met with only partial success. Examples include inhibitors of various receptor tyrosine kinases, farnesyl transferase inhibitors, which may or may not modulate RAS family members, and the RAF kinase inhibitor BAY43-9006 [25]. Cross-talk and redundancy between multiple components of the signal transduction cascade has been suggested to be one of the possible causes of failure of these novel signalling inhibitors and the combination of such agents with a more broad spectrum signal transduction inhibitor like 17AAG could provide additive or synergistic activity [26].

The combination of Hsp90 inhibitors with drugs acting on the PI3 kinase pathway is also an attractive possibility. As Hsp90 inhibitors deplete AKT/PKB, an important component in the PI3 kinase pathway, and also inhibit its phosphorylation [21], it will be interesting to explore combinations of Hsp90 drugs with PI3 kinase and m-TOR inhibitors. Only the latter are currently in clinical trial.

Equally of interest is the potential combination of Hsp90 inhibitors with drugs acting on various components of cell cycle control, which are also modulated by 17AAG and other Hsp90 blockers.

The possibility of the addition of Hsp90 inhibitors to proteasome inhibitors is an exciting one [27]. Hsp90 inhibitors block the normal folding of client proteins and promote their proteasomal degradation. Simultaneous treatment with a proteasome inhibitor would be expected to cause the accumulation of unfolded client proteins in the cell, leading to additive or synergistic effects.

Overcoming the Limitations of Hsp90 as a Target

Hsp90 constitutes about 1% or more of all proteins in the cell [28]. Hsp90 tends to be upregulated in cancer cells and these are likely to be more dependent than are normal cells on cancer-causing proteins because of oncogene addiction [29, 30]. Nevertheless there are potential risks associated with the side-effects of Hsp90 inhibitors as a result of effects on client proteins in normal cells. For example, the client protein LCK is involved in T cell signalling and chronic depletion of client proteins could potentially lead to immunosupression [31]. It remains to be seen whether normal tissue effects arising through Hsp90 inhibition will prove to be a real issue. If so, one possible solution may be to develop inhibitors to specific members of the Hsp90 family, such as GRP94 which may preferentially chaperone ERBB2 [32]. Such an approach may restrict the number of client proteins affected, and hence may help to increase the therapeutic index of Hsp90 inhibitors in the future.

Overcoming the Limiting Factors of the Present Generation of Hsp90 Inhibitors

A more immediate challenge is to overcome the potential limitations of the first-in-class Hsp90 drug 17AAG. Alternative Hsp90 inhibitors available in the laboratory include other benzoquinone ansamycins such as 17DMAG [36] derivatives of the macrolide antibiotic radicicol [37] and purine based inhibitors [38].

The present problems with 17AAG include hepatotoxicity and suboptimal pharmaceutical properties, particularly poor water solubility and oral bioavailability [13]. Although 17AAG has been shown to be less hepatotoxic than the parent compound geldanamycin, it still remains the dose-limiting toxicity. 17DMAG has greatly improved solubility and is a promising potential candidate for phase I clinical trials[13,14] [36]. Additional possible limitations of 17AAG include its metabolism by the polymorphic enzymes cytochrome P450 CYP3A4 and NQO1/DT-diaphorase, potentially leading to variability in pharmacokinetics, toxicity and response, together with the ability to act as a substrate for the P-glycoprotein MDR1 multidrug resistance efflux pump [4]. Potency on the Hsp90 target is also modest.

Radicicol has limitations because of unfavourable pharmacological properties, in particular caused by a potentially reactive epoxide group [4]. However, oxime derivatives of radicicol such as KF25706 have shown better pharmacokinetic properties [33].

Coumarin derivatives are able to inhibit Hsp90 [34], apparently via a locus distinct from the N-terminal ATP-binding site that is involved in the mechanism of action of benzoquinone ansamycins, radicicols and purines [35, 36].

Computerised modelling and X-ray crystallography are being used actively to aid the search for newer inhibitors of Hsp90 [35,36][15,16]. The combination of these techniques along with high throughput screening[17] should lead to the future development of improved Hsp90 inhibitors for clinical trials [4].

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CONCLUDING REMARKS

The present article has focused on the potential clinical applications of 17AAG and future Hsp90 inhibitory drugs. This class of agents has great potential because of the ability to hit simultaneously many multiple pathways that are important in cancer and to modulate all six hallmark traits of malignant cells. Early promise is indicated by the fact that 17AAG is generally well tolerated, that active plasma concentrations can be achieved, and that the molecular signature of Hsp90 inhibitors can be observed in peripheral blood lymphocytes and tumour biopsies from treated patients.

There are, however, many challenges ahead. We have discussed here a number of the key issues currently facing the clinical development of 17AAG and other potential Hsp90 drugs. In particular, we need to define the optimal dosing schedule, taking full account of pharmacokinetic/pharmacodynamic relationships and recognising the importance of building a pharmacological audit trail that links all the steps from the drug target, through drug exposure to drug effect [3, 4]. In terms of such PK/PD endpoints, although molecular assays based on blood samples and biopsies of normal and tumour tissues have been exceptionally useful, the development of surgically non-invasive molecular and functional spectroscopy imaging endpoints would be invaluable [37].

