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Novobiocin: Redesigning a DNA Gyrase Inhibitor for Selective Inhibition of Hsp90

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Abstract: Novobiocin is a member of the coumermycin family of antibiotics and is a well-established inhibitor of DNA gyrase. Recent studies have shown that novobiocin binds to a previously unrecognized ATP-binding site at the C-terminus of Hsp90 and induces degradation of Hsp90-dependent client proteins at ~700 μM. In an effort to develop more efficacious inhibitors of the C-terminal binding site, a library of novobiocin analogues was prepared and initial structure−activity relationships revealed. These data suggested that the 4-hydroxy moiety of the coumarin ring and the 3′-carbamate of the noviose appendage were detrimental to Hsp90 inhibitory activity. In an effort to confirm these findings, 4-deshydroxy novobiocin (DHN1) and 3′-descarbamoyl-4-deshydroxynovobiocin (DHN2) were prepared and evaluated against Hsp90. Both compounds were significantly more potent than the natural product, and DHN2 proved to be more active than DHN1. In an effort to determine whether these moieties are important for DNA gyrase inhibition, these compounds were tested for their ability to inhibit DNA gyrase and found to exhibit significant reduction in gyrase activity. Thus, we have established the first set of compounds that clearly differentiate between the C-terminus of Hsp90 and DNA gyrase, converted a well-established gyrase inhibitor into a selective Hsp90 inhibitor, and confirmed essential structure−activity relationships for the coumermycin family of antibiotics.

Introduction

The 90 kDa heat shock proteins (Hsp90) continue to emerge as a promising family of therapeutic targets,[1−4] not only for the treatment of cancer, but also for neurodegenerative diseases in which the accumulation of protein aggregates is lethal.[5] Hsp90 is a molecular chaperone that is responsible for the rematuration of misfolded/aggregated proteins and the maturation of nascent polypeptides into tertiary and quaternary structures.[6] Since the discovery that geldanamycin manifests its anti-kinase activity not through inhibition of Src Kinase, but instead by preventing the maturation of Hsp90-dependent kinases,[7] intense investigations have led to the discovery of numerous Hsp90 substrates and partial elucidation of the Hsp90-mediated protein folding process.[8−11]

The energy required for the Hsp90-mediated protein folding process is provided by the hydrolysis of ATP at the N-terminus of Hsp90,[12−14] which is the same location to which the natural products geldanamycin and radicicol bind (Figure 1).[15] These small molecules bind competitively versus ATP and prevent Hsp90 from completing the protein folding cycle.[16,17] Instead of folding the substrate, the heteroprotein complex becomes

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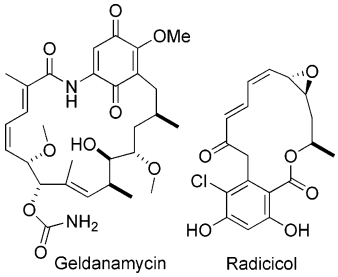

Figure 1. Geldanamycin and radicicol are inhibitors of the Hsp90 N-terminus.

destabilized in the presence of inhibitors and ultimately leads to the degradation of client proteins via the ubiquitin-proteasome pathway.[18,19] Consequently, small molecule inhibitors of Hsp90 transform the protein folding machinery into a catalyst for protein degradation.[6]

Because multiple oncogenic proteins are substrates for the Hsp90-mediated protein folding process, Hsp90 has emerged as an exciting target for the development of cancer chemotherapeutics.[20−23] Examples of client proteins dependent upon the Hsp90 protein folding machinery include the steroid hormone receptors, AKT, Her2, c-Raf, Bcr−Abl kinase, MAK, mutant p53, and telomerase.[1−4,20−23] Therefore, inhibition of Hsp90 results in the simultaneous disruption of multiple signaling nodes and leads to induction of apoptosis. Currently, there are more than 20 clinical trials in progress based on Hsp90-targeted drugs, and several reports have attempted to explain the high level of differential selectivity observed for Hsp90 inhibitors.[24−28] This combination of attributes makes Hsp90 a novel target for the development of new drugs.[29]

When the first cocrystal structure of the Hsp90 N-terminus bound to geldanamycin was solved,[15] it was observed that geldanamycin bound to Hsp90 in a bent conformation as opposed to its relatively flat, native structure.[30] A similar bent conformation was also observed for ADP, which normally binds to ATP-binding pockets in an extended conformation.[31,32] Upon a thorough search of the literature, it became clear that DNA gyrase,[33,34] MutL,[35] and histidine kinase[36] also bind ADP in a bent conformation and these proteins contain similar amino acids in the same proximity to ADP as Hsp90. This led to the hypothesis that inhibitors of these other proteins may also serve as Hsp90 inhibitors.

