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Discovery of benzamide tetrahydro-4*H*-carbazol-4-ones as novel small molecule inhibitors of Hsp90

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ABSTRACT

Hsp90 maintains the conformational stability of multiple proteins implicated in oncogenesis and has emerged as a target for chemotherapy. We report here the discovery of a novel small molecule scaffold that inhibits Hsp90. X-ray data show that the scaffold binds competitively at the ATP site on Hsp90. Cellular proliferation and client assays demonstrate that members of the series are able to inhibit Hsp90 at nanomolar concentrations.

Heat shock protein 90 (Hsp90) has emerged as a promising target for the treatment of cancer and other diseases.[1] The cellular function of Hsp90 is to chaperone the folding and then maintain the conformational integrity of multiple proteins. Hsp90's preferred clients encompass a range of signaling proteins involved in oncogenesis, including Her2, c-Kit, Met, Hif-1α, and androgen receptor. Additionally, mutated proteins that are implicated in cancer are often sensitive clients and are generally more dependent on Hsp90 chaperone function than their wild-type counterparts.[2] Hsp90 is an ATPase and this function is essential to its chaperone capability.[3,4] Inhibition of the ATPase function results in client proteins being ubiquinated and then degraded via proteosome pathways. The discoveries that the natural products geldanamycin and radicicol could competitively inhibit ATP binding to Hsp90[5,6] have prompted significant research into the discovery of small molecule inhibitors of Hsp90.[7,8] We report here the discovery of a novel class of small molecule carbazol-4-one benzamides that are unrelated to published ansamycins, resorcinols, or purine-based inhibitors. They potently inhibit Hsp90 and demonstrate nanomolar cellular inhibition of the target.

Screening of a focused library, designed to inhibit proteins with purine binding sites, yielded a novel benzamide hit for Hsp90 (Fig. 1). Synthetic and modeling analyses of this chemical scaffold

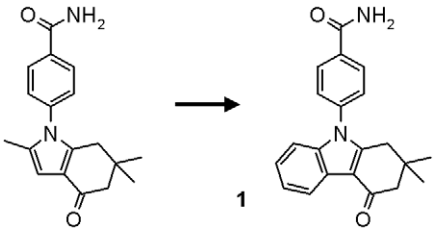

Figure 1. Hsp90 benzamide screening hit and the 1,2,3,9-tetrahydro-4*H*-carbazol-4-one analog **1**.

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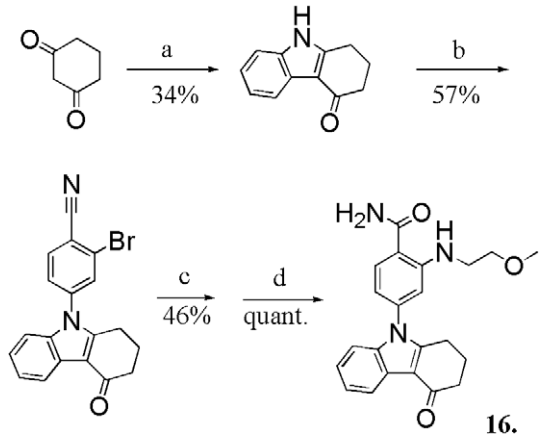
prompted effort to combine the benzamide with a 1,2,3,9-tetrahydro-4*H*-carbazol-4-one moiety. This carbazol-4-one functionality has demonstrated favorable pharmacokinetic properties as it is incorporated into ondansetron, a well tolerated anti-emetic medicine.

The benzamides in this study were obtained in a straightforward manner and a representative synthesis is shown in Scheme 1. The 1,2,3,9-tetrahydro-4*H*-carbazol-4-one ring system was established by means of combining 1,3-cyclohexanedione and phenyl hydrazine via the Fischer indole synthesis in a Personal Chemistry microwave apparatus.[9] Use of dimedone or the mono-methyl reagent instead of 1,3-cyclohexanedione yielded the related analogs described in the SAR. The purified tetrahydro-4*H*-carbazol-4-one was then reacted with the desired 4-fluorobenzonitrile in the presence of sodium hydride. For most analogs, the next step was to incorporate the amine side chain of interest via a palladium catalyzed aromatic amination utilizing $Pd(OAc)_2$ and DPPF with microwave irradiation.[10] Hydrolysis of the nitrile to the carboxamide gave the desired product.

