C-terminal Modulators of Heat Shock Protein of 90 kDa (HSP90): State of Development and Modes of Action

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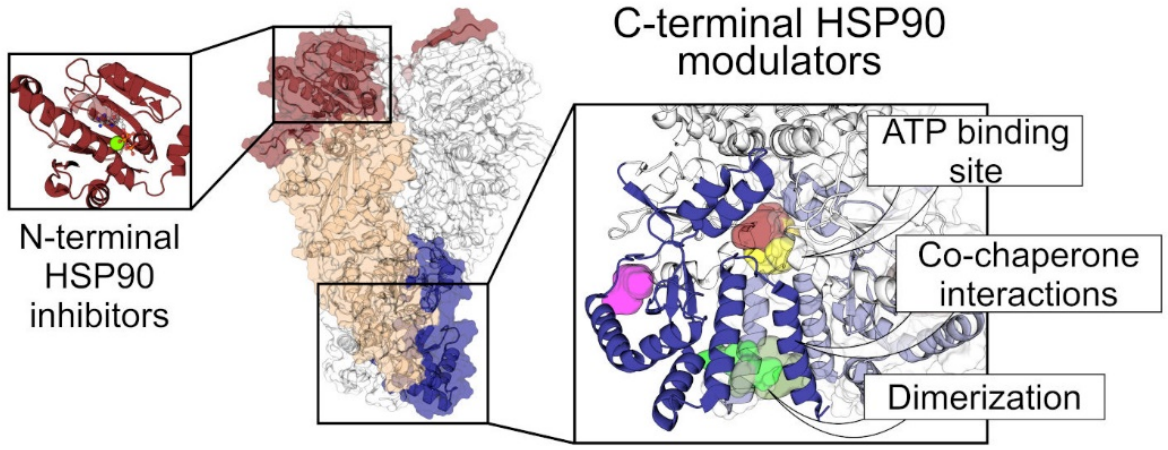
Graphical abstract



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Abstract

Cells constantly need to adopt to changing environmental conditions, maintaining homeostasis and proteostasis. Heat shock proteins are a diverse class of molecular chaperones that assist proteins in folding to prevent stress-induced misfolding and aggregation. The heat shock protein of 90 kDa (HSP90) is the most abundant heat shock protein. While basal expression of HSP90 is essential for cell survival, in many tumors elevated HSP90 levels are found, which is often associated with bad prognosis. Therefore, HSP90 has emerged as a major target in tumor therapy. The HSP90 machinery is very complex in that it involves large conformational changes during the chaperoning cycle and a variety of co-chaperones. At the same time, this complexity offers a plethora of possibilities to interfere with HSP90 function. The best characterized class of HSP90 modulators are competitive inhibitors targeting the N-terminal ATP-binding pocket. Nineteen compounds of this class entered clinical trials. However, due to severe adverse effects, including induction of the heat shock response, no N-terminal inhibitor has been approved by the FDA so far. As alternatives, compounds commonly referred to as “C-terminal inhibitors” have been developed, either as natural product-based analogues or by rational design, which employ multiple mechanisms to modulate HSP90 function, including modulation of the interaction with co-chaperones, induction of conformational changes that influence the chaperoning cycle, or inhibition of C-terminal dimerization. In this review, we summarize the current development state of characteristic C-terminal inhibitors, with an emphasis on their (proposed) molecular modes of action and binding sites.

Vitae

Holger Gohlke is Professor of Pharmaceutical and Medicinal Chemistry at Heinrich Heine University Düsseldorf and head of the NIC research group “Computational Biophysical Chemistry” at Forschungszentrum Jülich. He obtained his diploma in chemistry from the Technical University of Darmstadt and his PhD from Philipps-University, Marburg. He then did postdoctoral research at The Scripps Research Institute, La Jolla, USA. After appointments as Assistant Professor in Frankfurt and Professor in Kiel, he moved to Düsseldorf in 2009 and was appointed in Jülich in 2017. He was awarded the “Innovationspreis in Medizinischer und Pharmazeutischer Chemie” from the GDCh and the DPhG, the Hansch Award of the Cheminformatics and QSAR Society, and the Novartis Chemistry Lectureship. His current research focuses on the understanding, prediction, and modulation of interactions involving biological macromolecules from a computational perspective. His group applies and develops techniques grounded in computational pharmaceutical chemistry, computational biophysical chemistry, and molecular bioinformatics.

David Bickel has been a graduate student in the research group of Holger Gohlke at Heinrich Heine University Düsseldorf since 2017. He received his degree in Pharmacy from Leipzig University in 2015 and did his diploma thesis at the Fraunhofer Institute for Cell Therapy and Immunology IZI (Halle (Saale)) in the Department of Drug Design and Target Validation. His current research focuses on the modulation of protein-protein interactions by small molecules, employing molecular modeling and simulation techniques.

1 Introduction

Cells constantly need to adopt to changing environmental conditions, maintaining homeostasis. The heat shock proteins are a diverse class of molecular chaperones that serve this purpose. Originally discovered, and named, with respect to their elevated expression upon cell exposure to heat, they were also found to assist cellular function under basal conditions.[1-3] The heat shock protein of 90 kDa (HSP90) is the most abundant heat shock protein, constituting 1-2 % of the total cellular protein, which is increased to 3-5 % upon exposure to external stressors.[4,5] In eukaryotes, it forms the heart of a complex machinery involving multiple cofactors and cochaperones.[1,6] This "modular design" enables the HSP90 chaperoning complex to interact with a wide variety of client proteins along important cellular signaling pathways, such as steroid hormone receptors or protein kinases.[7]

1.1 HSP90 structure

In the cell, HSP90 exists as a homodimer.[8,9] Each monomer consists of three domains: a N-terminal domain (NTD), followed by a middle (MD) and C-terminal domain (CTD)[10] (Figure 1). The NTD is the main ATPase domain of HSP90 and belongs to the GHKL superfamily; it shares high structural similarity to histidine kinases, gyrases, and topoisomerases.[11,12] A highly charged linker region that varies in length and composition between species and isoforms connects it to the middle domain (MD),[13-15] which appears to play an important role in the ATP hydrolysis and client recognition.[15,16] The C-terminal domain forms the main dimerization interface of HSP90.[17,18] The C-terminus also contains the MEEVD motif, which is an important interaction site for a subset of co-chaperons containing tetratricopeptide repeat (TPR) domains.[19]

During the chaperone cycle, periodically transient interactions occur via the respective N- and C-terminal domains of the dimer.[20] The two monomers thus interact in a flexible clamp-like way, which is essential for the ATP hydrolysis.[1,21]