There is still much to understand in terms of the molecular mechanism of action of Hsp90 inhibitors and the genes that can control sensitivity and resistance. The ability to block multiple oncogenic pathways and overcome combinatorial oncogene addiction is consistent with *in vitro* and tumour xenograft data that point to a broad spectrum of activity. On the other hand, particular tumours may be especially sensitive because of their dependence on certain critical client proteins. Figuring out which tumour types will be most appropriate for phase II efficacy studies will be especially important. A number of possibilities have been discussed in this article, including malignant melanoma where biological and clinical activity has been seen in Phase I.[18]

The mechanism of action of 17AAG supports its evaluation as monotherapy. Single agent activity is certainly seen in animal models. However, the use of Hsp90 inhibitors in combination with either cytotoxic agents or other modern molecular therapeutics is also warranted. Some of the key issues have been discussed here, including the choice of the best drugs to use in combination with Hsp90 inhibitors and also the importance of the precise temporal scheduling of the agents in the selected combinations. Unfortunately, our laboratory models of drug combinations are probably not very predictive of clinical efficacy and utility.

Finally, we have discussed here the potential for the development of improved Hsp90 inhibitors for future clinical evaluation. The use of technologies such as computer-based design/X-ray crystallography and high throughput screening is likely to have a big impact. The next generation of Hsp90 inhibitors might be anticipated to show improvements with respect to some of the current limitations of 17AAG. In addition, drugs exhibiting greater molecular selectivity, perhaps based on inhibiting particular members of the Hsp90 molecular chaperone family, could also be extremely interesting.

During the clinical development and use of Hsp90 inhibitors, it is important to obtain and exploit the maximal amount of molecular and pharmacokinetic/pharmacodynamic information, while at the same time being pragmatic about the options available and the decisions made. In this way, it should be possible to realise the full potential of Hsp90 inhibitors for cancer treatment.

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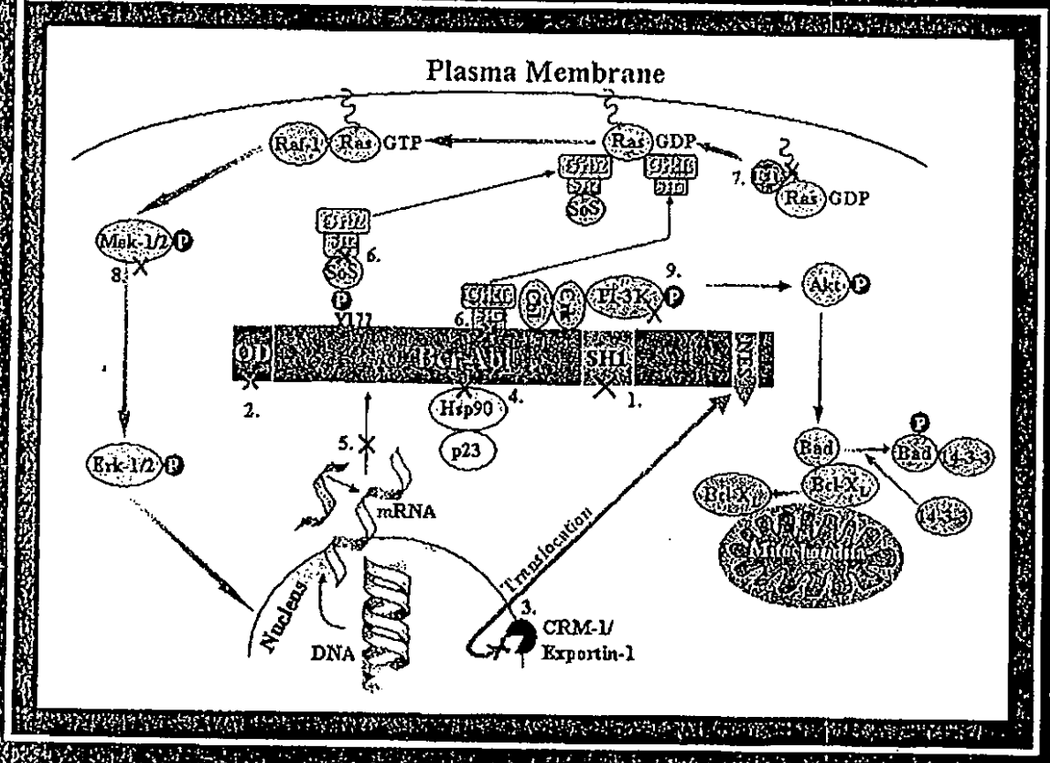

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Novel Targeted and Immunotherapeutic Strategies in Chronic Myeloid Leukemia