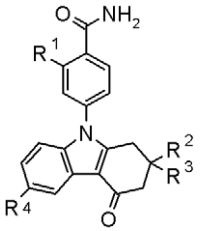
The SAR for this series of compounds is reported in Table 1. Shown are data for a non-enzymatic binding assay, previously described,[8] that allowed determination of binding affinity to monomeric Hsp90. An assay measuring effects on Her2 stability was also established to evaluate cellular effects of the compound.[11] It is important to note that Hsp90 function is complex, involving structural rearrangements as well as co-chaperone protein binding, and so inhibition of monomeric Hsp90 is necessary but not sufficient for cellular activity.[4] Compound **1** showed promising submicromolar Hsp90 binding although it lacked measurable cellular activity. Investigation focused initially on the importance of the gem-dimethyl group present in the hit. Removal of the methyl groups was viewed as potentially desirable for solubility and molecular weight reasons. Although compound **2** showed reduced activity, it still retained moderate affinity. Addition of either a bromo (**3**, **4**) or cyano (**5**) group at the benzamide 2 position effectively killed Hsp90 binding. The methoxy analog **6** was also inactive.

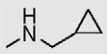
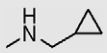
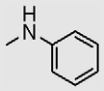
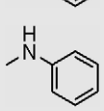
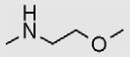
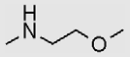
Compound **7** with an amino group showed reduced but still measurable affinity, and binding models suggested room for additional substitution at this position. The affinities of cyclopropyl analogs, **8**, **9**, and **10** were consistent with this observation, and moreover, these compounds showed the first cellular activity observed for the series. Compounds **9** and **10** demonstrated improved binding relative to compound **1**, and the gem-dimethyl analog **8** showed nanomolar cellular activity. Cylcopropyl methyl analogs



Scheme 1. Preparation of **16**: (a) phenylhydrazine (1.2 equiv), TFA, microwave/140 °C/600 s; (b) NaH (2 equiv), DMF, 2-bromo-4-fluorobenzonitrile (1.3 equiv); (c) methoxyethylamine (5 equiv), $Pd(OAc)_2$ (0.01 equiv), DPPF (0.01 equiv), NaO*t*-Bu (2 equiv), toluene, microwave/110 °C/1200 s; (d) DMSO (cat.), EtOH, KOH (ca. 10 equiv), H_2O_2 (32%, XS), 60 °C, 2.5 h.

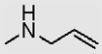
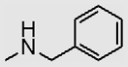
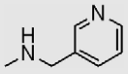
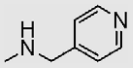
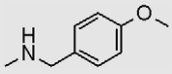
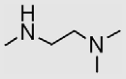
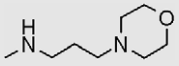
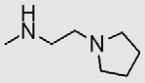
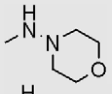
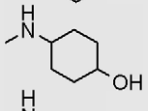
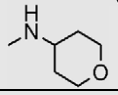
Table 1
Structures and activities for benzamide tetrahydro-4*H*-carbazol-4-one analogs



Compound	R^1	R^2	R^3	R^4	Hsp90[a]	Her2[b]
1	H	Me	Me	H	0.75	>50
2	H	H	H	H	1.8	>50
3	Br	Me	H	H	>10	ND
4	Br	H	H	H	>10	>10
5		H	H	H	>10	>50
6	–OMe	H	H	H	>10	>50
7	$–NH_2$	H	H	H	2.9	>10
8		Me	Me	H	0.35	0.61
9		Me	H	H	1.7	7.0
10		H	H	H	0.35	5.4
11		H	H	F	0.25	1.3
12		Me	H	H	2.9	6.9
13		H	H	H	0.27	1.3
14		Me	Me	H	>10	ND
15		H	H	H	1.0	2.1
16		H	H	H	0.29	8.9
17		H	H	F	0.78	4.1
18		H	H	H	0.35	5.3

(*continued on next page*)

Table 1 (*continued*)

Compound	R^1	R^2	R^3	R^4	Hsp90[a]	Her2[b]
19		H	H	F	0.25	5.4
20		H	H	H	0.31	6.8
21		H	H	H	>10	>10
22		H	H	F	1.1	6.5
23		H	H	H	0.82	5.0
24		H	H	H	>10	>10
25		H	H	F	1.2	34
26		H	H	F	1.1	2.8
27		H	H	H	1.9	31
28		H	H	F	0.27	1.6
29		H	H	H	0.90	1.2
30		H	H	H	0.41	0.56

[a] Values (μM) are estimated K_d's derived from an 8 point non-enzymatic ATPase assay as described in text and Ref. 13.

