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Development of Phenyl Cyclohexylcarboxamides as a Novel Class of Hsp90 C-terminal Inhibitors

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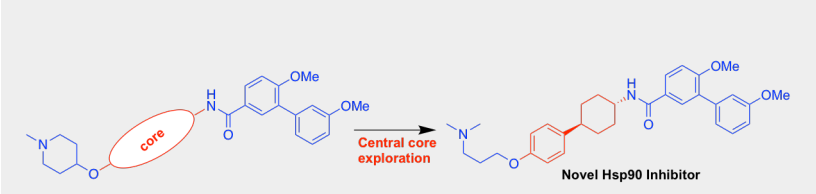

Backbone optimization: The central core was explored to identify an optimized scaffold for Hsp90 C-terminal inhibition. Structural investigations led to the development of phenyl cyclohexyl carboxamides as a novel class of Hsp90 C-terminal inhibitors. This new scaffold exhibits improved biological activity and provides a new template for future Hsp90 inhibitors.

Author(s), Corresponding Author

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Title

Abstract

Inhibition of the heat shock protein 90 (Hsp90) C-terminus represents a promising therapeutic strategy for the treatment of cancer. Novobiocin, a coumarin antibiotic, was the first Hsp90 C-terminal inhibitor identified, however, it manifested poor anti-proliferative activity (SKBr3, IC_{50} ~ 700 μM). Subsequent SAR studies on novobiocin led to development of several analogues that exhibited improved anti-proliferative activity against several cancer cell lines. Recently, we demonstrated that the biphenyl core could be used in lieu of the coumarin ring system, which resulted in more efficacious analogues. In continuation of previous efforts, the work described herein has identified the phenyl cyclohexyl core as a novel scaffold for Hsp90 C-terminal inhibition. SAR studies on this scaffold led to the development of compounds that manifest mid-nanomolar activity against SKBr3 and MCF-7 breast cancer cell lines through Hsp90 inhibition.

Keywords: Heat shock protein 90; Hsp90 C-terminal inhibitors; Phenyl Cyclohexylcarboxamides; Structure-activity relationship; Breast cancer.

1. Introduction

Heat shock protein 90 (Hsp90) is an evolutionarily conserved molecular chaperone that plays a critical role in the maintenance of protein homeostasis as well as adaptive response to cell stress.[1] Hsp90 is a core component of the protein folding machinery that regulates the folding, stability, function, and proteolytic turnover of more than 300 client proteins, including protein kinases, transcription factors, and signal transducers.[2] These client proteins control a wide range of cellular functions, such as cell signaling, protein trafficking, chromatin remodeling, cell proliferation and survival.[2-3] However, in cancer, these clients are frequently mutated and/or over-expressed to drive oncogenic processes, such as dysregulated proliferation, metastasis, and angiogenesis. In fact, ~25% of the Hsp90-dependent clients (eg. Her2, CDK6, Raf1, Akt, survivin, telomerase) represent oncoproteins that are directly associated with all ten hallmarks of cancer.[3a, 4] Moreover, Hsp90 is overexpressed in cancer cells to stabilize these oncoproteins against proteotoxic stresses and to fold mutants that enable the growth and/or survival of tumorigenic cells.[5] Consequently, Hsp90 inhibition provides an opportunity to simultaneously disrupt multiple oncogenic pathways that are required for cancer cell survival. As a result, Hsp90 has emerged as a promising therapeutic target for the development of cancer chemotherapeutics.[6] 17 Small molecules that target the Hsp90 N-terminus have entered clinical trials for the treatment of cancer, demonstrating proof-of-concept for Hsp90 inhibitors as potential anticancer agents.[7] Although these molecules have shown some promising clinical responses, several concerns have arisen such as concomitant induction of the pro-survival heat shock response (HSR), hepatotoxicity, and cardiotoxicity amongst others, which represent additional risks that must be overcome during the development of new inhibitors.[3b] Therefore, small molecules that modulate Hsp90 via alternative mechanisms represent a novel approach towards the evolution of Hsp90 inhibitors.[3b, 8]

In 2000, Neckers and co-workers discovered that novobiocin, a clinically used antibiotic and DNA gyrase inhibitor, binds the Hsp90 C-terminus, and allosterically inhibits Hsp90 function.[9] Importantly, novobiocin and related natural products do not induce the HSR and therefore, can avoid the clinical limitations associated with Hsp90 N-terminal inhibitors. Unfortunately, novobiocin exhibits low cellular activity (SKBr3 IC_{50} ~ 700 μM) and thus, has limited therapeutic potential. Preliminary studies with novobiocin identified key structural features required for Hsp90 inhibitory activity, which led to analogues with improved inhibitory activity.[10] Subsequent studies

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revealed that the benzamide side chain of novobiocin is important for anti-proliferative activity and modification of this side chain produced several promising compounds, such as KU174 (Figure 1).[11] Additional studies demonstrated that the stereochemically complex sugar moiety could be replaced with ionizable amines, which produced analogues that manifest mid-nanomolar to low micromolar activity against multiple cancer cell lines.[12] In contrast to these side chains, limited structural investigations have been performed on the coumarin core of novobiocin.

Recent investigation of the novobiocin scaffold demonstrated that an aromatic or heteroaromatic ring system could be used in lieu of the coumarin core without compromising activity, suggesting that the central core may serve as a backbone to orient both the benzamide and sugar/side chains within the binding pocket.[13] Consequently, it was proposed that the coumarin core could be replaced with other scaffolds to identify compounds upon which more potent inhibitors could be realized. Moreover, the development of a structurally diverse set of compounds is necessary to better understand the mechanism by which the C-terminal domain regulates the Hsp90 chaperone cycle. Towards this objective, we discovered that the coumarin core of novobiocin could be replaced with a biphenyl scaffold, which led to analogues that exhibit potent anti-proliferative activity.[14] More recently, we demonstrated that a stilbene core (Figure 1) could be used in lieu of the coumarin core as well.[15] In continuation of these studies, the current work identifies a novel core, which has led to the development of more efficacious Hsp90 inhibitors.

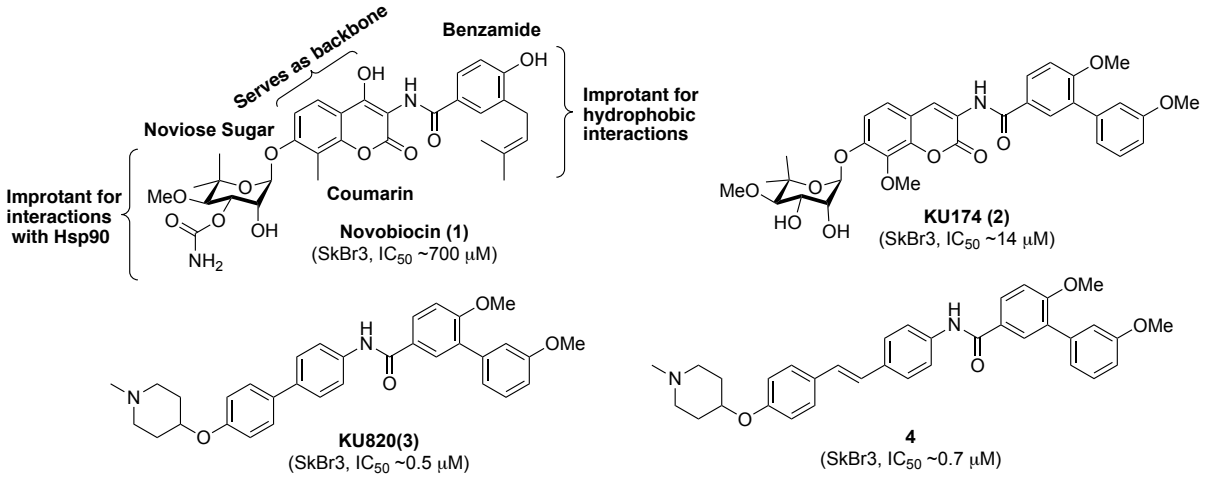

Figure 1. HSP90 C-terminal inhibitors.

