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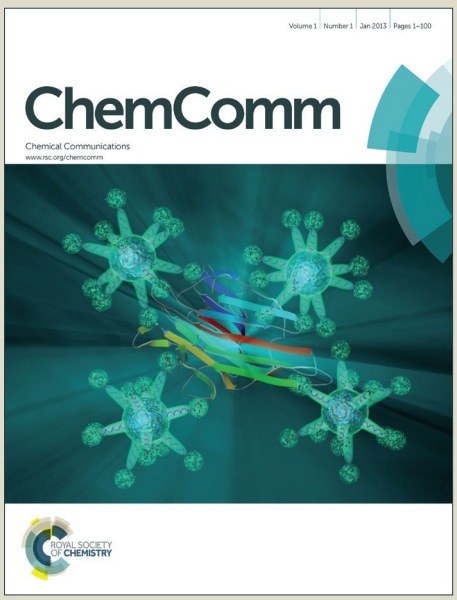

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ARTICLE TYPE

Targeting the Hsp90 C-terminal domain by the chemically accessible dihydropyrimidinone scaffold†

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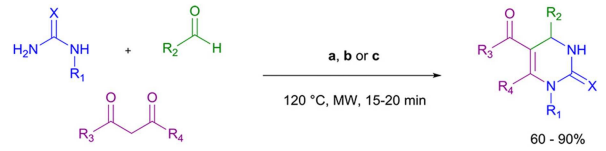
Hsp90 C-terminal ligands are potential new anti-cancer drugs alternative to the more studied N-terminal inhibitors. Here we report the identification of a new dihydropyrimidinone binding the C-terminus, which is not structurally related to other well-known natural and nature-inspired inhibitors of this second druggable Hsp90 site.

Heat shock protein 90 (Hsp90) is a key member of the multicomponent chaperone machinery that regulates a multitude of proteome-maintenance functions, such as *de novo* folding and refolding of stress-denatured proteins.[1] There are two main cytoplasmic isoforms of this chaperone: the inducible Hsp90α (major form) and the constitutive Hsp90β (minor form).[2] Hsp90α is involved in many human diseases, including neurodegenerative conditions[3] and cancer,[4] in which its expression is 2- to 10-fold higher than in normal cells, making tumor cells more dependent on its chaperoning function.[5] Since it mediates the folding and activation of many client proteins crucial for oncogenesis and malignant progression (p53, Raf-1, Bcr-Abl, Akt, Her-2, EGFR steroid receptors and other oncoproteins), an increasing number of experimental evidence is supporting its key role in tumor progression and neoplastic cell survival.[6] In recent years many natural and synthetic Hsp90 N-terminal inhibitors have been developed, some of which show excellent antitumor activity and have entered clinical trials, while only few C-terminal inhibitors have been identified so far.[7] In contrast to N-terminal modulators, which have some drawbacks in clinical application (high concentration for biological effect, poor solubility and toxic side effects),[8] the C-terminal inhibitors represent a promising therapeutic alternative for targeting malignant cells, as they do not induce the deleterious pro-survival heat shock response (HSR) commonly reported for N-terminal ligands.[9] The first identified C-terminal ligand was novobiocin (IC_{50} = 700 µM against Hsp90), a natural coumarin antibiotic which inhibits type II topoisomerases.[10] Since novobiocin's discovery, only few other C-terminal inhibitors have been found, including taxol, epigallocatechin-3-gallate, cisplatin, sansalvamide A derivatives, and novobiocin's structural related synthetic analogues.[11] Although the binding mode of Hsp90 N-terminal inhibitors has been well defined,[12] the structural elements required for Hsp90 C-terminus interaction are currently poorly characterized, due to the lack of a co-crystal structure with a ligand bound to this site. A recent work on novobiocin analogs by molecular dynamics approaches has provided additional information on the structural variations of Hsp90 C-terminal binding site; however, the vast conformational space of this flexible chaperone is still a strong limitation for the rational design of selective inhibitors.[13]

In an attempt to identify non-natural inspired modulators as new molecular scaffolds for Hsp90 C-terminus inhibition, we started from the evidence, reported by Csermely et al.,[14] that this domain is able to interact with both purine and pyrimidine nucleotides (GTP and UTP preferentially), unlike the N-terminus which is highly specific for adenine nucleotides. On the basis of the structural analogy between UTP and the privileged heterocyclic core 3,4-dihydropyrimidin-2-(1H)-one (DHPM),[15] we have synthesized seventeen different decorated DHPM derivatives (Fig. 1), by a microwave-assisted Biginelli multicomponent reaction,[16] a procedure that we have successfully used in the exploration of the pharmacological effects of this versatile scaffold.[17]



