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Discovery and development of heat shock protein 90 inhibitors

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ABSTRACT

Heat shock protein 90 (Hsp90) is an important target in cancer because of its role in maintaining transformation and has recently become the focus of several drug discovery and development efforts. While compounds with different modes of action are known, the focus of this review is on those classes of compounds which inhibit Hsp90 by binding to the N-terminal ATP pocket. These include natural product inhibitors such as geldanamycin and radicicol and synthetic inhibitors comprised of purines, pyrazoles, isoxazoles and other scaffolds. The synthetic inhibitors have been discovered either by structure-based design, high throughput screening and more recently using fragment-based design and virtual screening techniques. This review will discuss the discovery of these different classes, as well as their development as potential clinical agents.

1. Introduction

Heat shock protein 90 (Hsp90) is a chaperone which functions in quality control in the cell. Unlike other components of the cellular chaperone machinery such as Hsp70, Hsp90 does not participate in primary protein folding events, but rather its function is restricted to the post-translational maturation and disposition of a relatively limited subset of conformationally labile client proteins.[1,2] The ATPase activity of Hsp90 drives the chaperone cycle and directs binding, induction of the active conformation and release of its client proteins. Most of its client proteins are involved in signaling pathways that are necessary for cell proliferation, regulation of cell cycle progression and apoptosis.[3,4] In addition, it functions in maintaining malignant transformation and elevating the survival and growth potential of cancer cells.[5–7] Its client proteins include kinases, steroid hormone receptors and transcription factors that are directly involved in malignancy, and also mutated oncogenic proteins required for the transformed phenotype (i.e. Her2, Raf-1, Akt, Cdk4, Polo-1 kinase, cMet, mutant B-Raf, mutant p53, AR, ER, Bcr-Abl, HIF-1 alpha, hTERT).[5,6,8]

Hsp90 is a family of chaperones and represents 1–2% of total cellular protein, which increases to 4–6% under stress.[7,9] Currently, four are known and include Hsp90α and Hsp90β, which are found in the cytoplasm, Grp94 in the endoplasmic reticulum and Trap-1 in the mitochondria.[10–13] The cytoplasmic forms exist predominantly as dimers within the cell, and each subunit is comprised of three domains;[14] a 24–28 kDa N-terminal ATP-binding domain; a 38–44 kDa middle domain; and an 11–15 kDa C-terminal dimerization domain. The N-terminal domain contains a unique nucleotide binding pocket that binds both ATP and ADP. Conformational changes that occur upon binding and hydrolysis of ATP regulate the ability of the chaperone to bind its client proteins.[14] When bound to this pocket, the nucleotides adopt a distinctive C-bent shape found only in ATPases belonging to the GHKL family (G = DNA gyrase subunit B; H = Hsp90; K = histidine kinases; L = MutL). Because this pocket is distinct from that of other ATPases it has become an attractive target for inhibition.[15] Chaperone activity is also regulated by the binding of co-chaperone proteins including Hsp70, Hip, Hop, CDC37/p50, immunophilins and Aha1.[14,16]

The role of Hsp90 in oncogenic transformation had not been appreciated until the discovery of pharmacological agents that selectively inhibit its function. The first known inhibitor, the ansamycin antibiotic geldanamycin (GM; **1**; Fig. 1), was found in a search for compounds able to revert the transformed phenotype of v-src transformed 3T3 cells.[17] Subsequently, it was shown to bind to Hsp90 and interfere with Hsp90–v-src heterocomplex formation.[18] GM alters chaperone function and drives the degradation of many Hsp90 client proteins by stimulating Hsp90-mediated presentation to the ubiquitin–proteasome machinery. Consequently, the client proteins cannot attain their active conformation and are degraded by the proteasome.[19] Included amongst these degraded proteins are some that are frequently mutated in human

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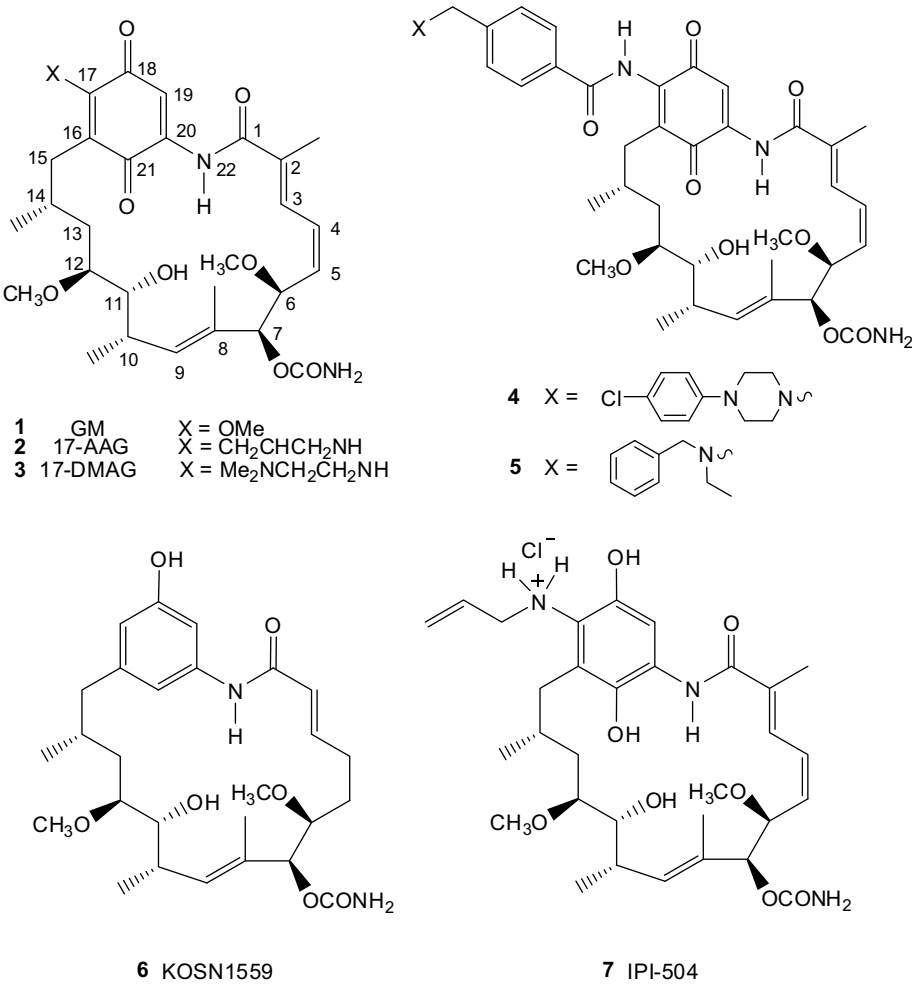


Figure 1. Structures of GM and related derivatives.

cancers.[6–9,20] Degradation of these proteins leads to cell-specific growth arrest and apoptosis of cancer cells in culture and to tumor growth inhibition or regression in animal models.

The usefulness of Hsp90 inhibitors in cancer lies in the fact that many of its client proteins are oncogenic and because their degradation simultaneously blocks a variety of cancer-causing signaling pathways. The diverse array of signaling pathways associated with its client proteins may be correlated with both wide-range anticancer effects[5,21] and a decreased propensity of developing resistance.[22] Additionally, Hsp90 inhibitors have shown selectivity for cancer cells and several suggestions have been proposed to explain this.[17,23] In tumor cells, Hsp90 exists predominantly in an activated state in complex with co-chaperones whereas in normal cells it exists primarily in an uncomplexed latent state,[24] leading to an increased accumulation of inhibitor in cancer cells. Also, Hsp90 is overexpressed in many types of cancers in humans.[5,25] Cancer cells show an increased dependence on Hsp90 for stabilizing client proteins involved in transformation and with mutated oncoproteins that drive the transformed phenotype.[26] Hsp90 interacts in a transformation specific manner with several oncogenic substrates, including v-src, which requires Hsp90 for function, whereas the non-oncogenic c-src has only a limited dependence on Hsp90.[27] The high stress environment of tumors brought about by hypoxia and nutrient deprivation contributes to make cancer cells more dependant on Hsp90.