[b] Values (μM) are IC_{50} determinations from a Her2 ELISA in SKBR3 cells as described in text and Ref. 11.

12 and **13** were also well tolerated. An interesting observation was that addition of a bulkier phenyl group, compounds **14** and **15**, was reasonably well tolerated when positions R^2 and R^3 were both hydrogen but was completely inactive for the corresponding gem-dimethyl substitution. The methyl ethyl ether analog, **16**, showed good Hsp90 binding, and at this point, X-ray crystallographic studies were undertaken for this compound to better understand the Hsp90 binding mode for this series.[12]

Co-crystallization of **16** with the N-terminal binding domain of Hsp90 proved successful, and a 1.74 Å high resolution structure was obtained (Fig. 2). The ligand binding orientation was unambiguous from the Fo–Fc omit map. Previous models were in overall good agreement with the structure, but the X-ray provided additional detailed information on the complex. As anticipated, the benzamide acted as an adenine mimic with the amide group forming hydrogen bonds with residues Asp 93, Thr 184, and a coordinated water molecule. These residues were observed previously to form a hydrogen bonding network in geldanamycin (GA) and ADP X-ray structures.[6,14] Sandwiching the benzamide ring were residues Met 98 and Asn 51 with the interaction of the asparagine involving pi stacking of the amide side chain moiety with the benz-

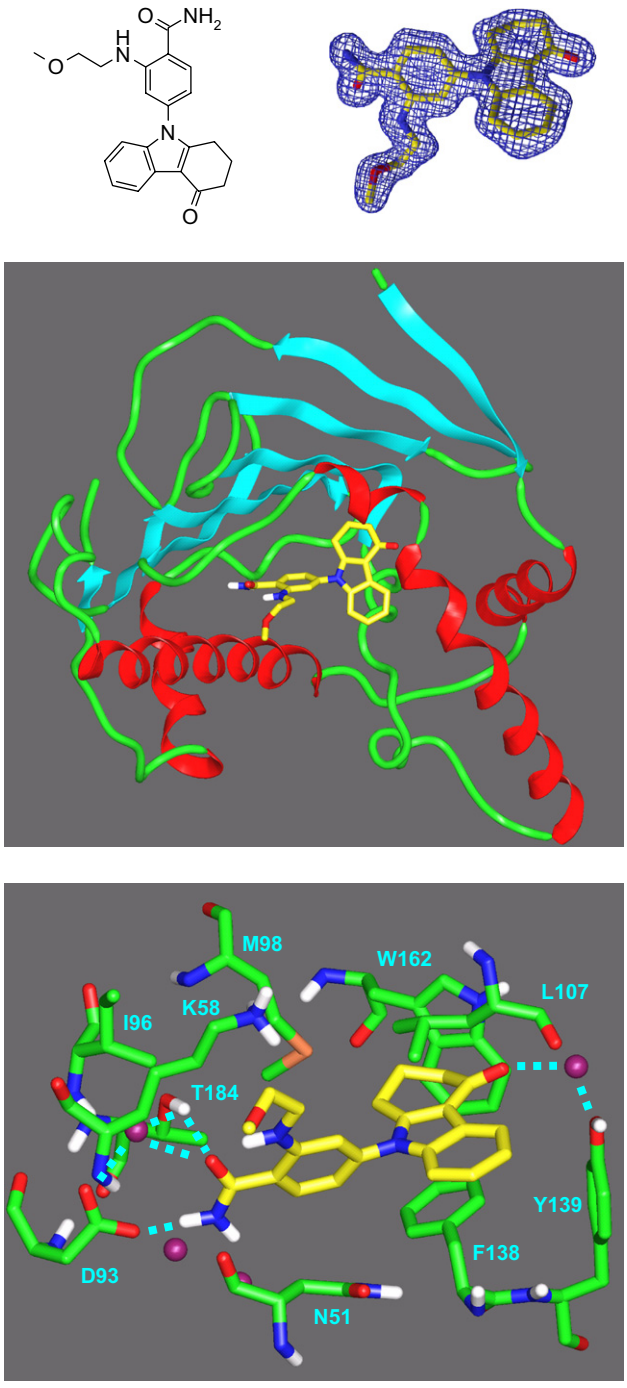


Figure 2. X-ray structure of compound **16** bound to the N-terminal domain of Hsp90. Fo–Fc map at 3σ is shown at top right; entire protein structure is shown in middle; close-up view of active site region is shown at bottom. Some of the active site water molecules are shown as purple spheres and ligand hydrogen bonds are indicated by cyan dashes.