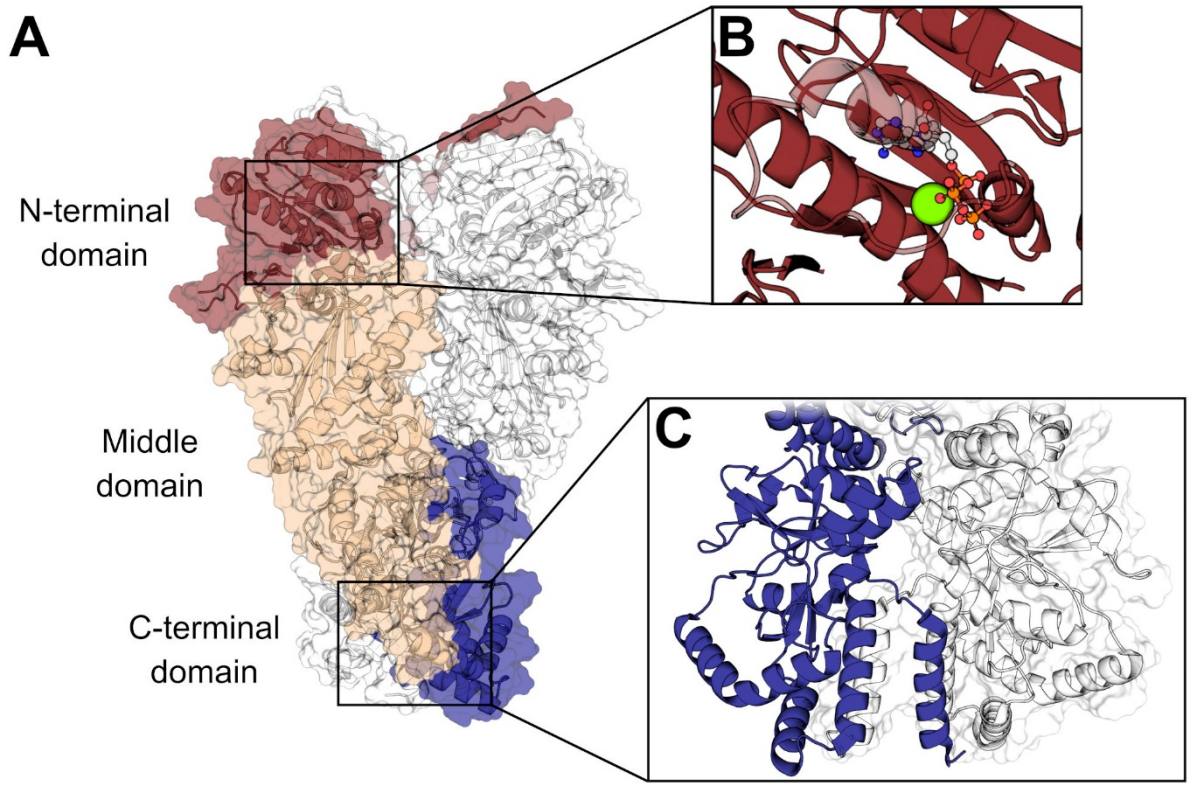

Figure 1. Surface representation of human HSP90β as resolved from cryo-electron microscopy (PDB ID: 5FWK). A. For monomer A the N-terminal domain is represented in red, the middle domain in beige, and the C-terminal domain in blue. The monomer B is shown in light grey. The charged linker region and the C-terminal fragment that interacts with TPR domains are not resolved, and therefore not shown in the graphic. **B**. Blow-up of the ATP binding site in the N-terminal domain with bound Mg^{2+}-ATP. **C**. Blow-up of the C-terminal domains of monomer A (blue) and monomer B (light). The helix-bundle in the center formed by the C-terminal helices 4, 4', 5, and 5'constitutes the primary dimerization interface in the C-terminal domain.

1.2 HSP90 isoforms

In humans four isoforms of HSP90 have been identified: cytosolic HSP90α and HSP90β, mitochondrial TRAP1 (tumor necrosis factor-associated protein 1), and GRP94 (94 kDa glucose-related protein) in the endoplasmic reticulum.[4,22] All isoforms share a conserved structure, consisting of a N-terminal ATPase domain (NTD), the MD, and the CTD. However, TRAP1 is lacking the charged linker region between NTD and MD, and both GRP94 and TRAP1 are missing the C-terminal MEEVD motif.[23] A fifth isoform − HSP90N (HSP89αΔN) − lacking the N-terminal ATP binding site was reported to be associated with tumor formation.[24,25] However, subsequent studies concluded that this isoform is most likely a chimeric translocation product of the *HSP90AA1* gene, limited to very few cancer cell strains.[23,26]

The two major cytoplasmic isoforms have a sequence identity of 86%[27] but vary in their expression. HSP90β is constitutively expressed at higher levels than HSP90α.[4,22] Yet, the expression of HSP90α is highly stress-inducible.[4,22] Differences in regulation as well as isoform specificity observed for some client proteins and co-chaperones suggest distinct cellular functions of the two isoforms.[28-31] Despite this fact, most of the functional assays are performed on cytoplasmic HSP90 (i.e., mixtures of HSP90 α

and β). Therefore, except in a few cases, there is no information on isoform selectivity of HSP90 modulators.

1.3 HSP90 in cancer

While basal levels of HSP90 are needed for maintaining protein homeostasis under physiological conditions, the HSP90 machinery can be exploited by cancer cells to cope with proteotoxic stressors, such as a high mutation burden, hypoxia, and acidosis that are frequently found in the tumor microenvironment.[32,33] Accordingly, elevated levels of HSP90 are observed in various types of malignancies[33-35] and correlate with poor prognosis in breast cancer,[36,37] gastric cancer,[38] and acute myeloid leukemia.[39] Furthermore, secretion of HSP90α into the extracellular space was shown to promote tumor invasiveness.[40,41] As a consequence, the last decades have seen increasing efforts to develop drug-like HSP90 inhibitors for clinical use.

1.4 Inhibitors of HSP90 in clinical trials

In 1994, the ansamycin antibiotic geldanamycin (**1** (Arabic numbers in bold are only assigned to "first-in-class" molecules in this review), Figure 2) was reported to elicit antiproliferative effects by inhibiting HSP90, which subsequently led to depletion of tyrosine kinases and cell death in cancer cell lines.[42,43] In the following years, the natural product radicicol (**2**, Figure 2) was shown to have the same effect but higher potency.[44,45] Both compounds bind to the ATP-binding site in the N-terminal domain of HSP90.[46,47] Subsequent structure-activity relationship (SAR) studies in concert with the resolution of co-crystal structures of ADP,[11] geldanamycin,[47] and radicicol[46] with HSP90 led to the development of a set of drug candidates with improved pharmacodynamic and pharmacokinetic properties compared to the original natural products. For geldanamycin, particularly the semi-synthetic modification of the 17-methoxy group was found to lead to active compounds that exhibited less toxicity with respect to the natural compound,[48-50] leading to the four ansamycins that entered clinical trials so far (https://clinicaltrials.gov, accessed Feb 2019) (Figure 2).

Structure-based drug design, based on the unconventional conformation that ATP adopts in GHKL-ATP binding sites, led to the development of fully synthetic purine analogues[51-54] as inhibitors of HSP90 ATPase activity, which could be administered orally.[51,53,55] Of these, six entered clinical trials (Figure 2). In a high throughput screening against yeast HSP90 ATPase activity, the first synthetic resorcinolic HSP90 inhibitors were identified.[56] Since then, a set of diversely substituted resorcinols was found to inhibit HSP90,[57-60] of which five entered clinical trials in the following years (Figure 2). Finally, a rather diverse class of HSP90 inhibitors entered clinical trials, where all compounds feature a substituted benzamide substructure (Figure 2). All compounds share a similar binding mode in the ATP binding site.[61-63]

In summary, nineteen structurally distinct HSP90 inhibitors that exert their activity by targeting the N-terminal HSP90 binding site have entered in clinical trials (https://clinicaltrials.gov, accessed Feb 2019)

