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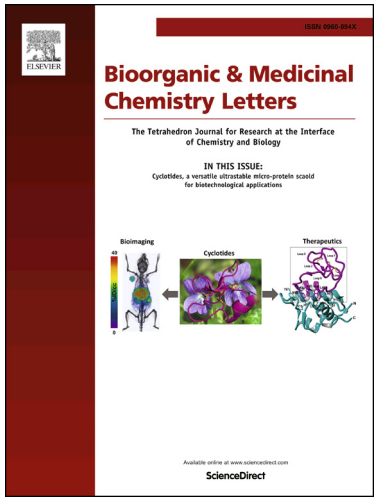

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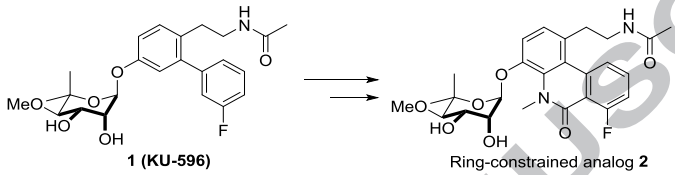

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Synthesis and evaluation of a ring-constrained Hsp90 C-terminal inhibitor that exhibits neuroprotective activity

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ABSTRACT

KU-596 is a second-generation C-terminal heat shock protein 90 KDa (Hsp90) modulator based on the natural product, novobiocin. KU-596 has been shown to induce Hsp70 levels and manifest neuroprotective activity through induction of the heat shock response. A ring-constrained analog of KU-596 was designed and synthesized to probe its binding orientation and ability to induce Hsp70 levels. Compound 2 was found to exhibit comparable or increased activity compared to KU-596, which is under clinical investigation for the treatment of neuropathy.

The 90 KDa heat shock protein (Hsp90) is a molecular chaperone that is responsible for the folding and maturation of more than 300 client proteins and consequently, represents a promising target for the development of both cancer and neurodegenerative agents.[1-7] Six candidates that target the Hsp90 N-terminus are currently under clinical investigation to assess their efficacy for the treatment of cancer.[8,9] Despite these efforts, Hsp90 N-terminal inhibitors induce an undesired, pro-survival heat shock response (HSR), which has impeded their clinical potential for cancer, as increased Hsp90 levels result from the administration of such inhibitors.[10] This same HSR is also reponsible for the upregulation of other heat shock proteins, including Hsp70, which exhibit pro-survival activity and can refold protein aggregates that accumulate during the pathology of several neurodegenerative diseases.[11,12]

In contrast to N-terminal inhibitors, Hsp90 C-terminal inhibitors can segregate the HSR from the degredation of client proteins, allowing the opportunity to develop small molecules that manifest selective activity towards cancer or neurodegeneration.[13-21] KU-32 is derived from novobiocin, the first Hsp90 C-terminal inhibitor identified (Figure **1**), and was shown to exhibit cytoprotective activity at concentrations that induce the HSR.[22,23] In fact, KU-32 can induce the HSR at ≥ 500-fold lower concentrations than that required to promote client protein degradation. Consequently, KU-32 represents a novel probe to investigate the potential neuroprotective activity of such compounds.

New analogs of KU-32 have since been developed and structure-activity relationships of these inhibitors have clearly defined attributes required for neuroprotective versus anti-cancer activity.[24,25] For example, the acetamide moiety of KU-32 is necessary to induce the HSR, but replacing this group with a benzamide results in client protein degradation without induction of the HSR. Although no co-crystal structure has been solved for inhibitors bound to the Hsp90 C-terminus,[26-28] models have been developed that support the binding of KU-32 at this location. One model suggests an unexplored pocket to reside in close proximity to KU-32, which provides an opportunity to design compounds that exhibit increased activity. Accordingly, a second generation of novobiocin analogs was developed to contain a biaryl core, which led to small molecules that manifest enhanced neuroprotective activity.[24] Among the novologues prepared, KU-596 was found to be most active in both luciferase reporter and mitochondria bioenergetic assays (Figure 1).[24, 25] When KU-596 was docked into the Hsp90 C-terminal model, the noviose sugar was found to project into a subpocket that contains amino acid side chains that contribute hydrogen bonding interactions. In addition, the fluorine atom on KU-596 appeared to form a hydrogen bond with Lys560 (Figure 3A), which is supported by prior structure-activity relationship studies.[25]

Since it is well-known that conformationally constrained molecules often exhibit lower entropic penalties as well as

improved affinity upon binding, an analog of KU-596 was pursued. The inclusion of a lactam to constrain the biaryl ring system also represented an opportunity to introduce hydrogen bonding interactions with the peptide backbone while rigidifying the biaryl ring system. Prior studies with KU-32 analogs supported substitution at this position, as replacement of the 8-methyl group with other functionalities led to improved activity (Figure 2).[20, 29] Such data supports the existence of unoccupied space in the binding site and correlates well with the proposed model.