Entry	R_1	R_2	R_3	R_4	X
1	phenyl	3-ethoxyphenyl	$CO_2C_2H_5$	CH_3	O
2	H	3-ethoxyphenyl	4-(methoxyphenyl)	$CO_2C_2H_5$	O
3	H	3-ethoxyphenyl	4-(methoxyphenyl)	$CO_2C_2H_5$	S
4	CH_3	phenyl	$CO_2C_2H_5$	CH_3	O
5	CH_3	3-formylphenyl	$CO_2C_2H_5$	CH_3	O
6	phenyl	3-formylphenyl	$CO_2C_2H_5$	CH_3	O
7	H	3-formylphenyl	4-(methoxyphenyl)	$CO_2C_2H_5$	O
8	H	3-formylphenyl	4-(methoxyphenyl)	$CO_2C_2H_5$	S
9	CH_3	(4-cyanophenyl)pyridin-2-yl	$CO_2C_2H_5$	CH_3	O
10	phenyl	(4-cyanophenyl)pyridin-2-yl	$CO_2C_2H_5$	CH_3	O
11	H	(4-cyanophenyl)pyridin-2-yl	4-(methoxyphenyl)	$CO_2C_2H_5$	O
12	H	(4-cyanophenyl)pyridin-2-yl	4-(methoxyphenyl)	$CO_2C_2H_5$	S
13	CH_3	5-(3-(trifluoromethyl)phenyl)furan-2-yl	$CO_2C_2H_5$	CH_3	O
14	H	5-(3-(trifluoromethyl)phenyl)furan-2-yl	$CO_2C_2H_5$	CH_3	O
15	H	5-(3-(trifluoromethyl)phenyl)furan-2-yl	4-(methoxyphenyl)	$CO_2C_2H_5$	O
16	H	5-(3-(trifluoromethyl)phenyl)furan-2-yl	4-(methoxyphenyl)	$CO_2C_2H_5$	S
17	phenyl	3-((cyclopentylamino)methyl)phenyl	$CO_2C_2H_5$	CH_3	O

Fig. 1. Structures and synthesis of **1**-**17** by microwave-assisted Biginelli reaction. Reagents and conditions: **a**) TMSCl (1 eq), MeCN **b**) $Yb(OTf)_3$ (10 mol %), AcOH/EtOH (3:1) **c**) $FeCl_3$ (10 mol %), AcOH/EtOH (3:1).