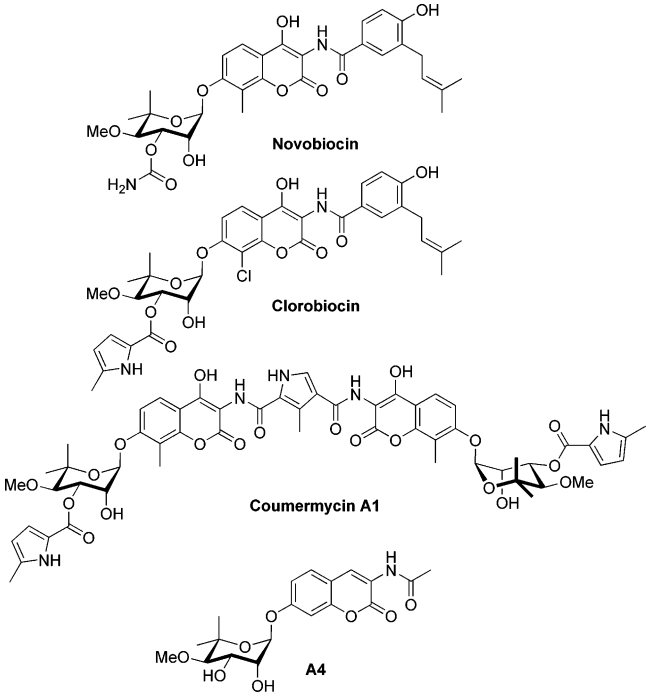

Figure 2. Members of the coumermycin family of antibiotics.

DNA gyrase is the therapeutic target for several antimicrobial agents including the quinolones and the coumermycin family of antibiotics (Figure 2).[37−40] In contrast to the quinolones, which prevent religation of double-stranded breaks via binding to the gyrase−DNA cleavage complex,[41] the coumarin antibiotics prevent ATP hydrolysis by binding to the DNA gyrase ATP-binding pocket.[42−45] Cocrystal structures of N-terminal fragments of GyrB bound to novobiocin and clorobiocin have been previously solved and found to bind these coumarin antibiotics in a bent conformation,[33,34] reminiscent of the manner in which Hsp90 binds ADP.[15] Furthermore, it is known that novobiocin manifests cytotoxicity and has been used for the treatment of cancer for several years.[45−49] Therefore, Neckers and co-workers proposed that novobiocin could be manifesting these activities

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via Hsp90 inhibition,[50,51] because DNA gyrase is not a mammalian protein.

In an effort to test this hypothesis, SkBr3 breast cancer cells were treated with novobiocin and Western blot analyses were performed for several well-known Hsp90 clients. It was found that novobiocin induced the degradation of Hsp90-dependent clients in a concentration-dependent manner.[50,51] However, when truncated variants of Hsp90 were eluted from an immobilized novobiocin solid-support, only the C-terminus of Hsp90 was shown to bind novobiocin, suggesting an additional ATP-binding pocket was present in Hsp90. Furthermore, it was found that ligands bound to the N-terminus of Hsp90 were readily displaced by novobiocin binding to the newly identified C-terminal binding site, suggesting an alternative mechanism for Hsp90 inhibition that may have additional therapeutic applications.[50,51]