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2. Results and Discussion

2.1 Design, synthesis and evaluation of biphenyl mimics-

Prior SAR studies on novobiocin have revealed the benzamide side chain and sugar are critical for Hsp90 inhibitory activity and that modification to these moieties has produced several promising compounds, such as **5**, which manifests improved activity against several cancer cell lines.[11-12, 16] Recently, it was demonstrated that a biphenyl scaffold could be used in lieu of the coumarin core, which also led to potent inhibitors, such as KU820 (Figure 2), presumably due to the favorable conformation adopted by the relatively flexible biphenyl core within the binding pocket.[14a, 17] In addition, it was determined that the planarity and flexibility of the central core are important for Hsp90 inhibitory activity.[15] Therefore, it was hypothesized that the central core could be modified to optimize orientation of the side chains for increased inhibitory activity. Since biphenyl analogues manifest superior activity, this scaffold was chosen as the starting point for the discovery of more efficacious Hsp90 inhibitors.

Increasing evidence suggests that molecules with aromatic scaffolds offer limited spatial diversity, while the incorporation of a saturated ring system can improve drug-like properties, such as ligand/receptor interactions as well as solubility.[18] As a flexible structure, the saturated ring system has the ability to adopt multiple conformations and to project substituents into optimal orientations within the binding pocket. Therefore, optimization of the core began by replacement of the A- and/or B-ring of the biphenyl core with a saturated ring system. The previously optimized biaryl amide side chain and *N*-methylpiperidine present in **3** were appended to these new cores for evaluation of preliminary structure-activity relationships.

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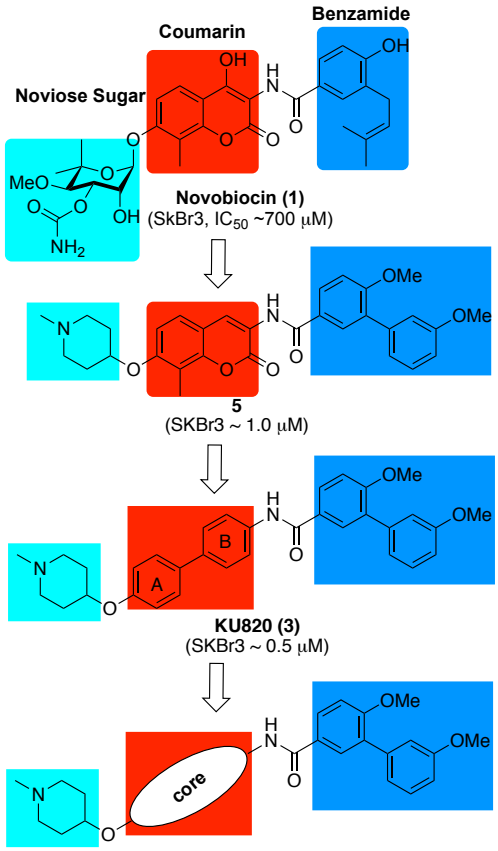

Figure 2. Rationale for proposed molecules.

As shown in Scheme 1, the preparation of analogues that contain a saturated B-ring (**15a** and **15b**) commenced via benzyl-protection of phenol **6** to afford ketone **7**, which was then stereoselectively reduced with either L-selectride or sodium borohydride to give the syn (**8a**) or anti (**8b**) diastereomers in >90% de, respectively. Inversion of stereochemistry was made possible by mesylation of the alcohols (**8a**, **8b**), followed by S_N2 substitution with sodium azide to yield **10a** and **10b**. Reduction of the azides with palladium on carbon under a hydrogen atmosphere gave amines **11a** and **11b**, which were then coupled with biaryl acid **12** using standard coupling conditions to afford the corresponding amides, **13a** and **13b.** Mitsunobu etherification of the resulting amides with 1-methyl-4-hydroxypiperidine (**14a**) gave the desired products, **15a** and **15b**, in moderate yields.

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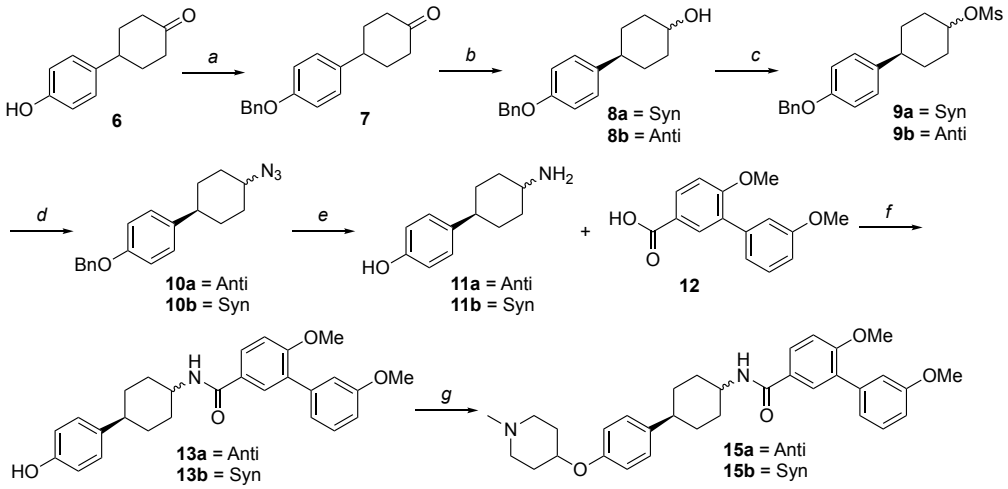
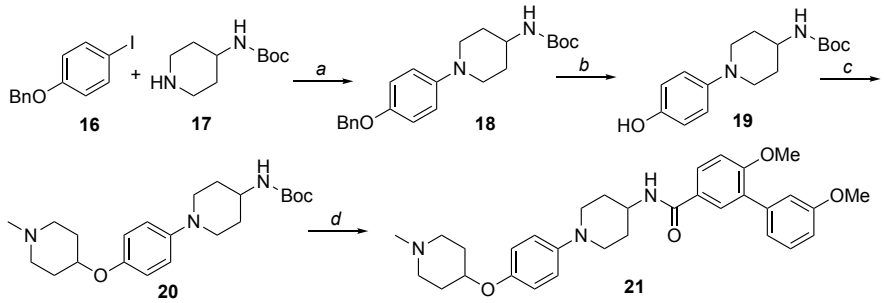


Scheme 1. Synthesis of phenylcyclohexyl carboxamides. Reagents and conditions: a BnBr, K_2CO_3, acetone, 0 °C to rt, 12 h, 95%; b L-selectride, THF, -40 °C to rt, 12 h, 92% or $NaBH_4$, MeOH, 0 °C to rt, 12 h, 90%; c MsCl, Et_3N, DCM, 0 °C to rt, 4 h, 92 − 94%; d NaN_3, DMF, 100 °C, 12 h, 80 − 85%; e 10% $Pd(OH)_2$, H_2, MeOH/THF, rt, 12 h, ~100%; f EDCI•HCl, Et_3N, DCM, 0 °C to rt, 12 h, 45 − 50%; g 1-Methylpiperdin-4-ol (**14a**), DIAD, PPh_3, THF, 0 °C to rt, 12 h, 36 − 40%.

In addition, an analogue that contains a piperidine ring (**21**) was designed to determine whether orientation of the cyclohexyl ring is important for activity. Synthesis of **21** was achieved via a Buchwald coupling between aryl bromide **16** and piperidine **17** to produce **18**, which then underwent hydrogenolysis with palladium hydroxide under a hydrogen atmosphere to give the free phenol, **19** (Scheme 2). Mitsunobu etherification of **19** with 1-methyl-4-hydroxypiperdine (**14a**) yielded the *N*-Boc-protected amine **20**, which was then treated with trifluoroacetic acid to remove the Boc- protecting group before the resulting amine was coupled with biaryl acid **12** to afford the desired product, **21**.



