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Natural Product Inspired Hsp90 N-Terminal Inhibitors for the Treatment of Cancer: From Bench to Bedside

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Abstract

The 90 kDa heat shock proteins (Hsp90) are responsible for the conformational maturation of nascent polypeptides and the rematuration of denatured proteins. Proteins dependent upon Hsp90 are associated with all six hallmarks of cancer. Upon Hsp90 inhibition, protein substrates are degraded via the ubiquitin-proteasome pathway. Consequentially, inhibition of Hsp90 offers a therapeutic opportunity for the treatment of cancer. Natural product inhibitors of Hsp90 have been identified *in vitro*, which have served as leads for the development of more efficacious inhibitors and analogs that have entered clinical trials. This review highlights the development of natural product analogs, as well as the development of clinically important inhibitors that arose from natural products.

Keywords

Hsp90; chaperone; geldanamycin; radicicol; natural product-based drug design

Introduction

Natural products cover a vast amount of chemical space and their unique, complex structures represent an excellent platform for the optimization of biological activities. Natural products or compounds derived from natural products comprise the majority of currently marketed drugs, in addition, there are others undergoing pre-clinical and/or clinical development.[1-3] Recent advances in bioanalytical techniques, DNA sequencing, and bioinformatics have increased the rate at which new natural products can be discovered.[4] Consequently, natural products remain a major source for lead compounds in drug discovery. Although natural products are common sources for leads, library and fragment based screens have gained popularity in recent years.[5,6] Recently, Zeilinger and co-workers reported a microarray-based screening method for rapid screening that utilizes low protein and compound concentrations.[7]These methods have also resulted in drugs that have been

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FDA approved, as well as others undergoing clinical development. However, natural products continue to provide complex three-dimensional scaffolds that serve as leads for drug development. Such scaffolds manifest unique biological activities that continue to expand the number of validated therapeutic targets.[8]

Geldanamycin (GDA), a member of the ansamycin class of antibiotics, is a classical example of natural product-based drug discovery in medicinal chemistry.[9] Analysis of the biological activity elicited from the administration of GDA led to the discovery that its primary biological target is the 90 kDa heat shock protein, Hsp90.[10] Many drug development campaigns with GDA have provided drug candidates that have entered clinical trials and provided biological probes that have increased the understanding of Hsp90 biology.[11,12] In the last two decades, other natural products have also been shown to bind and inhibit the Hsp90 chaperone machinery (Figure 1).[10,13-24] These natural products have served as a starting point for numerous drug development campaigns; which have led to the discovery of highly efficacious inhibitors that have now entered clinical trials.[9,25-27] This review gives a synopsis of natural product-based Hsp90 N-terminal inhibitors.

Molecular chaperones represent a class of proteins that are responsible for the conformational maturation of nascent polypeptides as well as the rematuration of denatured proteins.[28-30] These chaperones work together with co-chaperones and partner proteins to facilitate the folding of substrate proteins (referred to as clients). Many of these chaperones are upregulated in response to conditions that cause cellular stress, such as exposure to heavy metals, hypoxia, acidosis, and increased/decreased temperature.[31] The heat shock proteins were originally identified after cellular exposure to elevated temperatures, which led to their overexpression to refold proteins that denatured at such temperatures.[32] Hsp90 is responsible for the maturation of more than 200 proteins, including several therapeutically sought after anticancer targets such as Her2, Raf, ALK, Src, and Akt.[33,34] Hsp90 is highly conserved in eukaryotes and represents 1-2% of the total cellular protein at ambient temperatures, but can represent up to 6% in stressed cells.[34-36] Hsp90 contains an ATP-binding site that is conserved within the GHKL superfamily of proteins, such as histidine kinase, DNA gyrase B, and MutL. The GHKL superfamily is characterized by the presence of Bergerat ATP-binding fold in which ATP binds in a unique, bent conformation that is in contrast to the typical extended conformation.[17,37] In cells, Hsp90 exists as a homodimer and each monomer consists of an N-terminal ATP-binding domain, a middle domain connected to the N-terminus by a charged linker, and a C-terminal dimerization motif. ATP binds to the highly conserved N-terminus and its hydrolysis provides the requisite source of energy to facilitate client protein maturation. The middle domain plays a crucial role in client protein recognition, as well as interactions with co-chaperones. The C-terminus mediates dimerization and contains a second nucleotide binding motif.[38]. The Hsp90 family of proteins consists of four isoforms in mammals: Hsp90α and Hsp90β are localized in the cytosol, however, Hsp90β is constitutively expressed and Hsp90α is inducible upon exposure to cellular stress. Grp94 is found in the endoplasmic reticulum and Trap-1 resides in the mitochondria. [39]

The Hsp90 chaperone cycle is complex, but advances in technology and small molecule probes have provided insights into the catalytic cycle. The Hsp90-mediated protein folding

process is catalytic and protein maturation is driven by ATP-hydrolysis (Figure 2). The Hsp90 chaperone cycle begins with the delivery of nascent polypeptides to Hsp90 by Hsp70 (**1.3**). This process is mediated by the Hsp70-Hsp90 organizing co-chaperone, Hop (**1.2**). Upon delivery of the nascent polypeptide, immunophilins, co-chaperones, and partner proteins interact with the Hsp90 homodimer to form a heteroprotein complex (**1.4**).[40] ATP is recruited to the N-terminus (**1.5**) and then hydrolyzed upon p23 association (**1.7**), which stabilizes the closed conformation of Hsp90.[38] Following maturation, the client protein is released and the heteroprotein complex dissociates to regenerate the Hsp90 homodimer (**1.1**).[41]

Inhibition of either the N- or C-terminus can disrupt the Hsp90 chaperone cycle. The N-terminal binding site is very specific for ATP, however the C-terminus of Hsp90 binds both purine and pyrimidine nucleotides.[42] Neckers and co-workers demonstrated that GDA does not bind to the C-terminus and is a specific, competitive inhibitor of ATP binding at the N-terminus.[43] Alternatively, novobiocin binds exclusively at the C-terminus of Hsp90. Upon disruption of the chaperone cycle, the client protein is degraded primarily through the ubiquitin-proteasome pathway (**1.6**).[44] Because malignant cells undergo a constant rate of proliferation, increased rates of protein synthesis and metabolism are required for survival. As a consequence of the increased dependency upon Hsp90, Hsp90 exists solely as the heteroprotein complex (**1.4**) in malignant cells. While in normal cells, Hsp90 exists as the homodimer (**1.1**). The heteroprotein complex possesses more than 200-fold greater affinity for ATP compared to the Hsp90 homodimer.[27,45] Therefore, inhibitors that bind the ATP-binding pocket display an inherent selectivity for the heteroprotein complex, resulting in differential selectivities of ~200-fold for malignant verses normal cells. Not surprisingly, Hsp90 inhibitors accumulate at higher concentrations in tumor tissue than in normal tissue.[45]