Joseph Schwartz, Javier Pinilla-Ibarz, Rui Rong Yuan, and David A. Scheinberg

The advanced understanding of the molecular biology and immunology of chronic myeloid leukemia (CML) has led to novel therapeutic strategies unique to this disease. CML responds to immune-mediated therapies, including stem cell transplantation, donor lymphocyte infusion (DLI), and interferon alfa. T cells and other immune effectors are implicated in the mechanisms of action of these immune therapies. Recently, clinical observations supported by laboratory data have demonstrated the presence of CML-specific T cells in patients. Several proteins may potentially act as leukemia-specific antigens for major histocompatibility complex (MHC)-restricted cytotoxicity in CML, and active specific therapies (vaccines) are in development. Antigens under investigation include bcr-abl, PR1, Wilms tumor protein (WT1), minor histocompatibility antigens (mH), GML-66, GML-28, and survivin. Other strategies target vascular endothelial growth factor (VEGF) and heat shock protein 90 (Hsp90) inhibitors or make use of CML-derived dendritic cells (DC).

Semin Hematol 40:87-96.

CHRONIC MYELOID LEUKEMIA (CML) is a malignant hematopoietic stem cell disorder characterized by the presence of the Philadelphia (Ph) chromosome.[65] The Ph chromosome represents a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11).[73] The molecular consequences of this translocation are juxtaposition of the c-abl oncogene from chromosome 9 into the breakpoint cluster region (bcr) within the *bcr* gene on chromosome 22, resulting in a chimeric *bcr-abl* gene.[24,43,73] The fused genes encode a 8.5-kb chimeric mRNA, which is translated into a 210-kd or 190-kd protein.[9,40,76] This bcr-abl protein shows tyrosine kinase activity, is uniquely present in the leukemia cells of CML patients, and is necessary and sufficient for transformation.[46] The molecular biology of CML makes it a useful model for the development of therapeutic strategies that target unique or highly restricted antigens. CML responds to immune-mediated therapies, including stem cell transplantation, donor lymphocyte infusion (DLI), and interferon alfa. A tyrosine kinase inhibitor STI-571 (Gleevec) was recently shown to block the Abl-coded kinase activity to provide significant clinical benefit in patients with CML.[28,45] Novel targeted and immunotherapeutic strategies in CML will be discussed (Tables 1 and 2).

True Tumor-Specific Antigens: p210-b3a2, p210-b2a2, and p190-e1a2

The p210 (expressed in the majority of patients) and p190 are new proteins uniquely expressed in CML cells; the junctional regions of p210 and p190 contain a sequence of amino acids that is not expressed on any normal protein. In addition, as a result of the codon split on the fused message, a new amino acid (lysine in b3a2) versus glutamic acid in b2a2 or e1a2 is present at the exact fusion point in the b3a2 proteins. Therefore, the unique amino acid sequences encompassing the b3a2, b2a2, or e1a2 breakpoints can be considered truly tumor-specific antigens. Despite the intracellular location of these proteins, short peptides produced by cellular processing of the fusion protein products can be presented on the cell surface within the cleft of human leukocyte antigens (HLA) molecules, and in this form they can be recognized by T cells.[62,81,97]

CML p210-b3a2

Recent data support the hypothesis that peptides that bind to HLA with moderate to high affinity are capable of T-cell stimulation after natural processing and cell surface presentation within the cleft of the appropriate HLA. We and others have demonstrated the immunogenicity from fusion region–derived peptides of p210-b3a2 in the context of major histocompatibility complex (MHC) class I. Screening large numbers of fusion peptides from the junctional sequences of CML has identified p210-b3a2–derived fusion protein amino acid sequences with appropriate anchor motifs for binding to class I and II HLA

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Table 1. Strategies for Specific Immunotherapy of CML

- Peptide vaccines
- Protein vaccines
- DNA vaccines
- Dendritic cells
- Antibodies
- Anti-angiogenesis

molecules. We found four peptides derived from the b3a2 CML breakpoint that bound with high or intermediate affinity to HLA A3, A11, and B8.[12,13,15,39] A fifth nonapeptide that binds to HLA A2.1 molecules was recently identified.[97] Moreover, lysis of bcr-abl b3a2 leukemic cells by $CD8^+$ b3a2 peptide-specific T cells in a MHC class I–restricted and –unrestricted manner has been described in humans.[20,23,62,64,69,97] However, the lysis of bcr-abl b3a2 tumor cells (which present peptides endogenously processed) appears to be inefficient and not clearly demonstrable in all systems.[62,69,97]

Recent elegant mass spectrometry studies have demonstrated the presence of cell surface HLA-associated bcr-abl peptides previously described as binders of HLA A0301 in primary CML cells from HLA A3–positive patients. In addition, these patients mounted a cytotoxic T-cell response to this peptide that killed autologous CML cells and yet, more interesting, a peptide-specific HLA-tetramer demonstrated the presence of circulating peptide-specific T cells. This was the first direct evidence for expression of a bcr-abl–derived peptide on the cell surface in an HLA molecule.[21]

