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Novologues Containing a Benzamide Side Chain Manifest Anti-proliferative Activity Against Two Breast Cancer Cell Lines

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The heat shock protein 90 (Hsp90) folding machinery is essential for the maturation of nascent polypeptides into their biologically active three-dimensional-structures and for the rematuration/clearance of misfolded proteins that form under cellular stress.[1-3] As a pro-survival chaperone, Hsp90 overexpression is commonly observed in transformed cells, which is required to sustain the hostile tumor micro-environment associated with nutrient deprivation and hypoxia. Pharmacological inhibition of Hsp90 has been shown to induce the degradation of oncogenic proteins associated with all six hallmarks of cancer that rely upon Hsp90.[4-8] Consequently, Hsp90 represents a highly sought after target for the treatment of cancer. In fact, 17 small molecules that bind competitively to the N-terminal ATP-binding pocket are under clinical evaluation against various cancers.[9,10] However, heat shock factor 1 (HSF-1), the master regulator of the pro-survival heat shock response also binds Hsp90. Ultimately, Hsp90 N-terminal inhibition results in HSF-1 release, and upon phosphorylation, trimerizes and translocates to the nucleus wherein it binds the heat shock elements to activate the pro-survival, heat shock response (HSR). The HSR serves to expand the cellular buffering capacity and to assist in the maturation of mutated and oncogenic substrates.[11] This concomitant heat shock response is detrimental to the treatment of cancer and may lead to

drug resistance and tumor metastasis.[12] Recent studies have demonstrated that allosteric modulation of the Hsp90 C-terminus can separate the pro-survival heat shock response from pro-apoptotic, client protein degradation.[13-20] Two classes of small molecules derived from novobiocin **1**, (Figure 1) the first identified Hsp90 C-terminal inhibitor, were discovered via the structure-activity relationship studies. KU-32 (**2**), which lacks a 4-hydroxyl, the 3'-carbamate, and contains an acetamide in lieu of a prenylated benzamide, represents a lead compound that induces the heat shock response at concentrations much lower than that needed for client protein degradation.[2,21] Consequently, this class of analogues has been evaluated as neuroprotective agents to refold protein aggregates.[22-24] In contrast, KU-174 (**3**) contains a biarylamide side chain in lieu of the acetamide, and induces Hsp90 client protein degradation without induction of the heat shock response.[25-26] Therefore, this class of novobiocin analogues manifests optimal properties for the treatment of cancer, as no HSR is observed with such compounds.

Recently, a second generation of novologues that contains a scaffold that mimics KU-32 was identified, and biological evaluation against primary sensory neurons showed these molecules to possess enhanced neuroprotective properties.[27] Novologue **4** contains a 3-trifluoromethylphenyl ring in lieu of the coumarin lactone present in novobiocin and incorporates the acetamide onto the flexible ethylene linker to maintain hydrogen-bonding interactions with the Hsp90 C-terminal binding pocket. As shown during the discovery and development of novobiocin-based Hsp90 C-terminal inhibitors, restoration of the benzamide side chain onto KU-32 transforms the molecule from manifesting neuroprotective activity into one that serves as an anti-cancer agent. Therefore, we hypothesized that replacement of the acetamide present in novologue **4** with a biarylamide should also transform this novologue into an anti-proliferative agent (**5a**).