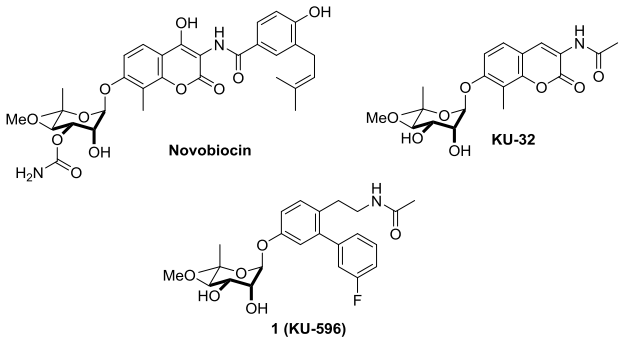


Figure 1. C-terminal Hsp90 inhibitors

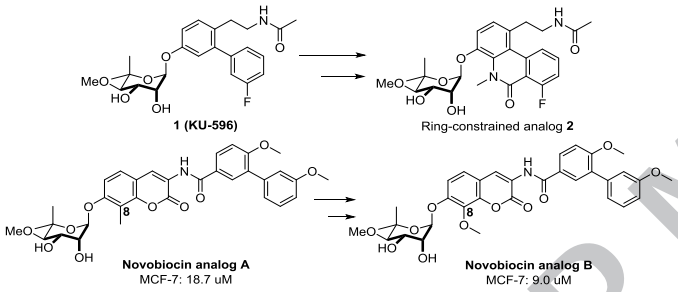
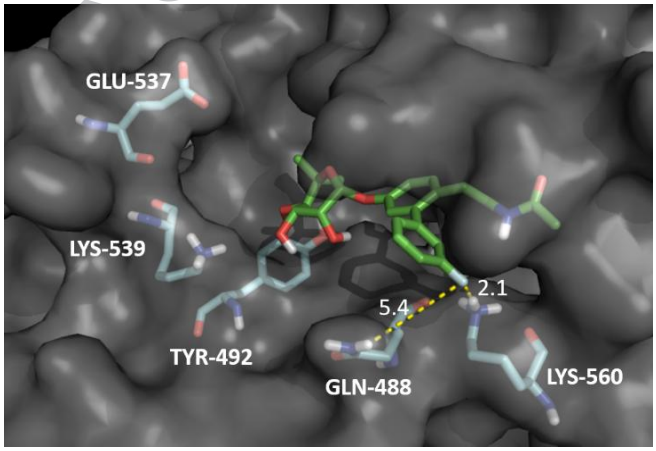


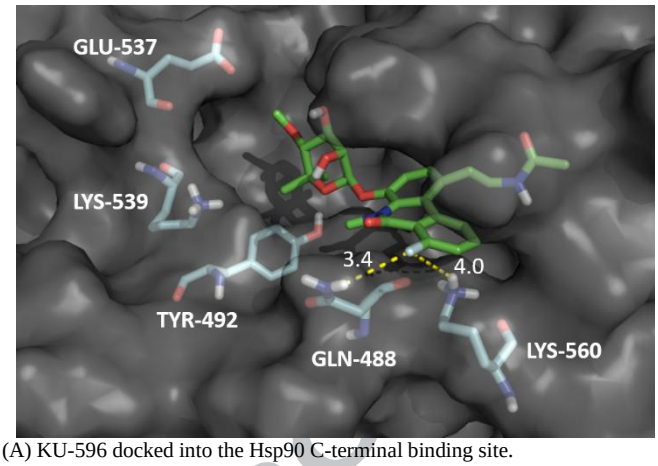
Figure 2. Design of ring-constrained novologue

The phenanthridone containing compound, **2**, was designed and then docked into the Hsp90 C-terminal binding site to further investigate the mode of binding (Figure 3B). Interestingly, the fluorine atom on **2** pointed towards Gln488 rather than the aforementioned Lys560 (Figure 3B), which can still form hydrogen bonding interactions via the amide NH.