Scheme 2. Synthesis of phenylpiperdin-4-yl carboxamide. Reagents and conditions: *a* $Pd_2(dba)_3$, X-Phos, ^tBuONa, toluene, 130 °C, 2 h, m.w., 37%; *b* 10% $Pd(OH)_2$, H_2, MeOH/THF, rt, 12 h, ~100%; *c* **14a**, DIAD, PPh_3, THF, 0 °C to rt, 12 h, 30%; *d* i. 30% TFA/DCM, 0 °C to rt, 4 h, ~100%; ii. EDCI•HCl, Et_3N, DCM, 0 °C to rt, 12 h, 55%.

In parallel, an analogue containing a saturated A-ring (**31**) was prepared as illustrated in Scheme 3. Synthesis of compound **31** was initiated by selective

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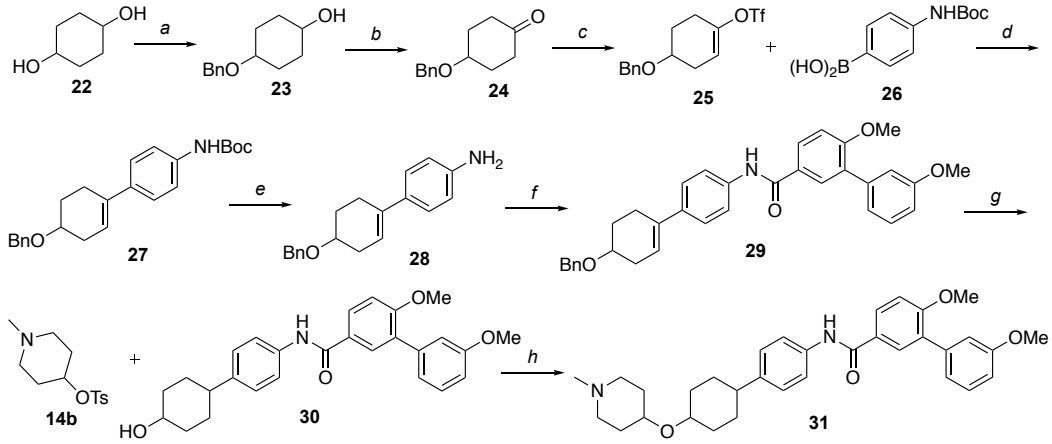
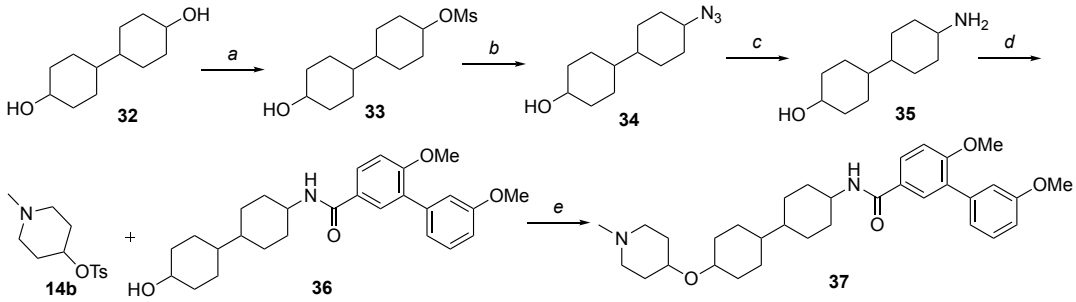
benzylation of cyclohexane-1,4-diol to give **23**,[19] which was then oxidized with pyridinium chlorochromate to yield ketone **24**.[20] The ketone was then converted to the vinyl triflate (**25**), before Suzuki coupling with boronic acid **26** to give the cyclohexyl phenyl core, **27**. Acid-catalyzed hydrolysis of the Boc-protecting group on **27** yielded aniline **28**, which underwent an amide coupling reaction with acid chloride **12** to afford **29**. Hydrogenolysis of **29** with palladium on carbon under a hydrogen atmosphere gave the free alcohol, **30**, which underwent an S_N2 substitution reaction with **14b** to afford **31** in moderate yield. Following a similar protocol as standardized for **15a** and **15b**, compound **37** was prepared to contain two cyclohexyl rings as shown in Scheme 4.



Scheme 3. Synthesis of a cyclohexylphenylamide. Reagents and conditions: *a* BnBr, NaH, DMF, 0 °C to rt, 12 h, 70%; *b* PCC, DCM, rt, 12 h, 50%; *c* N-Ph_2Tf, LDA, THF, 55%; *d* Pd(dppf)Cl_2, Cs_2CO_3, DMF, 100 °C, 12 h,30%; *e* 30% TFA, DCM, rt, 12 h, ~100%; *f* **12**, EDCI•HCl, Et_3N, DCM, 0 °C to rt, 12 h, 80%; *g* Pd$(OH)_2$, H_2, MeOH, 12 h, 40%; *h* K_2CO_3, DMF, 90 °C, 48 h, 25%.



Scheme 4. Synthesis of a cyclohexyl derivative. Reagents and conditions: *a* MsCl, Et_3N, DCM, 0 °C to rt, 12 h, 90%; *b* NaN_3, DMF, 100 °C, 12 h, 30%; *c* Pd/C, H_2, MeOH, 12 h, ~100%; *d* **12a**, DIPEA, DCM, 0 °C to rt, 12 h, 60%; *e* K_2CO_3, DMF, 90 °C, 48 h, 25%.

Upon construction, analogues containing saturated A- and/or B-rings were evaluated for their anti-proliferative activity against two cancer cell lines, SKBr3

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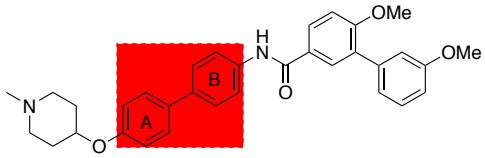
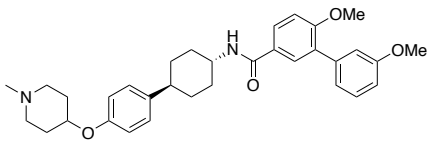
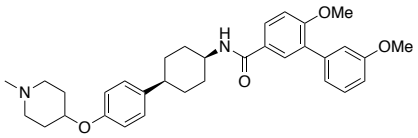
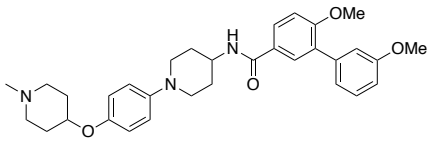
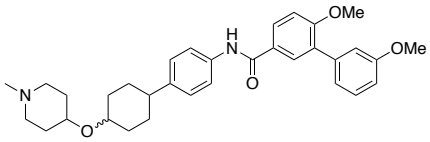
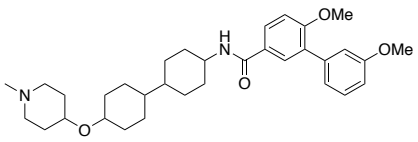
(estrogen receptor negative, Her2 overexpressing breast cancer cells) and MCF-7 (estrogen receptor positive breast cancer cells). As shown in Table 1, compound **15a** (*anti*) exhibited 4-fold greater anti-proliferative activity than the lead compound **3**, and 10-15 fold better activity than **15b** and **21**, indicating the *anti*-stereochemistry is important for anti-proliferative activity. Incorporation of a saturated ring into the A-position was detrimental, as compounds **31** and **37** were both inactive up to 50μM, suggesting that planarity of the A-ring is important for anti-proliferative activity.

Table 1. Anti-proliferative activity of analogues with the modified A- and/or B-rings.