(Figure 2). Another putative N-terminal HSP90 inhibitor (DS-2248, Daiichi Sankyo, structure undisclosed) was registered for phase I clinical trial, but discontinued (https://clinicaltrials.gov, identifier: NCT01288430). Altogether, none of the classical HSP90 inhibitors was approved by the FDA for therapeutic use as yet. One reason is the induction of the heat shock response (HSR), a well-known side effect of this class of inhibitors,[33,64-69] which was already described for geldanamycin. According to current model of HSR mechanism, the binding of N-terminal HSP90 inhibitors releases heat shock factor-1 (HSF-1).[70,71] The transcription factor becomes phosphorylated, trimerizes, and translocates to the nucleus, leading to overexpression of multiple heat shock proteins, including HSP70, HSP40, and HSP27.[72,73] As HSR is a prosurvival mechanism, it can be detrimental in an anti-cancer therapy.[74] In addition, other severe adverse effects, such as hepatotoxicity found for ansamycins[50,75-77] and ocular toxicity,[68,75,78] have been hampering the clinical success of HSP90 inhibitors until now. Further information on the clinical development of N-terminal HSP90 inhibitors can be found in excellent reviews by Neckers and Workman,[77] Avsar,[76] and Jhaveri *et al.*.[75,79]

In the meantime, many groups have started investigating alternative strategies to inhibit HSP90 without inducing HSR and/or ocular toxicity. The first compound to reach that goal was novobiocin, an inhibitor binding to the CTD of HSP90 (see also chapter 2.1.1).[80,81] A first phase I clinical trial of a C-terminal HSP90 inhibitor (RTA 901) has been performed on non-cancer patients (https://clinicaltrials.gov, identifier: NCT02666963). This compound has been reported to be based on novobiocin,[65] although the structure has not been released yet (see chapter 2.1.1 for further details). Similar to the molecular origins of N-terminal inhibitors, many of the non-N-terminal inhibitors are natural products. In the following, we review some of the successes in the discovery and development of non-N-terminal HSP90 inhibitors. In doing so, we categorize the compounds according to their (proposed) binding site and molecular mode of action.

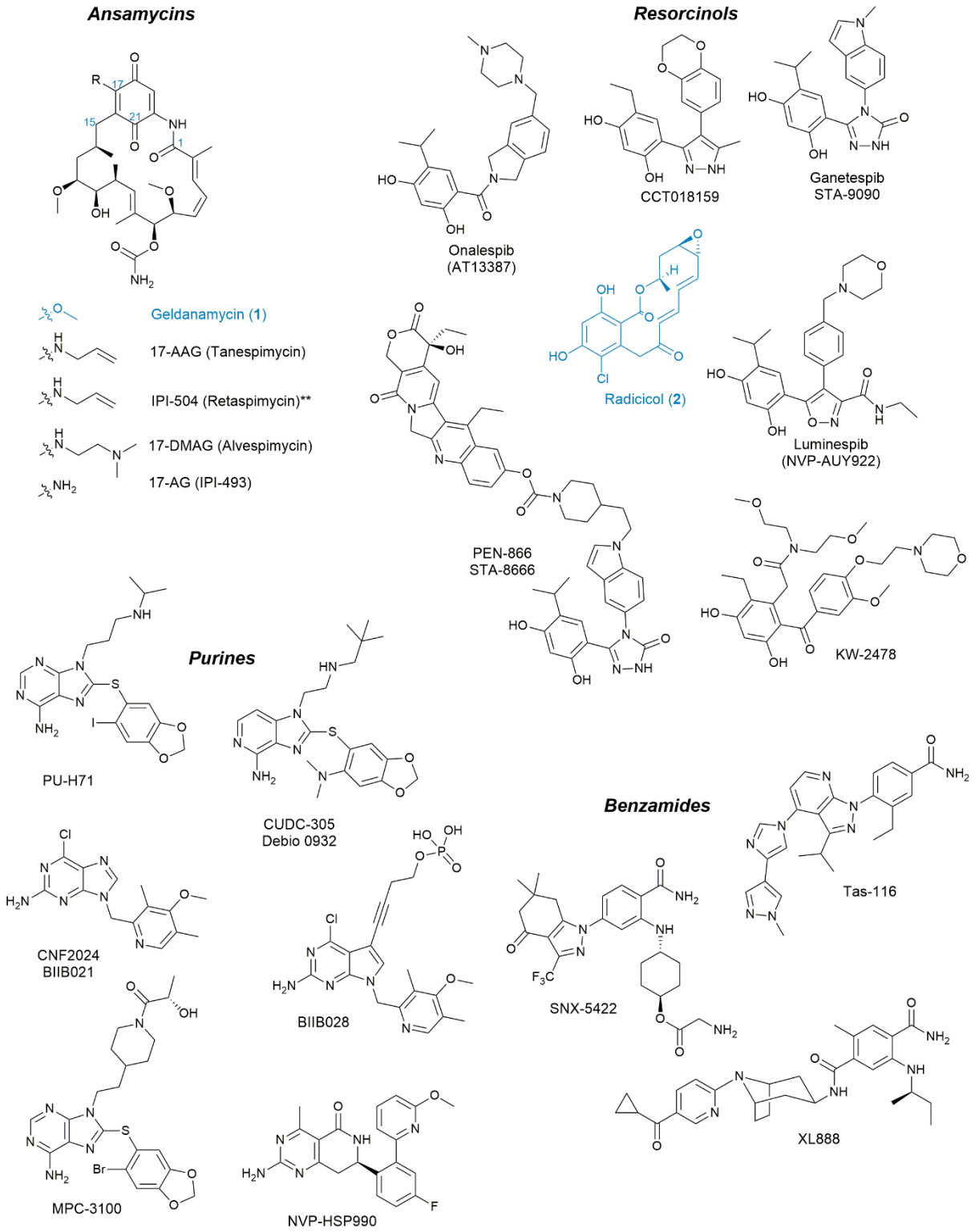

Figure 2. N-terminal HSP90 inhibitors that entered clinical trials. For the natural compound-derived classes, we also show their predecessors (**1**, **2**). Ansamycins: Four compounds derived from geldanamycin (**1**) entered clinical trials so far (** the quinone moiety in retaspimycin is reduced to a dihydroquinone moiety). Resorcinols: Five structurally distinct resorcinols derived from radicicol (**2**) entered clinical trials. The compound PEN-866 is a special case because it is a drug conjugate of the HSP90 inhibitor STA-9090 and an irinotecan metabolite that inhibits topoisomerase I. Purines: Six purine and purine analogues entered clinical trials. These compounds share similar binding modes mimicking the bend conformation of ATP in the N-terminal ATP-binding site. Benzamides: Three compounds entered clinical trials. Despite their structural diversity, they exhibit similar binding modes with a conserved placement of the benzamide motif in the ATP binding site.

2 Non-N-terminal inhibitors of HSP90

2.1 Inhibitors binding to the C-terminal nucleotide binding site

The discovery of the C-terminal ATP binding site is tightly linked to the evaluation of novobiocin (**3**) as a HSP90 inhibitor. The coumarin antibiotic was previously shown to interfere with nucleotide binding in bacterial gyrase B. Yet, in HSP90, no binding to the NTD was observed, and pull-down assays suggested a binding mode different from geldanamycin.[80,81] Further studies revealed a second ATP binding site in the CTD.[81-83] This binding site is only accessible when the N-terminal ATP-binding site is occupied.[82] Furthermore, the C-terminal binding site is able to bind purine as well as pyrimidine nucleotides, while the NTD is rather specific for adenine.[84]

To our knowledge, to date, no experimentally validated structure of an inhibitor binding to this site has been released. Yet, there have been several approaches to predict the C-terminal ATP binding site using molecular modelling (Figure 3).[85-88] In the following, we will discuss HSP90 inhibitors that are considered to bind to the C-terminal ATP binding site.