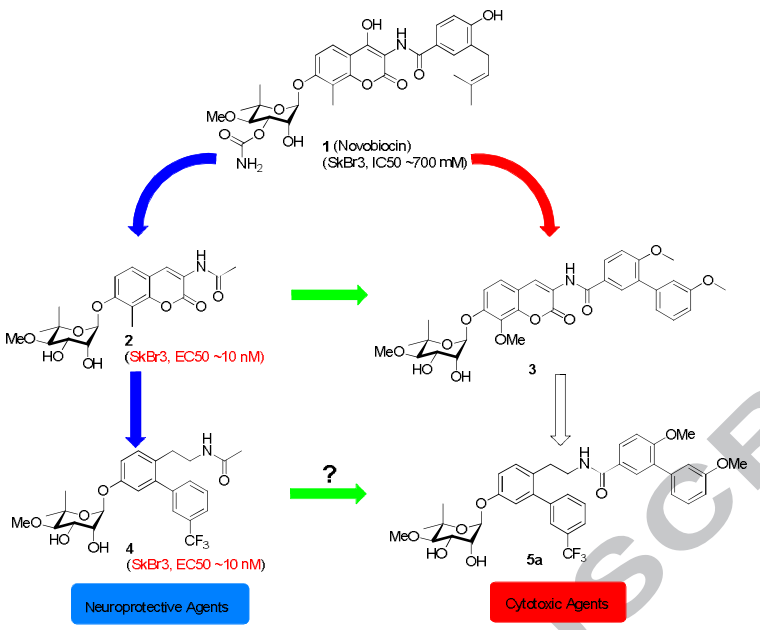
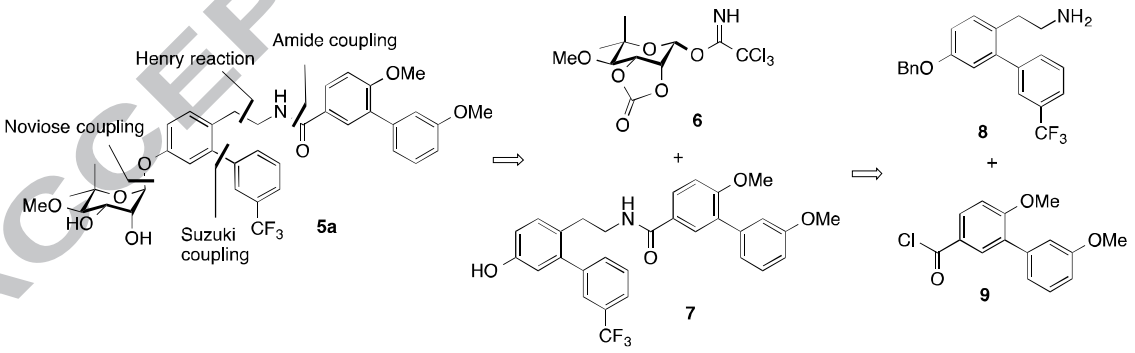


Figure 1. Hypothesis for the design of cytotoxic novologues.

As shown in Scheme 1, retrosynthesis of compound **5a** (and related analogues) was envisioned for construction via noviosylation of phenol **7** by activated noviose carbonate, **6**. Intermediate **7** could be assembled via an amide coupling reaction between amine **8** and acid chloride **9**, followed by hydrogenolysis. As reported previously,[27] the synthesis of amine **8** could be achieved from 2,4-dihydroxybenaldehyde through phenol protection, Suzuki coupling, a Henry reaction, and reduction of the corresponding α,β-unsaturated nitro styrene.

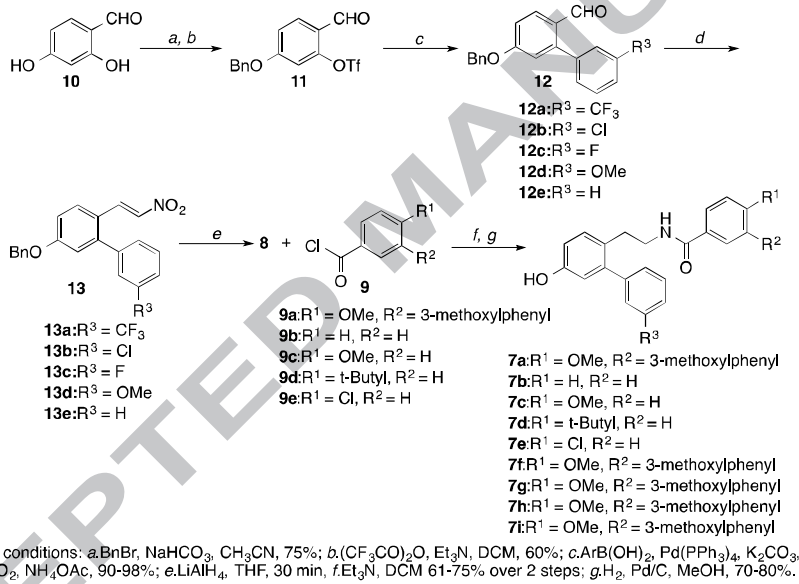


Scheme 1. Retrosynthesis of cytotoxic novologues.