The recognition of Hsp90 as a target for cancer therapy has catalyzed the discovery of inhibitors from a variety of classes that interfere with chaperone function. This review discusses the various classes of natural product and synthetic molecules that inhibit Hsp90 function through direct binding to the chaperone regulatory pocket.

2. Inhibitor classes

2.1. Natural products

Two general classes of natural product inhibitors of Hsp90 have been discovered which bind to the N-terminal ATP pocket, and are based on GM and radicicol (RD). Interestingly, each was originally isolated as an antibiotic from fermentation broths. These natural products, especially GM, played a vitally important function in the elucidation of the biological role of Hsp90 in cancer. They affect Hsp90 chaperone function in a similar manner and possess comparable biological activity.

2.1.1. Geldanamycin and derivatives

GM (**1**; Fig. 1) is a benzoquinone ansamycin that was first isolated as an antibiotic in 1970 from *Streptomyces hygroscopicus*.[28] Its structure includes a benzoquinone ring fused to a macrocyclic ansa ring. It was identified along with the ansamycin herbimycin A as an agent that was able to revert the phenotype of v-src oncogene transformed cells and had potent broad based and selective anti-cancer activity against a panel of humor tumor cell lines as well as in tumor xenografts.[17] In the National Cancer Institute's

(NCI) in vitro screen against 60 tumor cell lines, GM achieved 50% growth inhibition at 13 nM in highly responsive cell lines and at an overall average of 180 nM.[29]

The co-crystal structure of GM with yeast Hsp90 shows that it binds tightly to the ATP pocket of the N-terminal domain with K_d = 1.2 μM.[30] The benzoquinone ring is found near the entrance of the binding pocket and the ansa ring is directed towards the bottom of the pocket. When bound to Hsp90, GM adopts a C-shaped conformation similar to that of ADP. Figure 2A shows the key hydrogen bonding interactions between GM and yeast Hsp90.[31] Of particular note are those of the carbamate group, which is essential for activity[32,33] and stabilizes the complex through direct and indirect bonding with Leu34, Asp79, Gly83 and Thr171.

GM is a potent cytotoxic agent, but its clinical translation has thus far been precluded by a number of factors.[29,34] First, it exhibits severe hepatotoxicity, which has been associated with the benzoquinone ring and imposes strict dosing limitations. Secondly, it is metabolically and chemically unstable. Also, it has very low solubility in aqueous media resulting in formulations requiring DMSO. As a result, a substantial effort has been made in modifying its structure to improve safety, stability, potency and water solubility.

Much effort has been made at modifying the quinone ring, especially at the 17-position but also at the 19-position.[35,36] This is partly as a result of the relative ease with which the C-17 methoxy group may be substituted for by amine nucleophiles. While substitution at the 17 position is generally observed, in some instances where the amine is very bulky or when forcing conditions are used, 19-alkylamine or 17,19-bis(alkylamino) derivatives may be obtained.[35,36]

Improved in vitro cellular activity over GM was observed with substitution of the C-17 methoxy group by amino and small unhindered alkylamino groups.[35] Activity decreased with the addition of an acidic group to the alkyl amino chain, whereas incorporation of basic or a hydroxyl group to the alkylamino chain was tolerated. The derivative 17-allylamino-17-desmethoxy-geldanamycin (17-AAG; **2**; Fig. 1) has an IC_{50} = 31 nM for inhibition of Her2 in SKBr3 breast cancer cells. 17-AAG has potent in vivo activity and is less toxic than GM.[37,38] 17-AAG entered clinical trials in cancer patients in the US and UK.[39–42] Although initial trials were disappointing, development of an improved formulation of 17-AAG by Kosan Pharmaceuticals (http://www.kosan.com) resulted in KOS-953 (Tanespimycin), a clinical agent with promising activities. Under the new formulation, encouraging clinical results were noted in trastuzumab-refractory HER2-positive breast cancer and in multiple myeloma, inclusive in bortezomib-refractory patients.[43] Despite its promising activity in clinical studies, 17-AAG has several limitations that restrict its optimal clinical development. A lack or reduced activity of this agent in certain cells has been observed due to drug efflux by multidrug resistance elements or metabolic inactivation of these agents by nucleophiles, such as glutathione. In addition, 17-AAG has a liability with respect to metabolism by the polymorphic cytochrome P450, CYP3A4 which is likely to contribute to variable pharmacokinetics. The compound is also reductively metabolized by the polymorphic oxidoreductase NQO1 or

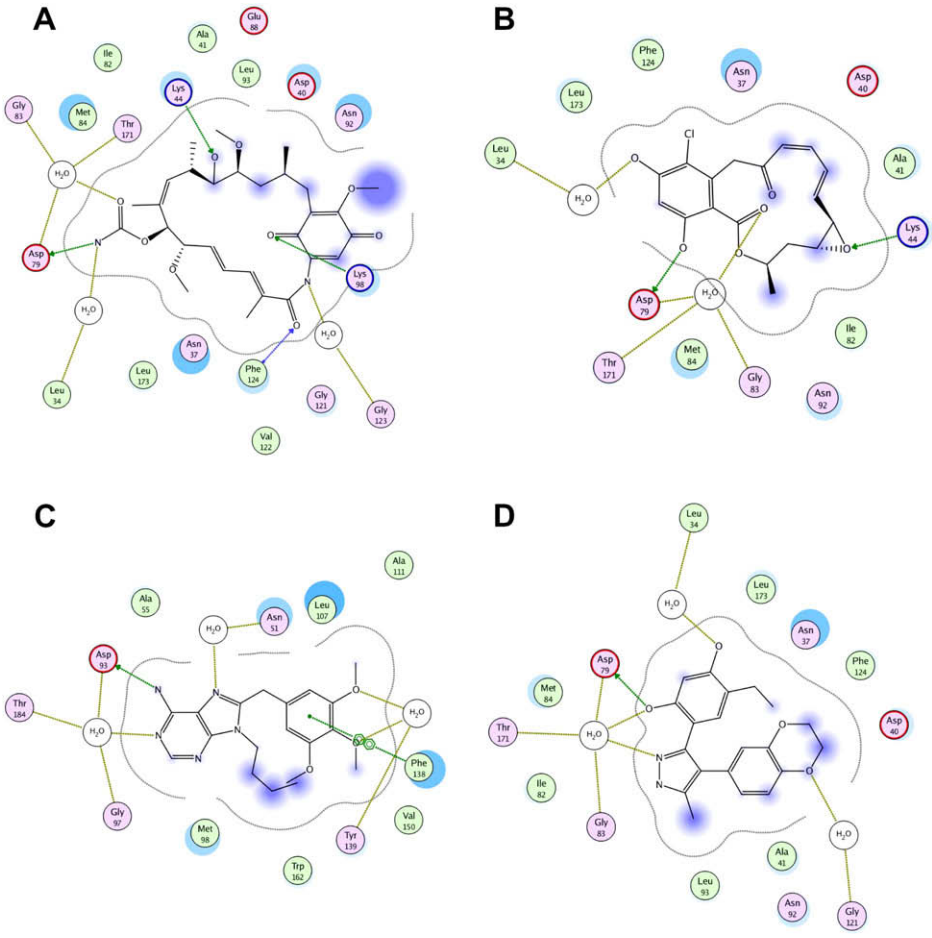


Figure 2. (A) GM (**1**) bound to yeast Hsp90 (1a4h.pdb).[31] (B) RD (**8**) bound to yeast Hsp90 (1bgq.pdb).[30] (C) PU3 (**19**) bound to human Hsp90 (1uym.pdb).[70] (D) CCT018159 (**35**) bound to yeast Hsp90 (2brc.pdb).[88]