Hsp90 inhibition exhibits therapeutic potential for the treatment of many different diseases; most notably Hsp90 inhibitors are sought after as cancer chemotherapeutics.[27,31,45,46] Since many of the Hsp90-dependent clients are associated with signal transduction pathways that become hijacked during cancer transformation, Hsp90 inhibitors manifest a single drug combination approach toward the treatment of cancer.[47] As shown in Table I, Hsp90 client proteins are present in every hallmark of cancer, therefore, inhibition of Hsp90 provides a unique opportunity to simultaneously target multiple oncogenic pathways, and thus provides a multidimensional attack on cancer.[37,48,49] N-terminal inhibitors represent the most developed class of Hsp90 inhibitors and will be discussed in detail. In addition, Hsp90 C-terminal inhibitors have shown promise as therapeutic agents.[50-54] Novobiocin, a member of the aminocoumarin class of antibiotics was identified as the first C-terminal Hsp90 inhibitor.[43,55,56] In 2009, polyphenol epigallocatechin-3-gallate, EGCG, was shown to also bind the C-terminus and inhibit Hsp90 function.[57] The tetranortriterpenoid, gedunin and cyclic peptide sansalvamide A bind at sites other than the N- or C-terminus and manifest anti-cancer activity through alternative modulation of the Hsp90 chaperone cycle.[21,23,58-60] In addition, alternative approaches to target Hsp90's interaction with partner proteins and co-chaperones have also emerged as novel strategies to target cancer.[41,61] For example, Celestrol, disrupts the Hsp90-Cdc37 complex and leads to inhibition of the chaperone

cycle.[62] Cdc37 is a co-chaperone that mediates the loading of protein kinases to Hsp90. Additionally, the macrolide natural product, Cruentaren A, disrupts the interaction of F_1F_0 ATP synthase with Hsp90 and manifests low nanomolar efficacy against various cancer cell lines.[16,63] Structure-activity relationship investigations of these natural products have produced highly efficacious anti-cancer agents.[64,65]

Geldanamycin-based Inhibitors

GDA (**1**, Figure 1) was originally isolated from the *geldanus* variant of the filamentous soil bacterium, *Streptomyces hygroscopicus*, in 1970 and subsequently shown to manifest potent antibiotic and growth inhibitory activity against HeLa derived KB cancer cells.[66] In subsequent years, GDA was shown to manifest potent anti-tumor activity against a variety of cancer cell lines. However, Hsp90 was not identified as the biological target of GDA until 1994, when Whitesell and co-workers demonstrated that GDA binds Hsp90 to induce the degradation of v-Src, an oncogenic client protein dependent upon Hsp90.[10] Although GDA manifests excellent potency, its hepatotoxicity, low chemical stability, poor bioavailability and solubility, prevented GDA from advancing to clinical trials.[67] Structure-activity relationship (SAR) studies on GDA have been pursued along with total synthesis, semi-synthesis, and genetic engineering of its biosynthetic pathway to generate new analogs. Such efforts have led to the development of improved derivatives that manifest superior pharmacokinetic and pharmacodynamic profiles.

The first total synthesis of GDA was reported by Andrus and co-workers in 2002, followed by other groups that developed more succinct routes.[68-72] Ultimately, the most efficacious analogs of GDA were produced by semi-synthesis that modified the 17-position. Due to the labile nature of the β-methoxy-α,β-unsaturated quinone, the methoxy could be easily replaced with various nucleophiles to provide C-17 substituted analogs. The greatest biological activities were observed by replacing the 17-methoxy with various alkyl amines, which further stabilized the reactive quinone moiety.[11,73,74] Many of these analogs (**12-17**, Figure 3) manifest improved biological activity over GDA. The hydroquinone analog (**18**, Figure 3) of GDA was also synthesized by reducing GDA with sodium hydrosulfite, which was stable and isolable.[75,76] The acetylated derivate of the hydroquinone was inactive (IC_{50} > 2.9 μM).[76] The anti-cancer activity exhibited by these analogs was evaluated by measuring the degradation of the oncogenic, Her2 receptor. Among the analogs tested, 17-allylamino-GDA (17-AAG, **16**) entered clinical trials.[76,77]

In 2004, Conforma Therapeutics further modified the 17-position of GDA and developed a cell lysate-based competition assay to evaluate the efficacy of new analogs. Among the reported analogs, **19** and **20** (Figure 4), manifested significant inhibition of cancer cell proliferation, however, they also manifested high clearance and low bioavailability.[74] Kosan Biosciences reported the synthesis of more than 60 analogs of the 17-position as well as the C-7 carbamate during their pursuit of GDA analogs. Modifications to the carbamate led to a loss in potency, as the carbamate provides key hydrogen bond interactions with the N-terminal ATP-binding site of Hsp90 (see Figure 10).[78] 17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin (17-DMAG, **21**, Figure 4) was identified from these studies and

was shown to manifest excellent potency (IC_{50} = 24 nM, in SkBr3 breast cancer cells) and solubility (1.4 mg/mL), and was selected to undergo clinical trial evaluation.[78]

In 2008, Panek and co-workers reported a total synthesis of GDA and utilized this approach to prepare the phenolic ansamycins, **23-24** (Figure 5).[72,79] The phenolic compounds were shown to exhibit increased affinity for Hsp90, compared to 17-AAG. Andrus and co-workers further simplified their total synthesis and reported the 8,9-amido-GDA derivatives, **25** and **26** (Figure 5), however, these compounds were determined inactive (ED_{50} > 20 μM in Her2 degradation assay).[80]

17-AAG undergoes cytochrome P450 3A4-mediated oxidation of the alpha methylene on the 17-amino substituent, resulting in subsequent hydrolysis of the C-N bond to produce 17-AG (**27**, Figure 5).[81] This metabolite manifests growth inhibitory activity against breast cancer cells (SKBr3) at 33 nM, which is similar to that of 17-AAG.[77] Consequently, Infinity Pharmaceuticals developed two water soluble GDA analogs, IPI-493(**27**)[81] and hydroquinone **28** (IPI-504).[11] It was determined that the hydroquinone of IPI-504 was more water-soluble than the corresponding quinone and upon subsequent preclinical studies, IPI-504 was found to exhibit excellent properties, which led to its clinical evaluation against various solid tumors, including non-small cell lung cancer, however IPI-504 is not currently being investigated in the clinic.[73,82,83,84] Porter and co workers reported the hydroquinone analogs (**29**-**30**, Figure 5) to exhibit increased binding affinity for human Hsp90 compared to the quinone form, which paralleled prior studies with radicicol and geldanamycin chimeras.[85]

Throughout the last ten years, genetic engineering has provided additional GDA derivatives via biosynthetic manipulation.[86,87] Kosan Biosciences reported the biosynthesis of GDA analogs by mutating the polyketide biosynthase genes present in the GDA biosynthetic pathway.[88] The most significant derivative obtained from this work was **31** (Figure 6), which manifested a K_d of 16 nM against Hsp90, highlighting that the quinone/hydroquinone is not required for inhibitory activity. In 2008, Zhang and coworkers prepared derivatives of macbecin utilizing altered biosynthesis. Their efforts identified **32** (Figure 6), which was shown to exhibit improved Hsp90 binding affinity by ~80-fold compared to macbecin (3 nM v. 240 nM, respectively).[89] **32** also manifested a reduced toxicity profile; however, additional preclinical studies with this compound have not yet been reported.