Entry	Compound	SKBr3 (IC_{50}, μM)	MCF-7 (IC_{50}, μM)
3	-	0.47 ± 0.06	0.71 ± 0.02
15a		0.17 ± 0.02 [a]	0.22 ± 0.01
15b		2.57 ± 0.08	2.43 ± 0.05
21		2.87 ± 0.13	3.37 ± 0.14
31		>50	>50
37		>50	>50

[a]Values represent mean ± standard deviation for at least two separate experiments performed in triplicate.

Preliminary SARs indicate that the phenyl A-ring is necessary for maintaining anti-proliferative activity, while modifications to the B-ring could improve activity. Since

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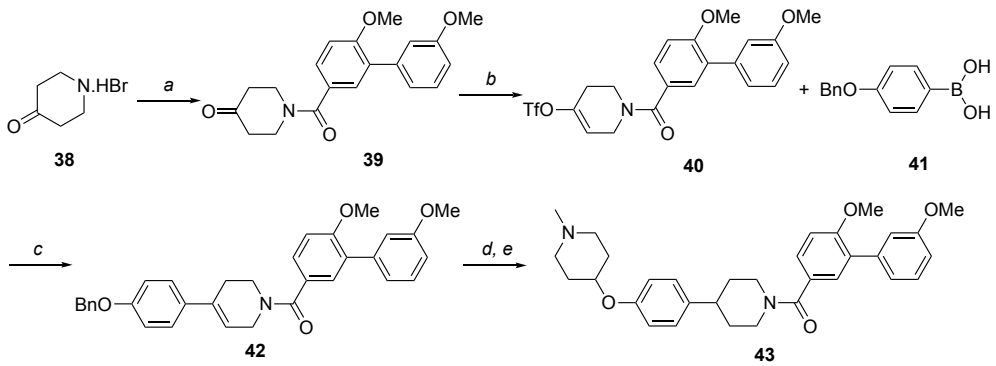
no co-crystal structure of Hsp90 bound to C-terminal inhibitors has been solved, there is limited knowledge about the size and nature of this binding pocket. Therefore, a library of analogues containing the phenyl A-ring attached to various appendages in lieu of the cyclohexyl B-ring was pursued to identify an optimal scaffold that could orient both side chains and maximize interactions for increased inhibitory activity. Recent studies have suggested that an optimal distance between 7.7 and 12.1 Å from the amine to the amide is important for Hsp90 inhibitory activity.[15] Therefore, analogues containing five-, six- and seven-membered rings with varying distances and orientations of both the amine and amide were pursued.

The phenylpiperidine analogue (**43**), which represents a shorter distance between the amine and amide, was prepared as described in Scheme 5. Synthesis of the phenylpiperidine-1-yl core commenced via an amide coupling between piperidine **38** and biaryl acid **12** to give amide **39**, which was converted to the triflate before Suzuki coupling with boronic acid **41** to give **42**. Hydrogenolysis of **42**, followed by Mitsunobu etherification of the resulting phenol with 1-methyl-4-hdroxypiperdine (**14a**) gave **43**.



Scheme 5. Synthesis of phenylpiperidin-1-yl carboxamide. Reagents and conditions: *a* **12**, EDCI•HCl, Et_3N, DCM, 0 °C to rt, 12 h, 75%; *b* N-Ph2Tf, LDA, THF, -78 °C to rt, 12 h, 55%; *c* $Pd(PPh_3)_4$, K_2CO_3, toluene/EtOH/H_2O, 110 °C, 12 h, 81%; *d* 1,4-cyclohexadiene, MeOH, 70 °C, 48 h, 85%; *e* **14a**, TMAD, PBu_3, benzene, 90 °C, 12 h, 35%.

Preparation of analogues (**53**) that contain a five-membered saturated ring is illustrated in Scheme 6. Synthesis began by a Heck coupling between aryl bromide **44** and cyclopent-2-enone to yield **45**, which was subsequently reduced via palladium on carbon and hydrogen gas to afford cyclopentanone **46**.[21] Reduction of **46** with sodium borohydride gave an inseparable mixture of *anti* and *syn* diastereomers, **47**, in a 7:3 ratio, respectively. The mixture of **47** was converted to the methanesulfonate ester, **48**, before nucleophilic substitution with sodium azide to produce **49**. Following reduction of the azide, the resulting amine was coupled with biaryl acid **12** to form the

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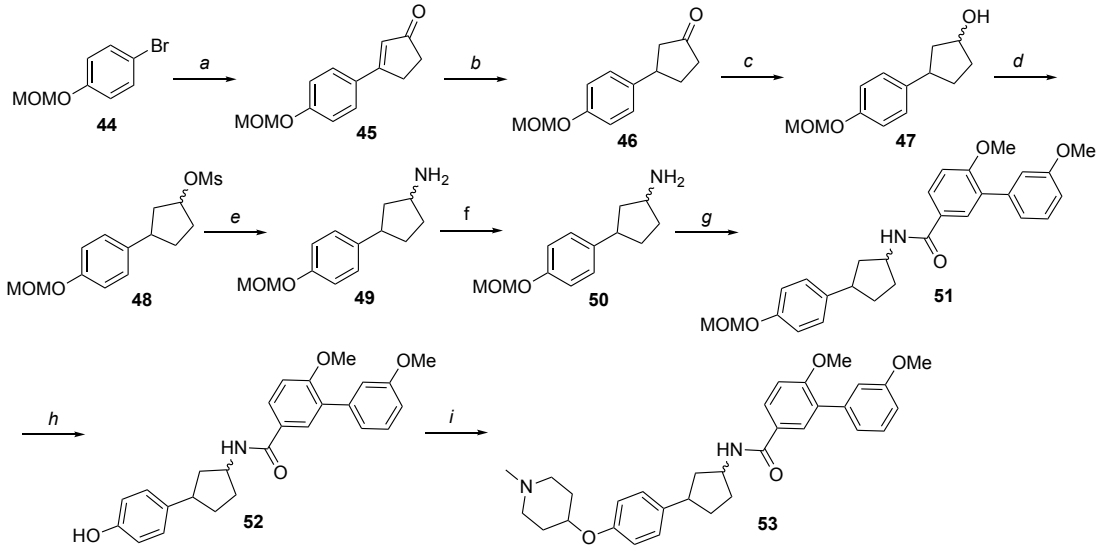
corresponding amide **51**. Removal of the methoxymethyl protecting group present in **51** provided the free phenol, **52**. Mitsunobu etherification of the resulting phenol with 1-methyl-4-hdroxypiperdine (**14a**) finally furnished the desired product **53** in moderate yield.



Scheme 6. Synthesis of phenylcyclopentyl carboxamides. Reagents and conditions: *a* Cyclopent-2-en-1-one, $Pd(OAc)_2$, triethanolamine, toluene, 110 °C, 12 h, 75%; *b* Pd/C, H_2, EtOAc, rt, 12 h, ~100%; *c* $NaBH_4$, MeOH, 0 °C to rt, 1 h, 90%; *d* MsCl, Et_3N, THF, 0 °C to rt, 1 h, 90%; *e.* NaN_3, DMF, 100 °C, 12 h, 85%; f. 10% Pd/C, H_2, EtOAc, rt, 12 h, ~100%; *g* **12**, EDCI•HCl, HOBt, DIPEA, DCM, 0 °C to rt, 12 h, 80%; *h* 6N HCl, MeOH/THF, 0 °C to rt, 12 h, 60%; *i* **14a**, TMAD, PBu_3, benzene, 90 °C, 12 h, 20%.