DT-diaphorase to a more potent hydroquinone inhibitor, which introduces another source of pharmacological variability and potential for drug resistance.[43]

In an extensive report of over 60 derivatives of 17-AAG, it was shown that Hsp90 binding affinity is not significantly affected by a diverse range of C-17 alkylamino groups.[36] However, differences in activity were apparent in the cell-based assay using SKBr3 cells, where small linear amino side-chains were preferable to bulkier groups, with a two carbon chain being optimal. C-17 alkylamine derivatives with additional amide, carbamate, urea, aryl and sugar groups gave compounds with weaker activity in depletion of Her2 than 17-AAG, despite their similar Hsp90 binding affinity. Efforts to improve water solubility included introducing charged groups such as amino and carboxyl. From this, the diamine 17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin (17-DMAG; **3**; Fig. 1) was reported to be slightly more potent in the cellular assay than 17-AAG (IC_{50} = 24 ± 8 nM as compared to 33 ± 10 nM), but is over 10 times more soluble in aqueous buffer at pH 7. The co-crystal structure of 17-DMAG with Hsp90 shows that it generally binds in a similar fashion to GM.[44] Clinical trials of 17-DMAG (also called alvespimycin or KOS-1022) started at several cancer centers and results from a phase I clinical trial showed promising results in patients with chemotherapy refractory acute myelogenous leukemia.[45] However, due to unacceptable side effects, Kosan terminated its clinical development in March 2008.

Geldanamycin has also been directly derivatized at the C-17 position with amides, carbamates, ureas and aryl groups.[46] While these derivatives have decreased ability to degrade Her2 as compared to 17-AAG, they retained good cell lysate Hsp90 binding affinity. Two amide derivatives (**4** and **5**; Fig. 1) had activity in several animal models when administered intraperitoneally (ip).

Modifications to the ansa ring have also been made in an effort to derive more potent derivatives.[32] Alkylation of the N-H group at the 22-position led to compounds with >100-fold lower activity, however, acylation gave compounds with activities comparable to GM. Acylation and alkylation of the 11-hydroxy resulted in analogs that were generally less potent than their unsubstituted derivatives, whereas oxidation to the ketone resulted in derivatives that were more potent. Substitution with an amino gave less active derivatives. The 7-carbamate is required for in vitro activity and any minor modification is not tolerated.[32,33] The crystal structure shows that it binds to Hsp90 through a network of four hydrogen bonds (Fig. 2A), and attempts to replace this group have led to derivatives with diminished activity with only substitution by a hydroxamate group retaining some affinity.[33]

Genetic manipulation of geldanamycin polyketide synthase gene gave GM derivatives lacking a methyl group at either C-2, C-8 or C-14 or a methoxy at C-6.[47,48] Each of these decreased binding affinity for Hsp90 and had inferior cytotoxic activity against SKBr3 cells. However, the 2-demethyl-4,5-dihydro-17-demethoxy-21-desoxy derivative of GM (**6**; KOSN1559; Fig. 1) binds to Hsp90 with 80-fold greater affinity (K_d = 16 nM) than 17-AAG and 40-fold greater affinity than GM. The increased affinity however, did not translate to better biological activity, and derivative **6** was 20–25 times less potent than GM and 17-AAG in an SKBr3 cell proliferation assay. On the other hand, the hydroquinone derivative of 17-AAG had approximately 2-fold tighter affinity for human Hsp90 than the quinone[49] and it also showed comparable activity in several cell-based assays including those testing proliferation of SKBr3 breast cancer and SKOV3 ovarian cancer cells, Her2 degradation and Hsp70 induction. The hydroquinone can be isolated as a hydrochloride salt (**7**; also called IPI-504, Fig. 1, developed by Infinity Pharmaceuticals), which is less prone to oxidation and more water soluble than 17-AAG (>250 mg/ml vs ~50 μg/ml). Both the quinone and hydroquinone exist in redox equlibria in vivo so that it is likely both are responsible for activity.[50] IPI-504 has en-

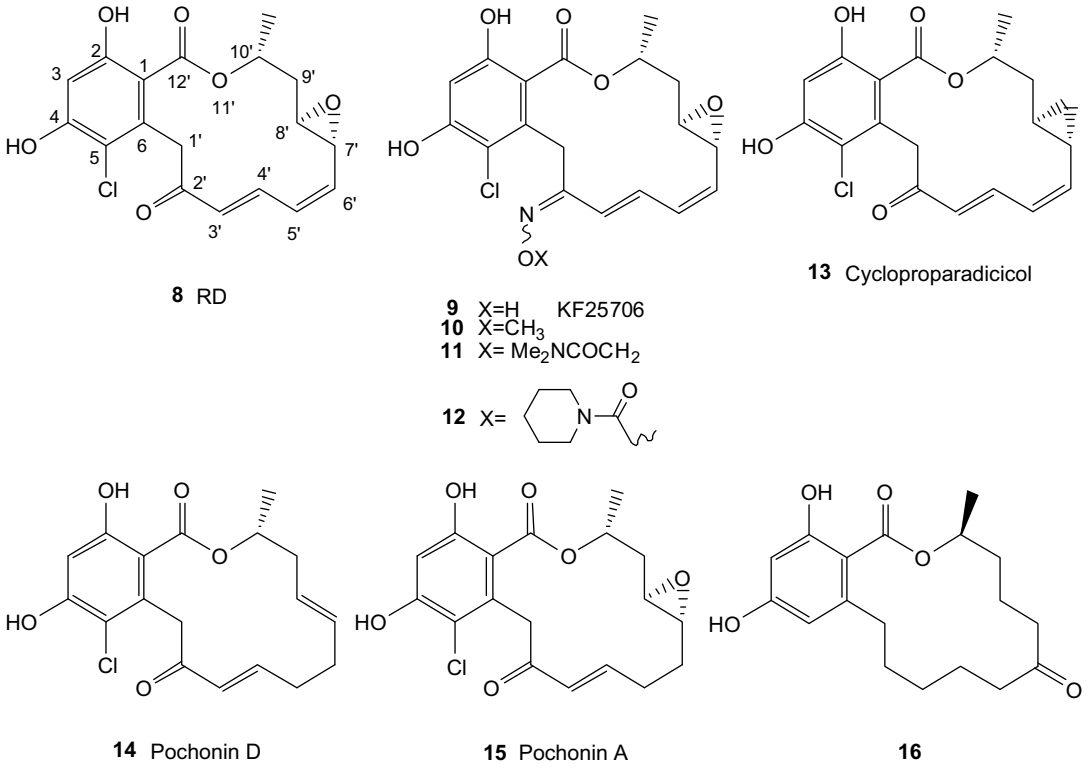


Figure 3. Structures of RD and related derivatives.

tered clinical trials in multiple myeloma and in relapsed, refractory gastrointestinal stromal tumors where it has demonstrated promising preliminary results.[51]

Despite improvements in the activity and physical/chemical properties of GM through synthetic manipulation, the presence of the benzoquinone moiety is likely to remain a problem in their clinical development. However, clinical studies with these agents have demonstrated proof of concept for Hsp90 inhibition as a viable target in cancer therapy.