Synthesis of these novologues commenced via selective phenol protection of 2,4-dihydroxybenaldehyde (**10**) with benzyl bromide. The resulting benzyl ether was converted to trifluoromethanesulfonate **11**, in the presence of trifluoromethanesulfonic anhydride and

triethylamine. Subsequent Suzuki coupling with commercially available aryl boronic acids (phenyl, *m*-CF_3, *m*-Cl, *m*-F and *m*-OMe) was employed to generate the respective biaryl ring systems, **12a-e**. These boronic acids were selected based on prior studies that suggested the inclusion of electronegative substituents at the meta-position of the B-ring exhibit favorable interactions with Lys539 in the Hsp90 C-terminal binding pocket.[27]

Benzaldehydes **12a-e** were subjected to a Henry reaction to afford the corresponding nitrostyrenes, **13a-e**. Subsequent lithium aluminum hydride reduction of the nitrostyrenes to the corresponding amines, followed by acylation with benzoyl chlorides **9a-e** gave the benzyl ether containing benzamides, which upon hydrogenolysis produced phenols **7a-i**.



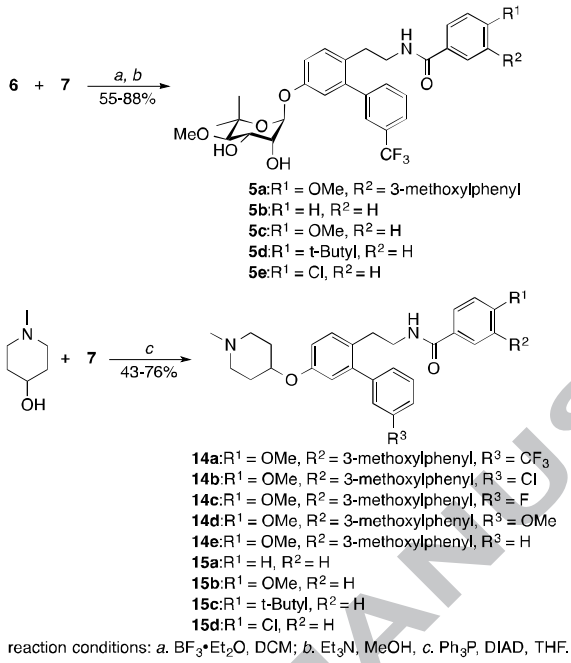
reaction conditions: *a*.BnBr, $NaHCO_3$, CH_3CN, 75%; *b*.$(CF_3CO)_2O$, Et_3N, DCM, 60%; *c*.$ArB(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, DMF, 60-90%; *d*.CH_3NO_2, NH_4OAc, 90-98%; *e*.$LiAlH_4$, THF, 30 min, *f*.Et_3N, DCM 61-75% over 2 steps; *g*.H_2, Pd/C, MeOH, 70-80%.

Scheme 2. Synthesis of phenol **7a-e**.

Preparation of the novologues required noviosylation of phenols **7a-e**, which occurred upon treatment with the trichloroacetimidate of noviose carbonate **6** in the presence of catalytic boron trifluoride etherate, followed by solvolysis of the cyclic carbonate with methanolic ammonia to generate the desired products, **5a-e**, in good yields.

In addition, novologues **14a-i** were synthesized to investigate prior observations that suggested the replacement of noviose with amines could further increase inhibitory activity

and solubility.[28-31] Therefore, Mitsunobu etherification of phenols **7a-i** with 1-methyl-4-hydroxypiperidine was performed to yield the amino containing novologues, **14a-i**.

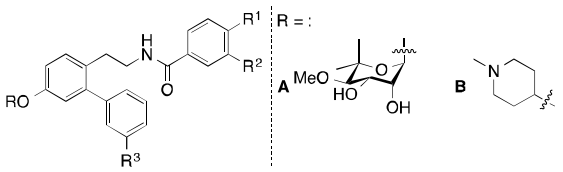


Scheme 3. Synthesis of cytotoxic novologues.

Upon construction of the library, these novologues were evaluated for anti-proliferative activity against two breast cancer cell lines: SKBr3 (estrogen receptor negative, HER2 over-expressing breast cancer cells) and MCF-7 (estrogen receptor positive breast cancer cells). As shown in Table 1, the novologues manifested anti-proliferative activity against both breast cancer cell lines. Novologues containing a piperidine ring were shown to exhibit greater activity than their noviose containing counterparts (**14a-e** vs **5a-e**), which is consistent with prior studies on novobiocin.[28] Although substitutions on the benzamide side chain were beneficial to anti-proliferative activity, 4-Cl (**5e**) and 4-methoxy (**5c**) were better than 4-*t*-butyl (**5d**) for the compounds containing noviose. However, compounds containing the piperidine ring manifested better activity when 4-*t*-butyl (1**5c**) or 4-Cl (1**5d**) were present, compared to the 4-methoxy derivative (**15b**). Substitutions on the B ring did not produce significant differences in anti-proliferative activity, however, as predicted, the inclusion of an

electronegative atom (F, Cl and CF_3) at the meta-position of the B-ring was most active. As hypothesized, all the novologues were more active than the parent, acetamide-containing, neuroprotective compound **4**.