As shown in Scheme 7, synthesis of analogues that contain a seven-membered ring began via a Grignard coupling reaction of commercially available ester **54** with allyl magnesium bromide to yield **55**.[22] Ring-closing metastasis using Grubbs first-generation catalyst, followed by removal of the hydroxyl group using triethylsilane provided the desired phenylcycloheptyl core, **57**. Oxidation of alkene **57** with meta-chloroperoxybenzoic acid resulted in an inseparable mixture of epoxides **58a** and **58b**, in a 1:1 ratio. Reduction of these epoxides with lithium aluminum hydride resulted in alcohol **59**, which upon acid-catalyzed cleavage of the methyl ether, provided **60**. Next, **60** was converted to methanesulfonate ester **61** before nucleophilic substitution with sodium azide and then subsequently reduced to afford amine **62**. Amide coupling of **62** with acid chloride **12a** produced **63**, which after base-catalyzed deprotection of the methanesulfonate group gave the free phenol, **64**. Finally, Mitsunobu etherification of the resulting phenol with 1-methyl-4-hdroxypiperdine (**14a**) furnished **65**.

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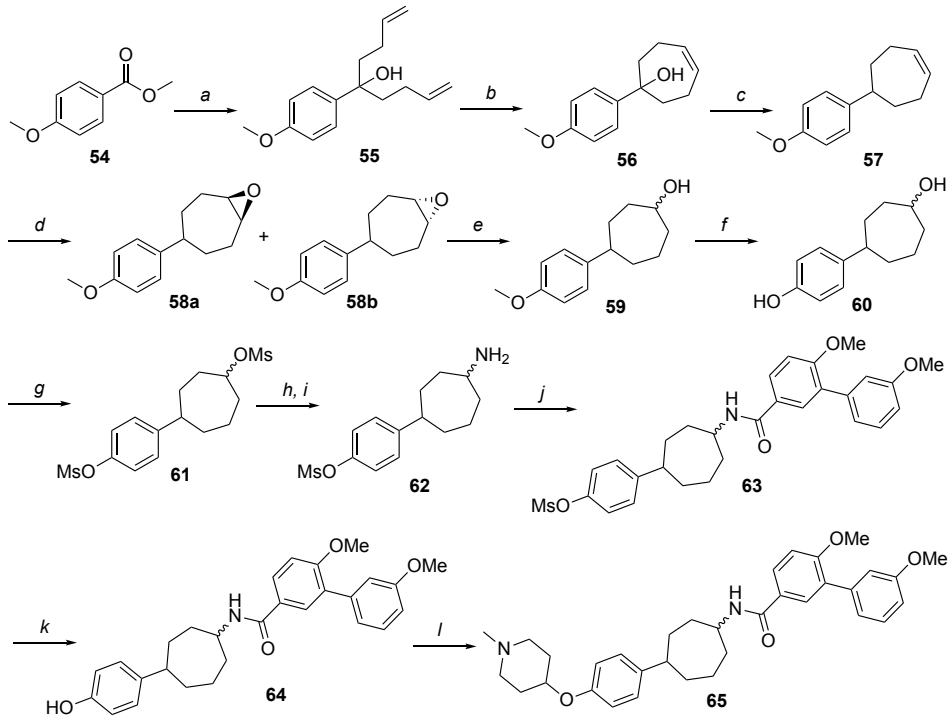

Scheme 7. Synthesis of phenylcycloheptyl carboxamides. Reagents and conditions: *a* AllylMgBr, THF, 0 °C to rt, 12 h, 84%; *b* Grubbs I, DCM, 40 °C, 12 h, 54%; *c* Et_3SiH, TFA, DCM, 48 h, 50%; *d* mCPBA, $NaHCO_3$, DCM, 0 °C, 12 h, 89%; *e* LAH, $AlCl_3$, THF, 0 °C to rt, 12 h, 60%; *f* BBr_3, DCM, -78 °C to rt, 2 h, 46%; *g* MsCl, Et_3N, THF, 0 °C to rt, 1 h, 90%; *h* NaN_3, DMF, 100 °C, 12 h, 40%; *i* Pd/C, H_2, EtOAc, rt, 12 h, 90%; *j* **12a**, DIPEA, DCM, 0 °C to rt, 12 h, 90%; *k* 3.2 N KOH, EtOH, 90 °C, 3 h, 60%; *l* **14a**, TMAD, PBu_3, benzene, 90 °C, 12 h, 32%.

Compounds (**70** and **78**) that contain a methylene linker were envisioned to impart additional flexibility and explore potential binding interactions as a consequence of the flexible benzamide side chain. As shown in Scheme 8, construction of the phenylcyclopentyl methyl derivative (**70**) was accomplished by employing the methanesulfonate ester **48** as a key intermediate. Briefly, **48** was first converted to cyanide **66** via a nucleophilic substitution reaction with tetra-butylammonium cyanide and then reduced with lithium aluminum hydride to afford the free amine, **67**.[23] Amide coupling of the resulting amine with acid chloride **12a** gave the corresponding amide **68**. Acid-catalyzed deprotection of the methoxymethyl-protecting group generated the free phenol, **69**, which underwent Mitsunobu etherification to yield an inseparable mixture of *anti* and *syn* diastereomers of **70** in a 6:4 ratio respectively.

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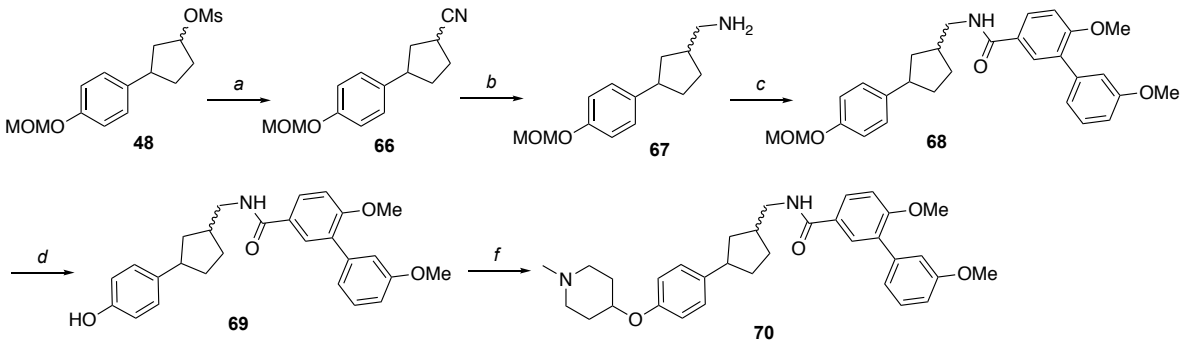
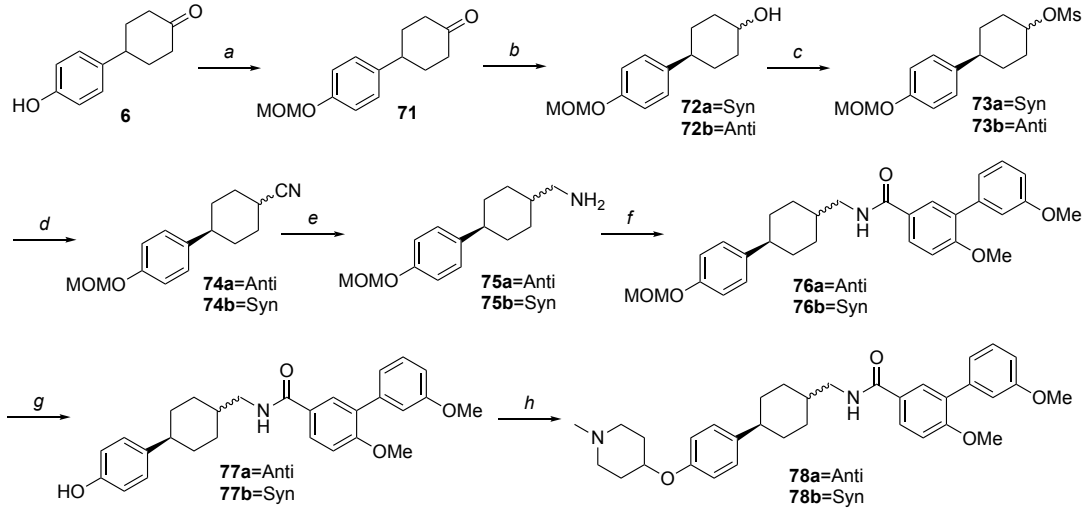


Scheme 8. Synthesis of phenylcyclopentyl methyl carboxamide. Reagents and conditions: *a* $(CH_3CH_2CH_2CH_2)_4N(CN)$, DMF, 85 °C, 12 h, 55%; *b* LAH, THF, 0 °C to rt, 3 h, 88%; *c* **12**, EDCI•HCl, HOBt, DIPEA, DCM, 0 °C to rt, 80%; *d* p-TSA, MeOH, rt, 12 h, 60%; *e* **14a**, TMAD, PBu_3, benzene, 90 °C, 12 h, 25%.