In 2007, Young-Soo Hong and co-workers reported a series of GDA analogs prepared from genetically engineered biosynthetic intermediates.[90] Later in 2009, they reported the preparation of non-quinone GDA analogs by mutagenesis of GDA polyketide synthase.[91] The lead compound, **33** (Figure 6), was shown to manifest greater affinity for Hsp90 and enhanced inhibition of Hsp90 ATPase activity than GDA. The 19-member macrocycle (**35**) and an unusual 20-membered macrocycle (**36**, Figure 6) of GDA were also prepared from an *S. hygroscopicus* strain that could not synthesize 3-amino-5-hydroxybenzoic acid, the initial substrate for GDA biosynthesis.[92]

These synthetic/biosynthetic efforts led to discoveries in the biology of Hsp90 and the development of assays for detection of Hsp90 inhibitors. As stated earlier, Conforma

Therapeutics developed a competition assay using immobilized GDA.[74] However, other groups also contributed to the development of biological assays that could rapidly identify new inhibitors. Blagg and co-workers synthesized biotinylated GDA-analogs (**37**-**38**, Figure 7) that connected GDA and biotin via photolabile and non-photolabile linkers, which served as both a tool to understand Hsp90 biology as well as to identify other biological targets of GDA.[93] More recently, Wuest and co-workers reported ^{18}F-labeled and rhenium-containing GDA analogs as probes for imaging Hsp90 expression and early tumor detection.[94] Their lead compound, **39** (Figure 7), manifests Hsp90 inhibitory activity comparable to GDA and the rhenium containing compound (**40**, Figure 7) is less active.

Recent synthetic efforts have attempted to address the issue of hepatotoxicity associated with GDA and its derivatives.[95,96] Shen and co-workers modified the 17-postion and made compounds containing a diamine linker.[97] The lead compound (**41**, Figure 8) produced lower levels of both aspartate transaminase and alanine transaminase in mice, as compared to GDA. **41** also manifests an IC_{50} of 190 nM against the MDA-MB-231 breast cancer cell line. This compound manifests increased *in vivo* tumor efficacy in a MDA-MB-231 xenograft model compared to 17-AAG and exhibits a MTD ≥250 mg/kg. In an alternative approach, Moody and co-workers envisioned the 19-position of the quinone ring to be susceptible to nucleophilic attack by biological thiols.[98] Therefore, a library of GDA analogs was synthesized by modification of the 19-position. Two analogs, 19-phenyl-GDA (**42**) and 19-methyl-GDA (**43**, Figure 8), were shown to be significantly less toxic than both GDA and 17-AAG. However, these modifications also led to a decrease in potency, wherein 19-methyl-GDA binds Hsp90 with a K_d of 16.3 μM, which is 5-fold higher than GDA (K_d = 2.9 μM).

The biological evaluation of GDA and its derivatives have provided significant insights into Hsp90 function, as well as to establish Hsp90 as a promising anti-cancer target.[27,33,99-102] GDA has served as a starting point for several medicinal chemistry campaigns and several GDA analogs have advanced into clinical trials. The current focus of GDA research has centered on addressing the toxicities associated with the benzoquinone moiety, as well as the use of GDA analogs in combination with other therapies in the clinic.

Radicicol-based Inhibtors

The resorcinol lactone, radicicol (RDC, **4**, Figure 9) was originally isolated from *Monosporium bonorden* in 1953.[103] RDC was found to manifest antifungal properties and later determined to exhibit antitumor properties. Similar to GDA, RDC was believed to be an inhibitor of the v-Src and Ras-Raf-MAPK signaling pathways.[104] RDC was known to exhibit a similar biological profile as GDA and in 1998, Schulte and co-workers demonstrated that RDC competes with GDA for binding Hsp90.[18] Subsequent experiments showed that RDC binds the N-terminal ATP-binding site of Hsp90, similar to GDA, however, in a different orientation.[105,106]

RDC manifests greater affinity for Hsp90 than GDA *in vitro* (K_d in ATPase assay, 19 nM vs. 1.2 μM, respectively).[107] Unfortunately, the administration of RDC *in vivo* does not produce anti-tumor activity.[104,108,109] Because RDC is rapidly metabolized to inactive

metabolites *in vivo* due to its electrophilic nature (allylic epoxide and α,β,γ,δ-unsaturated ketone), no *in vivo* activity has been observed with this natural product.[14,110] Consequently, RDC was not considered a viable candidate for clinical evaluation. However, this scaffold allowed for the development of new analogs that do not exhibit the detriments associated with RDC or GDA. In addition to RDC, less electrophilic natural products (Monocillin I, **44**, and Pochonins A, **45**, and D, **46**, Figure 9) have also been identified as Hsp90 inhibitors, although they manifest lower affinity.[111-113]

RDC attracted the attention of several synthetic groups beginning in the early 1990s, and the first total synthesis was reported by the Lett group in 1992.[114,115] Subsequent routes toward the natural product were also developed by the Danishefsky and Winssinger laboratories.[116-118] These total syntheses were developed for the preparation of analogs to reduce electrophilicity and to enhance metabolic stability *in vivo*. One strategy to reduce the metabolic susceptibility of RDC was to replace the epoxide with a cyclopropyl ring. Danishefsky and co-workers reported analogs that incorporated the cyclopropyl ring (**47**, Figure 9), which resulted in a two-fold loss in cellular efficacy.[117,119] However, replacement with a difluorocyclopropane ring (**48**, Figure 9) resulted in a significant loss of activity, indicating the epoxide oxygen does not exhibit strong hydrogen bond accepting interactions. A second strategy to increase the stability of RDC was to convert the 2′-ketone of RDC to the corresponding oxime.[25,120] The oxime analogs led to identification of the unsubstituted oxime analog, KF25706 (**49**, Figure 9), which manifested comparable potency to RDC against various human cancer cell lines.[104] KF25706 was determined metabolically stable, and was administered in xenograft rodent models of cancer and reduced tumor growth over a 30 day period.[25] Following this work, a series of substituted oximes was prepared and evaluated. The development of oxime derivatives was complicated by the mixture of *E* and *Z* isomers, however it was determined that the *E* isomer possesses higher affinity for Hsp90[121]. Metabolically stable RDC analogs represent a potential method to develop these natural product inhibitors, however, the complexity associated with this scaffold renders this compound difficult for large scale production. Fortunately, the data obtained from RDC analogs presented a new pharmacophore that could be utilized to design new Hsp90 inhibitors.[105,109,111,113,122]

As previously mentioned, GDA and RDC bind similarly within the N-terminus of Hsp90, however, the two natural products are not structurally similar. X-ray crystallographic studies have shown these structures to exhibit different interactions with Hsp90, which can be exploited for the development of more simplified scaffolds.[105] For example, the resorcinol moiety mimics the hydrogen bonding interactions manifested by the adenine ring of ATP and is necessary for Hsp90 inhibition. These phenols compliment a conserved hydrogen bonding network between Leu34, Asp79, Gly83, Thr171, and two conserved water molecules (Figure 10a). Disruption of this network reduces the affinity of inhibitors for Hsp90.[111] Hydrogen bonding interactions between Hsp90 and the RDC macrocycle are not critical, however the overall bent conformation appears important. While the hydrogen bonding network surrounding the resorcinol is important, RDC derives much of its affinity from entropic factors.[14] Molecular dynamic studies with RDC led to the identification of three major conformations: the bioactive “c-shaped” conformation, a planar conformation,

and a conformation in which the macrocycle is bent opposite the resorcinol ring. The bioactive conformation presents the lowest energy conformation by 2.4 and 3.3 kcal/mol, respectively.[113] This analysis suggests that only a minimal entropic penalty exists when RDC binds Hsp90. Unfortunately, the RDC bioactive conformation does not exhibit differential selectivity for the Hsp90 heteroprotein complex.[105,123]

The resorcinol ring of RDC serves as a valuable pharmacophore for several inhibitors undergoing clinical evaluation.[124,125] Ganetespib (STA-9090, **50**, Figure 11) is currently in phase I-III evaluation for the treatment of cancer. STA-9090 manifests greater affinity for Hsp90 than 17-AAG against various lung cancer cell lines (average IC_{50} = 6.5 nM vs. 30.5 nM, respectively). STA-9090 also exhibits increased tumor penetration and an improved side effect profile in preclinical models.[126,127] Currently, STA-9090 is undergoing nine different clinical trials for seven types of cancer.