Until recently, all of the p210 class I peptides described belonged to the bcr-abl junction. Peptides derived from the abl-bcr junction (detected by polymerase chain reaction [PCR] in about 80% of CML patients) can efficiently bind to several class I molecules (A1, A2, A3, A11, B7, B27, and B-35). The same group also demonstrated an HLA A0201–restricted cytotoxic T lymphocyte (CTL) specific for an abl-bcr peptide.[10]

Support for the immunogenicity of synthetic bcr-abl fusion peptides capable of interacting with class II MHC molecules has been accumulating as well. Peptides corresponding to the b3a2 fusion sequences were shown to bind DR3 (DRB1*0301), DR4 (DRB1*0402), and DR11 (DRB1*1101), and b3a2 peptides have been shown to induce HLA DR1(DRB1*0101)-, DR2 (DRB1*1501)-, DR4 (DRB1*0401)-, DR9 (DRB1*0901)-, and DR11 (DRB1*1101)-restricted proliferative responses of $CD4^+$ T lymphocytes and cytotoxic cell responses associated with DRB1*0901.[12,49,70,81,83,95]

Processing of endogenous bcr-abl protein by CML cells has been proved biochemically in the context of class I[21]; indirect evidence for processing of p210-b3a2 has been described within peripheral blood mononuclear cells (PBMC) in a patient treated with mitomycin C, as well as in a murine system.[19,90,95] Some investigators have suggested that bcr-abl–specific $CD4^+$ T lymphocytes can distinguish CML and normal cells directly via recognition of the bcr-abl fusion peptide in the context of the HLA class II expressed on leukemic cells. A b3a2 $CD4^+$ cell line proliferated in response to stimulation with b3a2-positive CML blasts in an HLA DR–restricted manner, suggesting that $CD4^+$ T lymphocytes can directly recognize a bcr-abl fusion peptide that is naturally processed and expressed on CML cells.[81] Furthermore, b3a2-specific $CD4^+$ CTL clones exerted strong specific cytotoxicity against b3a2 peptide-loaded B-lymphoblastoid cell lines (B-LCL).[95] This clone was able to specifically recognize dendritic cells (DC) from HLA-matched CML patients and produce interferon gamma, but it failed to show any cytotoxicity as measured in a chromium-51 release assay against the same DC.[96] Cytotoxicity as well as the interferon gamma production was characterized by b3a2 specificity and HLA DR9 restriction, implying that bcr-abl–specific $CD4^+$ T lymphocytes can

Table 2. Targets Evaluated in CML

Target	Rationale	References
BCR-ABL	t(9,22) chimeric fusion protein in CML	20,23,62,64,69,95,97
Proteinase 3	Normal protein overexpressed in myeloid leukemia cells	54-57
WT1	Normal protein overexpressed in AML, ALL, CML	6,38,66-68,94
Minor histocompatibility antigens	Normal proteins with small difference in amino acid sequence between individuals	31-33,78
CML66/CML28	Protein derived from CML cDNA expression library	92,93
Survivin	Inhibitor-of-apoptosis protein expressed in myeloid leukemia cells	5,16,58
Dendritic cells	Derived from bone marrow myeloid progenitor cells or PBMC with specific cytokine combinations	23,47,59,88,89
Vascular endothelial growth factor	Protein involved in tumor angiogenesis	1,48,86
Heat shock protein 90 (Hsp90) inhibitors	Hsp90 is a molecular chaperone required for stability of signal proteins	3,63,75

recognize the bcr-abl protein in the context of HLA DR molecules.[95,96] Similar results have been reported by others using a $CD4^+$ T-cell clone from a CML patient after DLI.[98]

CML p210-b2a2

A 23-mer b2a2 peptide that bound with intermediate affinity to HLA DR3 did not result in any peptide-dependent cells on in vitro sensitization of DR3 or any other donor class II molecules.[70] More recently, repetitive stimulation of T lymphocytes with a 17-mer peptide covering the fusion region in p210-b2a2 led to specific T-cell responses.[82] CD4 and CD4/CD8 double-positive clones obtained from a b2a2 peptide-specific cell line were cytotoxic and proliferative in an HLA DR2a (DRB5*0101)-restricted fashion. However, autologous Epstein-Barr virus (EBV)-transformed cells, transfected to express bcr-abl/b2a2, and allogeneic HLA D–matched p210-b2a2–positive cells from CML patients were not lysed. Finally, bcr-abl peptide-specific T-cell clones killed autologous EBV cells transfected with invariant chain (Ii) cDNA in which the HLA class II–associated invariant chain peptide (CLIP) was replaced by a bcr-abl b2a2 fusion oligonucleotide sequence, illustrating the potential of these T cells to recognize an endogenous bcr-abl b2a2 ligand. These data suggest that the b2a2 epitope for T-cell clones generated was presented by HLA DR2a molecules after endogenous synthesis and optimized processing for MHC class II. Recently, the generation of a DRB1*1406 was found to restrict a b2a2-specific clone, elicited with the same 17-mer b2a2 peptide, illustrating that presentation of b2a2 fusion peptide need not be restricted to HLA DR2a.[95]