Table 1. Anti-proliferative activity manifested by novologues containing a benzamide side chain

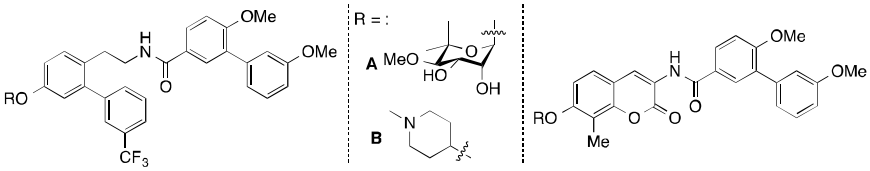


Entry	R	R^1	R^2	R^3	SKBr3	MCF-7
4	-	-	-	-	>500	>500
5a	A	OMe	*m*-methoxyphenyl	-	13.98±2.04	18.40±2.11
5b	A	H	H	-	41.90±7.69[a]	44.44±1.81
5c	A	OMe	H	-	14.02±0.64	12.10±0.37
5d	A	*t*-Butyl	H	-	21.78±0.62	23.69±0.91
5e	A	Cl	H	-	14.48±2.34	11.69±0.84
14a	B	OMe	*m*-methoxyphenyl	CF_3	1.52±0.05	1.62±0.15
14b	B	OMe	*m*-methoxyphenyl	Cl	5.63±1.45	6.87±1.05
14c	B	OMe	*m*-methoxyphenyl	F	2.82±0.93	3.19±0.15
14d	B	OMe	*m*-methoxyphenyl	OMe	11.54±0.75	12.36±0.86
14e	B	OMe	*m*-methoxyphenyl	H	6.77±1.35	8.12±0.35
15a	B	H	H	-	13.22±1.50	15.07±1.41
15b	B	OMe	H	-	2.71±0.62	4.82±0.11
15c	B	*t*-Butyl	H	-	1.16±0.10	1.48±0.26
15d	B	Cl	H	-	1.87±0.64	2.11±0.04

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

In order to gain further insight into this class of compounds, a comparison between the anti-proliferative activities manifested by novobiocin-derived inhibitors and these novologues was performed. Although the benzamide side chain is attached to a freely rotating ethylene linker in novologues **5a** and **14a**, they manifested similar activity to novobiocin derivatives **16** and **17**, suggesting that this new scaffold can serve as a surrogate for the coumarin ring system. More importantly, the entropic penalty introduced by incorporation of the ethylene linker appears to be compensated by interactions provided by incorporation of the trifluorophenyl ring. Alternatively, the rigid coumarin ring system may not be optimal for binding interactions.

Table 2. Comparison of anti-proliferative activities: benzamide-containing novologue versus novobiocin derivatives



Novologues			R	Novobiocin derivatives	
SKBr3	**5a**	13.98±2.04	A	7.50±0.80	**16**
	15a	1.30±0.19	B	1.38±0.18	**17**
MCF-7	**5a**	18.40±2.11	A	18.70±1.44	**16**
	15a	1.62±0.15	B	1.51±0.24	**17**

To confirm that Hsp90 inhibition was responsible for the observed anti-proliferative activities manifested by these novologues, Western blot analyses of several Hsp90 dependent client protein levels were examined in MCF-7 cell lysates treated with **14a**.[32] As shown in Figure 2, Her2, Raf-1 and *p*-Akt, all of which are well-validated Hsp90-dependent client proteins, were degraded in a concentration-dependent manner, while non-Hsp90-dependent actin levels remained constant. In addition, Hsp90 levels remained unchanged, indicating that these novologues bind the Hsp90 C-terminus.[33-36]