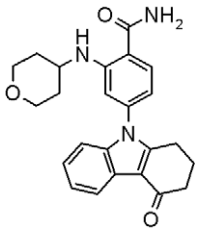
amide of **16**. Multiple tightly bound structural waters were resolved in the binding site.

The tetrahydro-4*H*-carbazol-4-one group was observed to make multiple hydrophobic interactions with Hsp90. More specifically, a tight binding pocket was formed on three sides involving Leu 107, Phe 138, and an edge to face interaction with Trp 162. Secondary interactions involved Val 150 and Leu 103. Additionally, the carbonyl group made a water-mediated hydrogen bond with Tyr 109, and several additional water molecules were observed in

the region of Tyr 109. Overall, the protein conformation resembled that previously seen for PU24FCl and related scaffolds in which Leu 107 is also seen to be displaced by ligand binding.[15] The methyl ethyl ether side chain of compound **16** was most proximal to the side chain of Met 98, forming favorable hydrophobic contacts with that residue as well as Ala 55 and Ile 96. The observed gauche conformation allowed the ether oxygen to form an intramolecular hydrogen bond with the amino hydrogen. The tight packing of the side chain with Met 98, coupled with the close fit of the tetrahydro-4*H*-carbazol-4-one into its region of Hsp90, indicated that longer range allosteric effects could be possible and may explain the divergent SAR for the gem-dimethyl analogs versus all-hydrogen analogs at position 2 of the carbazol-4-one. Superimposition of the X-ray structures of compound **16** and GA[6,14] bound to Hsp90 showed that the scaffold was able to effectively mimic key pharmacophore elements. The benzamide of **16** tightly overlayed the carbamate of GA while the methyl ethyl ether and indole components of **16** closely mapped to GA macrocycle atoms 8–11 and 2–5, respectively.

It was apparent that there was additional sterically accessible space that could be targeted in the region off the 2 position of the benzamide and that became the primary focus for additional analogs. Compounds **17**–**20** further explored the potential to make hydrophobic interactions with Met 98 and all showed good Hsp90 binding and moderate cellular potency. The introduction of a fluoro group at the 6 position (**17**, **19**) of the carbazol-4-one appeared to be well tolerated and this was observed for later analogs as well. It was anticipated that the fluoro would possibly interact favorably with the hydroxyl of Tyr 109 while potentially improving both metabolic and solubility properties of the series. Benzyl and anisole analogs **21** and **24** were inactive. One strategy was to target a proximal residue, Lys 58, for additional hydrogen bonding and this strategy was attempted via the pyridyl analogs **22** and **23**. Although much better than their benzyl counterparts, they were still not improved relative to earlier analogs. The incorporation of solubilizing groups was also attempted for a set of analogs, **25**–**27**; all showed reasonable Hsp90 binding, but only the less basic morpholino analog **26** had moderate cellular potency.

Table 2
Cellular potencies for compound **30**



Assay	IC_{50}[a] (μM)
A375 proliferation	0.40 ± 0.17
HT29 proliferation	0.37 ± 0.10
LNCAP proliferation	0.70 ± 0.26
MCF-7 proliferation	0.29 ± 0.05
MDAMB231 proliferation	0.96 ± 0.13
NCI-H460 proliferation	0.90 ± 0.15
PC-3 proliferation	0.82 ± 0.20
SKMEL5 proliferation	0.34 ± 0.09
SW620 proliferation	0.26 ± 0.06
Her2 degradation, SKBR3[b]	0.56 ± 0.13
Hsp70 induction, A375[b]	0.23 ± 0.07
pErk inhibition, AU565[b]	0.59 ± 0.24
pS6 inhibition, A375[b]	0.07 ± 0.02

[a] Values are means of four experiments with standard deviation shown.
[b] Both cellular readout and cell line are indicated.