Our group was unable to find b2a2 class I peptides that bound with MHC class I molecules, except for low-affinity binding to B8 and A11.[13] Additionally, others did not identify any A3 binders in b2a2 derived peptides.[39] Our results were recently confirmed by the demonstration of low binding of a b2a2-B8 peptide; however, an HLA B44–binding peptide was described.[15]

CML/ALL p190-e1a2

The e1a2 translocation, yielding a p190 fusion protein, occurs in up to 30% of adults with acute lymphoblastic leukemia (ALL) and in half of adults with ALL expressing the Ph translocation. By screening large numbers of e1a2 junctional sequences fusion peptides 6 to 14 amino acids in length, our group has identified p190-e1a2–derived fusion protein amino acid sequences with appropriate anchor motifs for binding to class I and II HLA molecules. We found six peptides derived from the e1a2 CML breakpoint with binding to HLA A0201, A3, A11, and A24 molecules on live or fixed normal and leukemic cells using a competitive radioimmunoassay. Some of these peptides generated peptide-specific CD8 T-cell responses in healthy human donors as assessed by chromium release and interferon gamma ELISPOT assay. These responses were peptide-specific and MHC class I–restricted.

Peptide-specific MHC class II–restricted proliferative responses were seen after stimulation with a peptide spanning the p190 fusion point in three donors (HLA DR types 0301/1104, 0701/1601, and 0101/0301). The generation of a $CD4^+$ cytotoxic T-cell clone from a healthy HLA-DRB1*1501 donor, able to recognize and kill autologous monocyte-derived DCs pulsed with e1a2 peptide, has been described.[79,80]

The present data do not provide proof of processing of endogenous p190 protein, but demonstrate that the e1a2 fusion region is potentially immunogenic and may serve as a target for the immune system.[17]

Clinical Trials With bcr-abl Breakpoint Peptide Vaccines

CML presents a unique opportunity to develop therapeutic strategies using vaccination against a true tumor-specific antigen that is also the oncogenic protein required for neoplasia.

We initiated a phase I dose escalation trial to evaluate the safety and immunogenicity of a multidose, multivalent bcr/abl breakpoint peptide vaccine in 12 adults with chronic-phase CML. We included the four peptides that we previously described as capable of binding to HLA class I molecules and eliciting T-cell responses.[12,13] In addition, because all of the class I and II motifs are not known, and certain motifs may be not entirely exclusive or inclusive with regard to the peptides that can bind to them, it is possible that other potential, unidentified, antigenic peptides existed within the 25-mer b3a2 junctional peptide we described as a class II binding peptide.[12] Hence this larger peptide was included both to elicit class II help and also because it may be processed by cells for class I presentation. Cohorts of three patients each received either 50 μg, 150 μg, 500 μg, or 1,500 μg of total peptide (equal amounts of five different amino acid sequences) mixed with 100 μg of QS-21 as an immunologic adjuvant. Delayed-type hypersensitivity (DTH), humoral responses, and unprimed ex vivo autologous proliferation (^{3}H-thymidine incorporation) and cytotoxicity (^{51}Cr release) responses were measured. All 68 vaccinations were well tolerated without significant adverse effects. Three of the six patients treated at the two highest dose levels of vaccine generated peptide-specific T-cell proliferative responses ex vivo (n = 3), and/or DTH responses

(n = 2), lasting up to 5 months after vaccination. CTL have not been identified.[71]

A safe and active dose was chosen for the phase II study. Adult CML patients with any HLA type and a b3a2 breakpoint were vaccinated five times over a 10-week period using a preparation of six peptides (100 μg each) and the immunologic adjuvant QS-21 (100 μg). Patients with an immunologic response received three additional monthly vaccinations, and those with a continued response received another three bimonthly vaccinations. We employed the same peptides used in the phase I trial plus the newly described HLA A2 peptide.[97] Immune and clinical responses were measured at pretreatment, several times mid-study, and at study endpoint. Immune response was defined as either DTH, ex vivo CD4 autologous proliferation (^{3}H-thymidine incorporation), interferon gamma ELISPOT specific for the peptides, or intracellular flow cytometry for interferon gamma. Clinical response was measured by bone marrow evaluation, cytogenetics, and PCR testing for bcr-abl. Eleven of 12 assessable patients had DTH or CD4 proliferative responses that developed after beginning vaccinations, and six of 12 patients have shown interferon gamma release by CD4 ELISPOT at one or more time points. The responding patients included six patients after bone marrow transplant with evidence of relapsed disease. Three patients demonstrated a decrease in Ph percentage (all concurrently receiving interferon alfa), and two patients vaccinated for recurrent disease after allogeneic transplant became PCR-negative after eight vaccinations. In conclusion, a tumor-specific breakpoint peptide-derived vaccine can be safely administered and can elicit measurable peptide specific immune responses in nearly all treated patients, even after bone marrow transplant.[18]