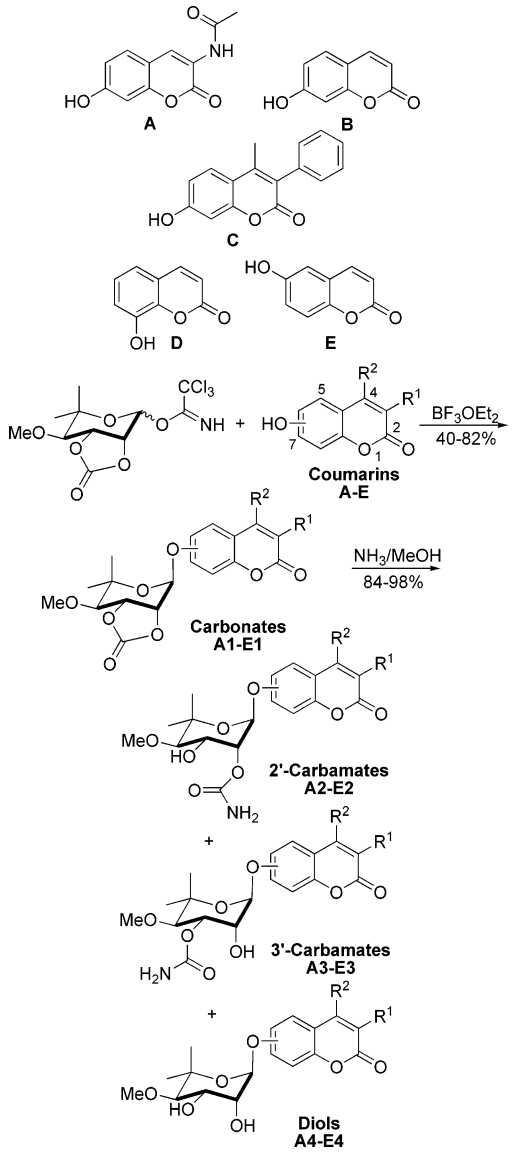
Unfortunately, novobiocin exhibits poor affinity for the Hsp90 C-terminus and manifests an IC_{50} value of $\sim$700 μM, suggesting that improved analogues are necessary to provide clinically useful compounds. Furthermore, it is necessary to determine structure−activity relationships for these molecules and to determine what modifications are necessary to convert a well-established, clinically used DNA gyrase inhibitor into a selective inhibitor of Hsp90. In this article, we provide structure−activity relationships for novobiocin based on a simplified scaffold and removal of key functionalities to provide the first example of a DNA gyrase inhibitor that has been converted into a selective inhibitor of the Hsp90 protein folding machinery.

Results and Discussion

A Library of Novobiocin Analogues. A library of novobiocin analogues was designed to probe the essential nature of several residues found on the natural product and to expeditiously reveal modifications that could enhance Hsp90 inhibition.[52] Toward this goal, it was decided that shortening of the amide side chain from a substituted benzamide to an acetamide, along with removal of the 4-hydroxy substituent, represented a relevant scaffold for elucidation of SAR, the **A**-sublibrary of coumarin molecules.[53] Likewise, it was determined that removal of both the 4-hydroxy and the amide linker to provide an abridged version of the coumarin ring in the form of sublibrary **B** would provide additional information about these functionalities. Steric replacements of both the 4-hydroxy and the benzamide ring were available in the **C**-sublibrary, whereas 1,2-positional isomers of the noviosyl linkage were present in both the **D**- and the **E**-sublibraries. Together, these sublibraries were designed to provide data in support of modifying both the coumarin ring and the amide side chain of novobiocin.

These carefully chosen coumarin rings were coupled with the trichloroacetimidate of noviose carbonate[54] in the presence of boron trifluoride etherate as described in Scheme 1.[55] The resulting cyclic carbonates (**1**) were treated with methanolic ammonia to provide 2′-carbamoyl (**2**), 3′-carbamoyl (**3**), and descarbamoyl products (**4**) in good yields. Upon purification by HPLC, the individual products were evaluated for their ability to induce degradation of the Hsp90-sensitive client protein, phospho-AKT (pAKT).[56] As can be seen in Figure 3, **A4** proved to be most effective at inducing pAKT degradation. **A4** differs from novobiocin in that it lacks the 4-hydroxy substituent on the coumarin ring and does not possess the 3′-carbamoyl group on the noviose side chain. In fact, the data obtained suggest that the 3′-carbamoyl group is detrimental to Hsp90 inhibition as evidenced by increased levels of pAKT upon incubation with **A3** versus **A4**.