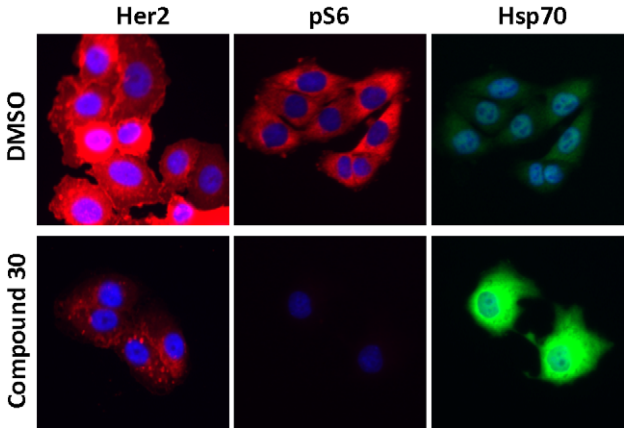


Figure 3. HCA images of AU565 cells showing effects of compound **30** inhibition at 10 μM on Her2 degradation and of A375 cells showing effects on pS6 and Hsp70 levels. Her2 and pS6 are colored red, Hsp70 is colored green, and DNA is colored blue. pS6 and Hsp70 images are of the same cells for the individual treatments, but pS6 is FITC exposure, Hsp70 is TRITC exposure. For each endpoint measured, length of exposures was identical for direct comparison.

The final members of the series compounds, compounds **28**, **29**, and **30** incorporated hydrophobic rings, coupled with hydrogen bond acceptors, in an effort to target both Met 98 and Lys 58. The improved potencies of these compounds, particularly compound **30**, supported this rationale. As a result, compound **30** was evaluated in multiple additional cellular assays.[11] These assays included cancer cell line proliferation as well as imaging assays for signaling pathways impacted by Hsp90 inhibition. Also evaluated was the induction of Hsp70 which is an expected result for Hsp90 inhibition as a mechanism of action. The results of these assays are summarized in Table 2 and illustrated in Figure 3. As can be seen, compound **30** potently inhibited cancer cell proliferation with IC_{50}'s below 400 nM in multiple cell lines. Potency was within a factor of 5–10 of the geldanamycin analog, 17-AAG, used as a positive control (data not shown). Of note is that both MAPK and AKT signaling pathways, as measured by phosphorylation of ERK and S6, respectively, were also inhibited. Additionally, Hsp70 was induced, fully supporting Hsp90 inhibition as a mechanism of action.

In conclusion, we have reported here a novel scaffold for competitively inhibiting the ATP binding site of Hsp90. The binding mode has been confirmed via X-ray crystallography. Syntheses of multiple analogs allowed for discernment of key SAR trends and led to potent Hsp90 inhibitors: **8**, **13**, **29,** and **30**. Detailed evaluation of compound **30** showed it to be potent across a range of cancer cell lines and able to cause degradation of oncogenic Hsp90 clients. The promising data presented here have prompted further evaluation of this scaffold and that work remains ongoing.

Acknowledgment

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11. All cell lines were purchased from ATCC. Proliferation rates were measured by seeding cells into 96-well plates, followed by compound addition 24 h later. After addition of compound, cells were allowed to grow for either an additional 72 or 144 h, depending on rate of growth. At harvest, media was removed and DNA content for individual wells was determined using CyQuant® DNA dye (Invitrogen). Her2 degradation in SKBR3 cells was measured by cellular ELISA using a mouse anti-Her2 primary antibody (Millipore) and anti-mouse peroxidase (Invitrogen) conjugated secondary antibody on cells treated for 24 h with compound followed by methanol fixation. High Content Analysis (HCA) was done using an ArrayScan 4.5 (Pittsburg, PA) or Becton Dickenson 435 (Rockville, MD) imager on cells treated 24 h with compound. After fixation in 4% PBS-buffered formalin, cells were probed with anti-Her2 (Millipore), anti-phospho-S6 (pS6) (Cell Signaling) and anti-Hsp70 (Assay Design) primary antibodies, followed by TRITC or FITC conjugated secondary antibodies.
12. An untagged N-terminal domain construct comprising residues 1–232 of Hsp90 was co-crystallized with compound **16**. Amino acids 16–224 and 281 water molecules were located. Number reflections = 27 945; % completeness = 95; R_{Cryst} = 0.200; R_{free} (5%) = 0.228; resolution (Å) = 1.74–66.52; space group = I222; unit cell (Å): a = 66.85, b = 90.60, c = 98.22, a = 90°, b = 90°, g = 90°. The PDB code is 3D0B.
13. Test compound affinity for Hsp90 was determined as follows: Hsp90 from porcine spleen extract was isolated by affinity capture on a purine-affinity media. The Hsp90 loaded media was then challenged with test compound at a given concentration, ranging from 0.8 to 500 μM, and the amount of Hsp90 liberated at each concentration was determined by Bradford protein assay. The resulting IC_{50}s were corrected for the ATP ligand concentration and presented as K_d values.
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