A high throughput screening campaign of 56,000 compounds led Workman and co-workers to identify the diaryl pyrazole resorcinol-containing compound, CCT018159 (**51**, IC_{50} 0.21 μM, Figure 11), as an Hsp90 inhibitor.[128,129] A medicinal chemistry campaign utilizing a structure-based approach led to the development of AUY922 (**52**, Figure 11), which is currently in phase II clinical trials for Her-2 positive and estrogen receptor positive metastatic breast cancer.[130] AUY922 is also being evaluated in phase II trials for non-small cell lung cancer and has produced 20% response rate.[131-133] Treatment of patients with multiple myeloma is also being evaluated with AUY922 in the presence and absence of the proteasome inhibitor, bortezomib.[134] However, results have not been promising due to the lack of efficacy as a single agent. In addition, dose tolerance appears to be an issue when administered in combination with bortezomib.[135]

KW-2478 (**53**) and AT13387 (**54**, Figure 11) are additional resorcinol-derived inhibitors that have advanced into clinical trials. KW-2478 was developed by Kyowa Hakko Kirin Pharma in Japan through a unique lead optimization strategy that included the combination of microbial screening, structure-based drug design, cell-based screening, and *in vivo* models.[67,125,136] This optimization strategy led to the identification of KW-2478, which manifests 3.8 nM affinity for Hsp90 and low nM antiproliferative IC_{50} values against various multiple myeloma cell lines. KW-2478 is currently being evaluated in patients with B-cell malignancies (phase I) and relapse or refractory multiple myeloma (phase I-II, in combination with bortezomib). Astex Pharmaceuticals developed AT13387 from a fragment-based screening approach. AT13387 manifests an IC_{50} of 0.71 nM via fluorescence polarization and antiproliferative IC_{50} of 48 nM against the HCT116 colon cancer cell line.[137] AT13387 is currently in phase I trials for patients with metastatic solid tumors as well as phase II trials for the treatment of gastrointestinal stromal tumors with or without Imatinib.

Chimeric Inhibitors

Toxicities associated with GDA and the lack of *in vivo* efficacy of RDC have proven difficult to overcome.[67] However, one approach to develop new Hsp90 inhibitory scaffolds is to combine the structural features found in both GDA and RDC. This approach offers the

potential to retain key interactions from both inhibitors with Hsp90, while simultaneously reducing the structural complexity, which enables rapid identification of structure-activity relationships for each natural product.

The first chimeric inhibitor of Hsp90 (radanamycin, **55**, Figure 12) focused on maintenance of the resorcinol-mediated hydrogen bonding network.[138] The quinone moiety manifests a key hydrogen bonding network near the solvent-exposed region of the ATP-binding pocket and allows for selective binding to the heteroprotein complex.[105] Amino acids within this region are responsible for isomerization of the GDA amide bond, which dictates the high differential selectivity manifested by GDA.[27,45,110]

Molecular docking studies suggested two potential linkers to connect the quinone and resorcinol ring systems. The first approach connected the resorcinol ring to the quinone via a two carbon-linker containing an amide bond, which led to the radanamycin seco-agent, radamide (**56**, Figure 12).[139] Radamide was found to inhibit Hsp90 ATPase activity at 5.9 μM compared to 2.5 μM for GDA.[107] *In vitro*, radamide induced the degradation of Her2, an Hsp90-dependent client protein, in MCF-7 cells. The second approach maintained the ester linkage of RDC and through a two carbon linker, connected it to the quinone, termed radester (**57**, Figure 13).[140] Antiproliferative activity of radester was determined in MCF-7 cells and was shown to manifest an IC_{50} of 13.9 μM and induced the degradation of Hsp90 client proteins, Raf and Her2.

Importantly, the hydroquinone derivatives of the seco agents of radanamycin were shown to manifest greater affinity for Hsp90 than the corresponding quinones.[139,140] The hydroquinone of radamide inhibited Hsp90's inherent ATPase activity at 1.8 μM and was shown to induce the degradation of Her-2 levels in MCF-7 cells. Similarly, the radester-hydroquinone manifested an antiproliferative IC_{50} of 7.1 μM and was also shown to induce the degradation of Her2 and Raf levels. Additionally, the macrocyclic chimera, radanamycin manifested antiproliferative activity against breast cancer cells at 1.2 μM and was shown to induce the degradation of Hsp90 client proteins, Her2 and Akt. These studies proved important as Infinity Pharmaceuticals was able to prepare a hydroquinone derivative of GDA, IPI-504 (see Figure 5). [11]

Co-crystallization studies of radamide bound to Grp94 and Hsp90 led to the discovery that radamide extends into a unique 5′-extension pocket present only in Grp94.[141] Although the four Hsp90 isoforms share >85% identity in the N-terminal ATP-binding site, this 5′-extension pocket results from a 5-amino acid insertion into the primary sequence of Grp94.[142-146] Extension into this pocket was shown with radamide, to result in Grp94-selectivity. As noted in the crystal structures, radamide projects into this region as a consequence of isomerization of the *trans*-amide to a *cis*-amide (Figure 13).[141] Therefore, *cis*-amide bioisosteres were pursued for incorporation into the radamide scaffold to design Grp94-selective inhibitors. This led to the development of BnIm (**58**, Figure 14), which represents the first Grp94-selective inhibitor as determined by inhibition of Grp94-mediated Toll-like receptor trafficking to the cell surface and cytosolic client protein degradation dependent upon Hsp90α and Hsp90β.[147]

BnIm was shown to exhibit no cytotoxic effects up to 100 μM, which is consistent with the non-essential nature of Grp94.[148,149] In addition to BnIm, a series of radamide analogs was also investigated for Grp94 inhibition. These compounds were shown to inhibit the migration of the highly metastatic breast cancer cell line, MDA-MB-231 at ~1 μM, while exhibiting minimal toxicity at 100 μM.[150] Grp94 is responsible for the trafficking of integrins and therefore Grp94 inhibition exhibits anti-migratory activity and represents a potential new target for the development of new anti-metastatic agents.[151] Finally, an analog of BnIm (**60**) was shown to exhibit low μM inhibition of mutant myocilin aggregation, which occurs through disruption of the Grp94-mutant myocilin complex, which allows for the autophagic degradation of mutant myocilin and may provide a nontoxic approach towards the treatment of primary open angle glaucoma.[152,153]

Purine-based Inhibitors

In addition to the natural products GDA and RDC, the natural substrate of Hsp90, ATP, has also been used as a starting point for the development of Hsp90 inhibitors.[154] Chiosis and co-workers utilized the purine moiety of ATP as a template to design small molecule inhibitors that bind the N-terminal ATP-binding site.[155] Using a structure-based approach, the purine moiety was linked to an aryl ring, which mimicked the unique shape adopted by ATP within the N-terminal binding pocket. The first inhibitor of this class, PU3 (**61**, Figure 15), was shown to inhibit Hsp90 with an EC_{50} of 15-20 μM, and manifested low micromolar anti-proliferative activity against various breast cancer cell lines.[155-157] This early study identified this class of purine based Hsp90 inhibitors and further SAR studies resulted in a significant improvement in affinity.