Self-Antigens

Proteinase 3

Proteinase 3, a serine protease stored in azurophilic granules, is a differentiation antigen associated with myeloid granule formation. Proteinase 3 is overexpressed in a variety of myeloid leukemia types including CML cells. Thus, it has been considered a possible target antigen for specific active Immunotherapy. CTL specific for an HLA A2.1 restricted nonpolymorphic peptide (PR1) derived from proteinase 3 showed HLA-restricted cytotoxicity and inhibited CML progenitors over normal marrow cells.[54,55] The degree of lysis and inhibition was proportional to the PR1 overexpression within the leukemic cells, and premature ontogenic expression of proteinase 3 was detected in CD34$^+$ leukemia cells.[54-56]

PR1-specific T cells could be identified by HLA A2–PR1 peptide HLA tetramers in the majority of CML patients who responded to either interferon alfa or allogeneic bone marrow transplantation.[57] Untreated patients and chemotherapy-treated patients as well as healthy donors had undetected PR1-specific CTL.[56,57] Furthermore, the appearance of these CTL correlated with cytogenetic responses to interferon alfa.[57] PR1-specific CTL isolated from these patients were capable of lysing fresh leukemia cells. Based on these positive initial investigations, PR1-based vaccine trials have been initiated and preliminary results of a phase I study recently reported.[90] PR1 peptide was administrated subcutaneously every 3 weeks (for a total of three doses) to two leukemia patients who relapsed after allogeneic bone marrow transplantation, one of them with CML. One hundred percent donor-derived PR1-CTL were identified in both patients using a PR1/HLA A2 tetramer. PR1-CTL from both patients showed PR1 peptide specificity in functional killing assay. The CML patient had no clinical response, however, with persistence of 95% Ph-positive cells.

The Wilms Tumor Protein

The Wilms tumor protein (WT1) is a self-protein expressed in a time- and tissue-specific manner during embryogenesis. WT1 is expressed at very low levels in the nucleus of some adult normal tissues, such as hematopoietic precursor cells, kidney cells, and gonadal cells. However, most human leukemia cells highly overexpress WT1 regardless of the leukemia subtype.[51-53] WT1 is highly expressed in CML cells in blast crisis, but not in the chronic phase. It is also expressed in normal CD34$^+$ hematopoietic progenitor cells, but at a low level compared with that in acute leukemia cells.[7,50] WT1 expression has been used as a leukemia cell marker for the diagnosis of minimal residual disease.[44] The suppression of WT1 expression by WT1 antisense oligonucleotide can inhibit the growth of leukemia cells,[2,91] suggesting that WT1 is involved in the process of leukemogenesis. If WT1 is preferentially expressed in a variety of human leukemia cells, but rarely in most normal cells, it may be possible to develop immunotherapeutic strategies for leukemia by targeting this protein.

Immunization in vivo of mice with a 9-mer WT1 peptide containing anchor motifs for binding to MHC class I molecules elicited WT1-specific CTL.[68] Further, immunized mice rejected challenges by WT1-expressing tumor cells and survived for a long time with no signs of autoaggression by the CTL. These findings indicate that WT1 protein is an attractive novel tumor antigen.

Recently, human CD8$^+$ T-cell clones (TAK-1, NIM-1) were generated directed against different WT1-derived peptide sequences.[6,66,94] The clones lysed autologous cells loaded with a WT1-derived

9-mer peptide in an HLA A24–restricted manner. Cells were also cytotoxic to HLA A24–positive leukemia cells expressing WT1, but failed to harm HLA A24–positive leukemia cells that did not express WT1, HLA A24–negative leukemia cells, or HLA A24–positive normal cells. The effects of TAK-1 and NIM-1 on cytotoxicity against leukemic cells were not synergistic, suggesting that recognition of a single epitope on the tumor-specific antigen by CTL is sufficient to exert maximal cytotoxic activity against tumor cells.[6] Other investigators have also generated WT1-derived peptide-specific and HLA A0201–restricted CTL.[38,67] HLA A0201–restricted CTL specific for the WT1 peptide lysed leukemia cell lines and inhibited colony formation by $CD34^+$ leukemic progenitors isolated from patients with CML, but did not affect normal $CD34^+$ cells. These data strongly imply that WT1 peptide is processed in leukemia cells, expressed in the context of the appropriate HLA, and recognized by $CD8^+$ CTL. For clinical applications targeting WT1, the development of effective means to elicit anti-WT1 responses in vivo via peptide vaccination, administration of peptide-loaded DC, adoptive transfer of WT1-specific CTL, and WT1 DNA vaccination[84] would be necessary.

There is also evidence for humoral immune responses against WT1. Two groups[29,37] found a high incidence of WT1-specific serum antibodies in patients with leukemia including CML. Antibodies reactive with WT1 full-length protein were detected in 19% of CML patients tested in contrast to 2% of normal individuals.[37] IgM and IgG isotypes of WT1 were frequent in CML and other hematologic malignancies, but rare in healthy volunteers, and the simultaneous production of IgM and IgG WT1 antibodies was limited to patients.[29] The higher incidence of antibody in CML patients is consistent with immunization to the WT1 protein occurring as a result of bearing WT1-expressing malignancy. Likely, strong and persistent stimulation by the WT1 antigen is needed to generate immunoglobulin isotype class switching from IgM to IgG WT1 antibodies. Furthermore, as this switching generally requires T-cell help, T-cell immune responses against WT1 protein should have occurred in the patients with IgG WT1 antibodies. These data should encourage further testing of vaccines against WT1 in order to elicit and/or boost the immune response to WT1.