Scheme 1. Preparation of a Novobiocin Library



Synthesis of Natural Product Analogues. To verify whether these initial structure−activity relationships paralleled those of the natural product, we elected to prepare two derivatives of

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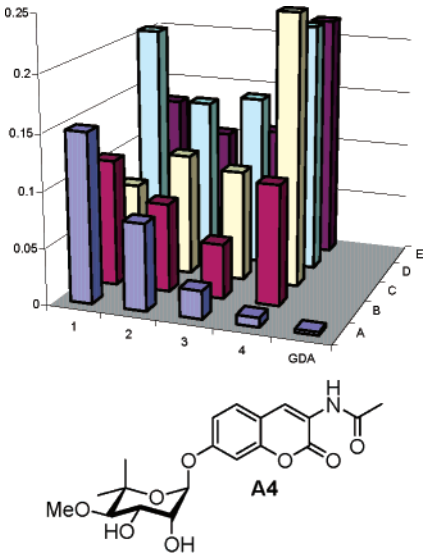


Figure 3. Relative ratios of phospho-AKT by Western blot analyses. Total protein concentration of each lysate was determined, and equal amounts of protein were run in each lane of the gels. For the graphs shown in Figure 3, the OD's (optical density) of the Western bands for phospho-Akt were measured, as were the OD's for actin probed as controls on the same blots. To obtain the graphed values, all specific OD's (for Hsp90 clients) were normalized to the respective actin OD.

novobiocin to confirm these findings. The first analogue prepared was 4-deshydroxynovobiocin (DHN1, Figure 4), which lacked the vinylogous acid moiety that is believed to be critical for gyrase inhibition due to its prescribed role in isomerization of the amide bond from trans to cis upon binding DNA gyrase.[57,58] Despite the number of papers describing the influential role of the 4-hydroxyl substituent in DNA gyrase inhibition, DHN1 had never been prepared. Upon testing our library of novobiocin analogues in various assays, the data suggested that this functionality played a negative role toward Hsp90 inhibition and that construction of this molecule could provide an understanding for its role in both DNA gyrase and Hsp90 inhibition, with the ultimate goal of identifying a compound that is specific for Hsp90. In addition, our studies

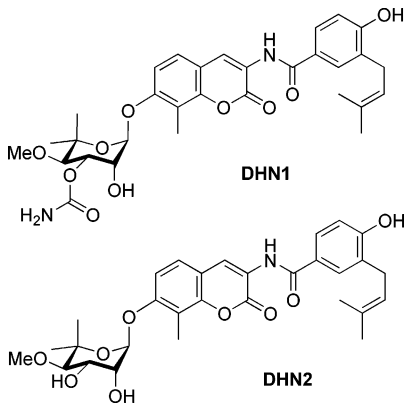
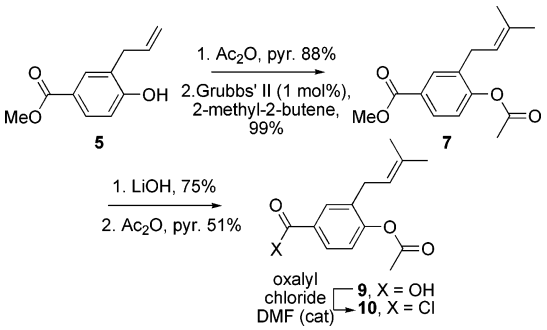


Figure 4. Chemical structures of 4-deshydroxynovobiocin (DHN1) and 3′-descarbamoyl-4-deshydroxynovobiocin (DHN2).

indicated that, similar to the 4-hydroxyl substituent, the 3′-carbamoyl group was also deleterious toward the generation of

Scheme 2. Synthesis of the Benzamide Side Chain



effective Hsp90 inhibitors. Therefore, the 3′-carbamoyl-4-deshydroxynovobiocin analogue (DHN2) was also pursued for the purpose of transforming a well-studied DNA gyrase inhibitor into a selective Hsp90 inhibitor.

The substituted benzamide side chain of novobiocin was prepared from methyl 3-allyl-4-hydroxybenzoate, **5** (Scheme 2).[59] Attempts to perform cross-metathesis on this substrate failed as complexation with the Grubbs' catalyst[60] appeared to have occurred with the orthophenol substrate. Therefore, the phenol was temporarily masked as the acetate, which allowed for a productive cross-metathesis reaction between 2-methyl-2-butene and the allyl appendage in excellent yield to provide the prenylated benzoic ester, **7**. The ester product (**7**) was then hydrolyzed and the phenol reprotected as the acetate to prevent subsequent ester formation.[61] Attempts to couple the unprotected phenol as well as the benzoic acid directly with the coumarin amine resulted in the formation of a complex mixture of products that produced only trace amounts of the desired amide. Therefore, acid **9** was converted to the corresponding acid chloride (**10**) in high yield following standard conditions.

