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Diverging Novobiocin Anti-cancer Activity from Neuroprotective Activity through Modification of the Amide Tail

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Key Words: Hsp90, Hsp90α, Aha1, anti-cancer, neuroprotection, novobiocin, structure-activity relationship.

Abstract: Novobiocin is a natural product that binds the Hsp90 C-terminus and manifests Hsp90 inhibitory activity. Structural investigations on novobiocin led to the development of both anti-cancer and neuroprotective agents. The varied pharmacological activity manifested by these novobiocin analogs prompted the investigation of structure-function studies to identify these contradictory effects, which revealed that modifications to the amide side chain produce either anti-cancer or neuroprotective activity. Compounds that exhibit neuroprotective activity contain a short alkyl or cycloalkyl amide side chain. In contrast, anti-cancer agents contain five or more carbons, disrupt interactions between Hsp90α and Aha1, and induce the degradation of Hsp90-dependent client proteins.

Introduction:

Hsp90 represents a highly sought after target for the treatment of cancer as substrates dependent upon Hsp90 are associated with all ten hallmarks of cancer.[1-3] Hsp90 is overexpressed in cancer to fold oncogenic substrates in the presence of various co-chaperones. While inhibition of Hsp90 can induce the degradation of oncogenic clients via the ubiquitin-proteasome pathway, it also induces the pro-survival heat shock response, which leads to concomitant induction of the heat shock proteins, including Hsp27, Hsp40, Hsp70 and Hsp90, and often results in cytostatic activity.[4] Although difficult to overcome for the treatment of cancer, increased chaperone levels are beneficial for the treatment of neurodegenerative disorders that result from the accumulation of aggregated or misfolded proteins, such as Alzheimer's and Huntington's disease.[5] Thus, Hsp90 is considered a target for both cancer and neurodegeneration.[6-9] Therefore, methods to segregate these activities represent a novel paradigm for which Hsp90 modulators can be developed to treat cancer or neurodegeneration.