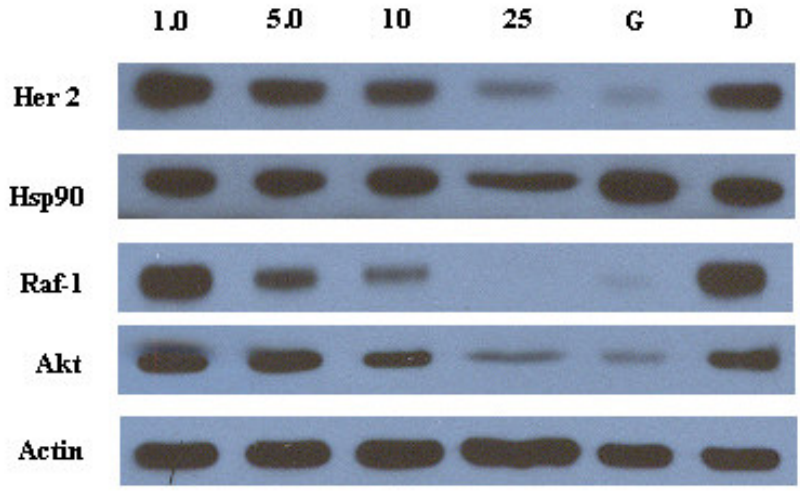


Figure 2. Western blot analysis of Hsp90-dependent client proteins from MCF-7 breast cancer cell lysate upon treatment with **14a**. Concentrations (in μM) were indicated above each line. Geldanamycin (G, 0.5 μM) and dimethylsulfoxide (D, 100%) were employed as positive and negative controls.

In conclusion, a library of benzamide-containing novologues was designed, synthesized and evaluated against two breast cancer cell lines. Initial structure-activity relationships for the amide appendage were elucidated and novologue **14a** was shown to exhibit lead-like activity. Western blot analyses demonstrated these compounds to manifest anti-proliferative activity through Hsp90 inhibition. Replacement of the acetamide side chain on the novologue scaffold with a benzamide side chain successfully transformed this class of neuroprotective agents into molecules that exhibit anti-proliferative activity. Further structure-activity relationship studies on this novologue scaffold are currently underway and will be reported in due course.

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32. MCF7 cells were grown to confluence and seeded at 0.4 x 10^6 cells/well/2 mL. Cells were incubated for 24 hours and treated with varying concentrations of **14a** in DMSO (0.25% DMSO final concentration), or vehicle (DMSO) for 24 hours. Cells were harvested in cold PBS and lysed using MPER (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche) according to manufacturer's directions. Lysates were clarified at 14,000g for 15 minutes at 4° C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal amounts of protein (5 µg) were electrophoresed under reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with the corresponding antibody. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.
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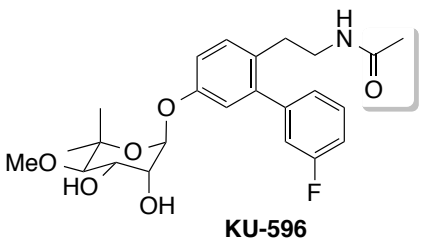
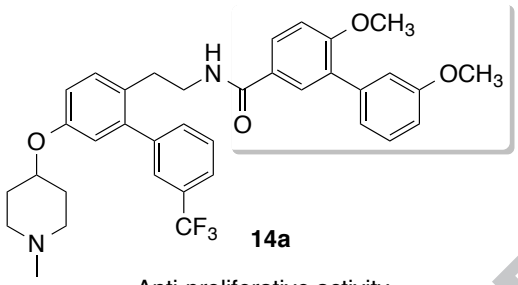


KU-596

Neuroprotective property



14a

Anti-proliferative activity

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Manuscript Draft

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Keywords: Heat shock protein 90, Hsp90 inhibitors, Novobiocin analogues, Breast cancer

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Order of Authors: huiping zhao; mercy anyika; antwan girgis; Brian S. J. Blagg, Ph.D.

Abstract: Hsp90 represents a promising target for the development of both anti-cancer and neuroprotective agents. Structure-activity relationship studies on novobiocin and novobiocin analogues, led to the development of KU-32 and recently, KU-596, as lead compounds for the potential treatment of neurodegenerative diseases. Similar to KU-32, we have demonstrated that upon replacement of the acetamide side chain present in KU-32 with a benzamide, this neuroprotective agent was transformed into a scaffold that manifests anti-proliferative activity. To assess structure-activity relationships for this new scaffold, a library of benzamide-containing novologues was prepared and evaluated against two breast cancer cell lines. Compound 14a manifested the most potent anti-proliferative activity from these studies and induced Hsp90-dependent client protein degradation in a concentration-dependent manner.