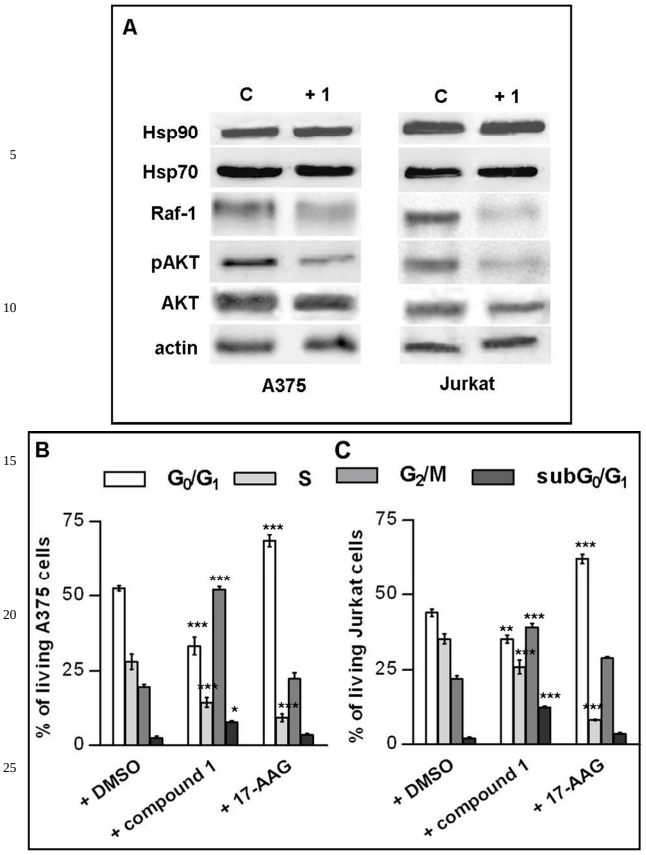


Fig. 2 Effect of compound **1** on Hsp90 client protein levels and cell cycle distribution in A375 and Jurkat cells. (A) Western blot analysis of total cellular proteins, extracted 24 h after treatment with **1** (50 or 20 μM), using specific antibodies. Actin was used as loading control. The shown blots are representative of three different experiments with similar results. (B-C) Quantification of cell cycle distribution of viable A375 (B) or Jurkat (C) cells treated with DMSO, compound **1** (50 or 20 μM, respectively) or 17-AAG (2 or 10 μM, respectively) for 24 h, evaluated by PI staining. Results are expressed as means ± SD of three independent experiments, performed in duplicate (***P < 0.001, ** P < 0.01, *P < 0.05 versus control).

This small collection was subsequently evaluated for the putative binding to the recombinant Hsp90α, by a Surface Plasmon Resonance (SPR)-based approach.[18] On the basis of this preliminary screening, 7 out of the 17 tested molecules with low K_D values were identified (Table S1, ESI). These included compounds with the less bulky 3-ethoxyphenyl and 3-formylphenyl substituents at R_2 of the DHPM ring (**1**, **2**, **6**-**8**), one molecule with a bulkier group at this position (**12**) and compound **17**, obtained from derivatization of **1** by reductive amination. In particular, the N-phenyl derivatives at R_1 position showed to bind to the immobilized protein with nanomolar (K_D values of 76 ± 7 and 30 ± 1 nM for **1** and **17**, respectively) or micromolar affinity (K_D = 3.86 ± 0.33 μM for **6**), with the exception of compound **10** which did not exhibit any binding to Hsp90α, probably due to the presence of the bulkier (4-cyanophenyl)pyridin-2-yl group. The presence of a sulfur atom at the C-2 position of the ring resulted in a non-homogeneous behaviour. Indeed, the thio-analogue of **2**, compound **3**, dropped the affinity to the immobilized protein; compound **12**, thio-derivative of **11**, was the only (4-cyanophenyl)pyridin-2-yl-containing molecule able to interact with Hsp90α; finally, compounds **7** and **8**, respectively obtained from urea and thiourea in the multicomponent reaction, were both tight binders (K_D values of 176 ± 9 and 363 ± 29 nM, respectively). The 5-(3-(trifluoromethyl)phenyl)furan-2-yl substituent at R_2 was not tolerated, since compounds **13**-**15** did not bind at all to the immobilized target protein.

We then tested the seven identified Hsp90α binders for their potential antiproliferative effect in A375 (human melanoma) and Jurkat (human leukemic) cell lines. Compound **17** exhibited IC_{50} values of 150 ± 0.3 μM in both cancer cell lines, while **2**, **6**-**8** and **12** had no cytotoxicy. The best result was reported for compound **1**, which showed moderate cytotoxic effects at micromolar concentration, with IC_{50} values of 50.8 ± 0.2 and 20.8 ± 0.3μM in A375 and Jurkat, respectively. Under the same experimental conditions, IC_{50} values for 17-AAG treatment were 2.1 ± 0.3 μM in A375 and 9.6 ± 0.15 μM in Jurkat cell lines, in agreement with those reported by Dal Piaz et al.[19] and Liu et al.[20] Interestingly, the cytotoxic effect found for compound **1** was in line with SPR analyses, which disclosed **1** as one of the most efficient binder to the immobilized recombinant Hsp90α (K_D of 76 ± 7 nM). It should be emphasized also that no negative effect was observed in PHA-stimulated proliferating PBMC, used as control non-tumor cell line, for which the percentage of non-viable cells after 24 h of treatment with 50 μM of compound **1** (about 8% ± 0.7) was similar to the value observed in DMSO treated control cells (about 7% ± 0.5). To ascertain that the cytotoxic activity of compound **1** was associated with changes in Hsp90 modulation, we verified the level of expression of some Hsp90 client oncoproteins in treated and untreated cancer cell lines, by western blot analysis (Fig. 2A). Following 24-h exposure to compound **1**, the levels of Hsp90 and Hsp70 proteins were unaffected, while the level of the client proteins Raf-1 and p-Akt was strongly down-regulated (about 50-70% less compared to untreated cells, by densitometric estimation) in A375 and Jurkat cell lines. This data suggests that the binding of compound **1** might cause conformational changes of Hsp90, thus preventing its chaperone activity, necessary for stabilizing the oncoproteins, which are subsequently addressed to the proteolytic degradation.[21] Interestingly, exposure to compound **1** did not cause any significant increase in the level of Hsp90 and Hsp70 in both cancer cell lines, demonstrating that the common and undesired HSR was not induced.