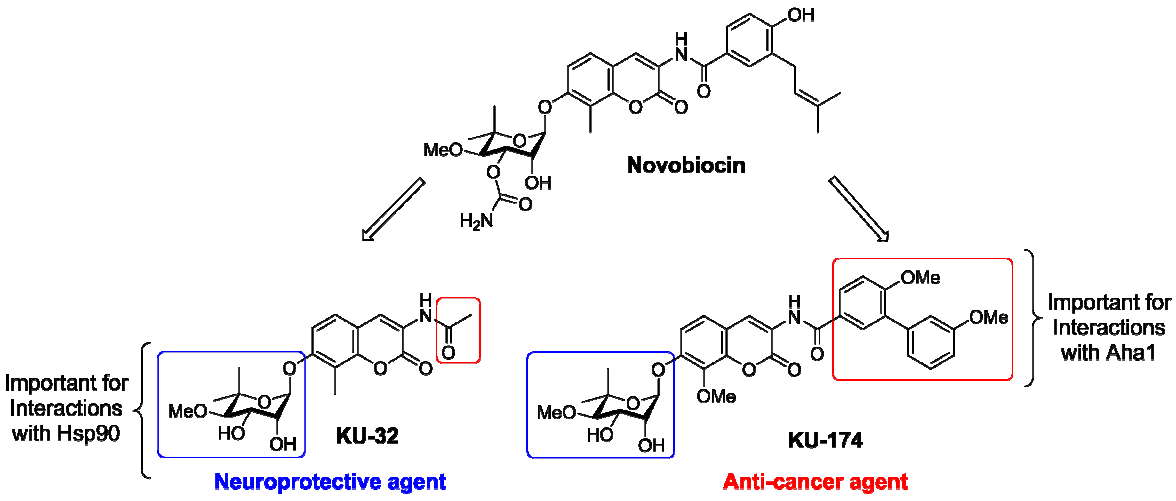


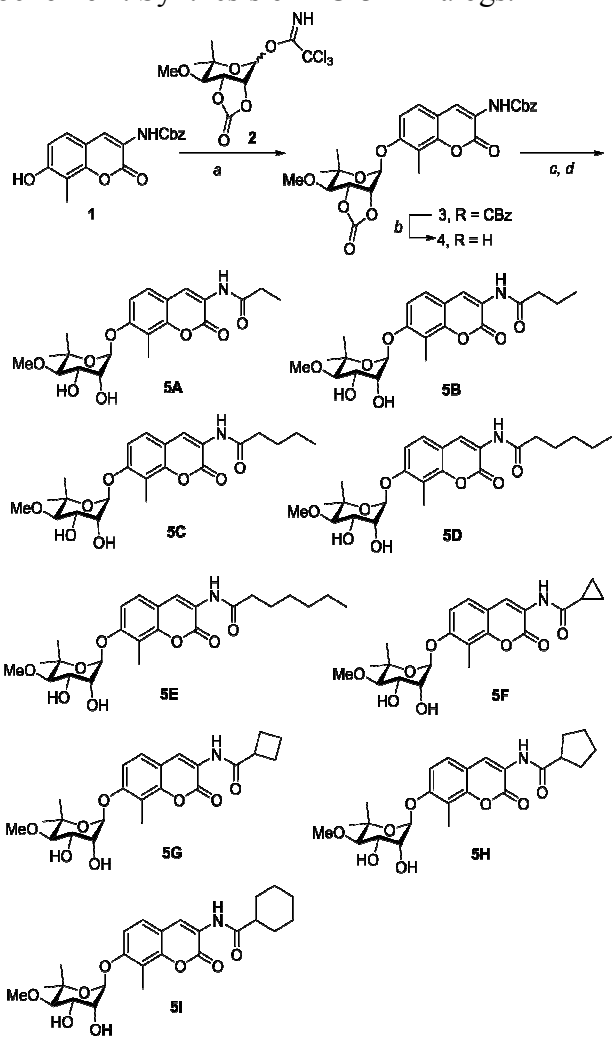
Figure 1. Structures of novobiocin based Hsp90 C-terminal inhibitors

KU-32 (Figure 1) is a neuroprotective novobiocin derivative that binds the Hsp90 C-terminal dimerization domain[10-12] and induces a robust heat shock response without concomitant client protein degradation. KU-32 manifests efficacy in attenuating the death of cortical neurons induced by β-amyloid peptides[12] and can improve multiple physiological indices of diabetic peripheral neuropathy.[9,13-15] The efficacy exhibited by KU-32 to protect against glucotoxicity correlates directly with an increase in mitochondrial bioenergetics.[9] KU-32 does not induce the degradation of Hsp90-dependent client proteins such as Akt and Raf until much higher concentrations than that needed to induce the heat shock response and promote neuroprotection.[13,14]

In contrast to the acetamide side chain on KU-32, KU-174 is a novobiocin derivative that contains an aryl amide side chain and exhibits potent anti-cancer activity by inducing the degradation of Hsp90-dependent client proteins without concomitant induction of the heat shock response.[16] Unfortunately, the mechanisms for distinguishing between these opposing activities remain unclear. Since Hsp90 forms a complex with several proteins to assist in the protein folding process, interactions between Hsp90 and its co-chaperones were investigated, which ultimately revealed the subtle nuances manifested by these two distinct classes of novobiocin analogs.

Activator of Hsp90 ATPase Activity (Aha1) is a co-chaperone that binds to and facilitates the ATPase activity of Hsp90, which is required during the protein folding process.[17-23] We have shown previously that a novobiocin-derived, Hsp90 C-terminal inhibitor could disrupt the Hsp90α/Aha1 complex.[22] Those studies indicated that the noviose sugar was responsible for binding Hsp90 while the benzamide side chain present in KU-174 (Figure 1) interacted with Aha1, and when combined, manifested anti-cancer activity.[22] In contrast, replacement of the benzamide with an acetamide chain as in the case of KU-32, did not disrupt the Hsp90α/Aha1 complex and consequently, did not exhibit anti-cancer activity.[22]

Scheme 1. Synthesis of KU-32 Analogs.



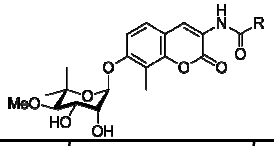
Reagents and conditions: *(a)* $BF_3 \cdot Et_2O$, DCM, rt, 2 h, 70%; *(b)* 10%Pd/C, H_2, EtOAc, rt, 4 h, ~100%; *(c)* R-COOH, EDCI•HCl, DMAP, Pyridine, DCM, rt, 12 h, 50-70%; *(d)* 2 % Et_3N/MeOH, rt, 12 h, 40%-70%.

In an effort to systematically investigate the differences manifested by the alkyl and aryl containing amide side chains, structure-function studies were investigated to identify the point of divergence in which a neuroprotective agent is transformed into an anti-cancer agent.