As outlined in Scheme 9, the phenylcyclohexyl methyl derivatives (**78a** and **78b**) were assembled following the general strategy described earlier.



Scheme 9. Synthesis of phenylcyclohexyl methyl carboxamides. Reagents and condtions: *a* MOMCl, DIPEA, DCM, 0 °C to rt, 12 h, 65%; *b* L-selectride, THF, -40 °C to rt, 12 h, 70% or $NABH_4$, MeOH, rt, 30 min, 85%; *c* MsCl, Et_3N, DCM, 0 °C to rt, 12 h, 85 − 90%%; *d* $(CH_3CH_2CH_2CH_2)_4N(CN)$, DMF, 85 °C, 12 h, 40 − 49%; *e* Pd/C, H_2, EtOAc, rt, 12 h, 65 − 70%%; *f* **12a**, DIPEA, DCM, 0 °C to rt, 12 h, 70 − 80%; *g* 6N HCl, MeOH, 0 °C to rt, 12 h, 66 − 72%; *h* **14a**, TMAD, PBu_3, benzene, 90 °C, 12 h, 31 − 39%.

Upon construction of the modified B-ring analogues, the compounds were evaluated for their anti-proliferative activity against both breast cancer cell lines as summarized in Table 2. In general, compounds containing other ring systems were less active than the lead compound **15a**, suggesting the 1,4-*anti*-cyclohexyl core is optimal. Incorporation of either a five- or seven-membered ring system resulted in less potent analogues as evidenced by **53** and **65**, which were 25-40 fold less active than **15a**, highlighting the orientation of the side chains for increased anti-proliferative activity. Inclusion of a methylene linker (**70**, **78a** and **78b** vs **15a**) did not improve activity, suggesting that flexibility is not necessary for maximal activity. Surprisingly,

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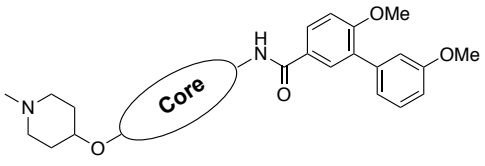
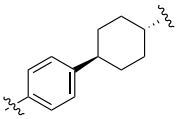
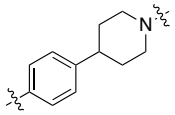
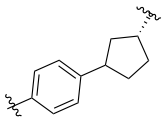
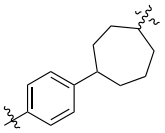
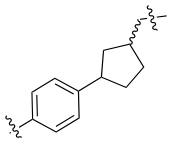
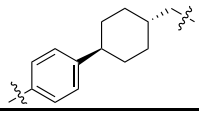
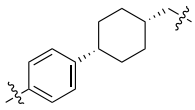
the stereochemistry of analogues **78a** and **78b** was not important for anti-proliferative activity, as both the *syn* and *anti*-diastereomers manifested similar activities.

Overall, these studies suggest that the 1,4-*anti*-cyclohexyl core is optimal for orientation of the appendages in a conformation that leads to increased anti-proliferative activity. Therefore, molecules containing the phenyl cyclohexyl core were further investigated.

Table 2. Anti-proliferative activity of analogues containing various central core.



Entry	Compound	SKBr3 (IC_{50}, μM)	MCF-7 (IC_{50}, μM)
15a		0.17 ± 0.02	0.22 ± 0.01
43		>10	>10
55		5.46 ± 0.94	9.14 ± 0.69
65		3.51 ± 0.14	4.17 ± 0.01
70		4.11 ± 0.61	6.62 ± 0.15
78a		2.98 ± 0.43	3.16 ± 0.31
78b		4.13 ± 0.74	5.0 ± 0.29

2.2 Exploration of the amine moiety. Prior studies on novobiocin have demonstrated that the noviose sugar or a surrogate is necessary for Hsp90 inhibitory activity and that modifications to this moiety can improve activity. Therefore, a concise library of

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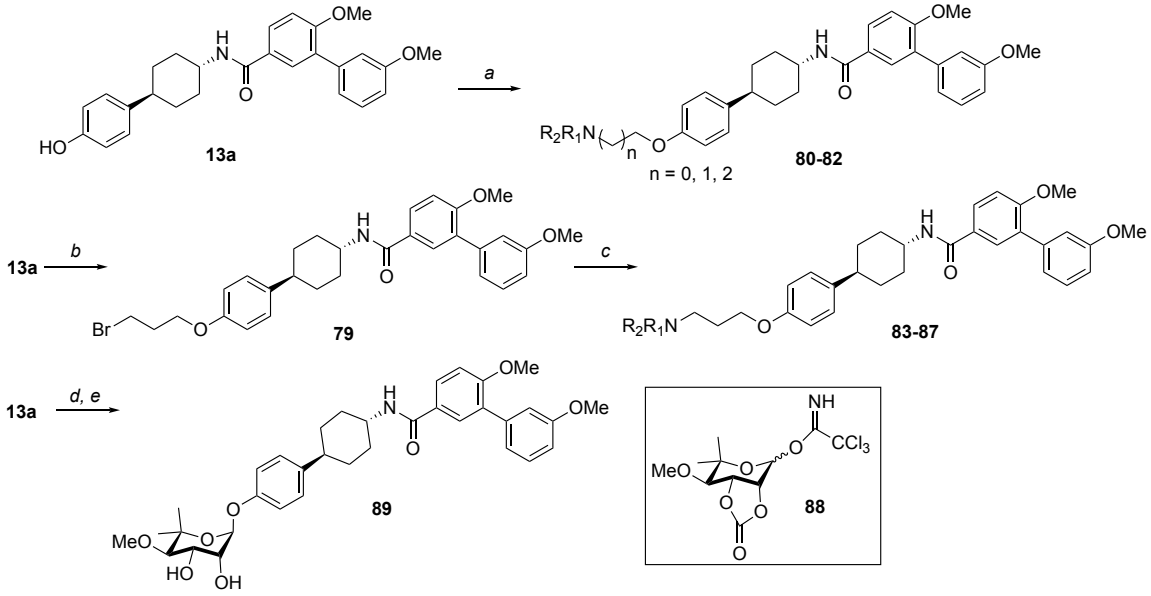
phenylcyclohexyl analogues containing various amines or the noviose sugar was designed to probe linker length, hydrogen-bonding interactions, and steric bulk. As shown in Scheme 10, analogues (**80**-**82**) containing various linker lengths were synthesized by Mitsunobu etherification of phenol **13a** with the corresponding amino alcohols. Synthesis of the propylene-tethered amines (**83**-**87**) was accomplished by *O*-alkylation of **13a** with 1,3-dibromopropane to give ether **79**, which was then treated with primary or secondary amines to yield the desired products in moderate yield. Compound **89** was prepared by coupling phenol **13a** with the trichloroacetimidate of noviose carbonate (**88**) in the presence of boron trifluroide etherate, followed by solvolysis of the cyclic carbonate.