2.1.2. Radicicol and derivatives

Radicicol (RD; **8**; Fig. 3) is a macrocyclic lactone antibiotic first isolated from the fungus *Monosporium bonorden*.[52] It can reverse the transformed phenotype of v-src transformed fibroblasts[53] and suppress transformation by the ras oncogene.[54] It specifically binds to the N-terminal domain of Hsp90 and depletes SKBr3 cells of the receptor tyrosine kinase Her2, Raf-1 and mutant p53.[55,56] It also inhibits *ras*-dependent phosphorylation of MAPK in K-*ras*-transformed rat epithelial cell lines by destabilization of Raf-1 protein.[57]

The co-crystal structure of RD with yeast Hsp90 shows that it binds tightly to the N-terminal ATP-pocket in a C-shaped conformation similar to ADP with K_d = 19 nM (Fig. 2B).[30] The aromatic ring is directed towards the bottom of the pocket whereas the macrocycle is at the top. Fig. 2B shows the key hydrogen bonding interactions between RD and yeast Hsp90.[30] The 2-hydroxy and 12′-carbonyl bind to Asp79, Gly83 and Thr171 primarily through a molecule of bound water. The 4-hydroxy makes an indirect interaction with Leu34 through a molecule of bound water and the epoxide bonds to Lys44. Occupancy of this pocket by RD disrupts its chaperone function resulting in an inactive Hsp90-protein complex, which is then degraded in a ubiquitin-dependent manner. It has cellular effects similar to ansamycins but lacks hepatotoxicity.[58]

RD is not stable in serum and thus has no in vivo activity.[59] Also, its effects are abolished in the presence of reducing agents such as DTT because of Michael addition to the conjugated diene.[53,60] One approach towards enhancing in vivo activity has been to synthesize oxime derivatives in an effort to reduce the electrophilicity of the Michael acceptor. 2′-oxime derivatives can be obtained by treating RD with hydroxylamine or *O*-alkyl hydroxylamines.[60] The oxime **9** (KF25706; Fig. 3) was more potent than RD in inhibiting v-src and in an antiproliferative assay with v-src transformed 3Y1 cells (SR-3Y1).[60] It had significant anti-tumor activity in vivo against various human tumor xenograft models, was shown to inhibit K-ras and v-src, and deplete the Hsp90 client proteins Raf-1, v-src, Her2, Cdk4 and mutated p53 in tumors.[59] It also showed no liver or renal toxicity in mice treated at 100 mg/kg twice daily for 5 days. Derivatives **10** and **11** (Fig. 3) were also more potent than RD in the tyrosine kinase assay as well as in an antiproliferative assay.

Efforts at further optimizing the oxime group of **9** to increase both in vivo antitumor activity and water solubility through the introduction of carbamoylmethyl groups resulted in several derivatives with enhanced activity.[61] Generally, the introduction of hydrophobic carbamoylmethyl groups led to enhanced activity whereas the introduction of hydrophilic groups or an aromatic ring decreased activity. One of the most potent analogs **12** (Fig. 3), inhibits v-src kinase (IC_{50} = 25 nM), decreased Raf-1 protein in the K-ras-transformed cell line KNRK5.2 and had significant antitumor activity in MX-1 adenocarcinoma and A431 epidermoid carcinoma xenograft models.[61]

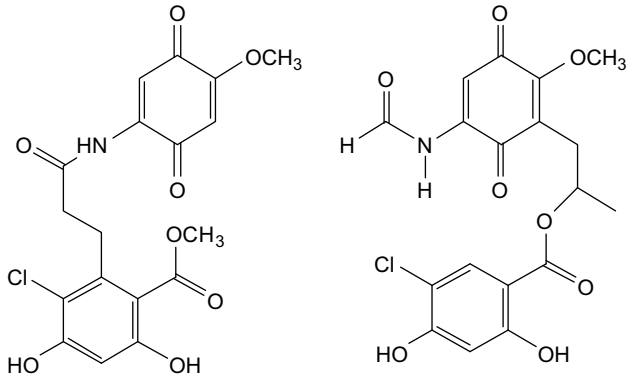
Another approach in enhancing in vivo activity of RD involved modifications to its epoxide ring. In cyclopropradicicol (**13**; Fig. 3) the epoxide is replaced by cyclopropane.[62] The crystal structure of RD shows that the epoxide forms a hydrogen bond with Lys44.[30] In spite of this, cyclopropradicicol (ED_{50} = 160 nM) still retained high binding affinity for Hsp90, although it was decreased 4-fold from RD (ED_{50} = 45 nM). It was concluded that the hydrogen bond between the epoxide and Lys44 is not critical for Hsp90 binding. Cyclopropradicicol degraded the oncogenic proteins Raf-1 and Her2 and retained potent cytotoxicity against MCF-7 breast cancer cells (IC_{50} = 43 nM, compared to RD IC_{50} = 23 nM). A number of stereoisomers of RD and cyclopropradicicol, with varied stereochemistry about the methyl group at C10′ and epoxide or cyclopropyl ring at C7′ and C8′ showed much lower binding affinity for Hsp90 and decreased biological activity. Additional analogs of cyclopropradicicol have been synthesized but none were more potent in the growth inhibition assay against MCF-7 cells.[63]

From a conformational analysis of RD, three low energy conformations were predicted.[64] The lowest of these is an L-shaped conformation adopted by RD upon binding to Hsp90. Pochonin D (**14**; Fig. 3) resembles RD in structure and was found to also adopt this mode of binding to Hsp90. It is isolated from *Pochonia chlamydosporia* and has an affinity for Hsp90 (IC_{50} = 80 nM), which is 4-fold less than radicicol (IC_{50} = 20 nM). Its activity suggests that neither an epoxide ring nor the dienone are critical for Hsp90 binding. The epoxide derivative, pochonin A (**15**; Fig. 3) was also found to be a good inhibitor of Hsp90 (IC_{50} = 90 nM) whereas the 7′,8′-diol analog was inactive.[65] A similar derivative (**16**; Fig. 3) with Hsp90 inhibitory activity was recently isolated from a high throughput screening effort.[66]

Finally, in an effort to identify natural product derivatives that are more amenable to structural modification, several chimeric molecules composed of RD resorcinol ring and GM quinone connected by amide[67] or ester[68] linkages have been synthesized. These molecules, termed radamide (**17**; Fig. 4) and radester (**18**; Fig. 4) respectively, retained affinity for Hsp90 and ability to degrade Her2 and Raf-1. In spite of the large synthetic efforts around the RD scaffold, no derivative with clinical applicability has yet been identified.

2.2. Synthetic inhibitors

To address the limitations of the natural product inhibitors, efforts have been directed by a number of groups, including our own, towards the discovery of novel compounds with better drug like properties, which bind to the N-terminal ATP pocket of Hsp90. Several unique scaffolds possessing favorable properties have been discovered either by structure-based design, high throughput screening, fragment-based design and virtual screening. Examples of these efforts are presented further in this review.



17 Radamide **18** Radester

Figure 4. Structures of chimeric molecules radamide and radester.

2.2.1. Purines and derivatives

The unique shape adopted by ATP when bound to the N-terminal nucleotide pocket of Hsp90 was used as the basis for the design of the first synthetic inhibitors.[69] These were ATP mimics composed of a purine-scaffold tethered by a linker to a neighboring aryl moiety, thus forming the C-shaped conformation. PU3 (**19**; Fig. 5), the first synthesized purine-scaffold inhibitor, bound Hsp90 with an EC_{50} of 15–20 μM, approximately 20-fold weaker than 17-AAG (EC_{50} = 1 μM) under similar conditions. PU3 elicited cellular effects that resemble those of GM, including degradation of Raf-1, Her2 and estrogen receptor.[69] PU3 also demonstrated antiproliferative effects against several tested breast cancer cell lines, MCF-7, SKBr3 and MDA-MB-468, at low micromolar concentrations. The co-crystal structure of PU3 bound to human Hsp90 (Fig. 2C) suggested that the purine-scaffold provides favorable interactions with the N-terminal ATP pocket: the C6-NH_2 interacts with an Asp93-Thr184-water triad, and two of the purine-ring nitrogens form hydrogen bonds with Asn51, a network of waters and the backbone of several other amino acid residues.[70] The aromatic ring of PU3, stacked between the side chains of Phe138 and Leu107, interacts with Met98 and Leu103. The methoxy groups make contacts with the aromatic rings of Trp162 and Tyr139 as well as the aliphatic carbons of Ala111 and Val150. The first and second methylene groups of the N9-alkyl chain provide additional hydrophobic interactions with residues Leu107 and Met98.[70] PU3 induces a unique conformational change in the ATP-binding site lid and opens a lipophilic pocket, a mode of binding which differentiates this class from other Hsp90 inhibitor chemotypes.