Results and Discussion:

Analogs containing increasingly larger alkyl and cycloalkyl groups on the amide side chain were pursued (Scheme 1) to identify the point at which KU-32 is transformed from a neuroprotective agent into an anti-cancer agent. As shown in scheme 1, synthesis of these analogs began by the noviosylation of phenol

Table 1. Anti-proliferative activity of KU-32 analogs.



Entry	R	SKBr3 (IC_{50}, μM)	PC3-MM2 (IC_{50}, μM)
KU32		>900	>500
5A		643 ± 50[a]	504
5B		313 ± 9	466
5C		118 ± 43	317
5D		84 ± 38	53.7 ± 0.35
5E		58.3 ± 2.1	34.7 ± 1.82
5F		517 ± 29	>500
5G		220 ± 16	284 ± 99.85
5H		67.0 ± 1.4	144.5 ± 10.6
5I		46.8 ± 0.7	54.55 ± 4.92

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

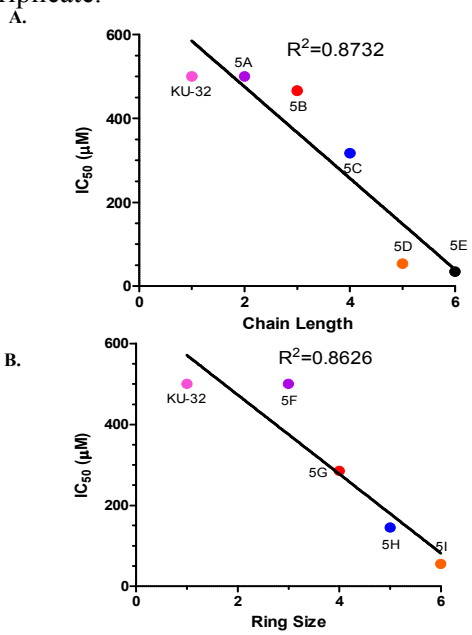


Figure 2. The IC_{50} (μM) values of PC3-MM2 cells were plotted against the chain length (A) or the ring size (B).

1[24] with activated noviose[25-29] (**2**) in the presence of catalytic boron trifluoride etherate to give **3** in good yield. Hydrogenolysis of the resulting benzyl carbonate furnished aniline **4**, which underwent amide coupling with acids containing sequentially larger alkyl substituents, followed by solvolysis of the carbonate to afford the desired diols, **5A-I**, in moderate to good yields.

The anti-proliferative activity manifested by these compounds was evaluated against the highly metastatic, Her2 over-expressing SkBr3 breast and the androgen-independent PC3-MM2 prostate cancer cell lines. Increasing the alkyl chain length or the inclusion of a cycloalkyl ring onto the amide side chain resulted in a size-dependent increase in anti-proliferative activity as shown in Table 1, which was dependent upon chain length (R^2=0.8732) or bulk (R^2=0.8626) (Figure 2).

Since rematuration of firefly luciferase is dependent upon the Hsp90 protein folding machinery, the refolding of denatured firefly

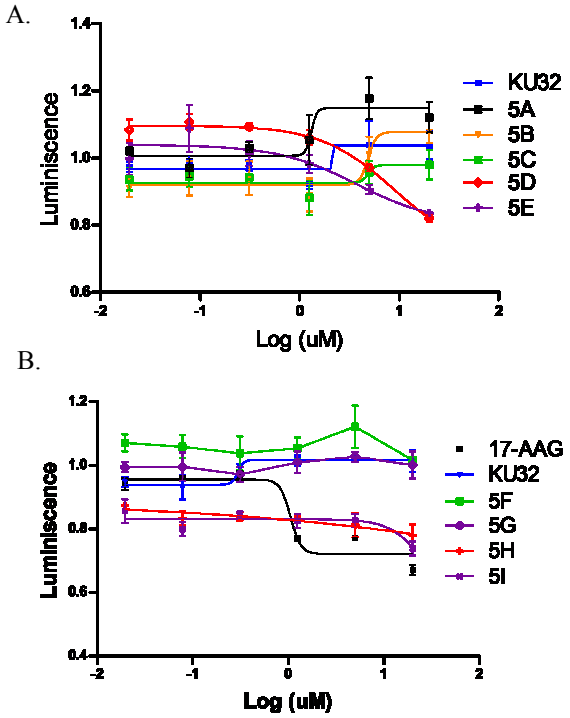


Figure 3. KU-32 analogs inhibit Hsp90-dependent luciferase refolding. Luciferase activity fold change compared to DMSO (1.0 fold) after incubation of heat-denatured luciferase with (A) KU-32, 5A, 5B, 5C, 5D, 5E, or (B) KU-32, 5F, 5G, 5H or 5I. 17-AAG was used as a positive control. The concentrations of KU-32 and analogs used during this assay ranged from 0.02 to 20 μM.