Scheme 10. Synthesis of phenylcyclohexyl carboxamides containing various amines. Reagents and conditions: *a* alcohol, TMAD, PBu_3, benzene, 90 °C, 12 h, 38 - 60%; *b* 1,3-dibromopropane, K_2CO_3, DMF, 0 °C to rt, 12 h, 68%; *c* amine, K_2CO_3, DMF, rt, 12 h, 48 − 68%; *d* **88**, BF_3OEt_2, DCM, rt, 12 h, 55%; *e* 2%Et_3N/MeOH, rt, 12 h, 68%.

Upon construction of the library, the individual compounds were evaluated against SKBr3 and MCF-7 cancer cell lines as summarized in Table 3. Acyclic amines (**80**, **81**) exhibited comparable anti-proliferative activity as the cyclic amine, **15a**, while homologation of the linker resulted in decreased activity (**82** vs **15a**), indicating the 3-carbon chain is optimal for anti-proliferative activity. The incorporation of bulky substituents (**83**, **86**, **87**) maintained activity, indicating that various substituents could be accommodated within the binding pocket. Interestingly, the inclusion of a second hydrogen bond donor/acceptor (**84**, **85**) produced activity comparable to **83**. In addition, secondary amines (**86**, **87**) manifested similar activity as the tertiary amines.

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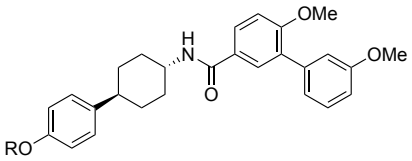
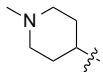
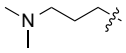
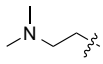
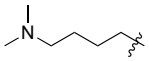
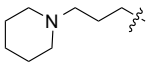
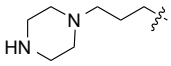
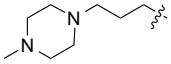
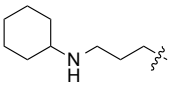
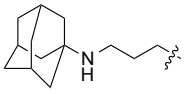
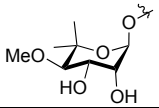
Surprisingly, compound **89** exhibited 5-20 fold decreased activity compared to **15a**, indicating the phenyl cyclohexyl core may bind differently than its coumarin counterpart.

Table 3. Anti-proliferative activity of phenylcyclohexyl carboxamides with modified side chain.



S. No.	R	SKBr3 (IC_{50}, μM)	MCF-7 (IC_{50}, μM)
15a		0.17 ± 0.02	0.22 ± 0.01
80		0.25 ± 0.05	0.34 ± 0.02
81		0.27 ± 0.05	0.36 ± 0.01
82		0.82 ± 0.05	1.28 ± 0.71
83		0.28 ± 0.09	0.34 ± 0.01
84		0.40 ± 0.04	0.41 ± 0.08
85		0.32 ± 0.02	0.46 ± 0.01
86		0.33 ± 0.04	0.36 ± 0.01
87		0.40 ± 0.06	0.57 ± 0.01
89		4.52 ± 0.25	1.20 ± 0.09

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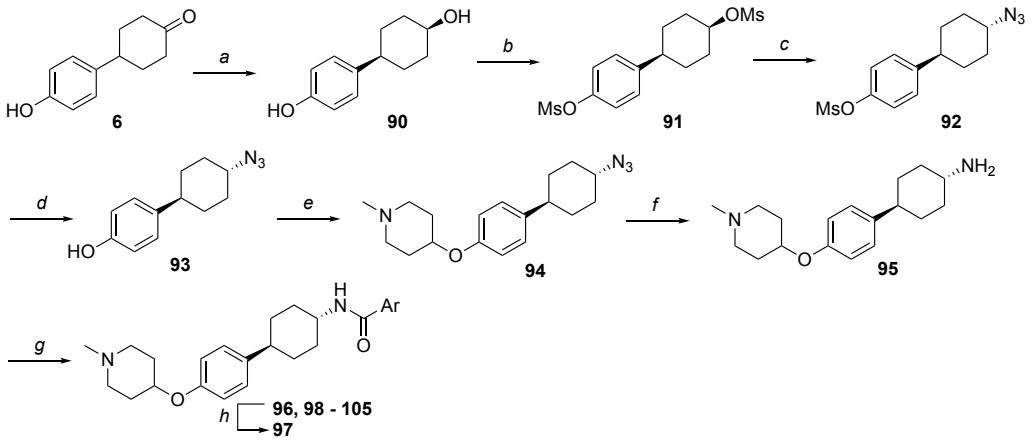
2.3 Exploration of the benzamide side chain using optimal core and amines.

Exploration of the benzamide side chain was also pursued with the optimized core and amine. Earlier structural investigations on the benzamide identified several side chains that contained either a prenyl, indole, triazole or biaryl moiety, all of which manifested mid-nanomolar to low micromolar activity against several cancer cell lines while attached to the coumarin scaffold. Therefore, a focused library of phenyl cyclohexyl derivatives was designed to contain these previously identified side chains in an effort to gain additional interactions with the binding pocket. In addition, structural investigation of the biaryl side chain was sought to identify locations to which modifications can be made. As shown in Scheme 11, synthesis of the phenylcyclohexyl analogues began by reduction of commercially available ketone **6**, with L-selectride to yield alcohol **90**, which was then converted to the methanesulfonate ester **91** before stereochemical inversion with sodium azide to afford **92**.[24] Base-catalyzed deprotection of the phenol, followed by Mitsunobu etherification with 1-methyl-4-hydroxypiperidine (**14a**) produced azide **94**. The azide was reduced via palladium on carbon under a hydrogen atmosphere to give amine **95**, which was then coupled with acid chlorides **12g**-**12p** to give amides **96**-**105**, in moderate to low yields.



Scheme 11. Synthesis of phenylcyclohexyl derivatives that contain modified benzamide side chains. Reagents and conditions: *a* L-selectride, THF, -78 °C to rt, 12 h, 90%; *b* MsCl, Et_3N, DCM, 0 °C to rt, 12 h, 90%; *c* NaN_3, DMF, 100 °C, 12 h, 65%; *d* 3.2 N KOH, ethanol, 90 °C, 12 h, 65%; *e* **14a**, PPh_3, DIAD, THF, 0 °C to rt, 12 h, 60%; *f* 10% Pd/C, H_2, MeOH/EtOAc, rt, 12 h, ~100%; *g* Ar-COCl, Et_3N, DCM, 0 °C to rt, 12 h, 65 − 80%; *h* K_2CO_3, MeOH, rt, 1 h, 78%.

Upon completion, the library of phenylcyclohexyl analogues that contain modified side chains was evaluated for antiproliferative activity against both breast cancer cell lines. As shown in Table 4, the incorporation of prenylated, triazole or indole (**96**-**99** Vs **15a**) side chains onto the phenylcyclohexyl core resulted in significantly decreased activity compared to the biaryl ring system, suggesting such

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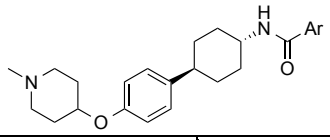
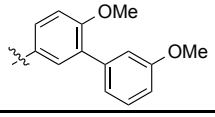
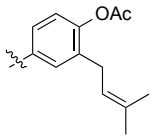
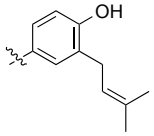
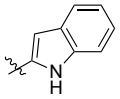
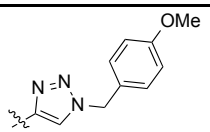
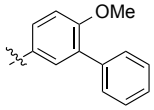
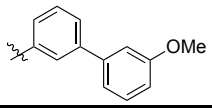
modifications are not well tolerated. These results suggest that the phenyl cyclohexyl core projects the amine and benzamide side chains into a different orientation as compared to the biphenyl and coumarin cores. The results also suggest that the 4-methoxy is important for activity as compound **100** showed similar activity, while compounds **101** and **102** exhibited 5-6 fold less activity compared to **15a**. Removal of the second phenyl ring resulted in a 20-40 fold loss in activity (**103** vs **15a)**, emphasizing a requirement for both aryl rings. In addition, the results suggest that a hydrogen-bond donor at the *para*-position is beneficial, as replacement of the methoxy group with either hydroxyl (**104**) or acetoxy (**105**) resulted in decreased activity.