The first library of purine analogs probed alkyl substituents at the 2-position, and incorporated halogens on the trimethoxyphenyl group as well as the linker connecting these two rings.[157] Various alkyl and aryl substitutions were also explored at the 9-position. Analogs containing pent-ynyl (**62**) and 2-isopropoxy-ethyl (**63**, Figure 15) linkers were most active and manifested inhibitory activities of 1.5 μM and 1.7 μM, respectively. Fluorine substitution at the 2-position (**64**, Figure 15) resulted in significant Hsp90 affinity.[156] Its enhanced potency (IC_{50}= 3.5 μM) correlated with its ability to increase hydrogen bonding of the C6-amine. Monobromo- (**65**) or monochloro-substitution (**66**, Figure 15) on the trimethoxyphenyl ring resulted in increased potency and the linker between the two rings was modified to contain a phenyl ether, secondary amine, sulfonyl, sulfinyl, benzyl ether and thioether.[156,158] However, the sulfur linker proved most valuable. These optimized features were combined, and the resulting analogs (**67** and **68**, Figure 15) inhibited Hsp90 with submicromolar activity. Compound **68** manifests low micromolar anti-proliferative effects against breast, colon, lung, and prostate cancer cell lines. **68** also induced the degradation of Hsp90-dependent client proteins, Her2, Akt, Raf-1 and mutant p53.[156] *In vivo* analysis of **68** revealed tumor specific inhibition of Hsp90, as this compound was retained in tumor tissues, but rapidly cleared from normal tissue. Administration of **68** resulted in a significant decrease in tumor mass upon dosing of 200mg/kg i.p. every other day for 30 days.[159] Preliminary SAR studies and encouraging *in vitro* and *in vivo* studies led to the development of more efficacious, second generation purine analogs. This series of analogs included derivatives of the phenyl ring and concluded that substitutions at the 2-, 4-

and 5-positions are important for Hsp90 inhibition.[158] Researchers at Conforma Therapeutics addressed the low bioavailability associated with purine analogs and incorporated an amino group into the N-9 alkyl chain.[160] The phosphoric acid salts of these amines were subsequently evaluated in murine tumor xenograft models. Ultimately, compounds **69-71** (Figure 16) became the first orally available Hsp90 inhibitors. Compound **71** manifests a 90 nM IC_{50} in Her2 degradation assays.[160] Another important water soluble analog (**72**, PU-H71, Figure 16) was reported by Chiosis and co-workers, which contained a 3-isopropylamino-propyl chain. This compound was shown to manifest a 16 nM binding affinity for Hsp90 and an IC_{50} of 50 nM in Her2 degradation assays.[161] This analog manifested significant efficacy *in vivo* and has advanced into clinical trials for the treatment of patients with low-grade non-Hodgkins lymphoma, as well as patients with advanced malignancies. Later, Conforma Therapeutics reported analogs with an amine at the 2-position and a chlorine at the 6-position. The most efficacious analog (**73**, Figure 16) also contained a pyridylmethylene group at the 9-position. Compound **73** (BIIB021) manifested 9 nM efficacy in Her2 degradation assays and exhibited 333-fold selectivity for tumor versus normal cells.[162] After successful preclinical studies, this compound became the first rationally designed Hsp90 inhibitor to enter clinical trials and is currently being evaluated in Phase II trials for gastrointestinal stromal tumors.[163] To increase tolerance, researchers at Conforma Therapeutics also reported the intravenously administered compound, **74** (BIIB028, Figure 16), a phosphate ester prodrug of the homopropargylic alcohol and it too, is currently under phase I clinical evaluation.[164]

A high throughput screen recently identified a purine-based compound that exhibits selective inhibition of Grp94.[165] PU-WS13 (**75**, Figure 16) manifested Grp94 selectivity over other Hsp90 isoforms in purified protein assays. *In* vitro, **75** was shown to inhibit interactions between Her2 and Grp94 at the cell membrane, which resulted in the decreased stability of Her2, leading to its degradation via the lysosome. Additionally, inhibition of Grp94 resulted in the apoptosis of myeloma cells. Grp94 is significantly upregulated in multiple myeloma resulting from increased ER stress. Multiple myeloma cell death was found to result from disruption of Grp94 and LRP6, which lowered LRP6 expression on the cell surface.[166,167] LRP6 is a co-receptor of Frizzled in the Wnt pathway and decreased interactions between these co-receptors results in caspase 9 activation and apoptosis.[168]

Conclusions & Future Directions

Hsp90 provides a unique opportunity to treat cancer due to the fact that Hsp90-dependent client proteins are associated with all six hallmarks of cancer. Consequently, Hsp90 has attracted the attention of research groups throughout the world. Beginning with the discovery of natural product inhibitors of Hsp90 (GDA and RDC), many analogs have been designed to probe the biological function of Hsp90, as well as to develop small molecule inhibitors that have advanced into clinical trials. Furthermore, key interactions within the N-terminal ATP-binding pocket have been identified, which has led to the discovery of new Hsp90 inhibitory classes. While GDA never advanced into clinical trials, 17-substituted analogs have proven superior candidates and exhibit fewer side effects while maintaining similar efficacy to GDA (**16**, **21**, **27**-**28**). Likewise, RDC did not advance into clinical trials, however the resorcinol ring of RDC emerged as a key pharmacophore. Several medicinal

chemistry campaigns have used the resorcinol moiety as a starting point to produce candidates that are currently undergoing clinical evaluation. The natural substrate of Hsp90, ATP, has also been used to develop inhibitors that have advanced into clinical trials (**72**-**74**). Many Hsp90 inhibitors have shown promise in clinical trials, however, no compound has yet been approved by the FDA. Some concerns associated with Hsp90 inhibitors (ocular, cardio, and hepatotoxicities) have arisen during clinical trials that resulted in the termination of some trials, suggesting new approaches to Hsp90 inhibition may be needed.[67,169] One method that may prove useful is the development of isoform-selective inhibitors.[147,150,152,165] By developing selective inhibitors for each isoform, toxicities associated with individual isoforms may be overcome. Furthermore, upon identification of client proteins that are dependent upon each isoform, perhaps more personalized treatments can be developed via this approach.

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Biosketches

Anuj Khandelwal earned a Bachelor's degree in chemistry in 2006 and received a Masters degree in organic chemistry in 2008 from the University of Delhi. He began pursuing his PhD in medicinal chemistry at the University of Kansas in 2011. Anuj received a Masters degree in medicinal chemistry in 2013. Since then he has worked on the optimization of EGCG for Hsp90 inhibition as well as the development of isoform-selective inhibitors of Hsp90.

Vince Crowley received a BS in Chemistry in 2012 from Creighton University (Omaha, NE). He joined the Department of Medicinal Chemistry at the University of Kansas in 2012. He is currently working under the supervision of Dr. Brian Blagg in pursuing Hsp90 isoform-selective inhibitors. Vince received a Masters degree in medicinal chemistry in 2014.

Dr. Brian Blagg earned his PhD at the University of Utah (C. Dale Poulter) then trained as a postdoctoral fellow under the guidance of Dr. Dale Boger (The Scripps Research Institute). In 2002, Dr. Blagg became a faculty member in the Department of Medicinal Chemistry at the University of Kansas. Since then he has become a leader in Hsp90 inhibition and medicinal chemistry.