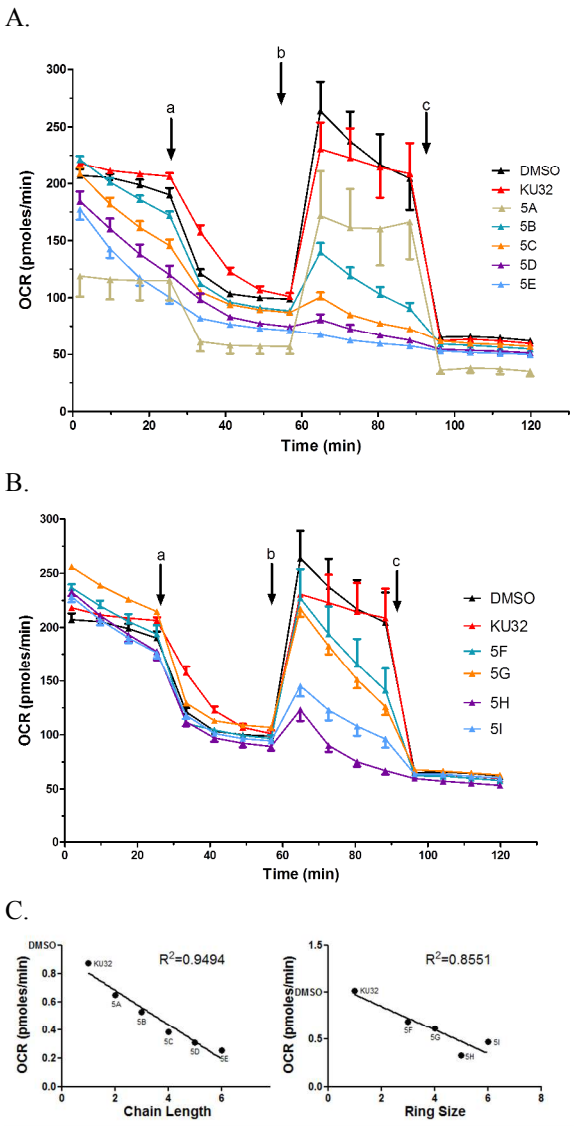


Figure 4. KU-32 analogs worsen mitochondrial bioenergetics in 50B11 neuronal cell line. A-B. 50B11 cells were treated for 24h with KU-32 or its analogs (5 μM each) for mitochondrial bioenergetic analysis. Basal oxygen consumption rate (OCR) was measured prior to the addition of oligomycin (a) to assess ATP-coupled respiration, FCCP (b) to measure uncoupled respiration, and (c) rotenone+antimycin A to assess non-mitochondrial respiration. **C.** The OCR values at the 88.3 min were plotted and compared to the DMSO value.

luciferase was assessed to confirm whether these analogs retained the ability to inhibit Hsp90.[10,29-31] As expected, KU-32 did not inhibit the ability of Hsp90 to refold luciferase, whereas compounds containing longer chains (5D and 5E) or carbocycles (5H and 5I) inhibited the re-maturation of firefly luciferase (Figure 3). In exciting contrast, analogs with shorter chains (KU-32, 5A-5C) or small carbocycles (KU-32, 5F-5G) stimulated the re-maturation of firefly luciferase. In fact, there was a clear point of divergence for the inhibition of luciferase refolding. These data suggest that the anti-proliferative activities manifested by these compounds (Table 1) result from modulation of the Hsp90 protein folding machinery (Figure 3).

Increased chaperone activity can produce neuroprotection that can be readily assessed through measurement of mitochondrial function via the seahorse bioassay. Previous studies revealed that KU-32 improved mitochondrial bioenergetics and could reverse sensory neuropathy *in vivo*.[9,12] Since the preliminary studies demonstrated that a systematic increase in chain length or the larger cycloalkyl rings resulted in anti-proliferative activity, analogs with larger side chains were expected to reduce the maximal respiratory capacity in the neuronal cells. As shown in Figure 4, the inclusion of propyl to heptyl alkyl chains led to a progressive decrease in maximal respiratory capacity, compared to KU-32.