Table 4. Anti-proliferative activity of cyclohexylphenylamides with modified side chain.



Entry	Ar	SKBr3 (IC_{50}, μM)	MCF-7 (IC_{50}, μM)
15a		0.17 ± 0.02	0.22 ± 0.01
96		3.21 ± 0.19	2.61 ± 0.21
97		1.54 ± 0.04	2.34 ± 1.41
98		>10	>10
99		1.16 ± 0.36	1.66 ± 0.31
100		0.39 ± 0.02	0.66 ± 0.01
101		1.03 ± 0.02	1.13 ± 0.15

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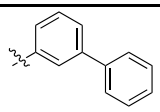
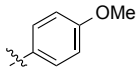
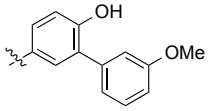
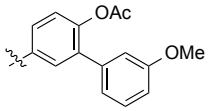
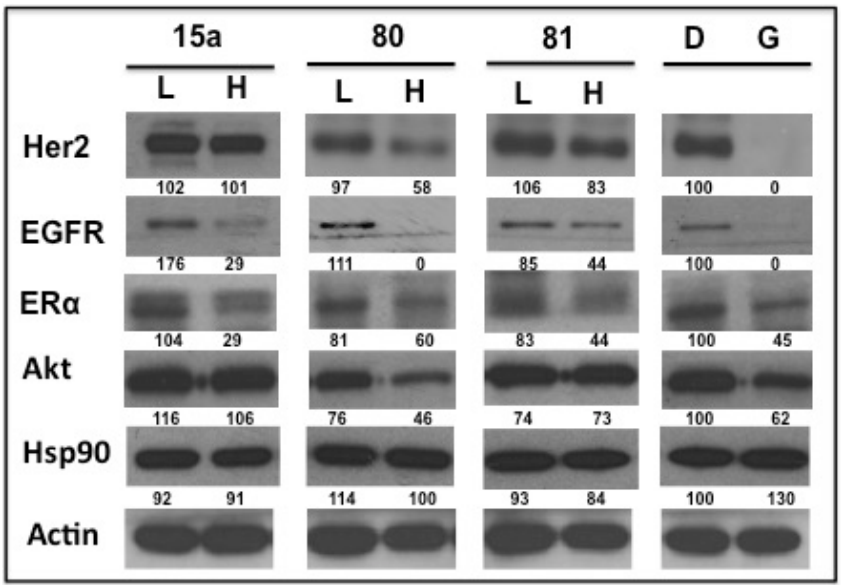
102		1.22 ± 0.12	0.72 ± 0.08
103		7.39 ± 0.58	5.83 ± 0.31
104		3.61 ± 0.14	1.81 ± 0.05
105		2.09 ± 0.05	1.75 ± 0.09

Validation of Hsp90 inhibition via Western Blot Analysis.

Western blot analyses were performed on MCF-7 cell lysates treated with five representative compounds (**15a**, **80**, **81, 55** and **100**) to confirm these analogues manifest anti-proliferative activity via Hsp90 inhibition. As shown in Figure 4A, incubation with compound **15a** caused degradation of Hsp90-dependent clients EGFR and ERα, while treatment with compounds **80** and **81** led to the degradation of Her2, EGFR, ERα, and Akt, clearly linking cell viability to Hsp90 inhibition. In addition, treatment of MCF-7 cells with **55** and **100** induced the degradation of the Hsp90-dependent client substrates ERα, and Akt (Figure 4B). The Hsp90-independent protein, actin remained unchanged, suggesting selective degradation of Hsp90-dependent client proteins. In addition, Hsp90 levels remained constant, which is a hallmark shared by Hsp90 C-terminal inhibitors.

A.



B.

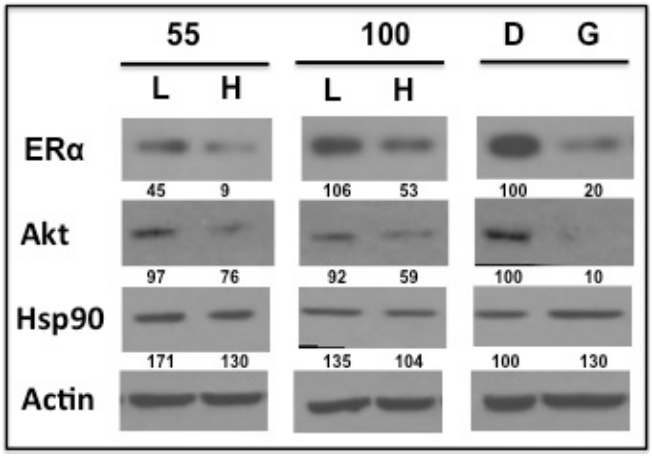


Figure 4 Western blot analyses of compounds after incubation with MCF-7 breast cancer cells for 24 h. (**A**) Western blot analyses of Hsp90-dependent client proteins (Her2, EGFR, ERα and Akt) degradation after treatment with **15a**, **55** and **80**. (**B**) Western blot analyses of Hsp90-dependent client proteins (ERα and Akt) degradation after treatment with **55** and **100**. L represents a concentration 1/2 of the anti-proliferative IC_{50} value, while H represents a concentration 5-fold of the anti-proliferative IC_{50} value. Geldanamycin (G, 0.5 μM) and dimethylsulfoxide (D, 100%) were employed as positive and negative controls, respectively. Protein levels were measured compared to the level of Actin.

Conclusions:

In summary, the biphenyl core was explored to identify an optimized scaffold for Hsp90 C-terminal inhibition. These studies led to the development of phenyl cyclohexyl carboxamides that manifest sub-micromolar to mid-nanomolar anti-proliferative activity against multiple breast cancer cell lines. Structural investigations suggest that the central core is important for projection of the amine and amide side chains as evidenced by increased anti-proliferative activity. Furthermore, SAR studies on these side chains indicate the phenyl cyclohexyl derivatives exhibit a different binding mode as compared to other scaffolds. Discovery of the phenyl cyclohexyl core provides a new platform on which the development of more efficacious Hsp90 C-terminal inhibitors can be explored.

3. Experimental section

3.1 Anti-proliferation assays. Cells were maintained in a 1:1 mixture of Advanced DMEM/F12 (Gibco) supplemented with non-essential amino acids, L-glutamine (2 mM), streptomycin (500 μg/mL), penicillin (100 units/mL), and 10% FBS. Cells were grown to confluence in a humidified atmosphere (37° C, 5% CO_2), seeded (2000/well, 100 μL) in 96-well plates, and allowed to attach overnight. Compound or GDA at

varying concentrations in DMSO (1% DMSO final concentration) was added, and cells were returned to the incubator for 72 h. At 72 h, the number of viable cells was determined using an MTS/PMS cell proliferation kit (Promega) per the manufacturer's instructions. Cells incubated in 1% DMSO were used at 100% proliferation, and values were adjusted accordingly. IC_{50} values were calculated from separate experiments performed in triplicate using GraphPad Prism.

3.2 Western blot Analyses. MCF-7 cells were cultured as described above and treated with various concentrations of drug, GDA in DMSO (1% DMSO final concentration), or vehicle (DMSO) for 24 h. Cells were harvested in cold PBS and lysed in RIPA lysis buffer containing 1 mM PMSF, 2 mM sodium orthovanadate, and protease inhibitors on ice for 1 h. Lysates were clarified at 14000g for 10 min at 4° C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal amounts of protein (20 µg) were electrophoresed under reducing conditions, transferred to a nitrocellulose membrane, and immunoblotted with the corresponding specific antibodies. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

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