Although the activity of PU3 was lower than the natural product inhibitors, its structure is amenable to extensive chemical modification in an effort to improve both potency and physical/chemical properties. Efforts were focused on probing the structure-activity relationship of the aromatic moiety on the purine at C8-position and on investigating the effects of various chains at N9-position. Additionally, the nature of the linker between the purine-scaffold and the substituted aromatic ring has also been investigated.

In the first described library of PU3 derivatives, about 70 compounds were synthesized which investigated the effects of both the nature and length of the N9-chain, substitution at position 2 of the purine moiety, addition of halogens on the trimethoxyphenyl moiety and the nature and length of the bridge between the purine and trimethoxyphenyl moiety.[71] The system was

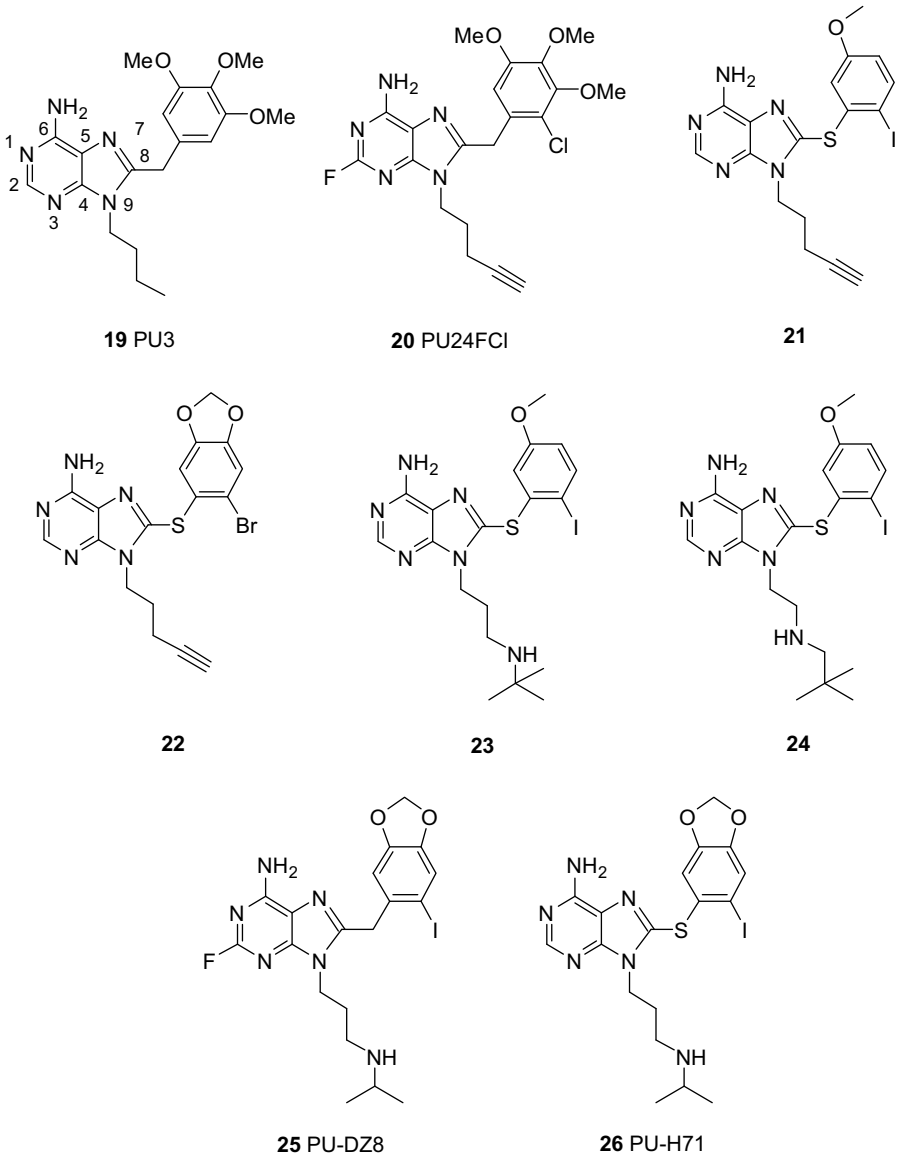


Figure 5. Structures of purine-scaffold Hsp90 inhibitors.

sensitive to variations on the N9-chain, with a preference for linkers with the first two to three methylenes of unbranched nature. Pent-4-ynyl and 2-isopropoxy-ethyl were favored from over 43 diverse chains and resulted in improved binding to Hsp90 by almost an order of magnitude (EC_{50} = 1.5 and 1.7 μM, respectively). The introduction of fluorine at C2 of the purine generally increased potency, likely through enhanced H-bond donor potential of C6-NH_2, and also increased water solubility for these compounds. The introduction of chlorine at C2 of the trimethoxyphenyl ring improves activity over PU3 greater than 3-fold (IC_{50} = 4.6 μM) by increasing the steric fit and decreasing rotation of the aryl moiety. Attempts in altering the bridge C with O, OCH_2 or NH gave inactive compounds, suggesting that the angle between the purine and trimethoxyphenyl moieties is important. Combining the favorable substitutions resulted in PU24FCl (**20**; Fig. 5) with an N9-pent-4-ynyl chain, which is 30 times more active than PU3 (IC_{50} = 1–2 μM) in degradation of Her2 in MCF-7 cells. PU24FCl exhibited antiproliferative effects against a broad panel of cancer cell lines with IC_{50} = 2–7μM and led to the degradation of Akt, Raf-1, Bcr-Abl, Her2, mAR and mutant p53 at similar concentrations.[21] The agent remained active in cancer cells resistant to 17-AAG. In addition, PU24FCl had 10- to 50-fold greater affinity for Hsp90 from transformed cells. When administered to mice bearing tumors, PU24FCl rapidly distributed to tumors and was retained in tumor mass, while being cleared from normal tissue. It also resulted in significant depletion of Her2, Akt and Raf-1 in tumors and a 72% reduction in tumor burden as compared to control, in mice bearing MCF-7 breast cancer xenografted tumors when administered 200 mg/kg ip on alternate days.[21]

Efforts aimed towards the identification of alternative linkers between the purine-scaffold and the aromatic moiety led to the discovery of sulfur as an allowable modification.[72,73] The introduction of O, OCH_2, NH, SO and SO_2 either reduced or abolished activity, probably due to the dramatic conformational change caused by these linkers between the purine and the phenyl moiety which interferes with the desired shape for binding to Hsp90.[71,74–76] Derivative **21** (Fig. 5) was reported to exhibit good potency in several cell-based assays, with an IC_{50} = 0.28 μM for degradation of Her2.[72,74] The SAR of the 8-arylsulfanyl series generally correlates with that observed for the methylene linker series.[73] The effects of substitution on the aryl moiety in this series have been investigated and it was determined that substitutions at positions 2,4,5 were most favored with regards to binding in the Hsp90 ATP-pocket.[73] These efforts identified 2-bromo-4,5-methylenedioxy derivative **22** (Fig. 5) as a potent Hsp90 binder (EC_{50} = 30 nM) and high selectivity (700- to 3000-fold) for tumor over normal Hsp90. Derivative **22** induced Her2 degradation with IC_{50} = 300 nM and inhibited the growth of SKBr3 cells with IC_{50} = 200 nM.