2.1.1 Novobiocin and derivatives

The amino-coumarine novobiocin (**3**) and the closely related compounds chlorobiocin and coumermycin A1 were the first C-terminal HSP90 inhibitors to be reported. Like geldanamycin, novobiocin leads to a depletion of HSP90 clients such as Raf-1 and HER2,[80,81] although in rather high concentrations compared to N-terminal inhibitors. Co-immunoprecipitation assays further revealed that novobiocin interferes with the formation of HSP90-HSC70, HSP90-p23, and HSP90-CDC37 complexes.[81,89]

In order to gain derivatives with improved activities, a series of SAR studies were performed,[90-93] where cytotoxicity and depletion of HSP90 client proteins served as optimization criteria. Further efforts were made to expand the chemical space by replacing the central coumarine moiety,[94-97] e.g., by biphenyls or stilbenes. Several analogues with improved activities were obtained, with some analogues exhibiting a more than 1,000-fold increase in cytotoxicity against the human breast cancer cell line SkBr3. Interestingly, also compounds that induce HSR were identified in these SAR studies, i.e., A4[93] and KU-32[98]. As the upregulation of heat shock proteins associated with the HSR might prevent protein aggregation and promote cell survival, the application of HSR-inducing HSP90 inhibitors in neurodegenerative diseases was investigated.[74,99,100] The first C-terminal HSP90 inhibitor RTA 901 that has been evaluated in a phase I clinical trial was described as an analogue of KU-32 and showed neuroprotective activity in preclinical tests.[65]

No crystallographic complex structure is available for any of the non-N-terminal inhibitors of HSP90. A cross-coupling experiment with novobiocin-azide derivatives revealed the formation of a covalent bond with K560 in the CTD.[86] Since in the closed state crystal structure of HSP90 no binding site is visible that would be able to accommodate novobiocin in this position, the authors used SAXS data to

generate a putative open state model with bound novobiocin. Other groups used computational methods, including binding site detection algorithms, molecular dynamics simulations, and molecular docking to elucidate the binding site of novobiocin [85-88]. In Figure 3, we depict the corresponding results on closed-state human HSP90β.

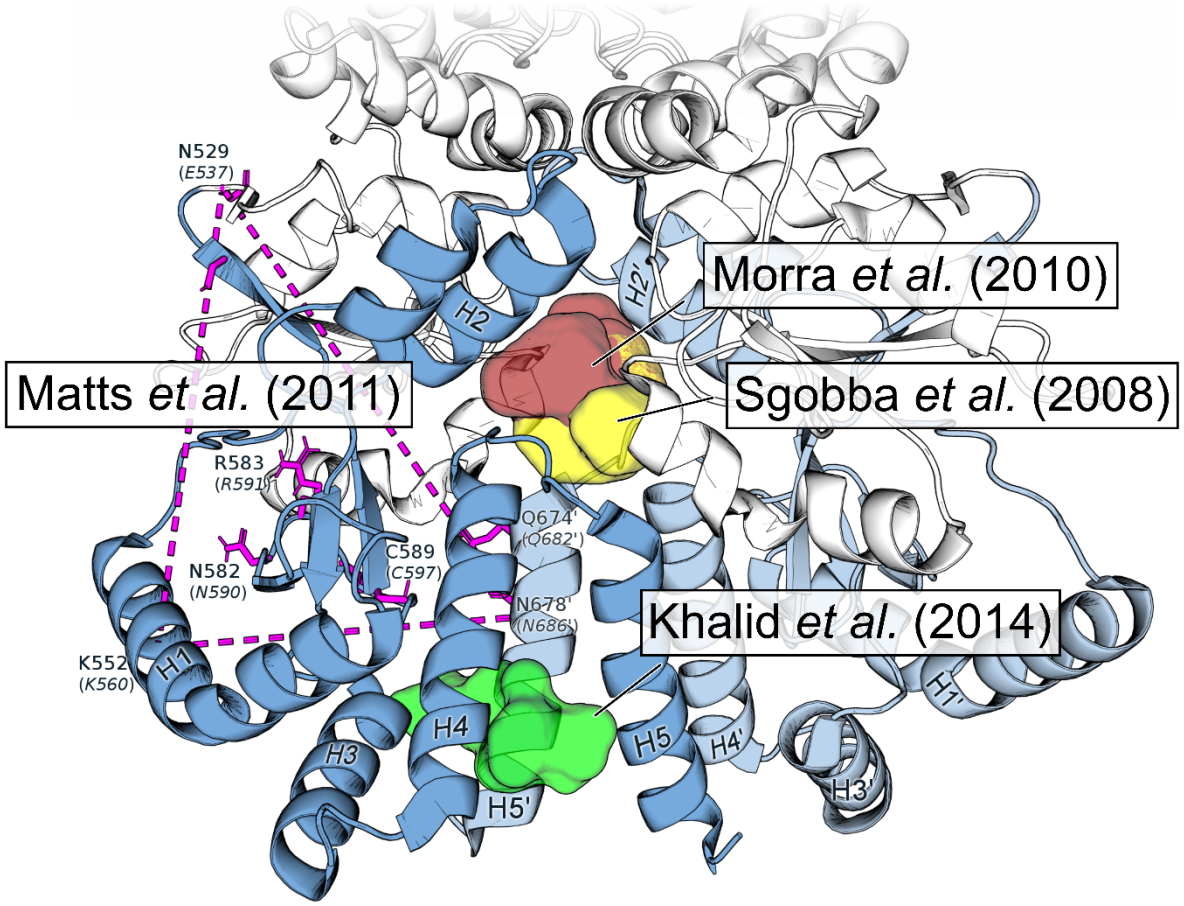

Figure 3. Overview over approaches to identify the C-terminal ATP binding site in HSP. The results were mapped on the structure of human HSP90β. Corresponding residue numbers in human HSP90α are given in brackets. The part of the C-terminal domain that was shown by Marcu *et al.* to contain the ATP-binding motif is colored in blue.[81] Sgobba *et al.* performed molecular simulations on a homology model of human HSP90α and used binding site prediction tools to predict the C-terminal ATP binding site.[88] Morra *et al.* used MD simulations and signal propagation analysis to identify allosteric binding sites on the structure of yeast HSP90 and molecular docking to obtain binding poses of novobiocin and analogues.[87] Matts *et al.* used an azide-analogue of novobiocin as a probe to identify the C-terminal ATP binding site. They found that this probe binds covalently to K560 (HSP90α) and built an putative open-conformation HSP90 model based on SAXS data that showed direct interactions of novobiocin with K560, E537, and N686.[86] Khalid *et al.* used web-server based tools to predict the C-terminal HSP90 binding site.[85]

2.1.2 Epigallocatechin gallate

The antiproliferative effect of the green tea catechin (-)-epigallocatechin-3-gallate (**4**, EGCG) is well described.[101,102] This activity was suggested to be due to direct inhibition of AKT and ERK1/2. By proteolytic footprints and pull-down assays with EGCG-conjugated sepharose beads, EGCG was found

to bind to a C-terminal fragment of HSP90.[101,103,104] Furthermore, EGCG inhibits luciferase refolding and leads to dose-dependent depletion of HSP90 client proteins ErbB2, Raf-1, and pAkt, which suggests that EGCG acts as a HSP90 inhibitor.[103] However, in pull-down assays also binding of EGCG to HSP70 was detected.[101] A set of SAR studies were performed leading to EGCG derivatives with enhanced antiproliferative activity.[105-107]

To further specify the binding site of EGCG, pull-down assays with ATP-agarose were performed. EGCG inhibited the binding of CTD-HSP90 to ATP.[101,103] In turn, free ATP was able to compete with the binding of HSP90 and HSP70 to EGCG-coupled sepharose.[101] These findings strongly indicate that the binding site of EGCG may overlap with the CTD ATP binding site.