To further investigate the cytotoxic effects induced by compound **1**, we analyzed the cell cycle progression of treated cancer cells versus normal cell PHA-stimulated PBMC, using flow cytometric analysis.[22] The A375, Jurkat and PBMC cells were incubated for 24 h with concentrations close to IC_{50} values of **1** or 17-AAG. Cell cycle distribution analysis indicated that compound **1** affects the cell cycle differently from 17-AAG inducing a G2/M arrest in both cancer cell lines, and a consequent increase of subG0/G1 DNA content, indicative of apoptotic/necrotic cell death, in the Jurkat cells (Fig. 2B-C). Compound **1** did not exhibit any pro-death or cytostatic activity in PHA-stimulated proliferating PBMC (data not shown).

In an effort to identify the Hsp90α region involved in the binding of **1**, we used a limited proteolysis-mass spectrometry-based strategy for the structural analysis of the Hsp90α/**1** complex.

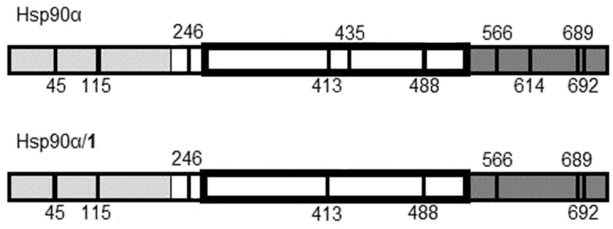


Fig. 3. Schematic representation of limited proteolysis experiments. The preferential cleavage sites detected on recombinant Hsp90α and on the Hsp90α/**1** complex are indicated in black. The Hsp90α N-terminal domain is highlighted in light grey, while the middle domain is boxed and the C-terminal domain is highlighted in grey.

The efficiency of this approach in the investigation of Hsp90α/inhibitor interaction relies on the evidence that exposed, weakly structured and flexible regions of a target protein can be recognized by a proteolytic enzyme and, therefore, observed differences in the proteolytic patterns, in the presence or in the absence of a putative protein ligand, can be useful to identify the protein regions involved in the molecular interactions.[19,23] The proteolytic patterns obtained, performed both on Hsp90α and on the Hsp90α/**1** complex, using trypsin or chymotrypsin as proteolytic agents, are summarized in Fig. 3. Comparison of the differential patterns derived from the digestion of native Hsp90α or of the Hsp90α/**1** complex confirmed a direct interaction between **1** and the chaperone. In addition, we observed that the peptide bonds following Lys435 and Lys614, preferential cleavage sites of the native chaperone in the absence of **1**, were protected in the complex, thus indicating that the middle and C-terminal domain of Hsp90α are preferentially involved in the molecule binding. The conformational changes of Hsp90 induced by compound **1** through apparent interaction with its C-terminus, prompted us to evaluate whether or not this binding could affect also Hsp90α oligomerization, as previously reported for other C-terminal inhibitors, such as some novobiocin-related compounds[24] or (-)-epigallocatechin-3-gallate.[25] To evaluate this hypothesis, an Hsp90α dimerization experiment, using a chemical cross-linking agent on the full-length protein and on the Hsp90α/**1** complex, was carried out.[25] Compound **1** inhibited chemically-induced oligomerization of the full-length Hsp90α (Fig. S2, ESI). Under the experimental conditions used and in the presence of the cross-linking agent, the protein tended to form tetramers, while incubation of Hsp90α with **1** clearly prevented protein tetramer formation. These effects on Hsp90α oligomerization closely resembles those observed for (-)-epigallocatechin-3-gallate,[25] thus confirming a similar interaction with Hsp90α for compound **1**. Taken together, the experimental data from limited proteolysis and oligomerization assays indicated that compound **1** interacts with the C-terminal domain of Hsp90α. Therefore we performed molecular docking (Glide Software)[26] to obtain a putative binding mode of **1** in the C-terminal region.[13] For our calculations, we used the ATP-bound active state of yeast Hsp82, an Hsp90α homologue (PDB code: 2CG9)[27] as model receptor and its sequence alignment with the human protein, reported by Lee et al.,[28] as reference during the comparative experimental/computational analysis. As recently reported by Colombo et al.,[13] the most frequent residues interacting with inhibitors are represented by Arg591, Asp503, Lys423, Gln596, and Arg599 of