The attachment of a primary or secondary amine on the N9-alkyl chain of the purine-scaffold allowed for their formulation as salts, thus dramatically increasing water solubility. Favorable substituents from previous SAR studies were assembled into derivatives of high binding affinity, good water solubility and oral bioavailability. Introduction of an unbranched amino alkyl chain, such as in compound **23** (Fig. 5; IC_{50} = 140 nM for Her-2 degradation in MCF-7 cells) and **24** (Fig. 5; IC_{50} = 90 nM for Her-2 degradation in MCF-7 cells) provided potent derivatives with improved water solubility.[74,75] Derivatives **23** and **24** exhibited antiproliferative effects against MCF-7 cells with IC_{50} = 200 and 500 nM, respectively. Both of these exhibit excellent water solubility (>10 mg/ml) as their phosphoric acid salt. In fact, when **24** was administered orally as its phosphoric acid salt, it induced approximately 70% tumor growth inhibition in an N87 stomach cancer xenograft mouse model at a dose of 200 mg/kg/day, 5 days/week after 30 days.[74]

Replacing the 5-methoxy group of the aryl moiety with 4,5-methylenedioxy gave derivatives such as PU-DZ8 (**25**; Fig. 5) which had an EC_{50} = 22 nM for Hsp90 and an IC_{50} = 90 nM for degradation of Her2 in SKBr3 cells.[77] Recently, inhibition of Hsp90 by PU-DZ8 and other purine-scaffold compounds in mouse models of tauopathies resulted in the reduction of aggregated tau[78] and the degradation of aberrant tau phosphorylated species.[79] Tauopathies, such as Alzheimer's disease and frontotemporal dementias are neurodegenerative diseases characterized by aberrant tau protein species. PU-H71 (**26**; Fig. 5), containing the 3-isopropylamino-propyl chain, was even more potent with EC_{50} = 16 nM for Hsp90 and IC_{50} = 50 nM for degradation of Her2 in SKBr3 cells.[77] PU-H71 has recently been shown to significantly inhibit tumor growth in mice bearing NCI-N417 small-cell lung cancer tumors at a dose of 75 mg/kg daily for 5 days administered ip.[80] In the NCI-N417 tumors, Akt inactivation and cleavage of PARP, demonstrating apoptosis, was detected as early as 6 h after PU-H71 administration, and the effects were still evident at 36 h. These effects occurred at pharmacologically relevant doses of PU-H71 that were retained in tumors (5.2 μM at 36 h). To our knowledge these data portray the first documented induction of apoptosis by an Hsp90 inhibitor in vivo. In several models of basal-like breast cancer, a tumor type reported to be less sensitive to GM-derivatives, administration on an alternate day schedule of 75 mg/kg PU-H71 resulted in over 95% tumor growth inhibition, with complete cures observed in several mice.[81] Due to its promising pharmacologic profile and potent anti-tumor activity in several cancer types, PU-H71 is scheduled for clinical translation in early 2009.

The efforts described thus far have been focused on compounds in which the purine-scaffold is linked to a polysubstituted phenyl group via a methylene or sulfide bridge. The replacement of the phenyl group with appropriately substituted benzothiazole or pyridinothiazole group has been reported to provide a new and potent series of Hsp90 inhibitory compounds.[82] The benzothiazole class requires a 7′-substituent with Cl being optimal. Derivative **27** (Fig. 6) induces Her2 degradation in MCF-7 cells with an

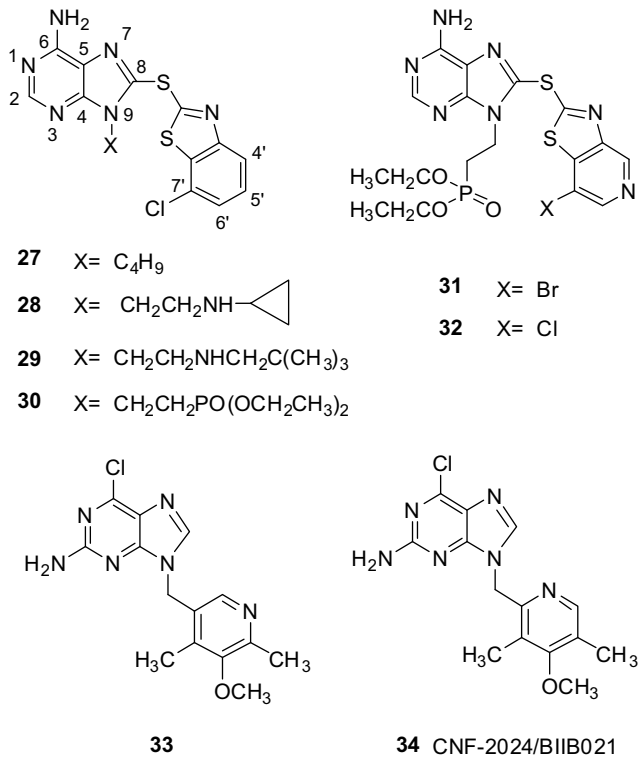


Figure 6. Benzothiazole, pyridinothiazole and N9-pyridylmethyl-substituted purine Hsp90 inhibitors.

IC_{50} = 180 nM, compared to 15 nM for 17-AAG. Potency was increased by substituting the N9-butyl with 2-cyclopropylamino-ethyl (**28**; Fig. 6), 2-neopentylamino-ethyl (**29**; Fig. 6) or 2-diethylphosphonate-ethyl (**30**; Fig. 6), which induce Her2 degradation in MCF-7 cells with an IC_{50} = 110, 35 and 30 nM, respectively. Similar to the benzothiazoles, the most active compounds in the pyridinothiazole class have a two-carbon linker substituted with a diethylphosphonate moiety. The 7′-Br (**31**; Fig. 6) and Cl (**32**; Fig. 6) analogs have comparable activity for Her2 degradation in MCF-7 cells with an IC_{50} = 28 and 30 nM, respectively. Derivative **28** exhibited 56% tumor growth inhibition in an N87 xenograft mouse model treated orally with 200 mg/kg/day, 5 days/week after 40 days.

Attachment of the benzyl or a pyridylmethyl group on position N9 gave another series of potent inhibitors which retained the critical interactions to Hsp90 as well as the C-shape conformation.[83] Several compounds with low nanomolar potency are reported, including the pyridylmethyl derivatives **33** (Fig. 6) and **34** (CNF-2024/BIIB021; Fig. 6), with IC_{50} = 20 and 30 nM, respectively, in the Her2 degradation assay. Derivative **33** exhibited 83% tumor growth inhibition in an N87 xenograft mouse model treated orally with 60 mg/kg/day, 5 days/week after 5 weeks. CNF-2024/BIIB021 demonstrated 87% tumor growth inhibition at 125 mg/kg in an identical model and dosing schedule. CNF-2024/BIIB021 is the first synthetic small molecule to enter Phase I clinical trials.[84] Dose-escalation studies of CNF-2024/BIIB021 administered orally to patients with relapsed B cell chronic lymphocytic leukemia, advanced solid tumors and lymphomas are currently ongoing at several centers.

A number of structures resembling the purine-scaffold have also been reported as Hsp90 inhibitors. Modification to the ring adjacent to the pyrimidine has led to triazolopyrimidines, pyrazolopyrimidines and pyrrolopyrimidines, which have demonstrated antiproliferative effects in MCF-7 cells and ability to induce the degradation of Her2.[85] Pyrimidothiophenes[86] and pyridothiophenes[87] have also been reported as Hsp90 inhibitors.