In total, these results indicate that EGCG inhibits HSP90 in a similar way as novobiocin. The inhibition of HSP90 and subsequent depletion of client kinases could also account for the activity of ECGC on various tumor pathways.[67] Still, for the further development of EGCG derivatives, a potential promiscuity with respect to kinases and transcription factors[108,109] needs to be addressed.

2.1.3 Deguelin

The flavonoid deguelin (**5**) exhibits potent antiproliferative and antiangiogenic properties in various cancer cell lines, as well as in xenograft mouse models.[110] Oh and co-workers found that deguelin downregulates the expression of hypoxia-inducible factor 1α. Subsequent experiments showed that this is caused by an inhibition of HSP90 via an ATP binding pocket.[111] In rats, however, deguelin was shown to induce a Parkinson's disease-like syndrome.[112] In order to overcome this potential adverse effect, structural analogues of deguelin were designed and tested. This resulted in the compound L80, which induces apoptosis in cancer cells and leads to reduced phosphorylation and degradation of HSP90 client proteins.[113,114]

The binding site of L80 was further characterized by a pull-down assay with ATP-agarose, where L80 was shown to inhibit the adsorption of the CTD and, partially, full-length HSP90, but not of the NTD.[114] This result suggests that L80 binds to a region overlapping the C-terminal ATP binding site. A molecular docking study based on the results from Sgobba *et al.*[88] further suggested a binding mode in between the homodimers, forming key interactions with K615, S677, and S677'.

2.1.4 Dihydropyridines

Dihydropridines have demonstrated neuroprotective properties. As a possible mode of action, the upregulation of the expression of HSP27, HSP40, and HSP70 upon heat shock in comparison to untreated control was proposed.[115] In a subsequent study, the dihydropyridin LA1011 (**6**) was shown to bind to HSP90 and, at millimolar concentrations, prevent refolding of thermally denatured luciferase. Unlike most other inhibitors of HSP90, no inhibition of HSP90 ATPase activity was observed, but an activation.[116]

Using isothermal titration calorimetry, LA1011 was demonstrated to bind to the HSP90 CTD. By molecular docking experiments and molecular dynamics simulations followed by sidechain substitutions, the authors suggested a binding site in between the C-terminal domains that roughly overlays with the location of the C-terminal ATP-binding site proposed independently by Sgobba *et al.*[88] and Morra *et al.*[87] for ATP and novobiocin.[116] However, to our knowledge, there is no experimental evidence that demonstrates that LA1011 inhibits the ATP binding to the C-terminal domain. Thus, investigations in that direction might be very interesting.

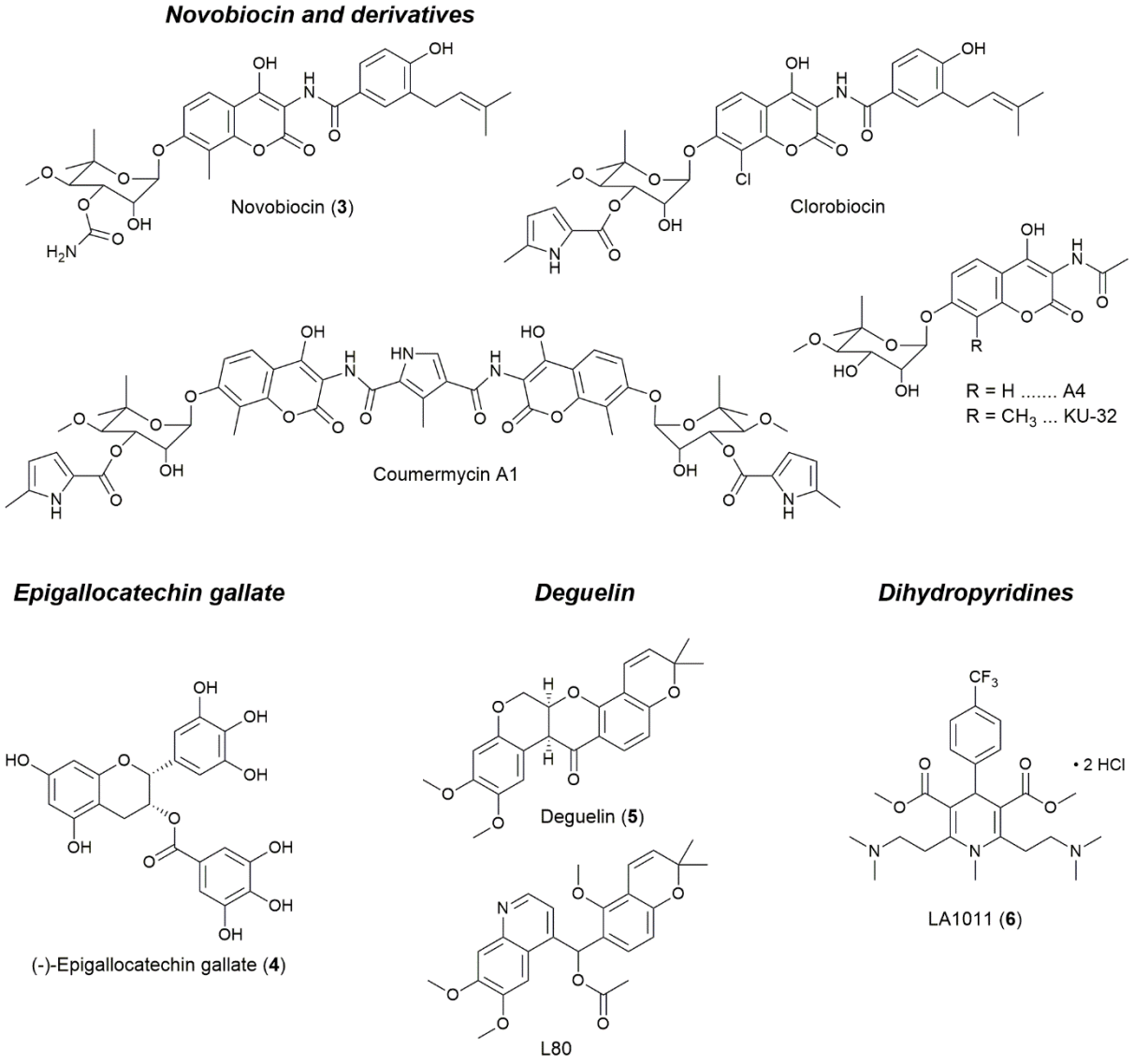

Figure 4. Inhibitors considered to bind to the C-terminal ATP-binding site of HSP90.