A



B



(A) KU-596 docked into the Hsp90 C-terminal binding site.
(B) **2** docked into the Hsp90 C-terminal binding site.
The dash line indicates the distance between the fluorine atom and hydrogen from the amino residue

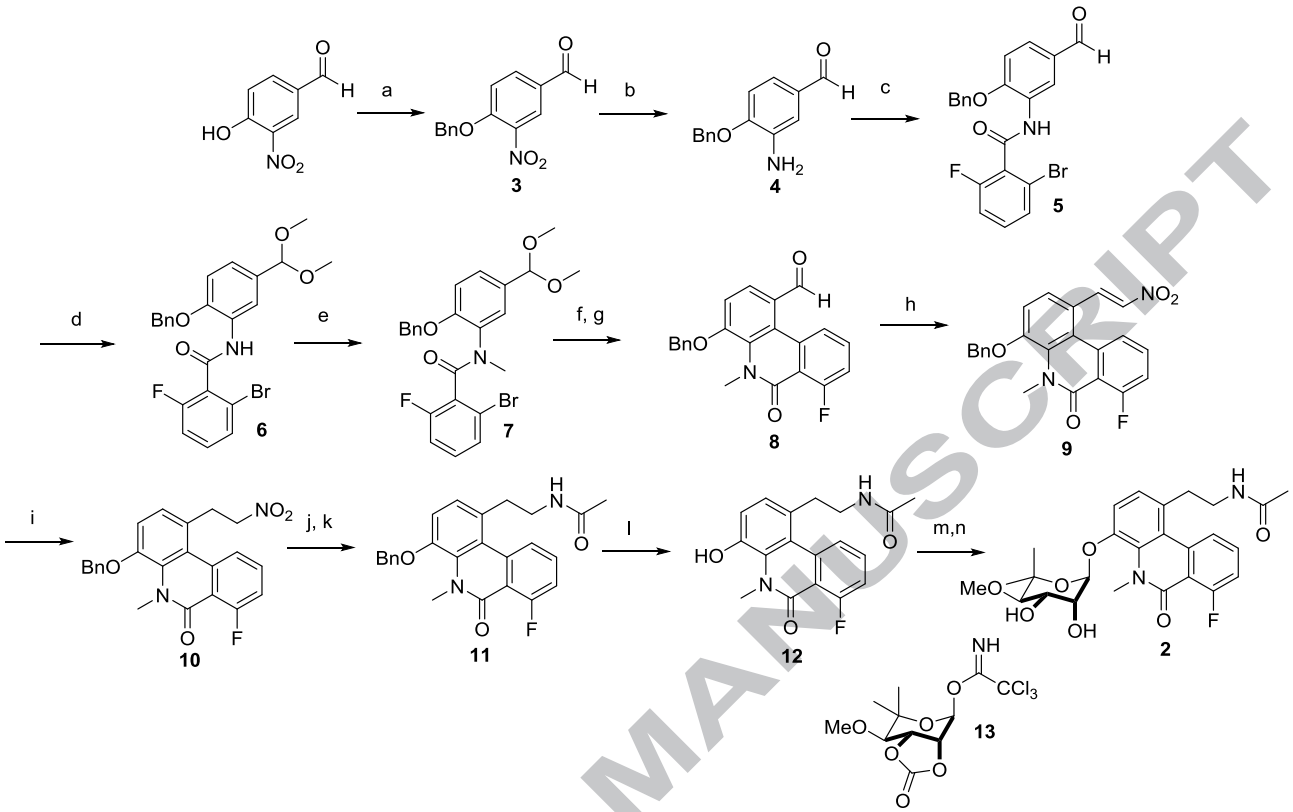
Figure 3. Molecular modeling for KU596 and compound 2

In addition, the lactam carbonyl participated in a hydrogen bonding network with Gln488. As expected, the sugar moiety maintained a similar orientaion inside the pocket as was predicted for KU-596 (Figure 3A). Similarly, the 3-amido side chain projected into the same hydrophobic region as observed with KU-596.