2.2.2. Pyrazoles and isoxazoles

Pyrazoles represent another novel scaffold to be identified and developed as Hsp90 inhibitors.[88–91] From high throughput screening against a library of 50,000 compounds, the 3,4-diarylpyrazole **35** (CCT08159/RBT0028535; Fig. 7) was shown to inhibit yeast Hsp90 ATPase assay activity with IC_{50} = 8.9 μM. Derivative **35** demonstrated cellular activity in a proliferation assay using HCT116 colon cancer cells (GI_{50} = 4.1 μM)[88] and its addition to HCT116 cells resulted in the depletion of Hsp90 client proteins Raf-1 and Cdk4. SAR studies showed that the ethyl group and both hydroxyls of the resorcinol ring were important for activity, as methylation or removal of one of the hydroxyls led to decreased or abolished activity. However, substitution of the ethyl group with a Cl as in **36** (CCT072440; Fig. 7) resulted in a more potent compound with an IC_{50} < 1 μM in the ATPase assay, but with comparable activity in the proliferation assay (GI_{50} = 4.0 μM).

Crystallographic data shows that CCT08159 binds to the ATP pocket in the N-terminal domain of yeast Hsp90 in a manner similar to RD (Fig. 2D).[88] The 2′-hydroxyl group of the resorcinol moiety and the N2 of the pyrazole form a network of hydrogen bonds with Asp79, Gly83 and Thr171 through a molecule of bound water. The 4′-hydroxyl of the resorcinol ring also forms a hydrogen bond interaction with Leu34 via a water bridge. The 5′-ethyl occupies a hydrophobic pocket, which is taken by the 5′-chloro in CCT072440. The crystal structure with human Hsp90α showed that the C5 methyl group of the pyrazole was close to the carbonyl oxygen of Gly97, which cannot form a hydrogen bond to the pyrazole NH because the peptide bond was out of the plane to it.[89] Incorporation of an amide side chain at C5 could potentially create another ligand-protein hydrogen bond interaction, thus enhancing binding affinity. In fact, compound **37** (VER-49009; Fig. 7) was identified as the strongest Hsp90 binder (IC_{50} = 25 nM) in a fluorescence polarization assay amongst a series of C5-amides and was able to reduce cellular levels of the client proteins Raf-1 and Cdk4.[89] It also showed better cellular activity over CCT018159 with GI_{50} = 260 nM, comparable to that determined for 17-AAG and

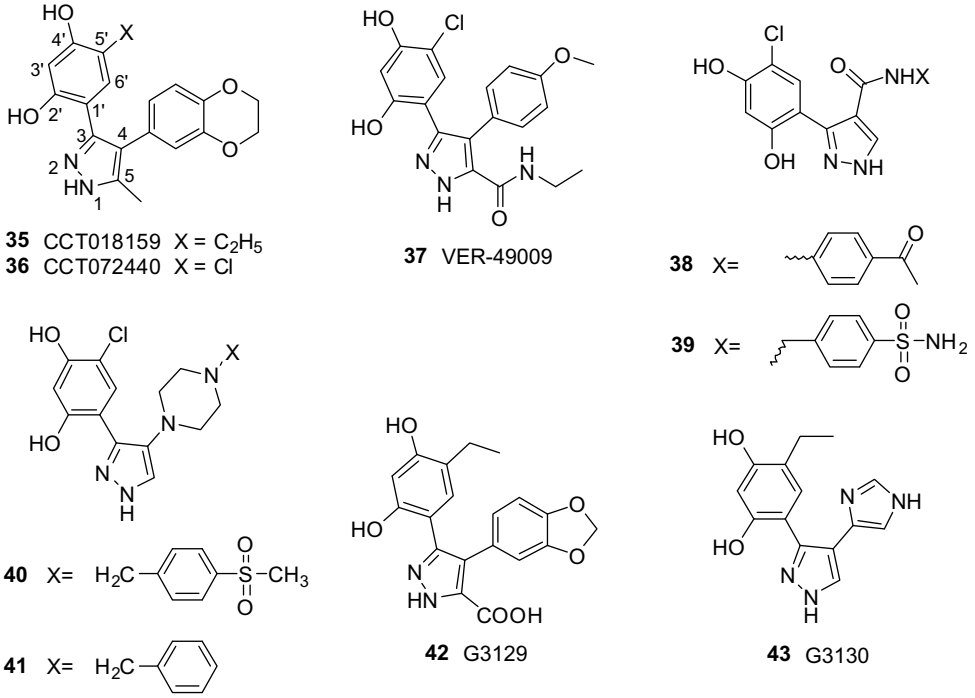


Figure 7. Structure of pyrazole-scaffold Hsp90 inhibitors.

RD, and exhibited antiproliferative effects against a wide range of human cancer cell lines.[92] The crystal structure of VER-49009 showed that the amide group made a hydrogen bond interaction with Gly97, suggesting a rationale for the tighter binding observed in the C5-amide series.

Substitution of the pyrazole 4-position of CCT018159 with amides[90] or amines[93] has also led to Hsp90 inhibitors. In the pyrazole-4-carboxamide series, compounds **38** (Fig. 7) and **39** (Fig. 7) were determined to be the most potent with IC_{50} = 0.258 and 0.461 μM, respectively.[90] These exhibit increased binding as compared to related compounds due to a hydrogen bond interaction of Phe138 with the ketone in **38** or the sulfonamide in **39**. Derivative **38** induced the degradation of Raf-1, Her2 and Cdk4 and inhibited the growth of HCT116 cells with GI_{50} = 11.6 μM. In the 4-aminopyrazoles, piperazinyl, morpholino and piperidyl derivatives were synthesized, of which the piperazinyl derivatives were most potent.[93] Derivative **40** (Fig. 7) and **41** (Fig. 7) were the most potent compounds in this series and had IC_{50} = 0.74 and 0.6 μM, respectively, in a fluorescence polarization assay designed to measure Hsp90 binding affinity. Derivatives **40** and **41** induced the degradation of Raf-1 and inhibited the growth of HCT116 cells with GI_{50} = 3.1 and 6.5 μM, respectively.

High throughput screening had been used independently from the efforts described above to also identify pyrazoles as Hsp90 inhibitors. From a diverse library of 1 million compounds, a number were identified as hits using a high throughput time-resolved fluorescence resonance energy transfer (TR-FRET) assay.[91] Among these compounds, **42** (G3129; Fig. 7) and **43** (G3130; Fig. 7) were confirmed as inhibitors in a surface plasmon resonance assay with K_d = 0.68 and 0.28 μM, respectively. The crystal structure of these compounds shows that they bind to the N-terminal ATP pocket in a manner similar to CCT018159. In G3129, the carboxylate at C5 provides a further salt bridge interaction in addition to hydrogen bond interaction, whereas in G3130, the imidazole at C4 of the pyrazole is involved in water-mediated hydrogen bonding. The higher affinity of G3130 suggests that the water mediated polar interaction of the C4-imidazole is the more important interaction.