2.2 Modulators of HSP90-CDC37 interaction

CDC37 (cell division cycle 37 protein) is a co-chaperone primarily associated with the chaperoning of protein kinases. It is essential for the stabilization of HSP90-kinase complexes.[117,118] The protein interacts with HSP90 via a large interaction surface, wrapping itself around the NTD and MD,[15] which

prevents the hydrolysis of ATP.[119] CDC37 knockout was shown to sensitize HSP90 for other inhibitors.[120] Thus, inhibiting the interaction between HSP90 and CDC37 might lead to kinase-specific HSP90 inhibitors and could provide a synergistic approach for the combination with other inhibitors.

2.2.1 Celastrol

Already in 2004 an activation of HSF-1 by the triterpenoid celastrol (**7**) was reported, leading to the expression of heat shock proteins, including HSP70, HSP40, and HSP27.[121] Later, Hieronymus and co-workers demonstrated properties of celastrol as an HSP90 inhibitor in a gene expression signature-based screening.[122] Co-immunoprecipitation assays and HSQC NMR studies revealed that celastrol prevents the interaction of HSP90 with its co-chaperone CDC37, leading to degradation of client kinases such as Akt and Cdk4.[123,124] Another study showed that celastrol is also able to disrupt the interaction of HSP90 and p23, leading to the selective degradation of client steroid receptors.[125]

Celastrol prevents proteolytic degradation of the CTD by trypsin, suggesting a binding site in the CTD.[123] Likewise, in DSF and DSC measurements, the stabilizing effect of celastrol on the CTD was confirmed.[126] By mass-spectrometric analysis of CDC37 after incubation with celastrol, it was shown that the compound forms covalent Michael adducts to CDC37-cysteins, which lead to the disruption of HSP90-CDC37 interaction.[124]

2.2.2 Withaferin A

The steroidal lactone withaferin A (**8**) possesses antiproliferative and antiangiogenic activity,[127,128] which at least partially is due to an inhibition of HSP90.[129] In cells, the natural product leads to degradation of client proteins (i.e., Akt, CDK4, and GR) in a dose-dependent manner via the proteasome-dependent pathway. In a pull-down assay with biotinylated withaferin A, binding to the C-terminal domain of HSP90 was confirmed.[129] Since withaferin A was only able to bind to the CTD when cysteine residues are present, the interaction of withaferin A with HSP90 is most likely of covalent nature. Furthermore, adding acetylcystein to the reaction buffer prevented client degradation completely.[129]

A pull-down assay with ATP-sepharose revealed that withaferin A does not inhibit ATP binding,[129] indicating a binding site different from the ATP binding sites. In a co-immunoprecipitation assay, the compound was shown to block the HSP90-CDC37 interaction. Two molecular docking studies performed by Grover *et al.* explored possible binding modes of withaferin A in the HSP90-CDC37 interface,[130,131] though in these studies the possible covalent binding via a cysteine was not addressed. The most likely covalent binding of withaferin A to its target proteins might compromise its selectivity.

2.2.3 Curcubitacin D

The triterpenoid curcubitacin D (**9**) has been known for its antiproliferative properties since the 1960s and inhibits several cancer cell lines at submicromolar concentrations.[132-135]. These cytotoxic properties can at least partially be attributed to an inhibition of HSP90. Curcubitacin D prevents the interaction of

HSP90 and its co-chaperones CDC37 and p23, which leads to degradation of the HSP90 clients Her2, Raf, Cdk6, and pAkt in a concentration-dependent manner.[136] Inhibition of the chaperone activity does not lead to increased levels of HSP27 expression.[136] This indicates that curcubitacin D is able to inhibit HSP90 chaperone activity without inducing HSR.

However, the molecular mode of action of this compound remains unknown. As such, it is not known yet whether the compound binds to HSP90 or the co-chaperones in order to prevent their interaction. Both CDC37 and p23 bind to the NTD and MD of HSP90, which might indicate that curcubitacin D binds there as well. However, also compounds binding to the CTD, such as novobiocin, inhibit interactions between p23 or CDC37 with HSP90.[81,89]

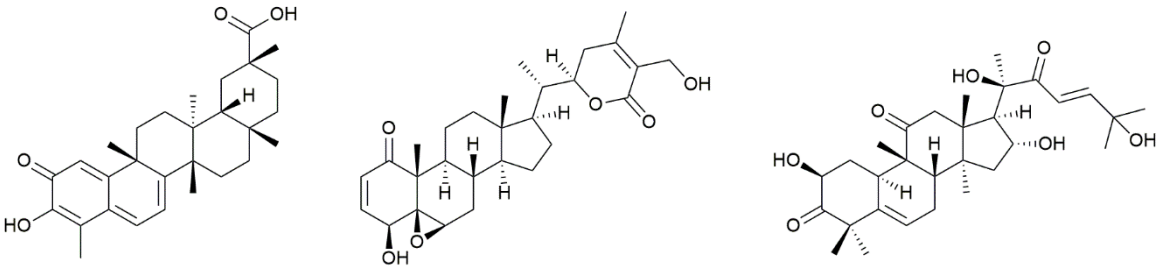

Figure 5. Compounds considered to inhibit the HSP90-CDC37 interaction.

2.3 Modulators of HSP90-p23 interaction

The small acidic protein p23 interacts with HSP90 via the NTD and MD. The interaction halts the HSP90 ATPase cycle and appears to facilitate HSP90 binding to client proteins.[137] While not essential for the maturation of HSP90 kinase clients, such as Cdk4 and Akt, p23 is necessary for the stabilization of steroid hormone receptors.

2.3.1 Gedunin

Gedunin (**10**) was first identified as an inhibitor of HSP90 by a connectivity map approach using gene expression patterns to identify targets of compounds with unknown mode of action.[122,138] The compound was shown to elicit insecticidal,[139] antimalarial,[140] and antiproliferative effects against cancer cell lines.[141-143] In early studies, degradation of HSP90 clients Raf and HER2 under gedunin treatment were observed.[144] Yet, a later study suggested that this degradation is mostly specific for steroid hormone receptors, while changes in HSP90 client kinase levels are insignificant.[145]

In pulldown and co-immunoprecipitation assays with biotinylated gedunin, it was shown that the compound binds to p23 and disrupts the interaction between HSP90 and this co-chaperone. Since p23 is an essential component of the HSP90 complex responsible for stabilization of steroid hormone receptors, these results align very well with the selective degradation of the stereoid hormone receptors. Interestingly, free dihydrocelastrol (**11**) inhibited the pulldown of p23 with gedunin, which might be an indication for similar binding sites.[145]

Like celastrol, gedunin has the potential to act as a Michael acceptor via the α,β-unsaturated ketone. However, SAR studies revealed that this is not the primary mode of action of the compound, although chemical modifications of the olefin generally resulted in less active derivatives.[144]

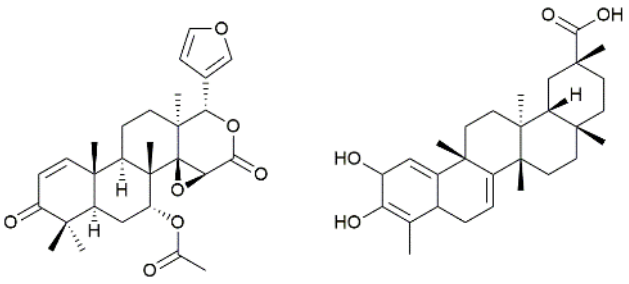

Figure 6. Compounds that modulate the HSP90-p23 interaction.