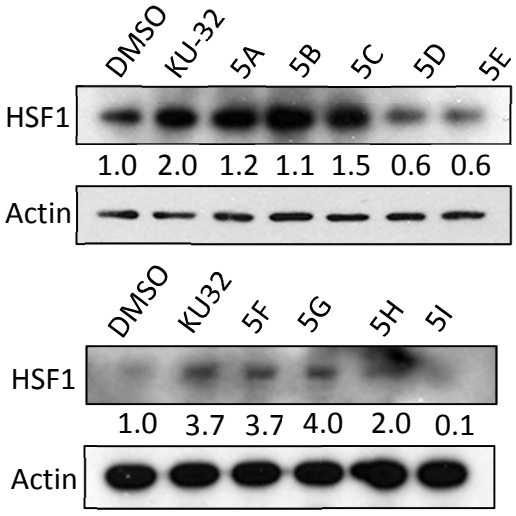


Figure 5. The expression of HSF1 upon treatment with the KU-32 analogs. PC3-MM2 cells were treated with KU-32 or its analogs (10 μM each) for 24 h and the levels of HSF1 were measured compared to the levels of β-actin.

Similarly, substitution of propyl to hexyl carbocycles also produced a decline in maximal respiration. Thus, the decrease in maximal respiratory capacity correlated directly with the anti-proliferative activity manifested by these analogs (Table 1).

Heat Shock Factor 1 (HSF1) is the transcription factor that serves to regulate the heat shock response, and is required to manifest neuroprotective activity.[32] Hsp90 binds HSF1 to form a stable complex, which prevents induction of the heat shock response under normal conditions. However, upon exposure to high temperature or Hsp90 inhibitors, this complex can disassemble and induce the heat shock response, which is necessary to refold proteins that become denatured at high temperature. Neuroprotective agents are sought that activate HSF1 and refold protein aggregates that occur in many neurodegenerative disorders.[32] Therefore, the levels of HSF1 were evaluated in PC3-MM2 cells treated with KU-32 and its analogs. The results clearly show that treatment with KU-32 activated HSF1 ~ 2-3.7 fold (Figure 5). However, as the size of the alkyl or cycloalkyl substitution increased, the levels of HSF1 decreased (Figure 5), once again suggesting that smaller groups (KU-32, 5A-5C, 5F-5H) induce the pro-survival response, whereas larger groups (5D, 5E, 5I) prevent induction of the heat shock response.

Compounds containing larger alkyl or cycloalkyl substitutions 5D, 5E, 5H, and 5I did not induce the heat shock response (Figure 5), but did exhibit decreased mitochondrial respiration (Figure 4), increased anti-proliferative activity (Table 1), and Hsp90 inhibitory activity (Figure 3). Consequently, these compounds were further evaluated to determine whether they could induce the degradation of known Hsp90-dependent client proteins. As shown in figure 6, the degradation of Hsp90-dependent client proteins such as Her2, Akt, Raf-1 occurred in the presence of 5D, 5E, 5H, and 5I without induction of Hsp90 levels, which is a hallmark of Hsp90 C-terminal anti-cancer agents.[26,33-35] Compounds 5A-5C, 5F and 5G were unable to induce Hsp90-client protein degradation at 100 μM.

Since an increase in luciferase maturation was observed in Figure 3 with shorter amide side chains, it was hypothesized that Aha1 remained bound to Hsp90 under these conditions, whereas in the presence of larger side chains, Aha1 association was disrupted. Therefore, Hsp90α/Aha1 interactions were investigated in PC3-MM2 cells treated with compounds 5A-I for 24 h, before Aha1 was co-immunoprecipitated and the percent of Hsp90α remained bound to Aha1 determined. As expected, Hsp90α/Aha1 disruption occurred more readily for analogs with large alkyl and cycloalkyl amide side chains (Figure 7). The percent of Hsp90α remained bound to Aha1 was plotted against the IC_{50} values obtained from Table 1, which once again demonstrated a