Isoxazoles are chemically related to pyrazoles and have recently been shown to be potent Hsp90 inhibitors by also binding to the N-terminal ATP pocket.[92,94] As has already been mentioned, the N2 of the pyrazole acts as an H-bond acceptor in a network of hydrogen bonds with Hsp90 through a molecule of bound water (Fig. 2D). Pyrazoles can exist in tautomeric forms and when this nitrogen is protonated it is unlikely to bind well to Hsp90. Therefore, it was rationalized that locking the molecule into a 100% H-bond acceptor by changing the nitrogen to oxygen might be advantageous for binding. The resulting isoxazole-scaffold proved to be a more than tolerable change, as demonstrated by the isoxazole anolog of VER-49009, **44** (VER-50589; Fig. 8), which has an IC_{50} = 28 nM in a fluorescence polarization competitive binding assay similar to that of VER-49009 (IC_{50} = 25 nM).[94] However, VER-50589 showed greater cell growth inhibition against each of six different human cancer cell lines than VER-49009. It also caused the depletion of Hsp90 client proteins Her2, Raf-1, CDK-4 and phosphorylated Akt in SKMEL2 and WM266.4 melanoma cells, and inhibited tumor growth by approximately 30% in mice bearing HCT116 xenografts when administered ip at 100 mg/kg daily.[92]

Isoxazoles substituted at the para-position of the 4-aryl ring with various amine groups had IC_{50} < 70 nM in the binding assay and were at least as potent as their pyrazole analogs.[94] Their ability to inhibit the growth of HCT116 cells was also greater than the corresponding pyrazoles. Methylation of the resorcinol hydroxyls caused a loss or drop in potency as was observed for the pyrazole class. The 5′-position of the isoxazoles proved to be more amenable to modification than in pyrazoles such as VER-49009. Phenyl,

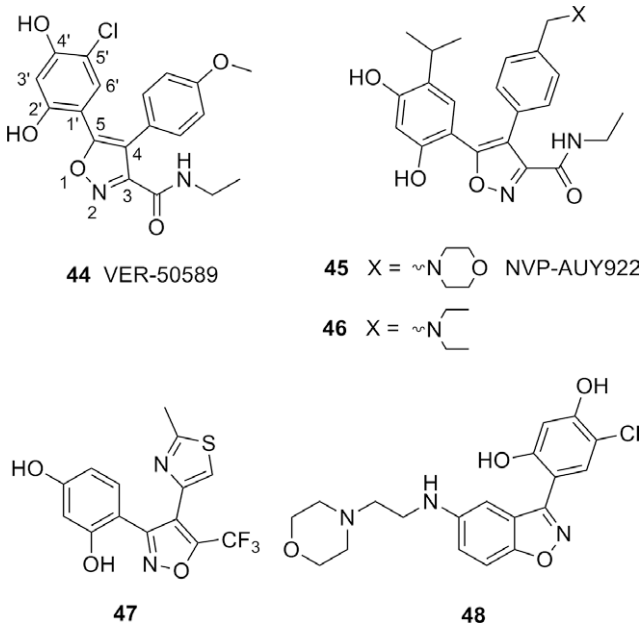


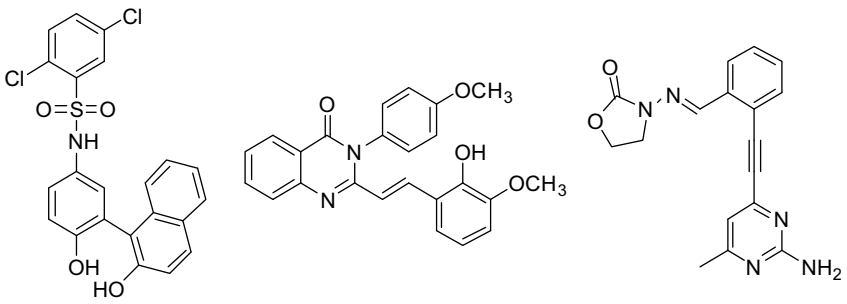
Figure 8. Structure of isoxazole-scaffold Hsp90 inhibitors.

phenylethyl, *tert*-butyl and isopropyl substituted derivatives retained the activity of their corresponding chloro or ethyl substituted analogs. Moreover, these derivatives had good activity in the cell growth inhibition assays, especially the 5′-isopropyl derivatives **45** (VER-52296/NVP-AUY922; Fig. 8) and **46** (Fig. 8), which have GI_{50} = 16 and 6 nM, respectively, against HCT116 cells. VER-52296/NVP-AUY922 inhibited tumor growth by approximately 50% in mice bearing HCT116 xenografts when administered ip daily at 50 mg/kg. NVP-AUY922 is currently in Phase I clinical evaluation in patients with advanced cancers.

Isoxazoles were also isolated in a high throughput screen using more than 15,000 compounds from the National Institutes of Health Molecular Libraries Screening Center Network.[66] This effort identified derivative **47** (Fig. 8) as a 9 μM potency Hsp90 inhibitor. Benzisoxazole-scaffold compounds with Hsp90 binding properties were also recently reported.[95] High throughput screening followed by optimization efforts led to the discovery of compound **48** (Fig. 8) as an inhibitor in the fluorescence polarization binding assay with IC_{50} = 30 nM, compared to 20 nM for GM. It was active in a panel of cell lines with submicromolar IC_{50} values and was able to induce degradation of Her2 and the androgen receptor. From crystallographic data with the N-terminal domain of human Hsp90α, the 2′-hydroxyl group of the resorcinol moiety forms an important interaction with Asp93, whereas the other hydroxyl substituent is involved in maintaining the water-mediated hydrogen bond network. The flexible morpholin linker was incorporated to increase water solubility and potency.

2.2.3. Other inhibitors

In addition to the purine, pyrazole, and isoxazole-based Hsp90 inhibitors, some other structures have also been reported, including 1-(2-phenol)-2-naphthols, which had been discovered using virtual screeening.[96] One such compound, **49** (Fig. 9) has an IC_{50} = 0.7 μM in a fluorescence polarization assay and a GI_{50} = 29 μM in a cell proliferation assay against HCT116 cells. Compound **49** was also shown to reduce Cdk4 at 34.9 μM. Virtual screening was also used to discover 3-phenyl-2-styryl-3H-quinazolin-4-one scaffold as Hsp90 inhibitors.[97] One of these compounds, **50** (Fig. 9), has an IC_{50} = 20 μM in an ATPase assay and a GI_{50} = 27.5 μM in a cell proliferation assay against MCF-7 cells. It



49 50 51

Figure 9. Structures of some novel Hsp90 inhibitors.

also induced Her2 degradation at 40 μM. Fragment-based drug design was used to discover aminopyrimidines as a new scaffold for Hsp90 inhibitors and following optimization led to derivative **51** (Fig. 9) with K_i = 4 μM in a fluorescence binding assay.[98]

3. Conclusion

Much of our understanding of the relationship between Hsp90 and cancer has come from the discovery of several classes of molecules that selectively inhibit chaperone function. The natural products GM and RD were the first inhibitors discovered and were critical in establishing this link. The GM derivative, 17-AAG, was the first inhibitor to have entered clinical trials and has served to validate Hsp90 as a target in cancer. However, because of considerable drawbacks associated with the natural product inhibitors, novel agents with improved drug-like properties were actively sought. The first synthetic inhibitor to be discovered was the purine class, which led to the development of CNF-2024, the first orally available synthetic inhibitor to enter clinical trials, and to PU-H71 which is currently in late-stage preclinical evaluation. The pyrazoles and related isoxazoles represent other novel scaffolds that resulted in VER-52296/NVP-AUY922, currently also in Phase I clinical evaluation. Considering the increasing interest in the target, it is likely that additional molecules that modulate Hsp90 activity will be discovered in the future. Serenex has developed an orally available synthetic inhibitor, SNX-5422, whose structure is undisclosed and claimed to be based on a novel scaffold unrelated to that of any published Hsp90 inhibitor to date. This agent has recently entered phase I clinical evaluation as well.[99] No clinical data on the synthetic inhibitors is yet available, but it is with great anticipation that we await the outcome of these studies. Finally, Hsp90 inhibitors have also recently been investigated for their role in neurodegenerative diseases. At this stage, it is tempting to suggest that, in correlation to cancer, Hsp90 inhibition may lead to correction in aberrant signaling involved in various neurodegenerative diseases and result in an extension in the survival of afflicted neurons.

Declaration of interest

Several purine-scaffold compounds disclosed in this review have been licensed out to Conforma Therapeutics (presently Biogen Idec). G. Chiosis has received a share of some payments by the licensee and may receive a share of eventual royalties.

Acknowledgments

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