2.4 Inhibitors of HSP90-HOP interaction

The HSP90 C-terminal domain ends in a most likely unstructured loop that contains the highly conserved MEEVD motif.[146] This motif forms the key interaction with tetratricopeptide repeat (TPR) domains, which are present in various HSP90 co-chaperones.[1] One of these is the HSP70/HSP90-organizing protein (HOP), which mediates the interaction between the HSP70-HSP40 complex and HSP90.[147] HOP contains three TPR domains, of which the domains TPR2A and TPR2B bind to the MEEVD motifs in HSP90 and HSP70, respectively.[148]

2.4.1 Sansalvamide A-amide and derivatives

The fungal depsipeptide sansalvamide A (**12**) and its derivatives have been investigated for their anticancer activity in various types of cancer.[149] A first mechanistic study performed with the amide analogue sansalvamide A-amide attributed the cytotoxic properties to the inhibition of the protein-protein interaction between HSP90 and its co-chaperone FKBP52 as well as its client IP6K2,[150] which are both associated to apoptotic pathways.[151,152] In a pull-down assay, sansalvamide A-amide was able to bind a NTD-MD construct lacking the CTD. In contrast, it showed only marginal affinity towards the individual domains and the CTD. This indicates that sansalvamide A-amide binds to the intersecting region between NTD and MD.[150] Since both FKBP52 and IP6K2 bind to the HSP90-CTD, McAlpine and co-workers suggested an allosteric mechanism for the modulation of this interaction.[150] Further studies investigating the properties of sansalvamide A-amide revealed that this compound and analogues induce the expression of HSP70 in a similar fashion as the N-terminal inhibitor 17-AAG.[153] However, other similar compounds were reported, where no induction of HSR was observed.[154,155]

Kawakami and co-workers further designed peptides based on the TPR sequence, to directly inhibit interactions of HSP90 via the MEEVD motif.[156,157] These peptides showed antiproliferative *in vitro* and in a xenograft mouse model.[157] McAlpine and co-workers combined this approach with the macrocyclic peptide scaffold of the sansalvamides.[158] This effort led to the recently published compound LB76,[159]

which was shown to bind to the CTD of HSP90 in a pull-down assay and inhibit the interactions of HSP90 with HOP, Cyp40, and, with lower potency, FKBP38 and FKBP51. Since HOP and Cyp40 bind to HSP90 via the MEEVD motif, while the interactions with the FKBPs are formed via a larger surface, the authors interpreted their findings as an indication for the specific binding of LB76 to the MEEVD motif.

2.4.2 Pyrimidotriazinediones

Regan and co-workers developed a high-throughput screening assay based on Amplified Luminescence Proximity Homogeneous Assay (AlphaScreen™) technology to identify small molecules that are able to inhibit the HSP90-TPR interactions.[160] They used this assay to identify a set of compounds featuring a common pyrimido[5,4-*e*][1,2,4]triazine-5,7-dione scaffold including the compound C9 (**13**). Subsequently, they showed that these compounds are able to decrease the levels of the HSP90 client HER2. Cytotoxicity of the compound was observed in various breast cancer cell lines. No overexpression of HSP70 was observed upon treatment with C9.[161] Combinational treatment with inhibitors of the N-terminal ATP-binding site 17-AAG or AUY922 (resorcinole class) reduced the lethal-IC50 of C9 with respect to the individually applied compound, and the HSR induced by the N-terminal inhibitors was dampened by C9.[162]

Using ITC measurements C9 was identified to bind directly to the TPR2A domain, while no interaction to a C-terminal HSP90-peptide was observed.[161] Thus, C9 elicits its inhibitory effect on the HSP90 machinery by binding to HOP and supposedly preventing the formation of the HSP90 supercomplex, rather than directly binding and inhibiting HSP90.

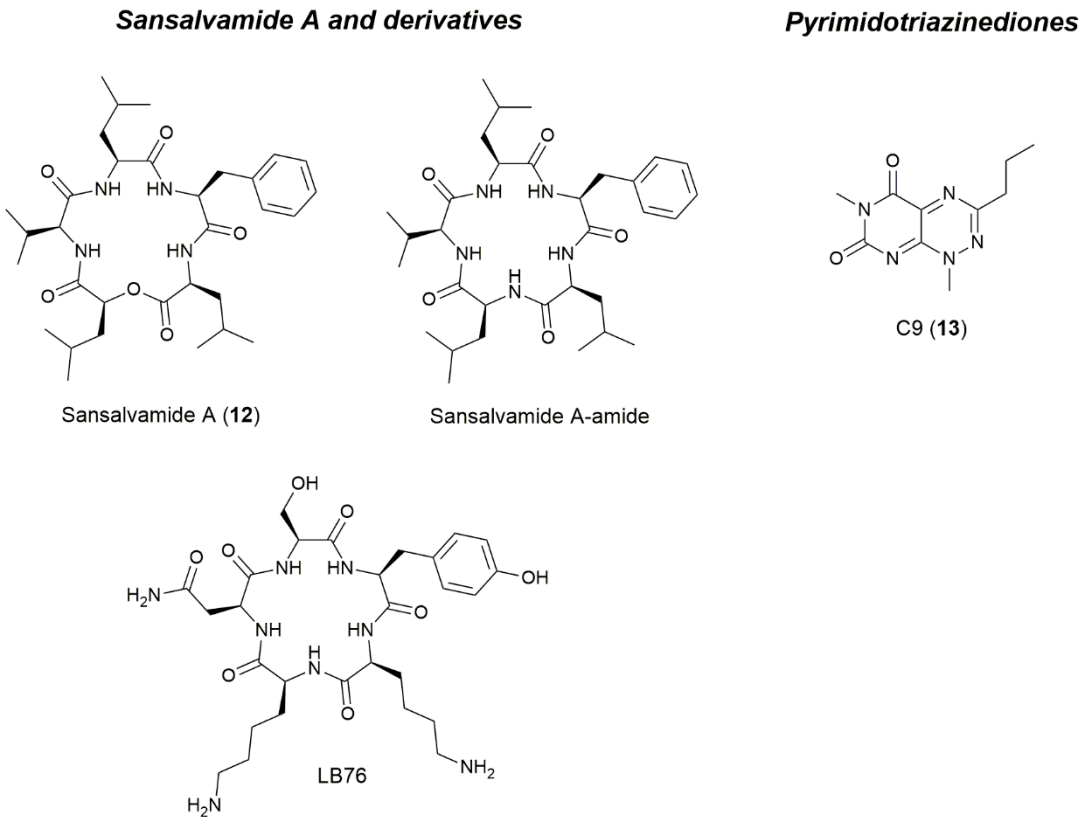

Figure 7. Inhibitors of the HSP90-HOP interaction.

2.5 Inhibitors of the C-terminal dimerization of HSP90

2.5.1 Aminoxyrone

The C-terminal domain of HSP90 is known to constitute the primary dimerization interface of HSP90, and HSP90 dimerization is essential for its chaperoning function.[17] Initially believed to be very stable, the interaction between the CTDs was later shown to exhibit a dynamic equilibrium of fast association and dissociation.[20] This finding offered the opportunity for small molecule compounds to bind to the dimerization interface.