Compound **2** was pursued via the synthetic route described in Scheme 1. Commercially available 3-nitro-4-hydroxy benzaldehyde was converted to benzyl ether **3** via benzyl bromide, followed by iron-mediated reduction of the nitro substituent to form the corresponding aniline, **4**. Subsequent amide coupling was performed via conversion of 2-bromo-6-fluoro benzoic acid to the acid chloride, followed by reaction with aniline **4** to ultimately yield **5**. The aldehyde of **5** was then converted to acetal **6** to mask its reactive nature during subsequent steps. The intramolecular Heck coupling was known to be catalyzed by palladium in the presence of silver carbonate.[30] However, attempts to perform an intramolecular Heck coupling without protection of amide **6** was unsuccessful. Therefore, the amide was methylated to give **7** before cyclization was successfully achieved to produce **8** under modified conditions.[31] Compound **8** was then converted to **9** via Henry olefination. Compound **11** was then synthesized from **9** via a two-step reduction before acetylation with acetyl chloride. Finally, the activated noviose sugar **13** was prepared[32] and appended to phenol **12** upon removal of benzyl group to yield the final product, **2**.

To evaluate the ability of **2** to promote Hsp70 induction compared to **1**, western blot analyses were performed in immortalized dorsal root ganglia sensory neuronal 50B11 cells. As depicted in Figure 4, compound **2** is more effective than **1** at the induction of Hsp70 levels. The slight decrease of Hsp70 at the highest concentration of **2** is a common attribute ascribed to this class of modulators, which typically give a U-shape curve upon dosing with Hsp90 C-terminal inhibitors.[24]

Scheme 1: Synthesis of 2



Reaction conditions: (a) BnBr, K_2CO_3, Acetonitrile, reflux, overnight, 94%; (b) Fe, NH_4Cl, THF/EtOH/H_2O, reflux, 2h, 75%; (c) 2-bromo-6-fluoro benzoyl chloride, Et_3N, CH_2Cl_2, rt, overnight, 83%; (d) trimethyl orthoformate, TsOH, MeOH, 50 °C, 2h, 87%; (e) NaH, MeI, THF, 0 °C to rt, overnight; (f) $Pd(PPh_3)_4$, Ag_2CO_3, dioxane, 90 °C, overnight; (g) TsOH monohydrate, acetone, rt, 2h, 76% over two steps; (h) CH_3NO_2, NH_4OAc, 80 °C, overnight, 86%; (i) $NaBH_4$, Dioxane/MeOH, 0 °C to rt, 2h, 83%; (j) Zn, 1N HCl, EtOH/CH_2Cl_2, rt, 4h; (k) Acetyl chloride, Et_3N, CH_2Cl_2, rt, 3h, 60% over two steps; (l) Pd/C, H_2, EtOAc, r.t., overnight, 75%; (m) **13**, $BF_3{\cdot}OEt_2$, CH_2Cl_2, rt, 3h; (n) Et_3N, MeOH, rt, overnight, 30% over two steps.

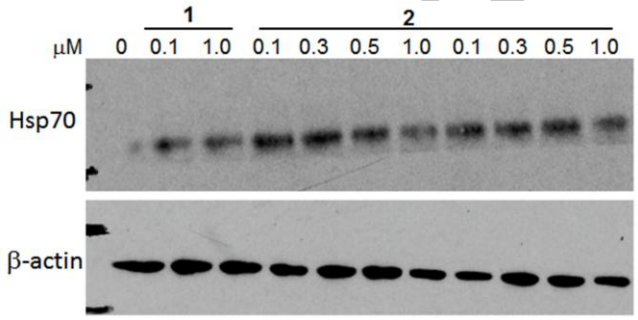
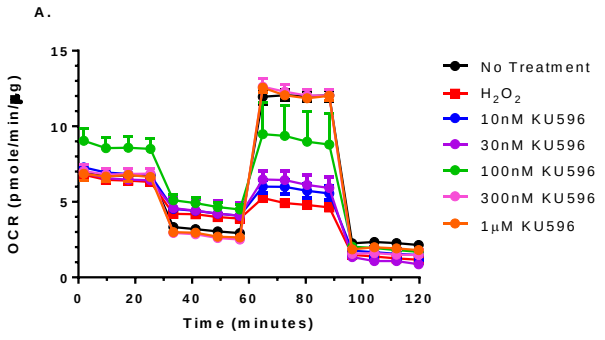


Figure 4. Western Blot analysis of Hsp70 induction for 2. 50B11 cells were seeded at 2 x 10^5 cells per well and cultured for 24hr at 37 °C prior to treatment with DMSO or the indicated concentrations of KU-596 or 3 for an additional 24 hr. The cells were harvested to determine Hsp70 protein expression level by immunoblotting with and Hsp70/Hsp72 biotin-conjugated monoclonal antibody.