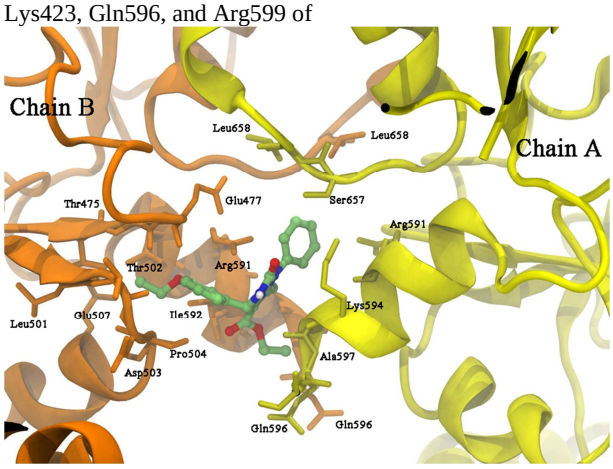


Fig. 4 Three dimensional model of **1** at interface of C-terminal domain of chain B (orange ribbon) and chain A (yellow ribbon) of the yeast Hsp90 (PDB code: 2CG9). **1** is depicted by sticks and balls colored by atom type (C green, O red, N blue, polar H white).

chain B, and Lys594 and Glu477of chain A (Hsp90 residue numbering as in the PDB entry 2CG9).[27] Fig. 4 clearly shows the interactions of **1** with the site located at the dimerization site interface (residues 587-594, chain A). In more detail, the contemporary π-cation interaction with Arg591 of chain B and Lys594 of chain A, and the hydrophobic contacts with the key residues, such as Gln596, Asp503, Glu477, account for its inhibitory activity, also with respect to the other compounds (Fig. S3-S8, ESI). These results are consistent with the data obtained from limited proteolysis and oligomerization assays, confirming that the C-terminal domain of Hsp90α ($Lys614_{Hsp90}$ ($Lys594_{Hsp82}$)) is involved in the molecule binding and in its inhibitory activity. In Table S2 (ESI), we have reported the most representative properties of compounds **1**-**17**, and among them, we have focused our attention on the calculated predicted apparent Caco-2 cell permeability (nm/sec).[26,29,30] In particular, **1** shows the highest predicted Caco-2 cell permeability with respect to the other Hsp90α binders (**2**, **6**-**8**, **12** and **17**) emerged from the SPR assay. Interestingly, the presence of a 3-ethoxyphenyl group in **1** at R_2 position increases the predicted Caco-2 cell permeability of ≈ 4 folds with respect to the related compound **6** which contains the 3-formylphenyl group, and this could explain their different in-cell activity together with the higher K_D value observed for **6**.

In conclusion, here we report that the new DHPM-based compound **1** is able to bind to the C-terminus of Hsp90α as indicated by limited proteolysis and oligomerization assay, and we propose a putative binding mode of DHPM core with Hsp90 by docking studies. The binding of **1** causes the degradation of key-oncoproteins, as revealed by western blot analysis, and a block of *in vitro* proliferation in two different cancer cell lines, without any apparent cytotoxic effect in non-tumor cells. The disclosure of this newly developed compound can be considered an important turning point in the development of Hsp90 C-terminal inhibitors which, together with the well-known N-terminal binders, could have a potential application in cancer therapy.[9] Indeed, the increasing interest toward the identification

of Hsp90 C-terminal modulators is motivated by the possibility of developing new molecules which could potentially overcome the negative side-effects connected to the induction of the deleterious HSR. Compound **1** has to be considered as a lead molecule for further structural optimization processes which, in light of the rapid, easy and high-yielding multicomponent synthetic procedure, could enable the development of structural related but more potent DHPM-based Hsp90 inhibitors. Finally, the results presented in this paper can be helpful to better understand the biological events associated with Hsp90 C-terminal inhibition, underlying the ability of a new chemical skeleton to interfere with the cellular endogenous chaperone machinery and to interact with Hsp90 to modulate client oncoproteins as well as to further elucidate the structural key elements required for the interaction with its C-terminal domain. On the basis of these findings, we are currently expanding our collection of DHPM derivatives in order to identify more powerful and safer Hsp90 inhibitors.

Notes and references

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