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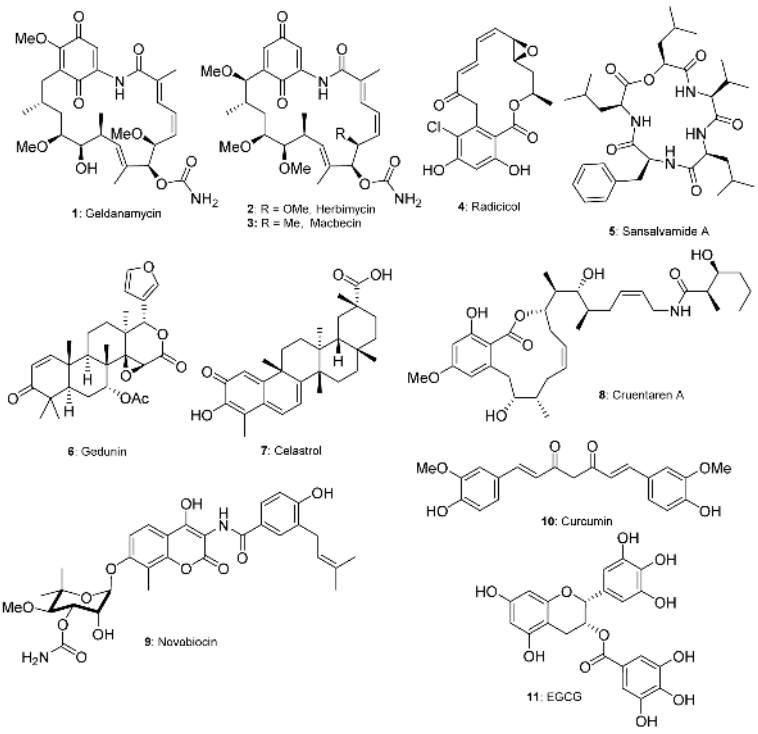

Figure 1.

Natural product inhibitors of Hsp90. **1**-**4** bind Hsp90 in the N-terminal ATP-binding pocket. **5** is an allosteric modulator of Hsp90. **6**-**8** disrupt the interaction of Hsp90 and co-chaperones. **9**-**11** bind the C-terminal ATP-binding motif.

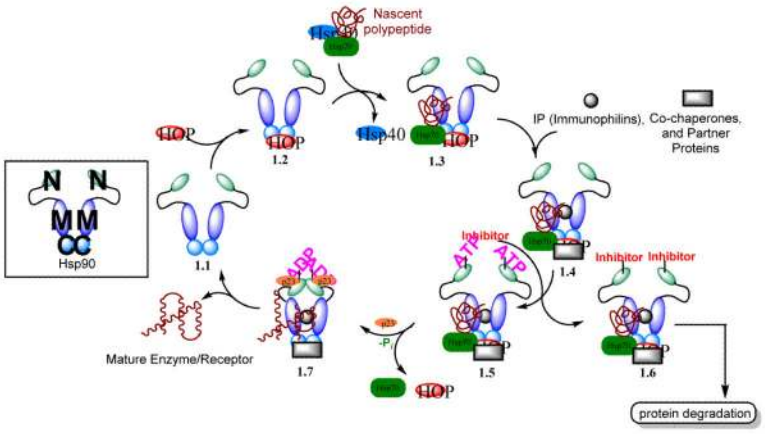

Figure 2.

The Hsp90 chaperoning cycle highlighting the effect of inhibitors on the cycle (**1.6**)

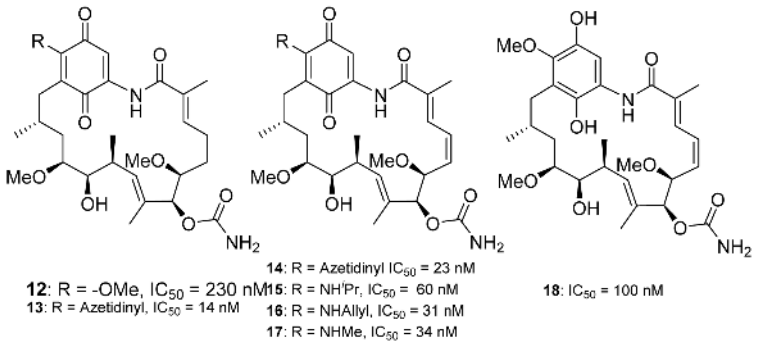

Figure 3.

GDA analogs reported from Pfizer. IC_{50}'s determine by measuring depletion of p185 (an client protein of Hsp90) in SkBr3 cells. IC_{50} of GDA reported at 70 nM.

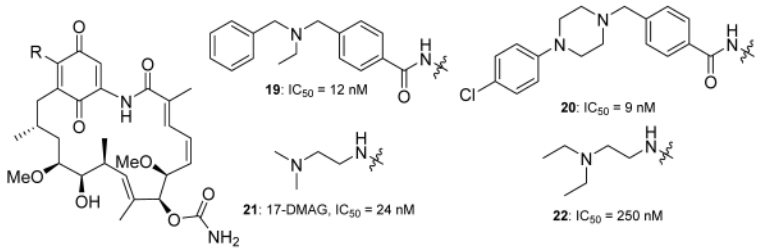

Figure 4.

19-**20** reported by Conforma Therapeutics. IC_{50} values were determined using a competitive binding assay measuring compounds binding to Hsp90 within the cell lysate of MCF-7 cells. The IC_{50} of 17-AAG was found to be 20 nM. **21**-**22** were reported by Kosan Biosciences. IC_{50} values were determined in SKBr3 cells and 17-AAG had a reported IC_{50} of 33 nM.

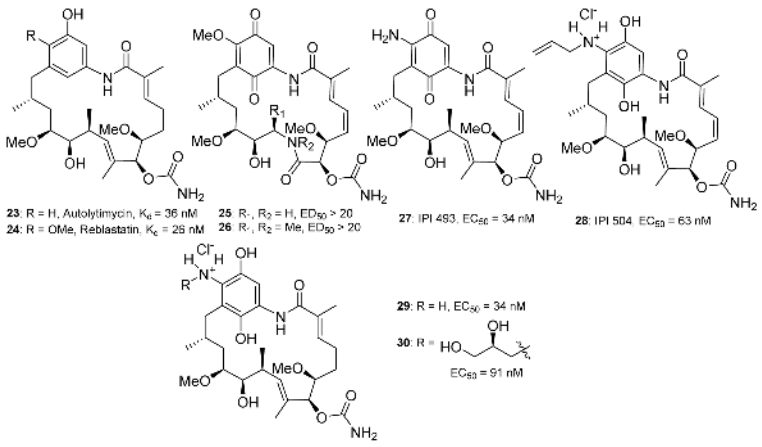

Figure 5.

GDA analogs prepared via semi-synthesis. K_d of **23-24** were measured in a competitive binding assay (17-AAG reported as 110 nM). ED_{50} values of **25-26** were determined via Her2 degradation in SkBr3 cells (GDA reported at 5 nM). EC_{50} of **27-30** were measured via fluorescence polarization with Hsp90α and BODIPY-GDA as a probe (17-AAG reported at 119 nM).

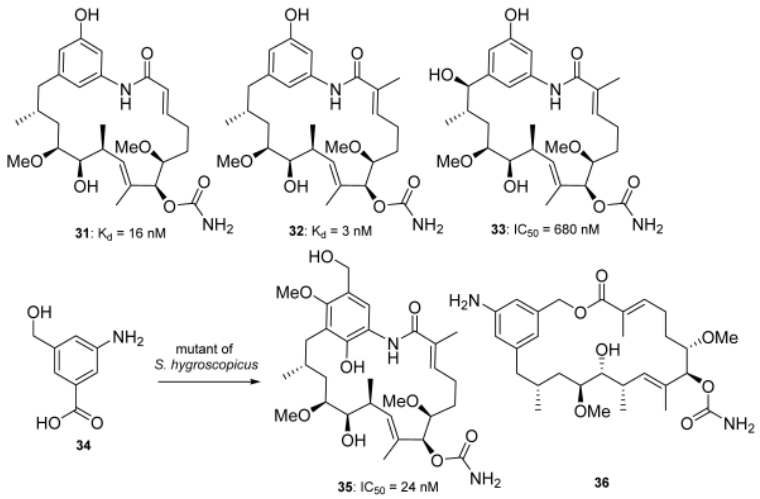

Figure 6.

K_d of **31** was determine using Scintillation Proximity Assay for Hsp90 binding (GDA = 670 nM). K_d of **32** was determined using ITC. IC_{50} of **33** was determined using an ATPase inhibition assay (GDA = 3.19 μM). IC_{50} of **35** was determined in a competitive binding assay using FITC-GDA. Biological data was not reported for **36**.

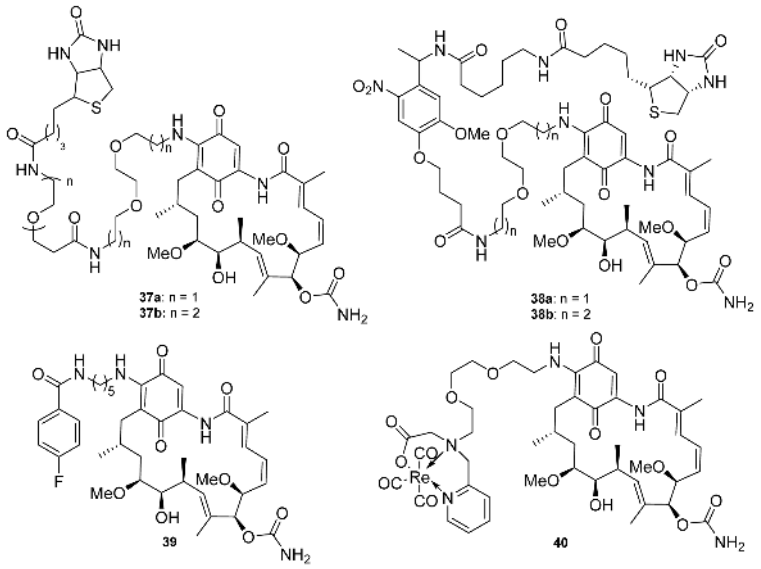

Figure 7.
Structure of probes synthesized to assess Hsp90 biology and function.

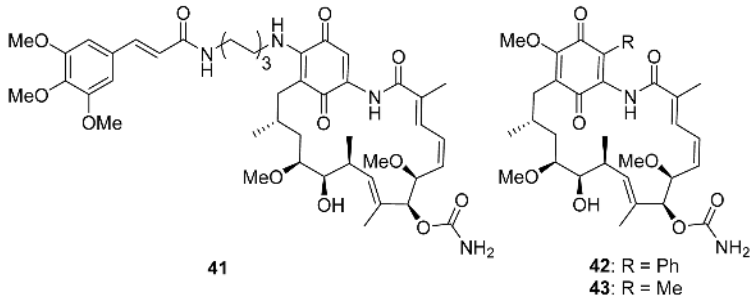

Figure 8.

Linker and 3,4,5-trimethoxycinnamyl group (**41**) provide decreased hepatotoxicity while maintaining efficacy similar to 17-AAG. 19-substitutions of GDA synthesized to mimic attack of biological nucleophiles, such as thiols (**42**-**43**).

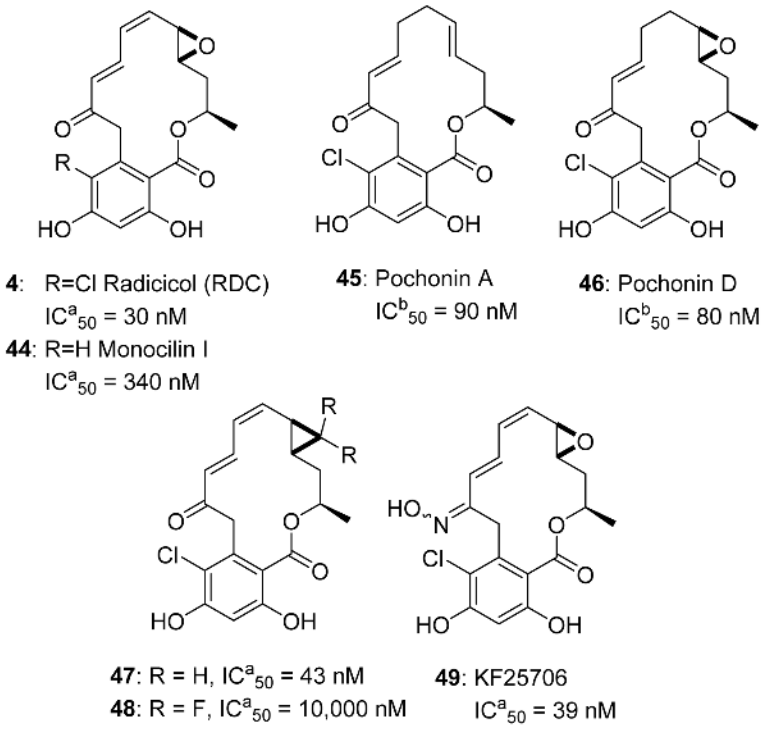

Figure 9.

Natural product resorcinol-based inhibitors of Hsp90 (**4**, **44**-**46**). **47**-**49** synthetic analogs of **4** to increase *in vivo* stability. IC^{a}_{50} represents IC_{50} values of antiproliferative activity against the MCF-7 breast cancer (**4**, **44**, **47**-**48**) and the KNRK5.2 (**49**) cell line. IC^{b}_{50} represents IC_{50} values of a time-resolved fluorescence resonance energy transfer (TR-FRET) assay.

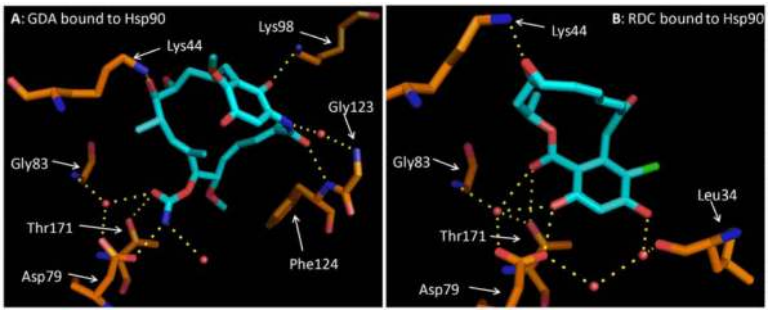

Figure 10.

Binding modes of GDA (**A**) and RDC (**B**) to the Hsp90 N-terminal ATP-binding site. The hydrogen bonding network of the carbamate and resorcinol with the binding site has been proven to be critical for Hsp90 affinity. PDB Codes: 1A4H for GDA and 1AH6 for RDC.