Minor Histocompatibility Antigens

Human minor histocompatibility (mH) antigens are polymorphic antigens in which the polymorphism results in single amino acid differences in a normally expressed protein among different individuals, giving rise to unique peptide antigens.[25,87] mH antigens, inherited independently from HLA, show broad or restricted tissue distribution and appear to play an important role in graft-versus-host disease (GvHD) after bone marrow transplantation as well as in the graft-versus-leukemia (GvL) effect after DLI. MHC-restricted mH antigen–specific CTL with tissue distribution limited to bone marrow have been isolated from allogeneic stem cell transplant recipients with the intention of developing protocols using them in adoptive immunotherapy after allogeneic bone marrow transplant.[25,26]

mH antigen-specific CTL can lyse freshly obtained myeloid leukemic cells and can inhibit their clonogenic growth; this recognition is MHC restricted. Furthermore, donor-derived leukemia-specific CTL clones were generated in vitro, using irradiated leukemic cells. Finally, a patient with accelerated-phase CML with leukemia-reactive CTL was successfully treated.[31-33,78] The amino acid sequence of a small number of antigenic mH peptides has been recently defined.[25,86] The identification of the hematopoietic lineage-restricted HA-1 and HA-2 antigens and the presence of their HLA A0201 binding motifs allows the possibility of ex vivo generation of mH HA-1– and HA-2–specific CTL from unprimed mH HA-1– and /or HA-2–negative healthy blood donors using HA-1 and HA-2 synthetic peptides pulsed onto antigen-presenting cells such as DC.[14,60] The ex vivo–generated HA-1– and HA-2–specific CTL efficiently lyse leukemic cells derived from patients with acute myeloid or acute lymphoid leukemia.[14,60] Furthermore, injection of T lymphocytes specific for a single immunodominant mH antigen eradicated leukemia cells without causing GvHD.[35] This observation was recently supported by in situ dissection of the graft-versus-host activities of CTL specific for mH showing little or no graft-versus-host induction by hematopoietic-restricted HA-1 and HA-2 mH antigens as opposed to ubiquitously expressed mH antigens.[27] Thus, effective nontoxic immunotherapy of hematologic malignancies may be achieved by targeting single immunodominant mH antigens.

Other New Tumor Antigens

CML66 and CML28

CML66 and CML28 are tumor-associated antigens initially cloned from a CML cDNA expression library. The CML66 antigen is localized to human chromosome 8q23, while CML28 is located to 19q13, a region associated with chromosomal abnormalities in some hematologic malignancies. They were found to be expressed in leukemias and a variety of solid tumor cell lines but not in normal hematopoietic tissue.[92,93] Expression in CML cells was 1.5-fold higher than in normal PBMC when quantitated by reverse transcriptase PCR (RT-PCR) for CML66 and

4.4-fold higher for CML.28.[92,93] The development of high-titer IgG antibodies specific for each of these antigens was detected by enzyme-linked immunosorbent assay (ELISA) and was correlated well with immune-induced remission of CML in patients who received a DLI.[92,93] Using ELISA, specific antibodies for both antigens were also detected in sera from 13% to 38% of patients with lung cancer, melanoma, and prostate cancer.[92,93] The immunogenicity of these antigens and their association with effective antitumor immunity in CML suggest that they may be a possible target for antigen-specific immunotherapy.

Survivin

Survivin, a member of the inhibitors-of-apoptosis gene family, is expressed in a cell cycle–dependent manner in most common cancers but not in normal differentiated adult tissues.[16] Survivin can be detected by different methods (Western blot, RT-PCR) in all myeloid leukemia cell lines and almost all primary acute myelogenous leukemia (AML) and ALL samples tested. There is very low or no expression in normal $CD34^+$ cells and normal PBMC.[16,58] Thus, survivin may be another candidate target for immunotherapy. Initially, survivin was found to be able to induce specific $CD8^+$ effector T cells in vitro using autologous pulsed DC as antigen-presenting cells.[74] Later, CTL responses to survivin were identified in chronic lymphoblastic leukemia patients.[4] The same group later described spontaneous CTL responses against survivin-derived MHC class I restricted T-cell epitopes in leukemic patients both in situ and ex vivo.[a] Moreover, survivin-reactive T cells isolated by magnetic beads coated with MHC/peptide complexes were cytotoxic to HLA-matched tumors of different tissue types.[5]