Excessive oxidative stress can also cause mitochondria dysfunction in diabetic sensory neurons, which is a key contributor to the pathogenesis of diabetic peripheral neuropathy (DPN).[33] Thus, oxidative stress can be induced by treating 50B11 cells with 0.5mM hydrogen peroxide to decrease mitochondrial bioenergetics (mtBE) as measured by the Seahorse assay and as indicated by a decreased response to the protonophore, FCCP. Both **1** (Figure 5A) and **2** (Figure 5B) can effectively improve mtBE in a dose-dependent manner. However, drug **2** may be more effective than **1**, since it can significantly increase cellular maximal respiratory capacity (MRC), starting at concentrations of ~100 nM (Figure 5C). The improvement of mitochondrial bioenergetics in sensory neurons may be one of the key mechanisms that contributes to the neuroprotective efficacy of both **1** and **2** as well as related molecules.



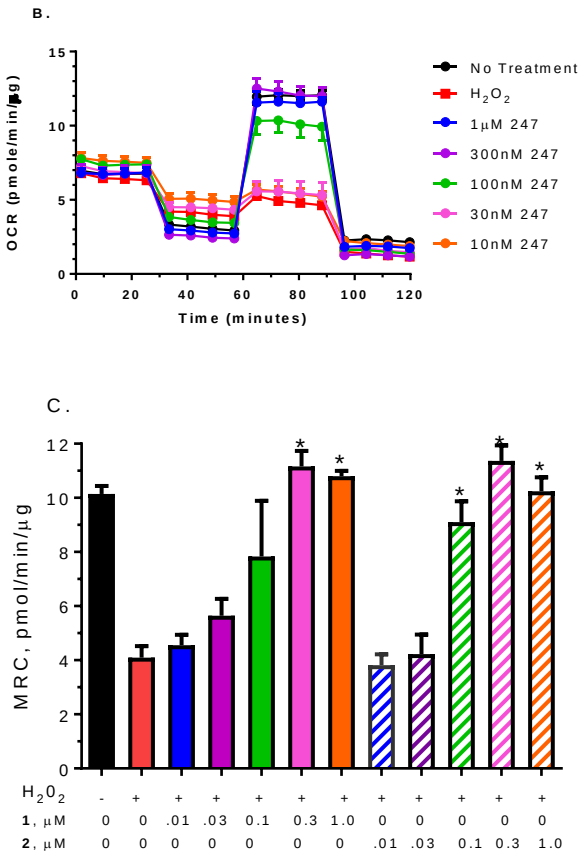


Figure 5. Improvement in mitochondrial bioenergetics by 2. The 50B11 cells were seeded into a 96 well plate and after 12 hrs the cells were changed into high glucose DMEM medium with 1% FBS, containing the indicated concentrations of **1** (A) or **2** (B) for 24 hr at 37°C. The cells were stressed with 0.5mM H_2O_2 for 2 hr, the medium changed to serum free unbuffered DMEM with 5.5 mM glucose and 1mM pyruvate and the cells incubated at 37°C for 1 hr prior to assessing oxygen consumption rate (OCR) with an XF96 Extracellular Flux Analyzer. (C) Maximal respiratory capacity (MRC) was calculated for each treatment. Results are from one experiment using 5 wells of cells per treatment. *, $p < 0.05$ vs H_2O_2 alone.

In conclusion, a ring-constrained novologue was synthesized and then evaluated by western blot analyses and mitochondrial bioenergetic assays. Compound **2** exhibited increased activity for mitochondrial bioenergetics against oxidative stress as compared to **1** at all concentrations except a slightly better activity was observed at 100 nM. This result is consistent with the proposed docking studies and supports a similar binding pose for both **1** and **2**.

Acknowledgments

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Supplementary Material

Procedure for the docking study, synthesis of **3-12**, western blot analysis, Seahorse assay, characterization and NMR spectra of **3-12**.