In order to rationally design inhibitors targeting this interface, Gohlke and co-workers identified key residues ("hot spots") in the dimerization interface of HSP90, using a combination of molecular dynamics simulations and free energy calculations.[163] The hot spots were mostly located on helices H4 and H5 of the CTD, and substituting these residues with alanine led to reduced thermostability of the protein with respect to the wild type.[163] Peptides resembling the sequences of H4 (aa 656-675) and a combined H4-H5 construct (aa 656-697) were shown to bind to the CTD of HSP90 with apparent K_d values of 1.02 µM and 1.46 µM, respectively. Furthermore, the peptides were analyzed by an Autodisplay assay,[164] where HSP90 was displayed on the surface of *E. coli*, forms dimers there, and is able to bind FITC-labelled p53. The peptides interfered with HSP90's capability to bind p53, most likely by inhibiting HSP90's dimerization.[165] Hence, these peptides were the first inhibitors of HSP90 C-terminal dimerization shown to bind to the CTD.

Based on these findings, α-aminoxy peptide-based inhibitors were designed exploiting the knowledge of the orientation and type of the hot spot residues.[163] α-Aminoxy peptides are a class of peptidomimetics that form stable 2_8-helical structures and are able to mimic certain side chain orientations of α-helical peptides.[166] The resulting hexamer aminoxyrone (**14**) mimics the key interactions of H5 in the C-terminal dimerization interface. To prove the ability of this compound to functionally inhibit HSP90, a luciferase refolding assay[167] was performed, were aminoxyrone inhibited refolding of firefly luciferase in a dose-dependent manner. Using microscale thermophoresis, the binding of aminoxyrone to the CTD was shown (K_d = 27.4 µM).[168] The compound showed antiproliferative activity against various cancer cell lines *in vitro* as well as in a chronic myeloid leukemia xenograft mouse model.[168] The low toxicity profile and lack of HSR[168] make his compound an attractive lead for further drug optimization.

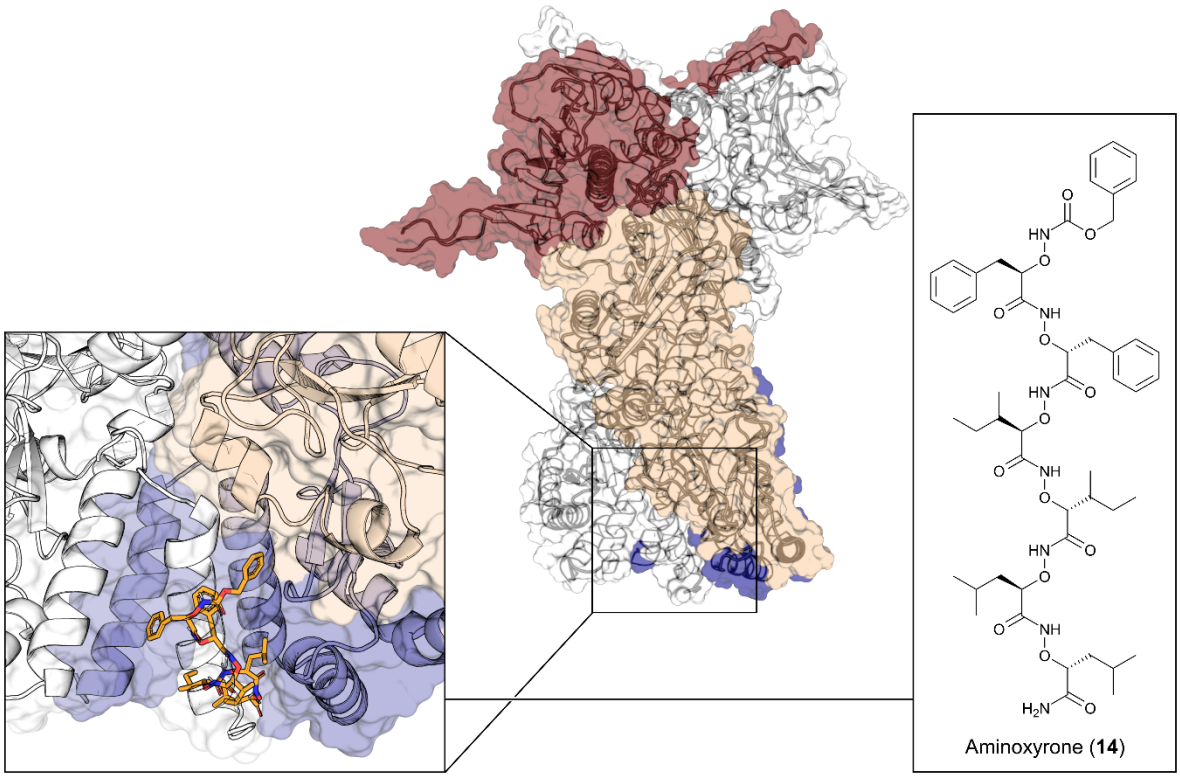

Figure 8. Peptidomimetic dimerization inhibitor aminoxyrone. The blow-up on the left side shows aminoxyrone in its bound pose as predicted by molecular dynamics simulations of free ligand diffusion.[168] The compound mimics helix 5 in the dimerization interface.

3 Conclusion

HSP90 has been a major target for drug discovery in academia and pharmaceutical industry for several decades, and many inhibitors have been published and patented. Most of these inhibitors target the N-terminal ATP binding site, and – except for RTA 901 – only compounds of this class have underwent clinical trials so far. However, a major drawback of this compound class is the induction of prosurvival HSR, which limits the compounds' efficacy and/or may lead to adverse effects.

Inhibiting HSP90 via allosteric binding sites, including the secondary ATP binding site in the CTD, or inhibiting protein-protein interactions between HSP90 domains, or HSP90 and interacting proteins essential for the function of the HSP90 machinery, are notable alternatives. Novobiocin was the first compound reported to inhibit HSP90 by an alternative mechanism, and by now a wide variety of compounds have been shown to elicit HSP90 inhibitory effects that way. Many of these newly identified compounds are natural products that show activity in the micromolar range. However, as a result of the complexity of the HSP90 machinery, only few details are known about the molecular mode of action of these compounds.

For the further development and optimization of these compounds, understanding the molecular mechanism behind their inhibitory effect will be essential. Given the complexity of the HSP90 chaperoning system, multivalent systems may be used to simultaneously target multiple binding epitopes.[169,170] In particular, since for many of the compounds, the inhibition of interactions between HSP90 and co-chaperones are assumed to be the mode of action. However, often it is not clear whether these compounds bind to HSP90 or to the respective co-chaperone. Furthermore, due to the flexible nature of HSP90, ligands could bind allosterically to the protein disrupting the co-chaperone interaction by imposing a certain conformation on HSP90 without binding to the protein-protein interface itself. Resolving complex structures of HSP90 and the ligands would be the optimal way to obtain structural information about the binding of such inhibitors. Alternatively, molecular modeling techniques such as molecular docking and molecular dynamics simulations of free ligand diffusion can provide valuable insights. In fact, for many C-terminal HSP90 inhibitors presented here, computational approaches have been successfully employed to guide ligand optimization.

A few approaches have been presented, where structure-based *de novo* design was applied to specifically induce particular effects on the HSP90 machinery. As such, LB76 is a rationally designed peptide-mimetic to inhibit interactions of HSP90 with HOP via the MEEVD motif, and aminoxyrone was developed as a peptidomimetic C-terminal HSP90 dimerization inhibitor. Although further optimization is required for both compounds to become potential drug candidates, they already now show that much can be gained by pursuing new routes towards HSP90 inhibition.

Conflict of Interest

The authors confirm that this article content has no conflict of interest.

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Author Contribution

HG conceived and supervised the study; DB and HG wrote the manuscript.

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