Dendritic Cells

DC are potent antigen-presenting cells that efficiently process and present antigen, which leads to effective sensitization of naive T lymphocytes.[8] Human DC can be obtained from myeloid progenitor cells in the bone marrow and also from human peripheral blood monocytes in the presence of cytokine combinations such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), tumor necrosis factor-α (TNFα), and CD40 ligand.[8] Several groups have successfully generated DC from the peripheral blood of CML patients using different combinations of cytokines. The cells obtained carried the morphologic and phenotypic characteristics of DC and continued to express the t(9;22) translocation.[30,42,59,77] Functional analysis of these cells demonstrated that they were potent stimulators of in vitro lymphocyte proliferation. The CML DC could stimulate autologous T lymphocytes in vitro, after which the stimulated T cells were shown to have developed strong cytotoxicity against autologous CML cells, but low reactivity against MHC-matched normal bone marrow cells.[24,59]

DC-stimulated T cells also inhibit growth of CML clonogenic precursors in colony-forming assays.[24] Reinfusion of adherent peripheral blood mononuclear cells treated with IL-4 and GM-CSF from two CML patients resulted in in vivo priming of T cells so that they could respond to subsequent in vitro rechallenge with fresh autologous leukemia cells. Although cytotoxic T cells against leukemia cells were not demonstrated, these primed reactive cells could proliferate and produce interferon gamma when cocultured with leukemia cells.[47] The subcutaneous vaccination of CML patients with DC generated from $CD34^+$ cells or monocytes in the presence of GM-CSF, IL-4, and TNFα, using keyhole limpet hemocyanin (KLH) as adjuvant, was well tolerated. DTH could be documented and was usually apparent after the third DC injection.[88,89] Similarly, in mice, immunization of mice with DC loaded with the synthetic specific bcr-abl chimeric nonapeptide, led to a 150-fold higher frequency of bcr-abl–specific CTL precursors in the spleen than in control mice immunized with peptide alone.[41] In vitro restimulation of DC peptide–primed splenocytes resulted in substantial secretion of interferon gamma and augmented cytolytic activity against a 12B1 leukemia target cell line. Finally, vaccination with peptide-loaded DC significantly prolonged survival in mice challenged with 12B1 leukemia cells. The ability to generate bcr-abl–specific CTL in vivo by DC-based immunization may have therapeutic benefit in the treatment of CML. One patient was treated with CML-derived DC vaccine following autologous peripheral blood stem cell transplantation[36]; the vaccination caused a decrease in the number of Ph-positive cells in the peripheral blood and bone marrow. A clinical trial of CML-derived DC vaccine for patients with late-stage interferon-refractory CML has been initiated.[22]

Other Novel Therapeutic Avenues

Vascular Endothelial Growth Factors

Increased microvessel density is present in the bone marrow of patients with hematologic cancers including CML. Plasma vascular endothelial growth factor (VEGF) levels are significantly higher in CML patients than in normal controls.[1] In addition, the number of VEGF-positive bone marrow cells is significantly higher in CML patients than in normal controls and correlates with bone marrow vascularity.[18] Furthermore, VEGF receptor–1 mRNA has been detected in all CML samples tested.[72] The im-

pact of elevated VEGF expression on the course of CML is unknown. Radioimmunoassay was used to demonstrate that high levels of VEGF exist in bone marrow samples taken from patients in all phases of CML, and there appeared to be a significant correlation with survival in patients with chronic-phase CML.[83] These data suggest that VEGF may play a role in the biology of CML and that targeting VEGF via antibody or soluble receptor strategies may prove to be a potential therapeutic option.

Heat Shock Protein 90 Inhibitors

Heat shock protein 90 (Hsp90) is a molecular chaperone whose association is required for the stability and function of signaling proteins on which cancer cells are highly dependent. Hsp90 client proteins include transcription factors such as mutant p53 and hypoxia-inducible factor 1α, transmembrane kinases such as erbB2, and soluble kinases including v-Src, Akt, Raf-1, and Bcr-Abl.[61] Hsp90 is constitutively expressed at 2- to 10-fold higher levels in tumor cells than in normal cells, suggesting that it may be important for the growth and/or survival of tumor cells.[84] Hsp90 inhibitors, by specifically interacting with a single molecular target, cause the destabilization and eventual degradation of Hsp90 client proteins. The most widely studied Hsp90 inhibitors are geldanamycin (GA) or 17-allylaminogeldanamycin (17AAG). In CML, in which one Hsp90 client protein, bcr-abl, is directly implicated in the pathogenesis of the disease, use of inhibitors may prove to be effective. The bcr-abl protein is dependent on association with Hsp90 for its stability and treatment of cells with GA or 17AAG leads to rapid destruction of bcr-abl.[3] High concentration of these two inhibitors as well as use of another Hsp inhibitor (radicicol) has been shown to induce apoptosis in CML cell lines (K562), as well as in cells transfected with the bcr-abl gene.[63,75] The Hsp90 inhibitors can also be used to increase potency of other therapeutic modalities. Low-dose GA promoted caspase activation causing sensitization of K562 and BCR-abl–expressing HL60 cells to previously ineffective levels of doxorubicin.[11] As 17AAG destabilizes Bcr-Abl protein by altering its association with Hsp90, its activity should not be affected by resistance mechanisms such as overexpression of the tyrosine kinase or point mutation.

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