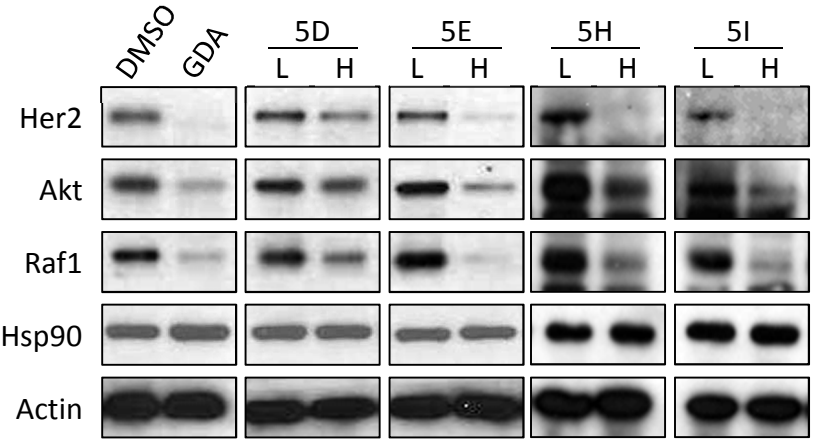


Figure 6. Cytotoxic KU-32 analogs down-regulate the expression of Hsp90 client proteins. PC3-MM2 cells were treated with the cytotoxic analogs of KU-32 (5D, 5E, 5H, and 5I) with the concentrations of H: $3xIC_{50}$ and L: $0.5xIC_{50}$ for 24 h and the levels of known Hsp90 client proteins were measured. Actin was used as the loading control.

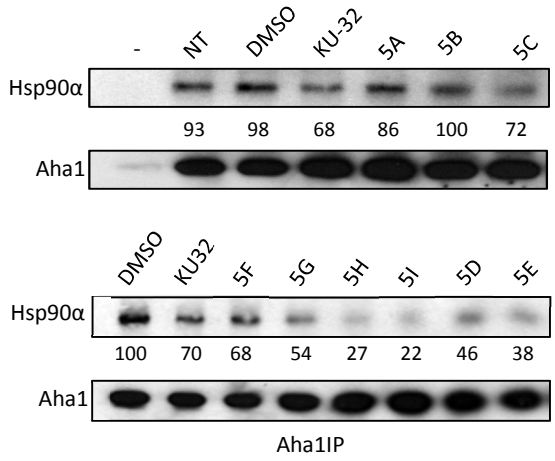


Figure 7. KU-32 analogs disrupt the Hsp90α/Aha1 complex. A-B. PC3-MM2 cells were treated with nothing (NT), DMSO (0.1%), KU-32 (100 μM), **5A** (100 μM), **5B** (100 μM), **5C** (100 μM), **5D** (50 μM), **5E** (35 μM), or **5F** (100 μM), **5G** (100 μM), **5H** (100 μM), **5I** (50 μM) for 24 h . Aha1 was immunoprecipitated and Hsp90α and Aha1 were analyzed by western blotting. Aha1 bound Hsp90α was quantified using Image J software and expressed as percent bound compared to the cells treated with 0.1% DMSO (control).

correlation between Hsp90α/Aha1 disruption based on chain length or ring size, as well as anti-proliferative activity, suggesting that larger side chains disrupt Hsp90α/Aha1 interactions to result in anti-proliferative activity, whereas smaller side chains promote protein folding and induction of the heat shock response to manifest neuroprotective activity (Figure 7).

Conclusion:

In summary, it was shown that KU-32 analogs containing an alkyl or cycloalkyl group with increased carbon atoms manifested a linear inhibition of Hsp90-dependent refolding of thermally denatured luciferase, disruption of Hsp90α/Aha1 interactions and increased anti-proliferative activity, while simultaneously decreasing mitochondrial bioenergetics. In fact, the results demonstrate that a shorter carbon chain or small ring retains the activities needed for neuroprotective activity (Figures 4 and 5), while an amide side chain containing at least five carbons exhibits anti-cancer activity by inhibiting Hsp90 function. These results suggest that a length of five carbons on the amide side chain is sufficient for the disruption of Hsp90α/Aha1 interactions and defines a point of divergence that transforms a neuroprotective agent into an anticancer agent.

Supporting Information

The supporting information is available free of charge on the ACS publication website at http://pubs.acs.org

Acknowledgements

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Lay Summary:

In this study, it was determined that the size of the amide side chain on novobiocin elicits a direct effect on several properties that are mediated by the Hsp90 molecular chaperone. The shorter amide side chains exhibited pro-survival activity that required assembly of the Hsp90/Aha1 complex, whereas longer side chains disassembled the Hsp90/Aha1 complex and led to more potent anti-proliferative activities.

TOC Graphic: