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REVIEW ARTICLE

Dual Targeting Strategies on Histone Deacetylase 6 (HDAC6) and Heat Shock Protein 90 (Hsp90)

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Abstract: The design of multi-target drugs acting simultaneously on multiple signaling pathways is a growing field in medicinal chemistry, especially for the treatment of complex diseases, such as cancer. Histone deacetylase 6 (HDAC6) is an established anticancer drug target involved in tumor cells transformation. Being an epigenetic enzyme at the interplay of many biological processes, HDAC6 has become an attractive target for polypharmacology studies aimed at improving the therapeutic efficacy of anticancer drugs. For example, the molecular chaperone Heat shock protein 90 (Hsp90) is a substrate of HDAC6 deacetylation, and several lines of evidence demonstrate that simultaneous inhibition of HDAC6 and Hsp90 promotes synergistic antitumor effects on different cancer cell lines, highlighting the potential benefits of developing a single molecule endowed with multi-target activity. This review will summarize the complex interplay between HDAC6 and Hsp90, providing also useful hints for multi-target drug design and discovery approaches in this field. To this end, crystallographic structures of HDAC6 and Hsp90 complexes will be extensively reviewed in light of discussing binding pockets features and pharmacophore requirements and providing useful guidelines for the design of dual inhibitors. The few examples of multi-target inhibitors obtained so far, mostly based on chimeric approaches, will be summarized and put into context. Finally, the main features of HDAC6 and Hsp90 inhibitors will be compared, and ligand- and structure-based strategies potentially useful for the development of small molecular weight dual inhibitors will be proposed and discussed.

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1. INTRODUCTION

Histone deacetylases (HDACs) are a family of enzymes playing a key role in the regulation of gene expression *via* the removal of acetyl functional groups from the lysine residues in histones and non-histone proteins [1]. Several studies demonstrated that aberrant epigenetic mechanisms, such as histone modification, have been linked to tumor development, as they contribute to a loss in cell cycle regulation, differentiation, and apoptosis [2]. As a consequence, HDACs inhibitors have been widely investigated as a class of anti-cancer agents able to restore normal gene expression levels and block tumor initiation and progression [3-5]. At present, four HDAC inhibitors have been approved by FDA for cancer treatment, including belinostat (PXD101), [6, 7] romidepsin [8-10] and vorinostat [11-13] for cutaneous T-cell lymphoma, and panobinostat [14-16] for multiple myeloma. Currently, 18 different isoforms of HDACs have been identified and classified into four classes: class I (HDAC1, 2, 3, 8), class II divided into subgroup IIa (HDAC4, 5, 7, 9) and IIb (HDAC6, 10), class III HDACs (sirtuins 1-7) and class IV (HDAC11) [17, 18]. Except for class III that requires the nicotinamide adenine dinucleotide (NAD) as a cofactor, the other isoforms are zinc-dependent deacetylases. Moreover, HDACs of different classes have shown differences in terms of structure, function and substrate preferences [1,

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19]. For instance, the therapeutic relevant HDAC6 is the unique HDAC isoform mainly located in the cytoplasm, both containing an N-terminal domain catalytic domain 1 (CD1) and a central catalytic domain 2 (CD2) (Fig. **1**) [20, 21]. HDAC6 CD2 demonstrates deacetylase activity of specific cytosolic non-histone substrates, such as α-tubulin, [22] heat shock protein (Hsp90), cortactin, peroxiredoxin, and heat shock transcription factor-1 (HSF-1), while CD1 showed much tighter substrate specificity for peptide substrates carrying C-terminal acetyllysine residues [23]. Besides, recent data on HDAC6 CD2 crystallography highlighted structural differences in the binding site topology with respect to the other known HDAC isozymes [19, 24]. These information are driving rational efforts to develop isoform specific HDAC6 inhibitors with greater therapeutic potential and fewer risks of adverse effects than the *pan*-inhibitors available on the market [25, 26]. However, the available therapeutic applications of HDAC inhibitors are limited and characterized by a lack of efficacy on solid tumors [27]. In this regard, an attractive new approach to develop HDAC inhibitors aims at designing a single polypharmacological agents that inhibits multiple oncogenic targets [28]. As detailed below, this would potentially help to overcome drug resistance mechanism, limit toxicity, provide synergy, and extend the therapeutic spectrum. Several studies have demonstrated the potential of multi-target strategies linking HDAC6 with other tumour targets. For example, HDAC6 is a client protein of Hsp90, which is very often upregulated in cancer cells, and is a downstream target of HDAC6 [29]. Hsp90 is a chaperone involved in the correct folding of many oncoproteins [30, 31]. Hsp90 inhibitors have proved to be effective in reducing tumour growth, induction of differentiation and activation of apoptosis [32]. However, despite more than 80 clinical trials performed on 22 Hsp90 inhibitors, none of them has met the therapeutic efficacy and safety required for FDA or EMA approval [33]. Recently, Jaeger *et al*. [34] suggested that an answer to achieve clinical benefit from Hsp90 inhibition might lie in a multi-target strategy to undermine the emergence of drug resistance. The benefit derived from a combined inhibition of HDAC6-Hsp90 have been studied on different types of cancer and neurogenerative diseases [35, 36]. For example, George *et al.* have shown that the coadministration of HDAC and Hsp90 inhibitors is able to increase apoptosis of resistant chronic myeloid leukaemia (CML) and acute myeloid leukaemia (AML), more than the respective single-agents [14]. On the same line, Chai *et al.* suggested that the combination of HDAC and Hsp90 inhibitors can overcome the acquired resistance observed in cancer cells [37]. On Anaplastic Thyroid Carcinoma Cells, the synergistic induction of apoptosis using Hsp90i and HDACi is mediated by the suppression of PI3K/Akt/mTOR signaling and DNA damage-related proteins [38]. Rodrigues Moita *et al.* have found that the preincubation of ovarian cancer cells with HDACi enhances the cytotoxic potency of Hsp90i [39]. Similarly, Zismanov *et al.* proved that the combined treatment with HDAC and Hsp90 inhibitors increase the level of endoplasmic reticulum stress leading to increased death of non-small lung cancer cells, with respect to the individual drugs [40]. Thus, the potential benefit in targeting HDAC6 and Hsp90 with dual-acting drug has garnered considerable interest from the scientific community. Rationally designing multi-target ligands is still a challenge, especially for targets as Hsp90 and HDAC6, which do not share significant binding site similarity [41-44]. However, computational approaches have proved to be of help in screening and selecting suitable candidates [45, 46]. The design could be facilitated by the resolution of several crystallographic structures of both targets, providing the framework for structure-based development of dual inhibitors. Moreover, the identification of an increased number of inhibitors with different chemotypes for the single targets would certainly facilitate the identification of overlapping chemical spaces to be exploited for the design of polypharmacological ligands. The strategies to design HDAC6-Hsp90 dual inhibitors and an up-to-date list of active molecules are herein reported and discussed.

2. DISCUSSION

2.1. The Hsp90-HDAC6 Interplay

Hsp90 and HDAC6 are the two established drug targets involved in the regulation of several biological processes controlling proliferation and metastasis in cancer cells [52]. Under physiological conditions, Hsp90 assists the correct assembly of hundreds of macromolecular structures, thus preventing their aberrant folding and aggregation into non-functional structures [53, 54]. Moreover, it also indirectly participates in signalling and intracellular transport, and in the regulation of the trafficking and degradation of many other proteins in the crowded cellular environment [31, 55]. In cancer cells, Hsp90 is responsible for uncontrolled proliferation and apoptotic resistance, by means of the regulation of several so-called *clients*, including steroid hormone receptors, kinases (*e.g*., Akt, Raf-1, Bcr-Abl, Cdk4 and Wee1), histone deacetylases (*i.e.*, HDAC1 and HDAC6), and other oncogenic proteins [56, 57]. Nevertheless, it has been demonstrated that Hsp90 is often overexpressed in several cancers, [58-61] its

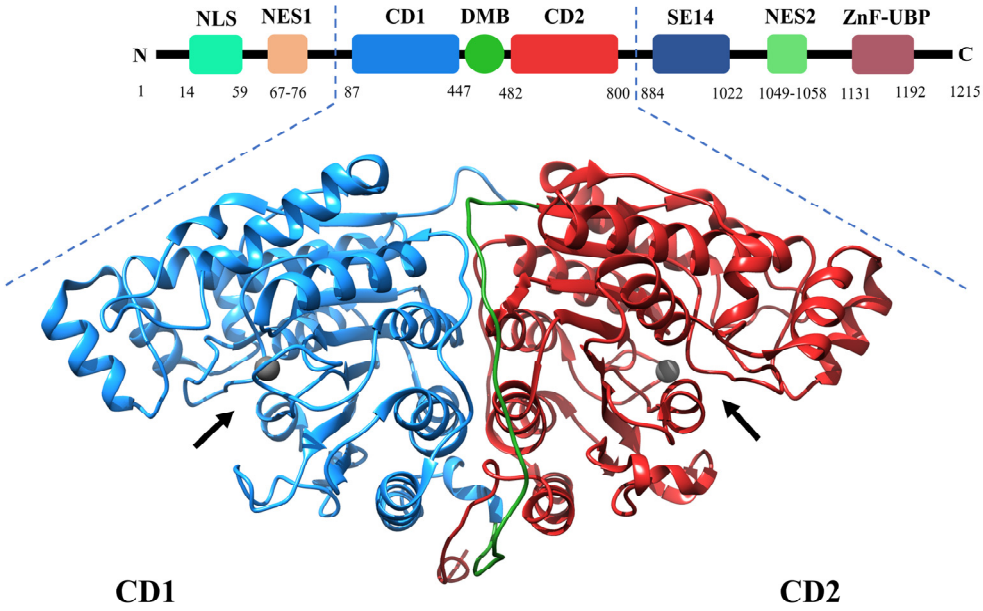


Fig. (1). Schematic representation of the HDAC6 domains framework, with the crystallographic structure of CD1-CD2 displayed (PDB code: 5G0J) [47]. In particular, CD1 is represented as a blue ribbon, the linker domain is coloured in green, while CD2 is shown as a red ribbon. The arrows point to the catalytic zinc metal in the binding site of the two domains. HDAC6 consists of 1215 amino acids sequence, characterized by a NLS and two leucine-rich nuclear export sequences (NES1 and NES2), important for the shuttling process from the cytoplasm to the nucleus and *vice versa* [48-50]. The two catalytic domains (CD1 and CD2) are required for the deacetylation enzymatic activity of the protein. Moreover, it also contains eight consecutive Ser-Glu-containing tetradecapeptide repeating units (SE14) and an ubiquitin C-terminal hydrolase-like zinc finger (ZnF-UBP) domain, which play an important role for intracellular retention and in the control of proteins ubiquitination [51], respectively. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

activity allowing tumour cells to overcome adverse environmental stress conditions that would normally limit their proliferation and metastasis to other tissues [60]. Although initial concerns on targeting a protein abundantly expressed also in normal cells, [60] several efforts have been addressed so far for the development of selective Hsp90 anticancer agents, some of them entering also late-stage clinical trials [60]. For example, ganetespib has been investigated up to phase III clinical trials both alone and in combinations for the treatment of several types of tumors, including breast cancer (*e.g.*, ClinicalTrials ID: NCT01560416), malignant pleural mesothelioma (*e.g.*, ClinicalTrials ID: NCT01590160) and non-small-cell lung cancer (NSCLC) (ClinicalTrials ID: NCT01348126, NCT01798485). Moreover, pimitespib (*i.e.*, TAS-116) has been recently investigated against advanced gastrointestinal stromal tumor (GIST), with promising results (*i.e.*, ClinicalTrials ID: NCT02965885) as shown below. The therapeutic potential offered by targeting Hsp90 for cancer treatment is testified by the continuous efforts on the design of novel inhibitors of this target. However, although a number of highly active and selective drug candidates modulating this protein have been reported, recent studies have also found an association between the insurgence of *de novo* chemoresistance in tumour cells, and prolonged treatments with Hsp90 inhibitors [37, 62]. For example, treatment of tumour cells with 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG) has been reported to induce acquired resistance to several structurally diverse Hsp90 inhibitors [37]. Moreover, drugs selectively acting on this target, such as SNX-5422, [63], have been withdrawn from clinical trials due to their non-optimal safety profiles; this effect probably deriving by the fact that Hsp90 is ubiquitously expressed in human cells and participates also in the regulation of several biological processes not strictly related to cancer [60]. Indeed, the activity of a number of client proteins results to be altered as a consequence of the Hsp90 inhibition. Considering that several types of cancers are driven by, or present overexpression of, specific oncoproteins, administration of Hsp90 inhibitors might results also in the modulation of clients not strictly involved in tumor pathology, thus potentially explaining some observed side effects [64]. Moreover, recent studies also demonstrated that some Hsp90 inhibitors do not show any selectivity

on cancer over normal cells, this potentially resulting in side effects [65, 66]. For example, Wang and co-workers demonstrated that luminespib is not selective for targeting Hsp90 in cancerous cells [66]. These findings fuelled research efforts in the identification of the causes related to toxicities and drug resistance very often observed in Hsp90 single-target therapies, and of alternative approaches potentially able to solve these issues. One among the most pursued alternative approaches is combination therapies [67-70]. Interestingly, some combinations of Hsp90 inhibitors with compounds binding to other therapeutically relevant targets have already entered clinical trials. For example, Hsp90 inhibitors, such as 17-AAG, have been clinically investigated in combination with Gemcitabine (ClinicalTrials ID: NCT00577889) and Bortezomib (ClinicalTrials ID: NCT00096005) for the treatment of stage IV pancreatic adenocarcinoma and advanced solid tumors or lymphomas, respectively [71]. In addition, these findings motivated the search of single molecules with multi-target activity, which is now mainly designed by means of computational methods [42, 43, 72]. HDAC6 is a member of the histone deacetylases protein family mainly present in the cell cytoplasm, [20, 73, 74], which regulates the acetylation levels of several cytoplasmic proteins, thus acting on cellular signalling and gene expression [20, 73, 75-77]. In particular, HDAC6 regulates microtubules dynamics through deacetylation of Lys40 in the α-tubulin subunit, thereby coordinating cell motility [76]. Moreover, it also modulates the activity of a number of substrate proteins, including Hsp90, by means of post-translational modifications [78]. Recent studies have also demonstrated a key role of histone deacetylases in the development and progression of several types of cancers [79], associating HDAC6 overexpression with oncogenic transformation of cells and metastasis [80]. Few compounds inhibiting the HDAC enzymes have been marketed so far (*e.g.*, Belinostat, Panobinostat and Vorinostat), or are currently undergoing clinical trials for the treatment of different solid tumours and lymphoid malignancies [81-83]. It should be noted that the majority of approved HDAC inhibitors show low isoform selectivity. Moreover, it has also been demonstrated that HDAC inhibitors might exert limited tumor selectivity in cell-based contexts, *i.e.* the compounds block the activity of histone deacetylases of cancerous and normal cells in a similar manner, potentially resulting in severe side effects. For example, Chang *et al*. demonstrated that trichostatin A shows different activity towards normal, lung cancer, breast cancer and melanoma cell lines, such effect not being directly linked to HDAC isoform selectivity [84]. In addition, some HDAC inhibitors provided limited efficacy in clinical trials as single agents [27, 85-87], pointing the attention to the need for combination therapies (*e.g.*, ClinicalTrials ID: NCT04703920, NCT04326764 and NCT03742245). Interestingly, approved *pan*-HDAC inhibitors bear a hydroxamate group that can provide coordination to other metalloenzymes, thus resulting in unwanted toxicities as thrombocytopenia, neutropenia, anemia, fatigue, diarrhea and nausea, which should be taken into account during the trials [88]. In this regard, it is worth noting that selective HDAC6 inhibitors provide marginal effects as single therapeutic agents, and they usually present negligible side effects and an overall better tolerability with respect to *pan*-HDAC drugs [77]. More importantly, selective HDAC6 inhibitors have proved to enhance the efficacy of other antitumor agents. As a consequence, this protein represents an ideal candidate for the design of polypharmacological ligands or combination therapies, *e.g.,* with Hsp90 [36, 77, 89, 90]. From a biological point of view, HDAC6 and Hsp90 activities are intimately biochemically linked at different levels in both tumour and non-cancerous cells [52]. In particular, HDAC6 is one among the most studied *clients* of Hsp90 [52]. Moreover, HDAC6 has been found to reversibly regulate the level of acetylation of Hsp90, thus disrupting its chaperone function [29, 91, 92]. Recent investigations have also highlighted a mutual indirect involvement of HDAC6 and Hsp90 in the physiopathology of different types of cancer. For example, it has been demonstrated that HDAC6 regulates androgen receptor (AR) hypersensitivity in castrate-resistant prostate cancer, which is mainly mediated through Hsp90 acetylation/deacetylation [93]. In addition, a link between Hsp90 and HDAC6 activities has also been found in the transcriptional regulation of the mutant p53 (mutp53) oncogene-driver, which is an established hallmark of different types of cancer [94]. In particular, it has been shown that Hsp90 forms stable complexes with mutp53 in cancer cells, while preventing its degradation by MDM2 and CHIP E3 ligase activity, which results in malignant progression [94, 95]. Moreover, recent studies have also showed that HDAC6 is required by mutp53 to properly interact with the Hsp70 and Hsp90 chaperone machinery [95]. Notably, administration of the HDAC inhibitor SAHA is reported to decrease the half-life of mutp53, MDMX and MDM2. Moreover, it induces the disruption of the Hsp90–mutp53 complex, thus resulting in mutp53 degradation in cancer cells [96]. Interestingly, combined administration of SAHA and 17-AAG resulted in synergistic effects, with loss of cell viability in mutp53 cancer cells, such as MDA231 and T47D [96]. Moreover, the combined inhibition of Hsp90 and

HDAC6 can induce mutp53 degradation, thus promoting apoptosis of cancer cells and preventing T-cell lymphomagenesis [97]. A strong interplay between HDAC6 and Hsp90 activities has also been observed for leukaemia cancers, as recently discussed by Krämer *et al.* [52]. In particular, the authors extensively discussed the mutual involvement of these structurally different proteins on the activity of a wide range of cancer-relevant targets and signalling pathways (*e.g*., the Bcr-Abl and Flt3-Itd proteins, and Egfr–Ras–Raf–Mek–Erk-related signalling paths, respectively), demonstrating that the disruption of the Hsp90-HDAC6 interaction may be an approach of high therapeutic value for cancer treatment [52]. Remarkably, recent findings have also showed that acquired resistance towards structurally diverse Hsp90 inhibitors may derive by alterations in the levels of HDAC enzymes, which most likely occurs through an epigenetic mechanism [37]. Although no drug combinations or even polypharmacological ligands inhibiting these proteins have entered clinical trials, the utility of a combination of Hsp90 and HDAC inhibitors appears to be particularly promising. Recent studies reported that the inhibition of HDAC6 allows to overcome drug resistance to Hsp90 inhibitors [37]. In particular, it has been demonstrated that the treatment of leukaemia cells with HDAC6 inhibitors reduces drug resistance to 17-AAG and 17-DMAG [98], two clinically relevant ansamycin Hsp90 inhibitors [99-101]. Moreover, it has been reported that HDAC inhibitors (*e.g.,* FK228) disrupt chaperone function *via* acetylation of Hsp90 [102], and the combined inhibition of histone deacetylases with 17-AAG provide synergistic effects in various types of cancers [14, 98, 103-107], which is an aspect of particular interest in polypharmacology [108]. Considering that Hsp90 and HDAC6 participate in the regulation of multiple pathways across different cellular compartments and act on overlapping signalling networks in cancer cells [52, 76], their combined inhibition would allow the simultaneous modulation of extended tumour-related networks of proteins [36, 52, 109]. For all these reasons, the development of candidate drugs endowed with dual Hsp90 and HDAC6 inhibitory activity would provide several advantages compared to independent targeting of the two proteins or combination therapies. In particular, the ability of multi-target ligands to simultaneously modulate the activity of Hsp90 and HDAC6 is expected to provide improved therapeutic effects, with respect to the single target approaches, as observed from the use of combination of drugs. Certainly, the development of multi-target ligands of Hsp90 and HDAC6 should properly take into account potential side effects deriving by modulation of single targets, as early as possible in the design process. However, such compounds would also help circumventing potential higher toxicities and unexpected target activity deriving by the simultaneous use of multiple inhibitors, these considerations being in line with the polypharmacology concept [108].

2.2. Structure of HDAC6 and Difference Between Other Isoforms

As mentioned above, HDAC6 belongs to the type IIb class of the HDAC family. HDAC6 is a zinc-dependent enzyme where the metal acts as a cofactor in the process of peptide deacetylation. HDAC6 is the only isoform of HDACs that shows two separate catalytic domains, *i.e.* CD1 and CD2 (Fig. **1**) [20]. Although the two domains share a similar sequence, they present a marked difference in substrate specificity, the CD1 being active almost exclusively on C-terminal acetyllysine [23]. Indeed, the X-ray structures of HDAC6 CD1 and CD2 revealed key features driving domains selectivity [47]. One of the main differences among the two catalytic domains lies in residue K330 of zebrafish (z)CD1 (replaced by L712 in zCD2), which leads to a wider pocket, with respect to zCD2. Moreover, K330 acts as a "gatekeeper", establishing a hydrogen bond with the α-carboxylate group of exo-acetyllysine. From a therapeutic point of view, HDAC6 CD2 is by far the most important, this domain presenting *in vitro* deacetylase activity of tubulin, tau, and several other cytoplasmatic proteins [24, 110]. To date, several crystallographic structures of HDACs in complex with active ligands have been solved, and their comparison has driven the structure-based drug design (SBDD) of selective HDAC6 inhibitors (Table S1). Over many years, the crystallographic structure of HDAC6 from zebrafish was the only model available for crystallographic studies. Recently, human (h)CD2 of HDAC6 crystallographic structure was solved (PDB code: 5EDU) [24]. X-ray data revealed that the two species share a high degree of conservation and structural similarity in the CD2 domain, confirming zHDAC6 as a valid surrogate for human isoform studies. The general architecture of HDACi is composed of three fragments joined together: a zinc binding group (ZBG), a linker, and a cap group. The isoform selectivity is closely guided by the nature and geometry of the substructures defining the pharmacophore. Moving to the active site structure, the catalytic Zn(II) is placed at the bottom of a narrow crevice approximately 10 Å long (Fig. **2**), establishing a tetrahedral coordination complex with D649, H651, D742 (D612, H614, D705 for zCD2) and a water molecule. In addition, H610 and H611 (H573 and H574 in *Danio rerio*) stabilize the

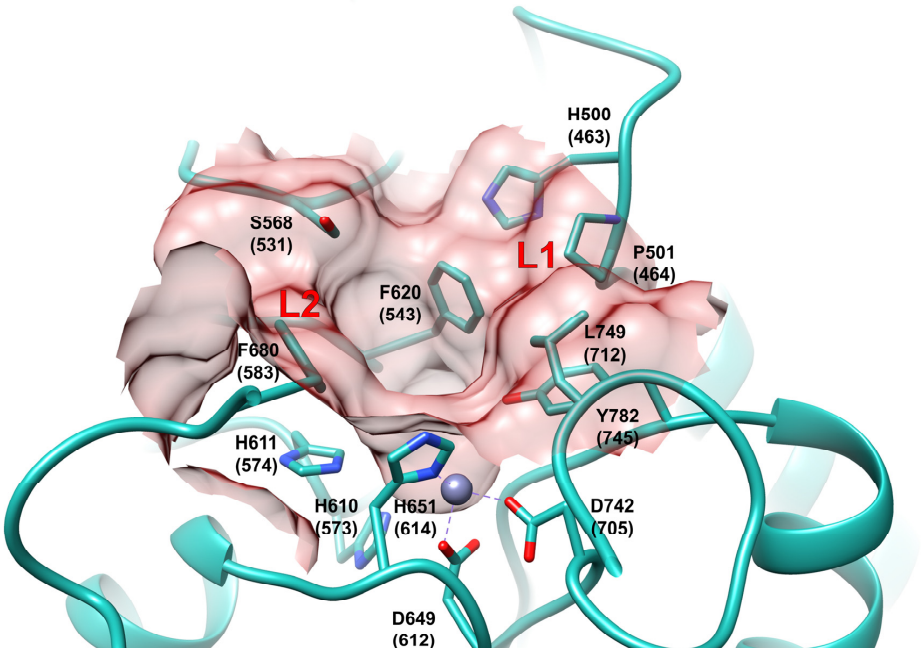


Fig. (2). Active site of human HDAC6-CD2 (PDB code: 5EDU). Crystallographic ligand was removed for convenience. Amino acid numbering for *Danio rerio* (zebrafish) HDAC6 is shown in brackets. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

water molecule, forming two hydrogen bonds, while Y782 (Y745 in zCD2) participates in the binding process by forming an additional H-bond interaction, *e.g.*, with the acetyllysine substrate. In particular, the tyrosine residue is conserved for class I and class IIb HDACs, while in IIa it is replaced by a histidine residue pointing outwards, which makes the crevice wider for this latter class of isoenzymes [111]. The linker group of HDAC6i binds between the two aromatic residues F620 and F680 (F543 and F583 in zCD2) that delineate the groove. The linker is fundamental to optimize interactions of the capping group with the protein residues and can be both a rigid aromatic or a more flexible aliphatic chain in HDAC6i, its length usually ranging between 3-7 Å. Finally, the rim of the crevice is formed by a sub-pocket lined by the L1 loop (D497-P501 in hCD2 and D460-P464 in zCD2), where HDAC6 inhibitors generally bind, and another shallow pocket defined by the L2 loop (G677−M682 and G640-N645 for human and zebrafish, respectively, Fig. **2**). According to thermodynamic studies, the binding of selective HDAC6 inhibitors is a process guided by the entropic contribution with the sub-pocket L1. Indeed, the L1 loop is relatively rigid and shifted around 1Å with respect to class I HDACs isoforms [112-114], which makes bulkier groups able to bind in this region without occurring in a conformational entropy loss while gaining a more favourable desolvation contribution. Thus, the analysis supports the importance of a capping group directed toward L1 sub-pocket in order to develop HDAC6 selective inhibitors. To date, few inhibitors interact with the L2 loop [47, 115-117], which are usually molecules with a bifurcated cap group that reaches both L1- and L2- sub-pockets. Interesting, the L2 sub-pocket is close to the "gatekeeper" residue S568 (S531 in zCD2), an amino acid peculiar to the HDAC6 isoform. The side chain of S568 is involved in acetyllysine catalysis [24], donating a hydrogen bond to the backbone NH group of the substrate. Likewise, S568 forms direct or water mediated hydrogen bonds with diverse ligands, such as ACY-1083, representing a key feature for the design of selective HDAC6 inhibitors.

2.3. The N-terminal Binding Pocket of Hsp90

Hsp90 belongs to a family of highly conserved proteins, with four isoforms classified in human cells: the inducible form Hsp90α and constitutive form Hsp90β are present in the cytosol, Grp94 in the endoplasmic reticulum, and Trap1 in the mitochondria. The Hsp90 chaperones associate in the cytoplasm forming flexible homodimers, each monomer being composed of three structurally and functionally domains: the N-terminal domain (NTD) associated with nucleotide binding and ATPase activity, the middle domain (MD) involved in the binding of client proteins and co-chaperons, and the C-terminal domain (CTD) fundamental for dimerization and co-chaperone binding. In the apo-state, Hsp90 assumes an open V-shape conformation predominantly. ATP binding to the pocket of the NTD triggers

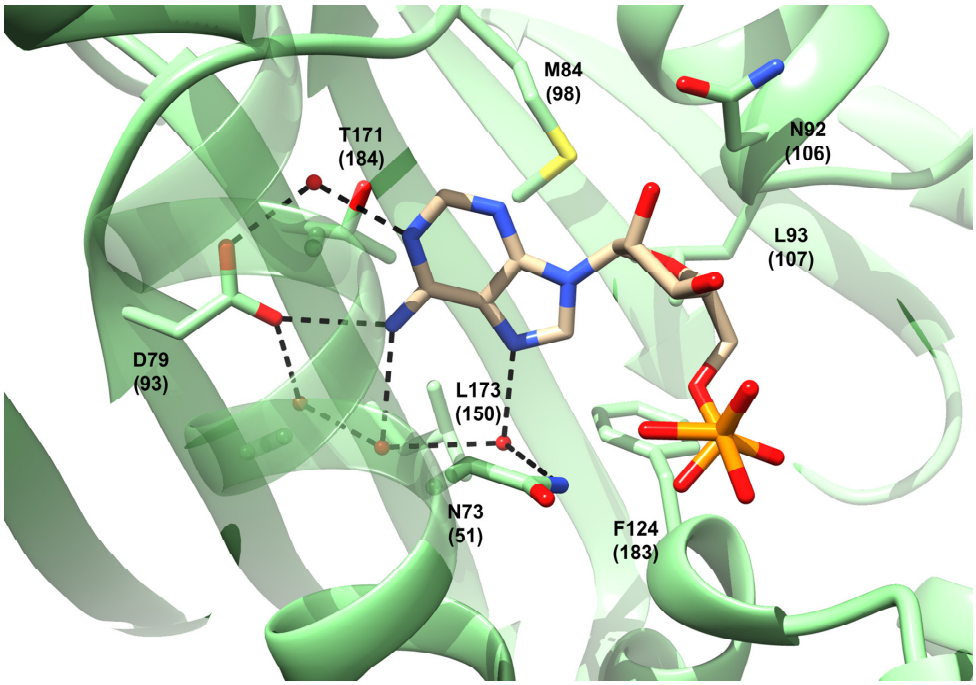


Fig. (3). The nucleotide binding pocket of yeast Hsp90 N-terminal domain in complex with ADP (PDB code:1AMW) [131]. Amino acid numbering for human Hsp90 is shown in brackets. Hydrogen bonds are highlighted with black dashed lines. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

Hsp90´s cycle, where large-scale conformational changes lead to a so-called "close" configuration in which NTD domains are dimerized and associated with the MD. During this process, Hsp90 explores two different intermediate states. Eventually, the cycle ends after the hydrolysis of ATP, the two NTD domains dissociate, ADP and Pi are released, bringing the dimer to its initial open conformation. In the last two decades, numerous crystallographic structures of Hsp90 have been obtained to investigate its flexibility and mechanistic cycle. In particular, the study of nucleotide binding pocket on N-terminal domain is crucial to design ATP-competitive inhibitors. Hsp90α and Hsp90β belong to the GHL ATPase family and presents a β-α-β ATP binding site [118-120]. The pocket consists of four motifs: Motif I and III interact with the phosphate group, Motif II and IV bind the adenine. Notably, Hsp90 creates a network of direct and water-mediated hydrogen bonds with the purine ring (Fig. **3**) [121]. The NH_2 group of adenine establishes a direct H-bond with the D93 side chain and a water molecule. The N7 atom forms a water mediated hydrogen bond with the N51 side chain, whereas N1 with an additional water bridge to D93. The shell of waters surrounding the adenosine binding site has a key role in ligand binding, therefore, it is important to be considered in the design of ATP-competitive inhibitors [122]. Indeed, the crystallographic structures of Hsp90 in complex with inhibitors generally show water-mediated interaction with adenine sub-pocket. On the opposite side the cavity opens, exposing the N3 and N9 of the adenine ring to solvent. As already mentioned, Hsp90 is highly flexible, which also affects the plasticity of ATP-binding site (Fig. **4a**) [123]. In particular, residues 104-111 on the α-helix3 can adopt a "loop-in" or "loop-out" conformations [124]. This conformational rearrangement, which is mainly driven by the shift of L107, results in the creation of a hydrophobic cavity (Fig. **4b**). Several studies reported inhibitors able to occupy the sub-pocket created by the rearrangement of residues 104-111, from the "loop-in" conformation into a helix shape [125-127]. Table **1** contains the PDB structures of N-term Hsp90, extracted from the RCSB Protein Data Bank [128] and classified according to the α-helix3 conformation (for details, see Supplementary Information). X-ray structures also demonstrate that the loop-in conformation is predominant for the apo N-term Hsp90, suggesting that the entropy gained by alteration of the protein conformation drives the thermodynamic binding profile for inhibitors occupying the α-helix3 region. Indeed, Amaral *et al*. [129] have shown that the loop-in binding compounds benefit mainly from the enthalpy contribution, while inhibitors inducing the helix conformation are favoured by entropy contributions. Another aspect to be considered for the design of Hsp90 inhibitors is the hydrophobic nature of the cavity adjacent to the adenine binding region. In particular, SAR studies have indicated that aromatic groups, such as substituted

Table 1. Hsp90 crystal structures classified according to the structural arrangements of the α-helix3 conformation.

Conformation	Cluster	Hsp90 complexes (PDB code _ Chain)
Helix	I	1UY6_A, 1UY7_A, 1UY8_A, 1UY9_A, 1UYC_A, 1UYD_A, 1UYE_A, 1UYF_A, 1UYG_A, 1UYH_A, 1UYI_A, 1UYK_A, 2FWY_A, 2FWZ_A, 2H55_A, 2QG2_A, 2WI4_A, 2WI7_A, 2XDS_A, 2XDU_A, 2YE6_A, 2YE7_A, 2YE8_A, 2YEE_A, 2YEI_A, 2YEJ_A, 2YJX_A, 2YK2_A, 2YK9_A, 2YKB_A, 2YKC_A, 2YKE_A, 2YKI_A, 2YKJ_A, 3B25_A, 3D0B_A, 3FT8_A, 3HYY_A, 3HZ1_A, 3HZ5_A, 3INW_A, 3INX_A, 3MNR_P, 3QDD_A, 3QTF_A, 3R91_A, 3R92_A, 3RKZ_A, 3WQ9_A, 4CWF_A, 4CWN_A, 4CWO_A, 4CWP_A, 4CWQ_A, 4CWR_A, 4CWS_A, 4CWT_A, 4EFT_A, 4EFU_A, 4HY6_A, 4L8Z_A, 4L90_A, 4L91_A, 4LWE_A, 4NH7_A, 4NH7_B, 4NH8_A, 4O0B_A, 4O04_A, 4O05_A, 4O07_A, 4O09_A, 4R3M_A, 4U93_A, 4XIP_A, 4XIQ_A, 4XIR_A, 4XIT_A, 5CF0_A, 5FNC_A, 5FNF_A, 5GGZ_B, 5GGZ_C, 5J9X_A, 5J20_A, 5J27_A, 5J82_A, 5J86_A, 5LNY_A, 5LNZ_A, 5LO0_A, 5LO1_A, 5LO5_A, 5LO6_A, 5LQ9_A, 5LR1_A, 5LR7_A, 5LRL_A, 5LRZ_A, 5LS1_A, 5NYH_A, 5OCI_A, 5OD7_A, 5ODX_A, 5T21_A, 5XQE_A, 5XR5_A, 5XR9_A, 5XRB_A, 5XRE_A, 5ZR3_A, 5ZR3_C, 5ZR3_E, 5ZR3_G, 6EI5_A, 6EL5_A, 6ELP_A, 6EY8_A, 6EY9_A, 6EYB_A, 6N8X_A, 6OLX_A, 6U9A_A, 6U9B_A, 6U98_A, 6U99_A, 4BQG_A, 3O0I_A, 6LR9_A, 6LTK_A, 7LSZ_A, 7LT0_A
Loop-out	II	4AWO_A, 4AWO_B, 4AWP_A, 4AWP_B, 4AWQ_A, 4AWQ_B
	III	5J8M_B, 5J8U_B
Loop-in	IV	1UYL_A, 1YER_A, 2BSM_A, 2BT0_A, 2CCS_A, 2CCT_A, 2CCU_A, 2JJC_A, 2QFO_B, 2UWD_A, 2VCI_A, 2VCJ_A, 2WI1_A, 2WI2_A, 2WI3_A, 2WI5_A, 2XAB_A, 2XDL_A, 2YE2_A, 2YE3_A, 2YE4_A, 2YE5_A, 2YE9_A, 2YEA_A, 2YEB_A, 2YEC_A, 2YED_A, 2YEG_A, 2YEH_A, 2YI0_A, 2YI6_A, 2YI7_A, 2YJW_A, 3B24_A, 3B26_B, 3BM9_A, 3FT5_A, 3HHU_A, 3K99_A, 3K99_D, 3OWB_A, 3T0H_A, 4EEH_A, 4EGH_A, 4FCP_A, 4FCQ_A, 4L93_B, 4L94_A, 4LWF_A, 4LWG_A, 4LWH_A, 4LWI_A, 4YKQ_A, 4YKR_A, 4YKT_A, 4YKU_A, 4YKX_A, 4YKY_A, 4YKZ_A, 5FND_A, 5J2V_A, 5J2X_A, 5J8M_A, 5J8U_A, 5J64_A, 5J80_A, 5M4E_A, 5M4H_A, 5NYI_A, 5VYY_A, 5XQD_A, 5XRD_A, 6B9A_B, 6CEO_A, 6CYG_B, 6CYH_B, 6ELN_A, 6ELO_A, 6F1N_A, 6FCJ_A, 6GP4_A, 6GPW_A, 6GR4_A, 6HHR_A, 6LSZ_A, 6LTI_A, 6TN4_A, 6TN5_A
	V	1OSF_A, 1YET_A, 2BYH_A, 2BYI_A, 2XHT_A, 2XHX_A, 2XJG_A, 2XJJ_A, 3B27_A, 3EKO_A, 3EKR_A, 3K97_A, 3K98_A, 3K98_B, 3K99_B, 3R4M_A, 3R4N_A, 3R4P_A, 3RLP_A, 3RLP_B, 3RLQ_A, 3RLR_A, 3T2S_A, 3VHA_A, 4B7P_A, 4JQL_A, 5GGZ_A, 5GGZ_D, 6LT8_A, 2K5B_A*, 1BYQ_A, 1YC3_A, 1YES_A, 2XDK_A, 2XDX_A, 2XJJ_B, 2XK2_A, 2YEF_A, 3EKO_B, 3R4P_B, 3T0Z_A, 3T1K_A, 3T1K_B, 3T2S_B, 3T10_A, 3VHC_A, 6GP8_A, 6GPF_A, 6GPH_A, 6GPO_A, 6GPP_A, 6GPR_A, 6GPT_A, 6GPY_A, 6GQ6_A, 6GQR_A, 6GQS_A, 6GQU_A, 6GR1_A, 6GR3_A, 6GR5_A, 2XJX_A, 3K99_C, 3R4O_A, 3R4O_B, 3VHD_A, 4EGI_A, 4EGK_A, 4FCR_A, 4YKW_A, 4YKW_B, 3VHD_B, 4BQJ_A
	VI	1YC1_A, 1YC4_A, 2BT0_B, 2BZ5_A, 2QFO_A, 2QG0_A, 2QG0_B, 2WI2_B, 2XAB_B, 2YEG_B, 2YI5_A, 3B24_B, 3B26_A, 3B28_A, 3HHU_B, 3HYZ_B, 3OW6_A, 3TUH_A, 3TUH_B, 3WHA_A, 4FCP_B, 4L93_A, 4W7T_A, 6B9A_A, 6B99_A, 6CYG_A, 6CYH_A
	VII	2QF6_A, 2QF6_B, 2QF6_C, 2QF6_D, 2WI6_A, 2XHR_A, 3B28_B, 3BMY_A, 3HYZ_A, 3WHA_B, 5J6L_A, 6EYA_A
	VIII	3EKR_B, 3HEK_A, 3HEK_B, 3OWD_A, 3R4N_B, 3RLQ_B, 3RLR_B
	IX	5J6M_A, 5J6N_A
	X	2BZ5_B

phenyls or benzofurans, forming π-π interactions with F138 generally favour the binding to the protein (Fig. **5**). Recently, Schuetz *et al*. combined experimental thermodynamic data and molecular dynamics simulations to define the binding kinetics of several classes of compounds [130]. These results showed that the presence of polar groups in the fragments that interact with the hydrophobic cavity generates a high enthalpic barrier due to the desolvation energy upon ligand binding. This results in a k_{on} slowdown of polar ligands and a great impact on the global association kinetics. Taken together, the information reported in this paragraph highlights key points to be considered for the design and development of potent N-Hsp90 inhibitors.

2.4. Features Required for HDAC6 Inhibition

Pan-HDACi showed important clinical benefits in some cancers, even though they displayed several side effects, which may be ascribed to their poor selectivity [133]. In 2006, the approval of SAHA made HDAC inhibitors debut in the anticancer drug arsenal, despite

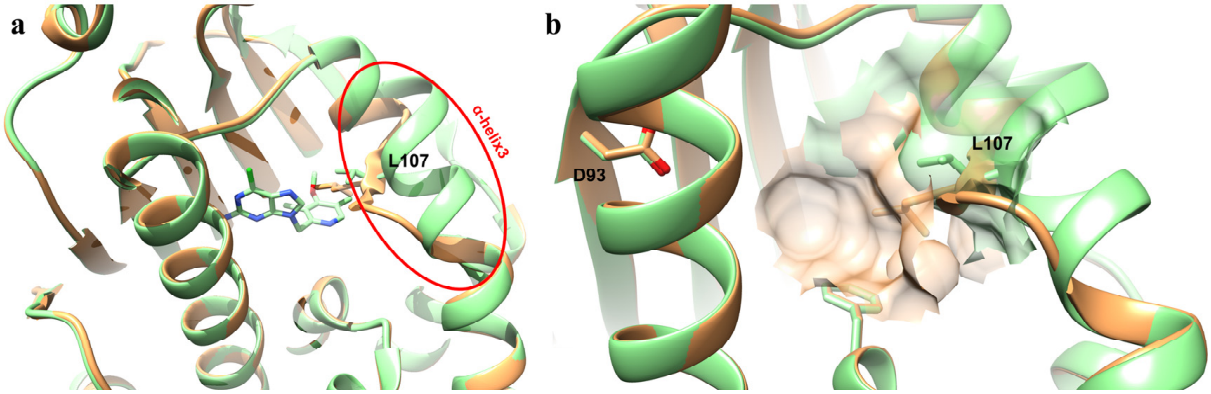


Fig. (4). Comparison between "loop-in" (orange, PDB code: 1UYL) [126] and helix conformation (light green, PDB code: 3QDD)[132] of N-term Hsp90α. Panel (**a**) shows the shift of alpha helix3 to the loop-in conformation. Panel (**b**) shows the difference in the surface area upon different conformational arrangements of L107. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

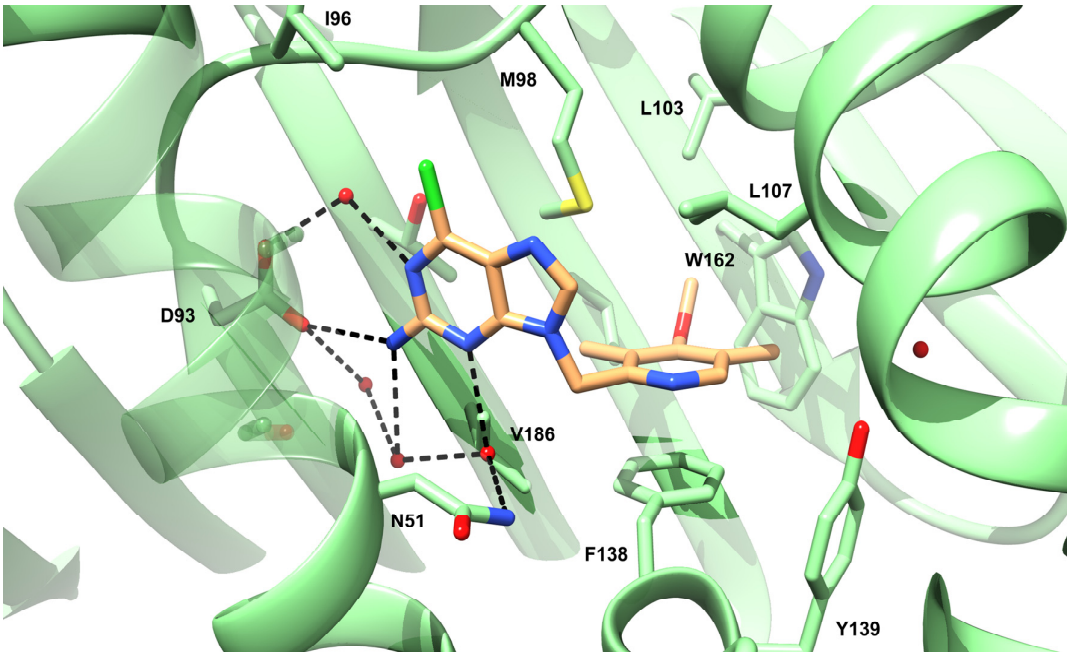


Fig. (5). Close up view of the ATP binding pocket of the human Hsp90 N-terminal domain in complex with BIIB021 (PDB code:3QDD) [132]. Hydrogen bonds are highlighted with dashed lines. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

issues related to low potency, cardiovascular concerns, and drug-drug interactions. The main structural framework required for inhibition of HDACs has been derived by means of extensive analyses on a multitude of HDAC inhibitors (Fig. **6a**). Indeed, all the reported HDAC inhibitors share the same structural motif including (i) a chelating group called zinc binding group (ZBG), which forms a complex with the catalytic Zn^{2+}; (ii) an hydrophobic spacer that fits into the catalytic tunnel of the active site, and; (iii) an external cap group that establishes different networks of interaction with the residues lining the binding site of the different HDAC, this portion of the molecules being often used for tuning isoform selectivity (Fig. **6b**) [1]. Due to the important role of HDAC6 in different biological pathways, the development of selective HDAC6 inhibitors is an urgent objective [134]. In order to design selective HDAC6 inhibitors, the pharmacophore model group is a crucial point to be considered, while the isoform affinity and selectivity are strictly correlated with the interaction between these three parts and the active site of HDAC6 [135, 136]. Indeed, the modification of the cap, the variation of the nature of the linker or the ZBG are usual strategies to improve selectivity, thanks to the unique three-dimensional structure of the HDAC6 binding cleft. Here, we summarize and describe the

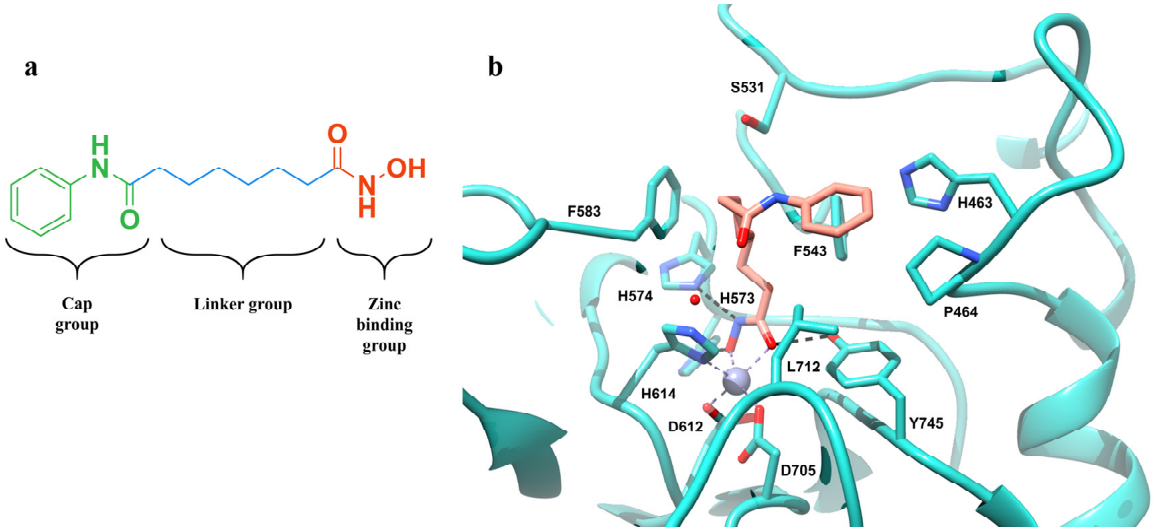


Fig. (6). Panel (**a**) shows the *pan*-HDAC inhibitor SAHA is divided into three representative pharmacophoric groups: cap group (green), linker group (sky blue), ZBG (red). Panel (**b**) shows HDAC6-CD2 in complex with SAHA (PDB code: 5EEI)[24]. Metal coordination and hydrogen bond interactions are indicated by dashed grey and dashed black lines, respectively. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

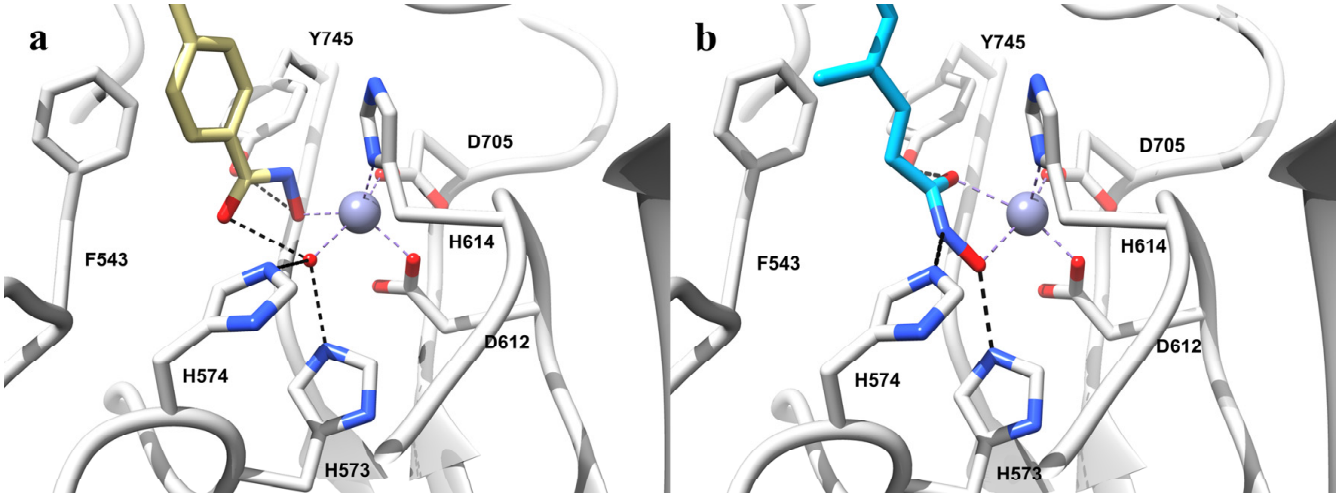


Fig. (7). Crystallographic representation of monodentate (Panel (**a**), PDB code: 6CW8)[111] and bidentate (Panel (**b**), PDB code: 5EEK) [24] zinc-binding coordination complex of hydroxamate derivates with HDAC6. Metal coordination and hydrogen bond interactions are indicated by dashed grey and dashed black lines, respectively. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

most common scaffolds employed for developing selective HDAC6 inhibitors. Among the reported ZBGs, hydroxamic acid is the most widely implemented moiety for the development of HDAC inhibitors. Indeed, currently marketed *pan*-HDAC inhibitors bear such a type of ZBG. Hydroxamic function is a strong Zn(II) binder that provides potent *pan*-HDAC inhibition [1]. Hydroxamic acids alone do not provide any isoform selectivity; the latter can be obtained through the use of the appropriate linkers and cap groups. X-ray studies on the different HDAC isoforms highlighted two hydroxamate-Zn^{2+} binding geometries. The canonical hydroxamate-zinc coordination geometry involves bidentate zinc chelation with C=O and N-O^-. H573 and H574 form hydrogen bonds with the deprotonated oxygen and hydroxamate NH. The carbonyl accepts an additional hydrogen bond from Y745 (Fig. **7a**). This bidentate binding mode seems preferred for flexible aliphatic linkers, such as Trichostatin A (TSA) and Ricolinostat. The alternative monodentate zinc chelation geometry is usually observed for hydroxamate attached to bulky aromatic linkers, such as phenylhydroxamates. In monodentate geometry the hydroxamate N-O^- coordinates the Zn^{+2} while the carbonyl oxygen accepts a H-bond from the zinc-bound water molecule, which is not displaced by the inhibitor and remains hydrogen bond-

ed to H573 and H574 (Fig. **7b**). The energy difference between monodentate and bidentate coordination has been studied on *pan*-HDAC inhibitor TSA (R-stereoisomer) (Fig. **8**), that showed both types of hydroxamate-Zn^{2+} coordination in available crystal structures. Bidentate geometry resulted in being only 0.5 kcal/mol favourite with respect to the monodentate [137]. Generally, alkyl linkers appear to be non-selective, while bulky aromatic and aliphatic rings show isoform selectivity toward HDAC6 and HDAC8, depending on the appropriate cap group. As for the hydroxamate moiety, genotoxicity and mutagenicity issues derived from the Lossen rearrangement must be taken into account. Therefore, alternative ZBGs have been designed; among these, 2-mercaptoacetamide and trifluoromethyl oxadiazole have yielded inhibitors modestly selective towards HDAC6. The mercaptoacetamide derivate **1** (Fig. **8**) reported by Porter *et al.*, showed an IC_{50} of 50 nM against HDAC6 with a 240-fold selectivity against HDAC8 [138]. In 2015 139. Segretti *et al.* reported selective and potent 2-mercaptoacetamides analogues as HDAC6 inhibitors, showing good pharmacological and drug-like properties [139]. In a different study, Porter *et al.* presented the X-ray crystal structure of the complex between the *N*-(5-(5,6-dichloro-1*H*-indol-1-yl)pentyl)-2-mercaptoacetamide **2**, (Fig. **8**) and HDAC6 catalytic domain from *Danio rerio* (zebrafish) to clarify the interaction with the Zn^{2+} [138]. The thiol group of the 2-mercaptoacetamide derivatives is probably mainly found as a negatively charged thiolate and coordinates the active site Zn^{2+} ion; also, the carbonyl oxygen accepts a hydrogen bond from the phenolic hydroxyl group of Y745 and the -NH group donates a hydrogen bond to the Nε of H574 **2**, (Fig. **9**). In our previous work, we studied dual HDAC6-Hsp90 inhibitors endowed with unique 5-(trifluoromethyl)-1,2,4-oxadiazole as ZBG, which uses the fluorine and oxygen atoms of trifluoromethyloxadiazole to bind zinc *via* weak electrostatic interactions instead of direct coordination as observed in hydroxamate derivatives **3**, (Fig. **9**) [43, 140]. Selective HDAC6 inhibitors with five-member heterocycles, such as oxazole, dihydroisoxazole, thiazole and oxadiazole were also explored. Compared with benzylhydroxamate, five-membered heterocyclic linkers alter the angle between the ZBG and the cap group, leading to a different orientation of the molecule in the binding pocket (Fig. **10**) [141]. Although oxazole and oxadiazole derivatives tend to be more active on HDAC6 than on other isoforms, selectivity is susceptible to changes of the cap group. For example, in 2016, Senger *et al.* described a series of hydroxamates-based HDAC6 inhibitors bearing five member heterocycles as a linker, proving that oxazole-based compounds displayed the best HDAC6 activity and selectivity compared to thiazole and oxadiazole derivatives **4**, (Fig. **8**) [142]. Moreover, Shen *et al.* [135] investigated isoxazole derivatives and developed SS-208 (Fig. **8**), which displayed an IC_{50} of 12 nM against HDAC6 and more than 100-fold selectivity over other isoforms. The isoxazole ring is well-known in drug design due to the non-covalent interactions, including hydrogen bonding (N and O as acceptors) or π–π stacking. As expected, the isoxazole ring in SS-208 established van der Waals interaction with residues F583, F643 and L712 in the hydrophobic channel of HDAC6 [135, 143]. Another important linker for HDAC6 selectivity is the ethylthiazolyl described by Nam *et al.* [144]. The most selective compound **5**, (Fig. **8**) showed IC_{50} of 43 nM on HDAC6 and 126-fold selectivity on HDAC1. Noteworthy, the analogue lacking the double bond on the fluorophenylethene cap group is ten-fold less selective, demonstrating the significant contribution of the cap group on selectivity. Above all, a hallmark of the CD2 active site in HDAC6 is the residue S531 located on the rim of the pocket; this amino acid is not present in any other HDAC isoform. Thus, inhibitors able to interact with S531 either directly or through water-mediated hydrogen bonds generally show isozyme selectivity. Ricolinostat and Citarinostat (Fig. **8**) are two selective HDAC6 inhibitors, currently in clinical trials, that displayed activity at low nanomolar concentration and more than 10-fold selectivity over other isoforms [134]. Both inhibitors hold hydroxamic acid as ZBG and are endowed with a six-methylene aliphatic linker. Crystallographic structures of Ricolinostat in complex with HDAC6 CD2 revealed water-mediated hydrogen bond between the amide carbonyl of the inhibitor capping group and S531 (Fig. **11a**). Another interaction with S531 can be observed in the complex between HDAC6 and the compound ACY-1083, which showed 260-fold selectivity towards HDAC6 *vs* other HDACs. Here, the hydroxyl group of S531 accepts a hydrogen bond from the secondary amino group on the linker motif of the inhibitor (Fig. **11b**). Finally, the appropriate cap group contributes significantly to the potency and affinity of HDAC6 inhibitors. The cap motif is the outermost and solvent-exposed portion of HDAC inhibitors, interacting with the edge of the binding pocket, a promising region for achieving isoform selectivity due to the large structural differences among HDACs. Regarding HDAC6, it is known that inhibitors preferentially position the cap against the L1 loop, which is slightly shifted with

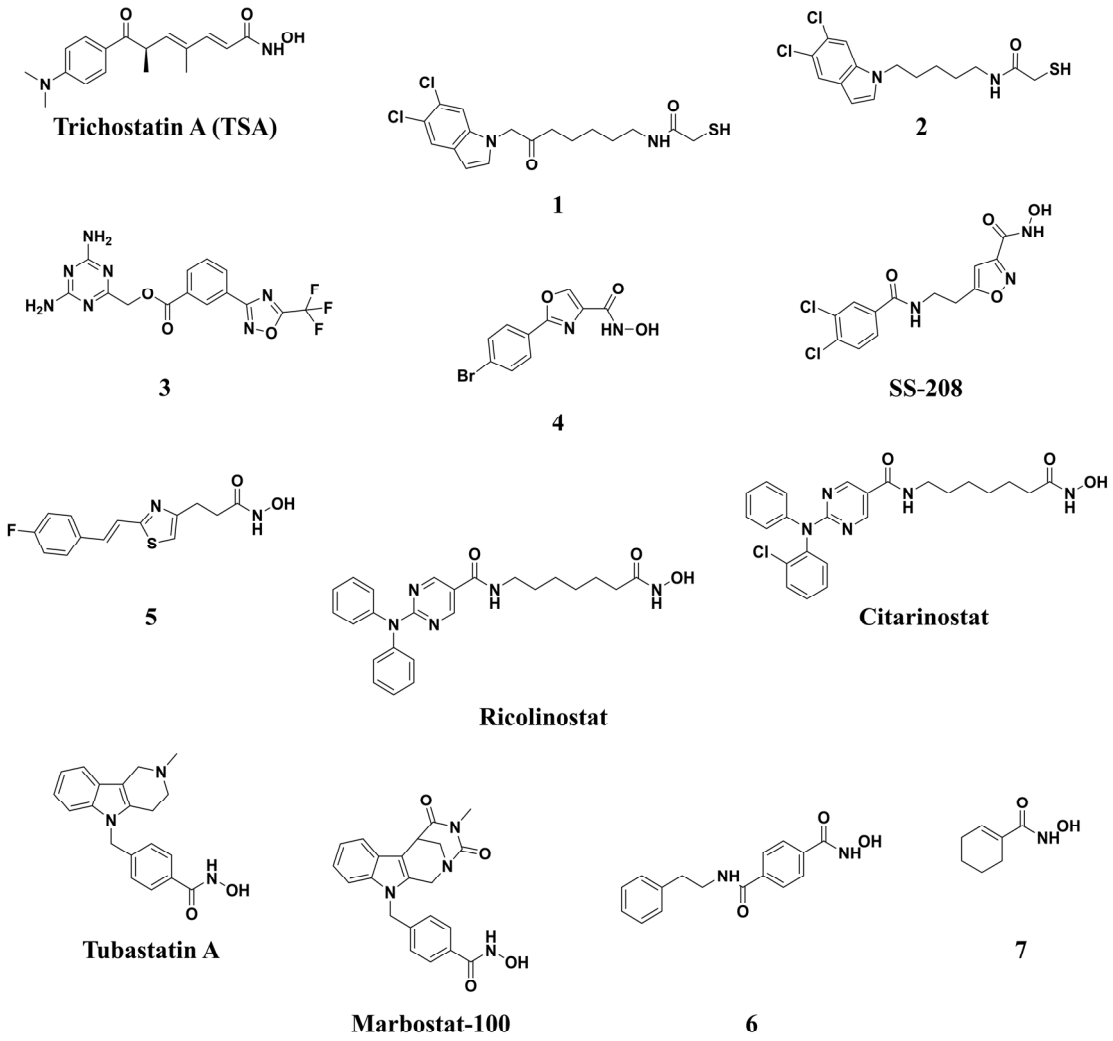
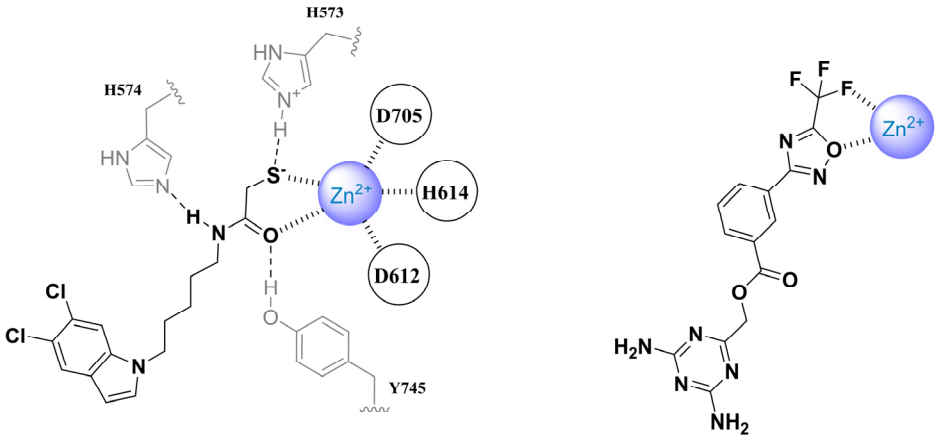


Fig. (8). Chemical structures of representative selective and nonselective HDAC6 inhibitors. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

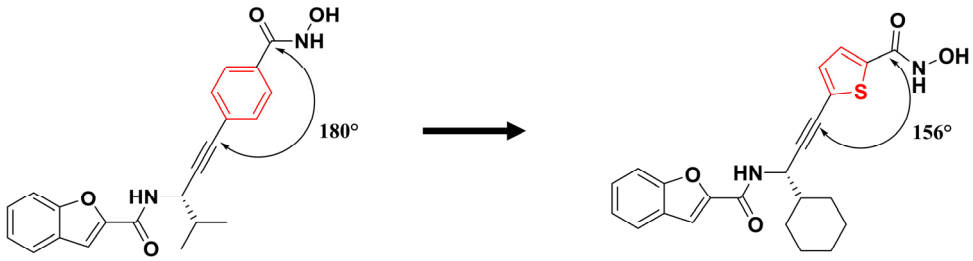
respect to class I HDACs (Fig. **12**). The structures of HDAC1, HDAC2 and HDAC3 show that the L1 loop adopts a different conformation, narrowing the binding site compared to HDAC6. Consequently, bulky cap groups usually present disadvantages in binding to class I HDACs due to steric clashes with the L1 loop. Ligands with large and rigid cap groups associated with short alkyl or aromatic linkers were used to design selective inhibitors of HDAC6. In 2010, Butler *et al.* described Tubastatin A (Fig. **8**), a phenylhydroxamate derivative with a tetrahydro-*γ*-carboline- cap group. Tubastatin A showed nanomolar activity and high selectivity with respect to other isozymes (IC_{50} = 15 nM, > 1000-fold selectivity over other HDACs, 57-fold over HDAC8) [145]. X-ray crystallography studies of the complex between Tubastatin A and HDAC6 clarified the main interactions responsible for activity and selectivity: the carboline group of the cap is able to interact with a hydrophobic region of L1 loop pocket defined by H463, P464, F583, and L712 side chains. Starting from Tubastatin A, several analogues have been developed, such as Marbostat-100 (Fig. **8**), which displayed superior potency toward HDAC6 (K*i* = 0.7 nM, more than 500-fold selectivity over HDAC1) [146]. Sellmer *et al.* extensively studied the SAR of Marbostat-100, demonstrating that its bulky cap group favourably occupied an extended hydrophobic region, establishing van der Waals interactions with the surface residues [146]. Interestingly, para-substituted benzohydroxamic acid derivatives are generally more selective compared to meta-substituted compounds. Olson *et al.* reported a series of meta-substituted benzohydroxamic acids inhibiting HDAC6 and HDAC8 in the low nM range. It was found that the para-substituted analogue **6** (Fig. **8**) was 9-fold more active on HDAC6, 150-fold selective over HDAC1, 300-fold selective over



2
2-mercaptoacetamide

3
5-(trifluoromethyl)-1,2,4-oxadiazole

Fig. (9). Schematic representation of the binding mode of HDAC6-CD2 inhibitors, bearing the mercaptoacetamide-based (2, left) and trifluoromethyloxadiazolyl-based (3, right) zinc binding groups. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*



8
IC_{50} HDAC6 15.6 nM

9
IC_{50} HDAC6 11 nM

Fig. (10). Difference between the angles of phenyl-based linkers and five-membered heteroaryl-based linkers of hydroxamic derivatives. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

HDAC8 and inactive on HDAC4 compared to the meta-substituted compound [147]. Another series of benzohydroxamic acids identified by Rodrigues *et al.* highlighted that shifting substituents from the *meta* to the *para* position can lead to a 100-fold increase of HDAC6 inhibitory activity, while maintaining selectivity over HDAC1 and HDAC2 [148]. However, Wagner *et al.* identified a series of cap-less hydroxamic acids with cyclic linkers that demonstrated high potency and HDAC6 selectivity [149]. The most active and selective compound of this series is the cyclohexene hydroxamic acid **7**, (Fig. **8**), with an IC_{50} of 12 nM on HDAC6 and 88-fold, 760-fold and 36-fold selectivity with respect to HDAC2, HDAC4 and HDAC8, respectively. These results evidenced that strong and selective inhibition of HDAC6 can be achieved by using a proper combination of linker and ZBG, without the need of bulky cap groups.

2.5. Current Scaffolds of Hsp90 Inhibitors

Over the last decade, a number of Hsp90 inhibitors have been identified, some of which entered clinical trials [150, 151]. Indeed, the search for Hsp90 inhibitors has deployed a massive amount of resources, resulting in vast documentation [64, 152] and the identification of ligands bearing peculiar molecular scaffolds. To date, human Hsp90α (UniProt ID: P07900) counts around 2500 tested molecules in ChEMBL [153] (https://www.ebi.ac.uk/chembl/, accessed on April 1st, 2021) and more than 300 crystallographic complexes (Table S2) in PDB (https://www.rcsb.org/, accessed on April 1st, 2021). Although 22 compounds (Fig. **13**) entered clinical trials or are currently under clinical evaluation as Hsp90 inhibitors [151], none of them has yet been approved either in combination or alone for the treatment of any types of cancer. However, there have recently been significant developments

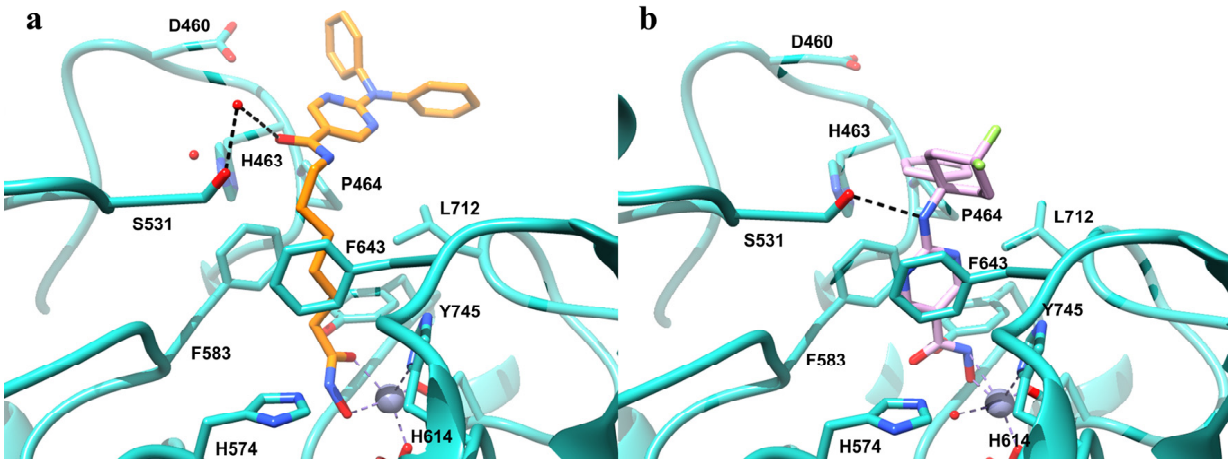


Fig. (11). Crystallographic complexes of HDAC6 CD2 with Ricolinostat (Panel (**a**), PDB code: 5WGL) and ACY-1083 (Panel (**b**), PDB code: 5WGM). Metal coordination and hydrogen bond interactions are indicated by dashed grey and dashed black lines, respectively. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

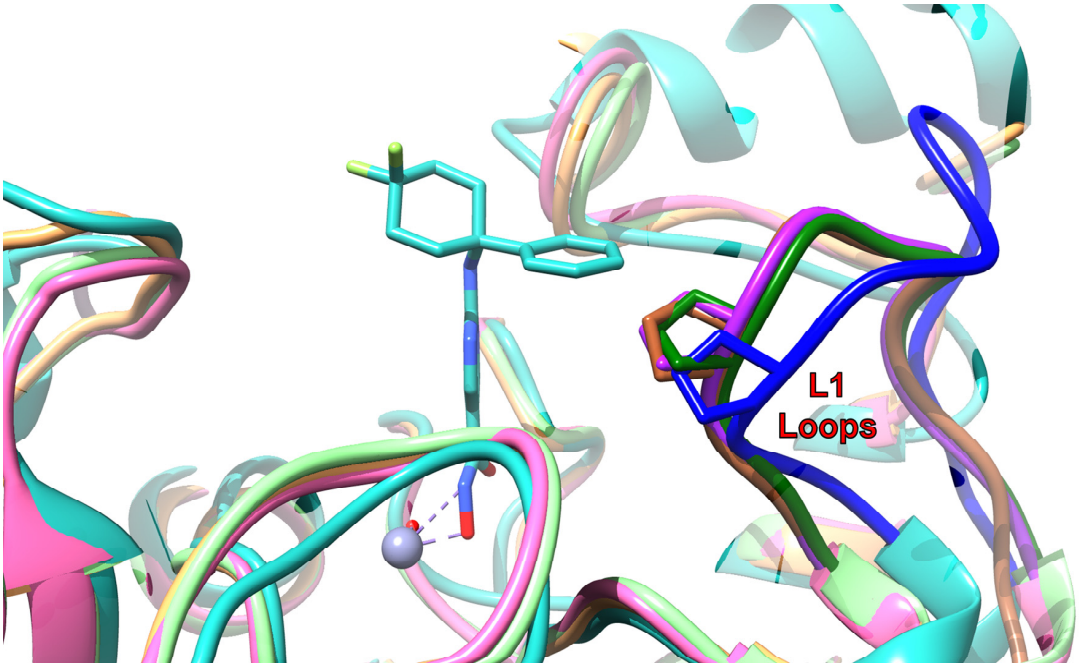


Fig. (12). Superposition of HDAC6 CD2 (light blue) in complex with ACY-1083 (PDB code: 5WGM), HDAC1 (pink, PDB code: 4BKX), HDAC2 (light green, PDB code: 4LY1), HDAC3 (orange, PDB code: 4A69). L1 loops of HDAC1, HDAC2 and HDAC3 are colored in purple, green and sienna, respectively. HDAC6 L1 loop (blue) is shifted around 1 Å with respect to class I HDACs. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

relating to clinical trials of Hsp90 inhibitors. Pimitespib (TAS-116), which very recently completed a phase III study for the treatment of advanced drug-resistant gastrointestinal stromal tumour (GIST), with promising outcomes [154]. Pimitespib benefits from high selectivity towards Hsp90α and β compared to the homologous Hsp90 family members GRP94 and TRAP1. The limited number of off-targets gives TAS-116 a successful safety profile and strong anticancer activity [155]. Moreover, a different strategy involves the use of Hsp90 inhibitors for clinical imaging of certain tumours. HS-196 and HS-201 are two ATP-competitive inhibitors of Hsp90 connected through a linker with a contrast and photosensitizing agent (verteporfin), respectively, that can be used for tumor imaging. Through overexpression of Hsp90 in tumor tissues, these molecules accumulate in malignant cells, thus enabling tumor imaging or selective photodynamic therapy [156]. HS-196 (ClinicalTrials ID: NCT03333031) and HS-201 (ClinicalTrials ID:

NCT03906643) recently started a phase I clinical trial for the treatment of solid tumors. However, the majority of Hsp90 inhibitors belong to a restricted number of scaffolds that bind to key residues of the protein, each of them being extensively studied through experimental SAR analyses. Fig. (**14**) shows a summary of the most proficient fragments that can be observed in Hsp90 inhibitors, with reported X-ray structure. Their scaffolds, mimicking the adenine interactions of the substrate, confer potency and selectivity to the inhibitors. Indeed, inhibition in the low nanomolar range has been achieved by many series of compounds thanks to extensive medicinal chemistry optimization efforts. The resorcinol-based compound AT13387 [157] was one among the first Hsp90 inhibitors derived by structural optimization of radicicol, which has been isolated from *Monosporium bonorden* [158]. Resorcinol is considered one of the most important pharmacophoric groups among Hsp90 inhibitors, especially in tandem with the isopropyl moiety, which significantly increases potency and affinity. Starting from AT13387, several research groups designed and synthesized Hsp90 inhibitors by maintaining the resorcinol scaffold and modifying the isoindoline moiety with various heterocyclic moieties [159-167]. The mechanism of action observed for resorcinol-based inhibitors derives from competition with ATP at the N-terminal domain of Hsp90 (Fig. **15a**). Purine and purine-like analogues were also developed with the help of crystallographic structural information [168]. Their similarity with ATP is clearly the key for their inhibition mechanism: the purine moiety acts as a competitive inhibitor at the binding site of ATP placed in the N-terminal domain. SAR of adenine-based ligands have been deeply studies and several derivatives entered clinical trials [126, 127, 169-171]. The 8-aryl adenine compounds maintain the same interaction of ATP, while the aryl ring binds into the hydrophobic cavity behind α-helix3 (Fig. **15b**). The N3 atom of the purine ring is shifted in imidazopyrazines-base compounds, although this scaffold maintains the same binding mode of adenine (Fig. **15c**) and the nitrogen is not involved in relevant interactions with the protein. The class of 2-amino purines was obtained by shifting the adenine -NH_2 group from the 6- to the 2-position. X-ray structures showed a different binding mode, with the purine ring flipped by 180° (Fig. **7d**). In order to preserve inhibitory activity, the aryl substituent must be placed at position 9-, suggesting the importance of a specific structural arrangement between the -NH_2 and the aryl ring of the hydrophobic pocket. Moreover, SAR studies on 2-amino purine derivatives showed a beneficial substitution of the nitrogen N7 with a carbon atom, while position 7- could be substituted with different groups to provide compounds with low nanomolar IC_{50} values. A variety of Hsp90 inhibitor classes mimic the adenine structure to ensure interaction in the ATP sub-pocket. For example, 2-amino purine, together with 5,6,7,8-tetrahydroquinazolin-2-amine, quinazoline, aminopyrimidine, 2-amino thienopyrimidine, and aminotriazoloquinazoline (Fig. **15d-i**) display similar binding patterns, the -NH_2 forming hydrogen bonds with D93 and with the conserved network of waters at the edge of the Hsp90 binding site [122]. Benzamide derivatives have been identified as promising Hsp90 inhibitors through *in vitro* target screening on a selected library of compounds [172]. The study resulted in the development of the clinical candidate SNX-5422, a potent and selective inhibitor. In the benzamide compounds, the -NH_2 and the amide oxygen mimic the adenine amine group and N1, interacting with D93 through direct and water-mediated H-bonds (Fig. **15j**). The design of benzolactam derivatives started from the idea of stiffening the structure of benzamides and eliminating an unnecessary hydrogen bond donor (Fig. **15k**). This strategy reduced the overall polar surface area of the benzamide scaffold, optimising the physicochemical characteristics of the series, and thus improving their central nervous system (CNS) activity. The hydroxy-indazole scaffold offers a different binding mode with respect to the ones discussed above. In this case, the hydroxyl group forms a hydrogen bond with D93, while the NH of indazole donates H-bond to a water molecule (Fig. **15l**). An additional type of interaction in the ATP sub-pocket is represented by the binding of azaindole derivatives [173]. The nitrogen atom of the azaindole scaffold establishes direct and water mediated hydrogen bond interactions with D93 (Fig. **15m**). Finally, the binding mode of 2-aminothienopyridine recalls that of the 2-aminothienopyrimidine-based compounds. It is important to note that similar interactions can be achieved with a wide range of scaffolds. For example, 2-aminothienopyridine decorated with a cyano group in the 5-position point toward A51, replacing a water molecule generally present in that position (Fig. **15n**). The large number of available crystallographic structures of N-Hsp90 underlines the interest in developing potent inhibitors. The numerous scaffolds studied as ligands of the adenine sub-pocket offer multiple pharmacophores with different binding modes and a wide range of pharmacokinetic properties. Moreover, the available SAR and computational methodologies applied to the study of N-Hsp90 are valuable resources that can speed up the development and optimization of new Hsp90 inhibitors.

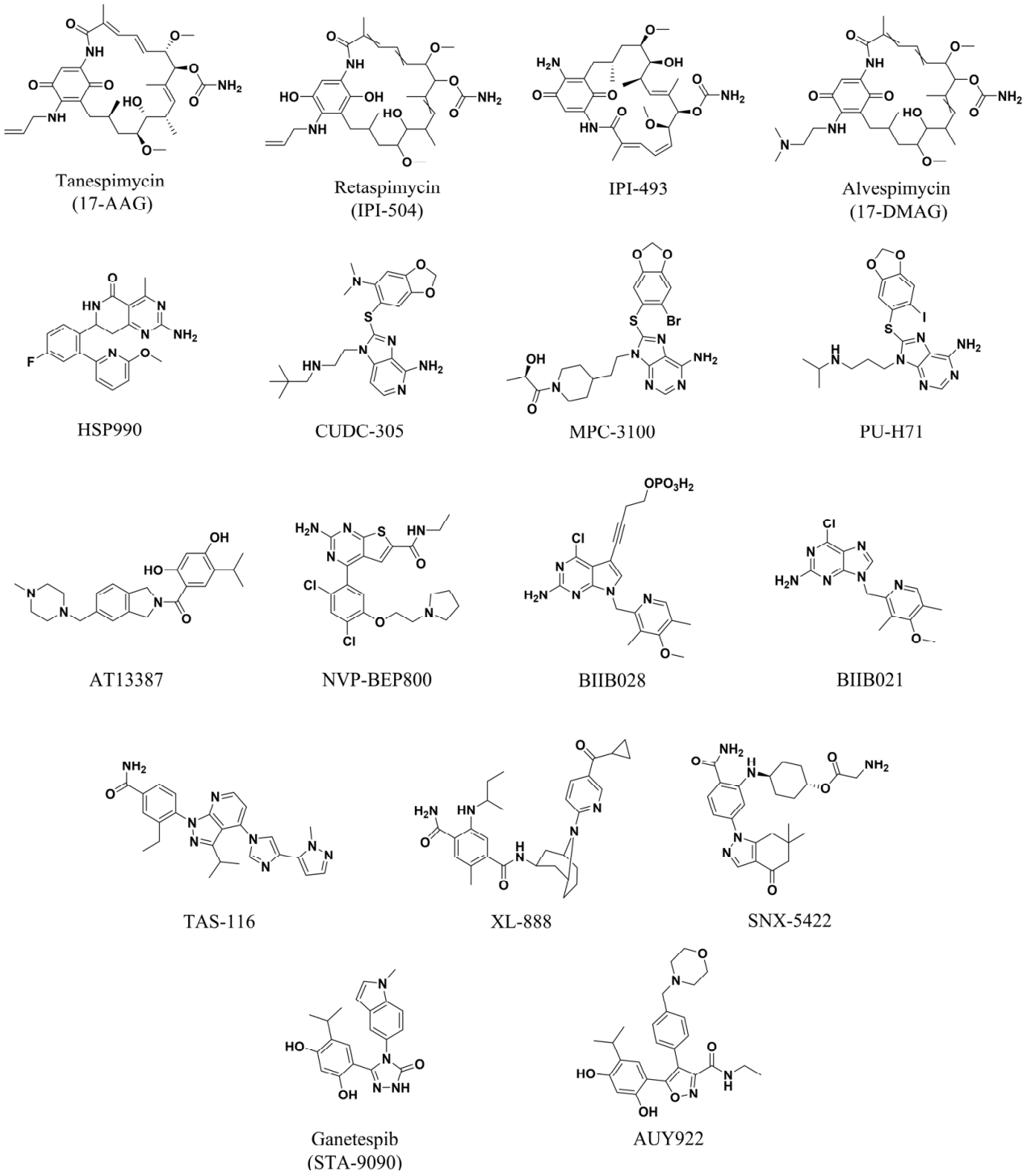


Fig. (13). Chemical structures of Hsp90 inhibitors under evaluation in clinical trials. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

2.6. Available Dual HDAC-Hsp90 Inhibitors

Several authors discussed the association of HDAC inhibitors with Hsp90, to synergistically increase the *in vitro* anticancer activity [14, 38, 104, 174]. However, few of them focused on the use of a single small molecule able to simultaneously block both enzymes. A single compound interacting with multiple targets may benefit of a safer profile with respect to the association of drugs highly selective towards a specific target [175]. The concomitant inhibition of both enzymes under a monotherapy regimen could provide several benefits, especially because synergistic activity dramatically

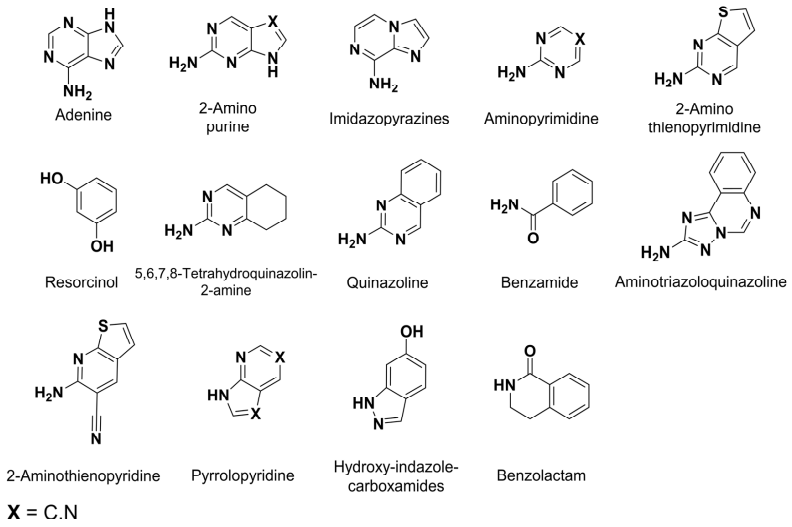


Fig. (14). Chemical scaffolds frequently used to design ATP competitive inhibitors of N-term Hsp90. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

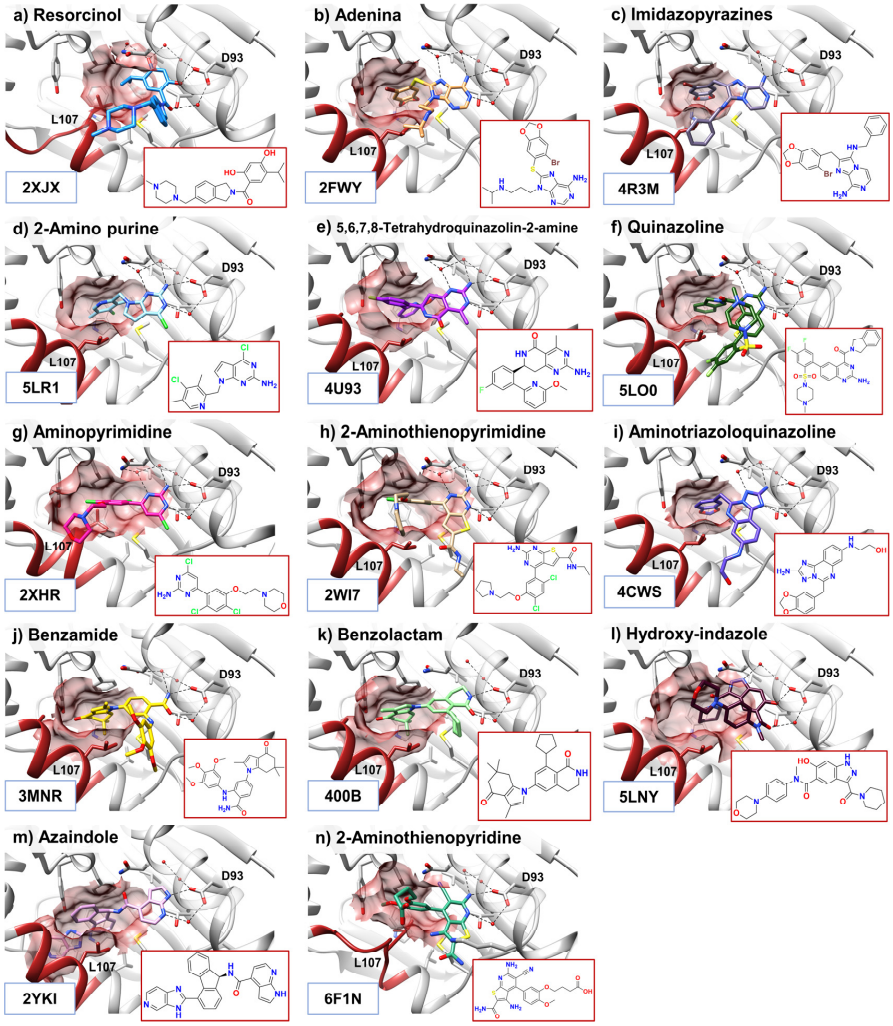
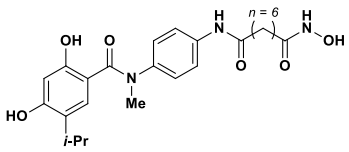
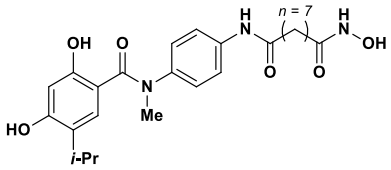
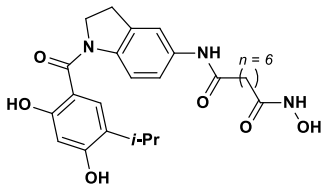
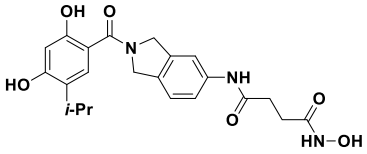
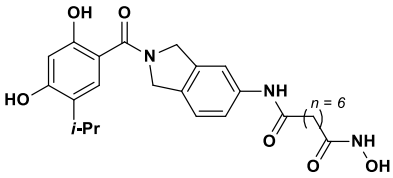
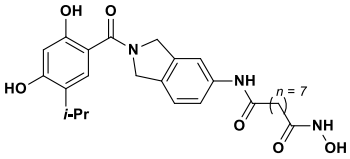
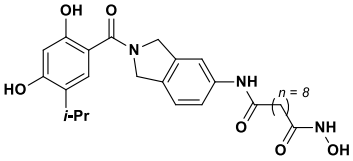
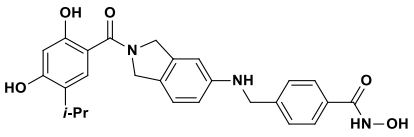
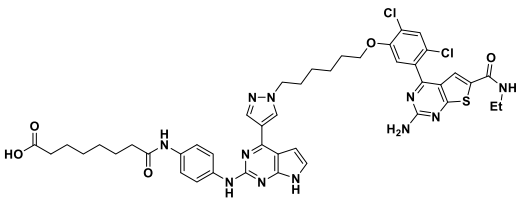


Fig. (15). Close up view of known inhibitors in crystallographic complex with N-term Hsp90. The α-helix3 is depicted in red, hydrogen bonds are highlighted with dashed lines. The surface area of hydrophobic cavity is also shown. PDB codes and 2D ligand structures are displayed at the bottom of the respective panel. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

reduces drug resistance [37, 108]. As discussed above, few molecules reported in the literature possess HDAC6/Hsp90 multi-target activity, mostly because of the high diversity of the two enzyme pockets and the different SARs of the two targets. An ideal drug must possess a chemical architecture that shares key features required for the inhibition of both targets. In this respect, the main strategies pursued so far include the analysis of the SARs of HDAC6 and Hsp90 inhibitors, and the combination of the respective pharmacophoric groups (or substructures) "head-to-tail" to generate a chimeric compound. Therefore, most of the known Hsp90-HDAC6 dual inhibitors reported to date possess a cap group that bears recognition motifs for Hsp90, connected *via* an alkyl chain, or an aromatic ring, to the ZBG (in most cases a hydroxamic acid) required for HDAC6. Molecular hybrids show the advantage of synergistic activity towards the two targets while reducing the dose, thus limiting potential undesirable side effects. However, the presence of non-polar carbon chains often increases the lipophilicity of the molecule leading to a reduced solubility in biological fluids [176]. Thus, the design of a dual inhibitor must be carefully studied, taking into account all the possible variables that could interfere with the activity of the drug (Table **2**). Mehndiratta *et al.* [177] identified two potent nanomolar dual inhibitors of HDAC6 and Hsp90 (Compound **6** (HDAC6 IC_{50} = 0.04 nM; Hsp90α IC_{50} = 153 nM) and compound **7** (HDAC6 IC_{50} = 0.316 nM; Hsp90α IC_{50} = 77 nM). The rational design of compounds **10** and **11** was based on the combination of the SARs of HDAC and Hsp90 inhibitors. Resorcinol was used as the recognition motif for Hsp90, also due to its high stability, and it was connected to the SAHA scaffold through an amide bond. Compound **10** proved to be highly selective towards HDAC6, with respect to other isoforms, and exhibited a potent apoptotic effect in human non-small H1975 lung cancer cells, by activating caspases 3,8,9, PARP, and γH2AX. Inhibition of Hsp90 was demonstrated by upregulation of Hsp70 and downregulation of Hsp90-associated client proteins, including EGFR, Src, FAK, and Rb. Compounds **10** and **11 (**MPT0G449**)** downregulated PD-L1 expression in IFN-γ treated lung H1975 cells in a dose dependent manner. Moreover, the cytotoxicity of compound **11** was recently evaluated on acute leukemia cell lines. MPT0G449 exhibited cytotoxicity on two AML cells higher than the pan-HDAC inhibitor SAHA and the Hsp90 inhibitor 17-AAG used as controls. The results proved that the ligand targets both HDAC and Hsp90, induces cell cycle arrest at the G2 phase, and is able to suppress oncogenic signalling in acute leukemia cells [178]. One of the most popular scaffolds employed in the development of dual inhibitors of Hsp90-HDAC6 is represented by (iso)indolines. The first indoline-based dual inhibitor was discovered by Ojha *et al.*[179]. Compound **12** is a potent HDAC6-HSP90 dual inhibitor (IC_{50} for HDAC6 is 1.15 nM, IC_{50} for Hsp90 is 46.3 nM), which markedly exerted antiproliferative activity *in vitro* (Table **2**). The same research group designed and synthesized several HDAC6-Hsp90 dual inhibitors by merging the resorcinol scaffold with an isoindoline moiety, linked to a terminal hydroxamic acid ZBG by alkyl or *N*-benzyl groups [180]. From the analysis of the structures, it emerged that the most potent HDAC6 inhibitor bears a carbon chain alkyl linker. Despite showing high antiproliferative activity towards human lung cancer cell lines, compound **13** with 2-C linker possesses no inhibition towards HeLa HDAC (IC_{50} > 1000 nM) and HDAC6 (IC_{50} > 10000 nM); the antiproliferative effect could be attributed to the exclusive interaction with Hsp90 (IC_{50} = 123.1 ± 33.9 nM). Compounds **14** and **15** proved to be the most potent dual inhibitors. This has been ascribed to the increase in length of the linker carbon chain, increasing the activity towards HDAC6. The best biological results for compounds with alkyl chain-based isoindolines were achieved with compound **16,** which possessed balanced antiproliferative (0.76 ± 0.13 μM and 0.52 ± 0.20 μM of GI_{50} in A549 and H1975 lung cell lines respectively) and enzymatic inhibition values (HDAC6 IC_{50} = 4.32 nM, Hsp90 IC_{50} = 46.8 nM). Moreover, **16** modulated the expression of biomarkers related to HDAC6-Hsp90 inhibition, and *in vivo* studies demonstrated its ability to reduce tumor growth both with single administration and in association with afatinib. The dual inhibitor **17** incorporates an isoindoline-based *N*-benzyl linker and markedly inhibited HDAC6 (IC_{50} = 33.3 nM) and Hsp90 (IC_{50} = 66 nM). *In vitro,* the molecule suppressed cell growth more markedly than SAHA and it was the most effective of the series (GI_{50} values of 0.37 μM and 0.13 μM in A549 and H1975 lung cell lines, respectively). Docking studies performed on compound **16** and **17** suggested that the compounds bind to N-term Hsp90 adopting a U-shape conformation. The resorcinol scaffold forms a series of hydrogen bonds inside the Hsp90 pocket with the side chains of N51, D93 and T184. The isoindoline ring extends close to the rim of the cavity, while the hydroxamic acid moiety establishes H-bonds with E47, N51 and G137. As for HDAC6, both compounds show similar interactions, except for the bidentate and monodentate zinc binding modes of **18** and **19** respectively. The rest of the molecule leans in the direction of the sub-pocket near L2,

Table 2. Activity data on HDAC6 and Hsp90 of dual inhibitors reported so far.

Compound ID	Structure	HDAC6 (IC_{50} nM)	Hsp90 (IC_{50} nM)	Antiproliferative activity (mM)
10		0.04	153.07	0.77 ± 0.08 A549 0.83 ± 0.07 HCT116 0.69 ± 0.06 H1975
11 (MPT0G449)		0.316	77.21	0.44 ± 0.19 A549 1.06 ± 0.03 HCT116 0.40 ± 0.04 H1975
12		1.15	46.3	1.04 ± 0.06 A549 0.91 ± 0.08 HCT116 1.11 ± 0.24 HL60 1.61 ± 0.91 H1975
13		>10000	123.1 ± 33.9	0.83 ± 0.25 A549 0.39 ± 0.08 HCT116
14		2.21	43.9 ± 3.5	2.92 ± 0.18 A549 4.34 ± 0.84 H1975
15		2.12	42.2 ± 3.2	2.35 ± 0.14 A549 1.07 ± 0.29 H1975
16		4.32	46.8 ± 6.4	0.76 ± 0.13 A549 0.52 ± 0.20 H1975
17		33.3	66.0 ± 5.7	0.37 A549 0.13 H1975
18		6300	20200	

donating hydrogen bonds to the backbones of S564 and F566. Yao L. *et al.* [181] designed multi-target inhibitors of JAK, HDAC and Hsp90 by combining the SARs of the respective targets. The authors took three reference known inhibitors, one for each enzyme, and made a combination of the pharmacophoric groups. Ruxolitinib, vorinostat and BEP800 were chosen as lead inhibitors for JAK, HDAC and Hsp90, respectively. Compound **18** was identified as their best polypharmacological inhibitor, blocking the three targets in the low micromolar range (HDAC6 IC_{50} = 6.3 μM, JAK2 IC_{50} = 3.76 μM and Hsp90 IC_{50} = 20.2 μM). Indeed, compound **18** is made up of three parts: a central core bearing a pyrrole-pyrimidine moiety, linked *via* a nitrogen atom to the scaffold of a suberoylanilide carboxylic acid, which encompasses the recognition motif for HDAC6 and JAK; the Hsp90 pharmacophore is represented by a thiophene-pyrimidine 2-*N*-ethyl carboxamide linked to the core by a n = 6 carbon chain and a 2,4 dichlorophenyl moiety. The micromolar potencies observed for this compound are counterbalanced by the simultaneous inhibition of the three targets, consistently with the polypharmacology concept [108]. Cumulative effects resulted in the desired biological activity, avoiding severe side effects. Therefore, the synergistic activity of compound **18** on the three different targets could make it an attractive lead compound for further optimization.

CONCLUSION

Cancer represents a multi-factorial disease with high adaptability, for which single-target therapeutic regimens show limited efficacy. To overcome such limitations, therapeutic treatments based on the administration of two or more drugs in a scheduled manner (combination therapies) are currently pursued to achieve improved efficacy. However, several limitations can also be envisioned, such as potential higher toxicities derived by drug-drug interactions, or potential negative synergistic effects [104]. The development of a single compound with multi-target activity is a rapidly evolving strategy to potentially limit drug resistance, avoid drug-drug interactions, and deliver more effective cancer therapeutics. HDAC6 and Hsp90 are extensively studied anticancer drug targets. Current literature has shown the importance of their interplay and participation in overlapping signalling and proliferation-related mechanisms in cancer cells. Indeed, the association of HDAC6 and Hsp90 inhibitors demonstrated a strong rationale and several advantages, supporting their selection as partners in multi-targeting approaches. At present, five HDAC inhibitors have been approved for clinical use as anticancer drugs. However, therapeutic treatments associated with HDAC inhibitors present considerable limitations, such as toxicity, drug resistance and lack of efficacy in solid tumors. On the other hand, despite several Hsp90 inhibitors have been tested in clinical trials, none of them has been approved, mainly because of poor efficacy and safety issues. The association of Hsp90 and HDAC6 inhibitors, or even better the administration of a single molecule endowed of dual activity, may provide next generation drugs with significantly better performances and less side effects. Most efforts should be directed toward this goal. The wealth of crystal structure information available for these two targets and the extensive SARs reported in public databases will greatly help to achieve this objective, which is at hand. HDAC6 and Hsp90 belong to different protein families and present two substantially different binding pockets. The design of dual inhibitors mainly relies on a chimeric approach that combines the features required for the binding to the two targets into a unique molecular entity. The zing binding group (*e.g.*, hydroxamic acid) is the key component of HDAC inhibitors, while different decorations at the linker and cap groups guide compounds' selectivity towards the HDAC6 isoform. Besides, the core scaffold of most Hsp90 inhibitors binds to the ATP pocket by mimicking adenine interactions, as observed for ATP. Appropriate linkage of ZBG to the scaffold of Hsp90 ligands has proven to be a successful strategy for the development of dual inhibitors. This strategy results in compounds that use the Hsp90 binding motif as a cap group in HDAC6, while the ZBG is solvent-exposed at the rim of the N-term Hsp90 binding pocket. To date, compounds based on the association of the resorcinol and the hydroxamic acid scaffolds achieved balanced inhibition in the low nanomolar range for both HDAC6 and Hsp90, and demonstrated significant antiproliferative effects. The results obtained so far clearly indicate that the potential therapeutic benefits of selective HDAC6/Hsp90 dual inhibition are high. It is somehow intriguing that despite the huge amount of structural information available for these two targets, relatively few possibilities to design dual inhibitors have so far been explored. Future efforts should be directed toward generating small molecular weight (*e.g.* drug-like) chimeras, keeping a special eye on HDAC6 isoform selectivity, potential off-targets, metabolic stability and toxicity.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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