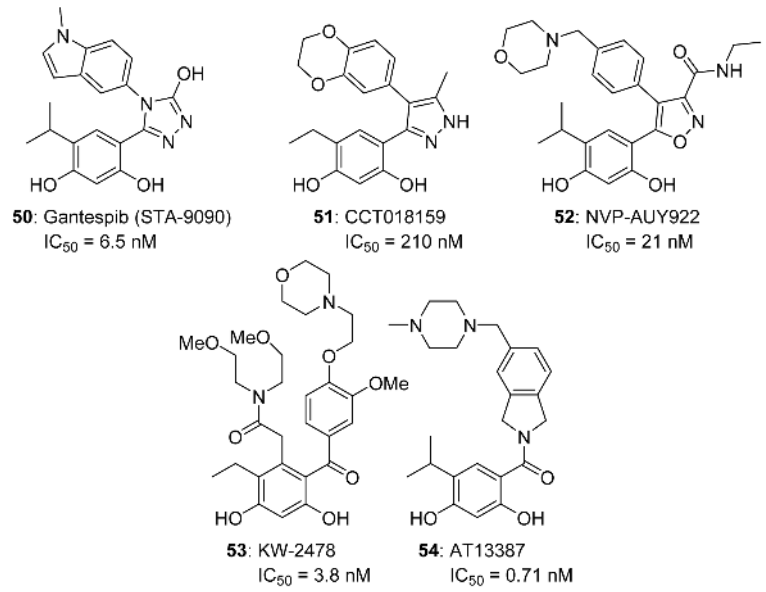

Figure 11.

Resorcinol containing Hsp90 inhibitors currently in clinical trials. IC_{50} values represent antiproliferative activity against various cancer cell lines.

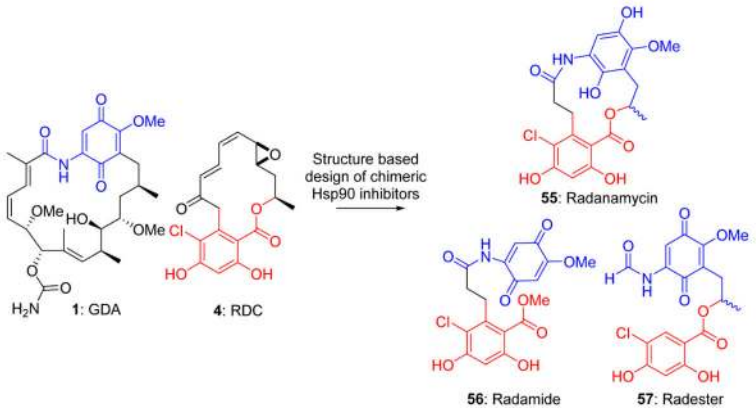

Figure 12.

Development of the first chimeric Hsp90 inhibitor (**55**) and the radanamycin seco agents, radamide and radester.

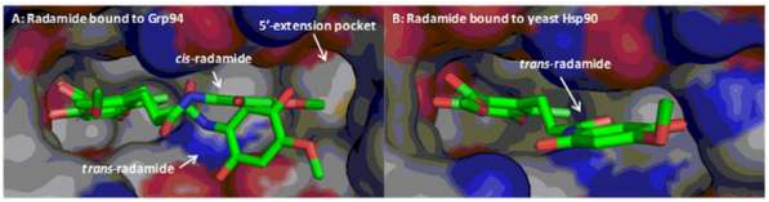

Figure 13.

Radamide bound to Grp94 (**A**) and yeast Hsp90 (**B**). **A** depicts 5′-extension pocket present in Grp94. *Cis*-Radamide projects towards the unique pocket of Grp94.

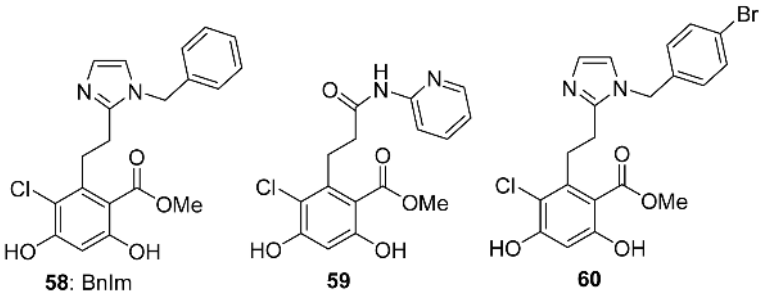

Figure 14.

Grp94-selective inhibitors. Incorporation of a *cis*-amide bioisostere led to the development of BnIm. **59** represents the most active compound from the Grp94-selective radamide analogs. **60** is an analog of BnIm that has been shown to reduce mutant myocilin aggregation *in vitro*.

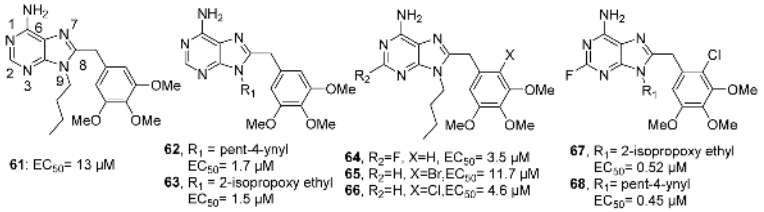

Figure 15.

Structures of purine-based Hsp90 inhibitors. EC_{50} values were determined in a competitive assay with GDA on Affi-Gel resin.

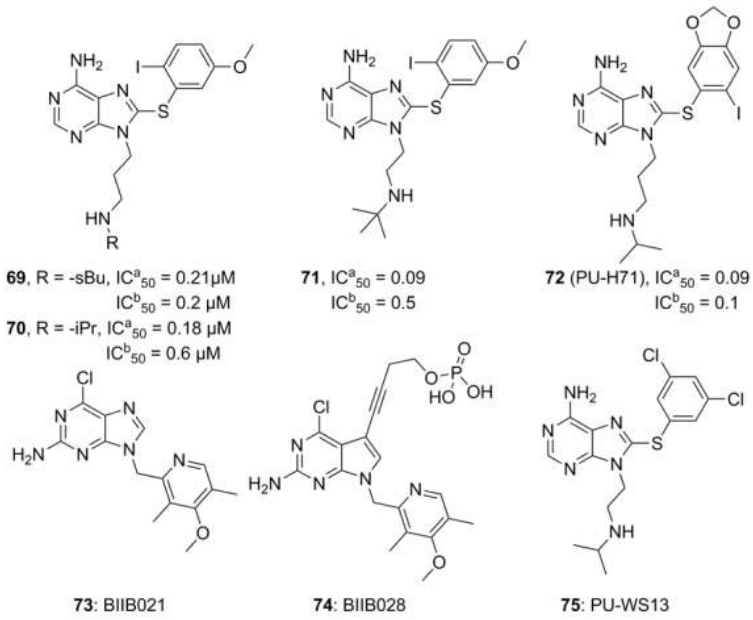

Figure 16.

Second generation purine analogs with enhanced potency and solubility. $IC_{50}{}^{a}$ values for **69**-**71** correspond to anti-proliferative activity in MCF-7 cells (MTS assay) and SkBr3 cells (sulforhodamine B assay) for **72**. $IC_{50}{}^{b}$ corresponds to the Her2 degradation assay **69**-**72** in the same cell lines used for determining anti-proliferative activity. **73**-**74** represent rationally designed purine analogs. **75** is a purine-based Grp94-selective inhibitor

Table I

The six hallmarks of cancer and Hsp90-dependent client proteins associated with each hallmark.

Hallmarks of Cancer	**Hsp90 Client Protein(s)**
1) Self-sufficient growth signals	Raf-1, AKT, Her2, MEK, Bcr-Abl
2) Insensitive to anti-growth signals	Plk, Wee1, Myt1, CDK4, CDK6
3) Evasion of apoptosis	RIP, AKT, mutant p53,c-MET, Apaf-1, Survivin
4) Limitless replicative potential	Telomerase (h-Tert)
5) Sustained angiogenesis	FAK, AKT,Hif-1α, VEGFR, Flt-3
6) Tissue invasion and metastasis	C-MET