Preparation of the 4-deshydroxy coumarin ring was achieved by the condensation of 2-methylresorcinol (**11**) with the CBz-protected vinylagous carbamate **12**,[62] which produced the desired coumarin **13**, in modest yield (Scheme 3).[63] The phenol was then noviosylated with the trichloroacetimidate of noviose (**14**)[54] in the presence of catalytic amounts of boron trifluoride etherate to generate **15** in good yield.[55] Hydrogenolysis of the benzyl carbonate afforded the amine **16**, which was readily coupled with the acid chloride **10** to give **17** in good yield. Both the acetate and the cyclic carbonate were removed and modified, respectively, to give the desired 3′-carbamoyl product, 4-deshydroxynovobiocin (DHN1), in good yield. Alternatively, the acetate and cyclic carbonate could be readily hydrolyzed to yield the desired 3′-descarbamoyl-4-deshydroxynovobiocin product (DHN2) in a single step upon treatment with methanolic triethylamine.

Biological Investigations. Previous studies have demonstrated that novobiocin manifests weak activity against the Hsp90 protein folding process as demonstrated by its ability to

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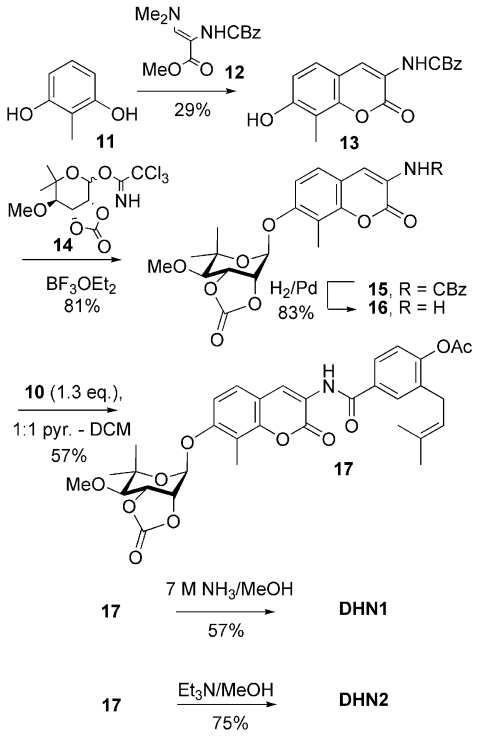
Scheme 3. Synthesis of DHN1 and DHN2



induce degradation of ErbB2 in SkBr3 breast cancer cells at ~700 μM concentration.[50,51] Therefore, both DHN1 and DHN2 were evaluated by the same procedure, and, as shown in Figure 5, both compounds exhibited improved activity as compared to novobiocin. Western blot analyses of Hsp90-dependent client proteins ErbB2 (Her2) and p53 were investigated as well as the related heat shock protein, Hsp70.[64] As can be seen in lane 6, DHN1 induced the degradation of both ErbB2 and p53 between 5 and 10 μM (lanes 5 and 6), whereas DHN2 induced the degradation of these clients between 0.1 and 1.0 μM (lanes 9−12), clearly indicating that DHN2 is more effective than DHN1, which itself is ~70× more active than novobiocin. Levels of actin were unaffected by inhibitor concentration in these immunoblot assays (data not shown). When compared to structure−activity relationships obtained from our combinatorial library approach, the data from these studies were found to be in good agreement with and support our hypothesis that both the 4-hydroxyl substituent on the coumarin ring and the 3′-carbamoyl group on the noviose side chain are detrimental to Hsp90 inhibition.

N-Terminal inhibitors of Hsp90 are known to induce the degradation of client proteins at concentrations that mirror that needed for anti-proliferative activity.[64] Therefore, both DHN1 and DHN2 were evaluated for their ability to inhibit the growth of SKBr3 breast cancer cell lines. As shown in Figure 6, neither treatment with DHN1 nor DHN2 resulted in substantial cytotoxicity, suggesting that C-terminal inhibitors of Hsp90 exhibit a mechanism of action that differs from N-terminal inhibitors or perhaps that a different set of client proteins that are responsible for cell growth are selectively targeted by inhibitors of the N-terminus. A multi-investigator effort is now underway

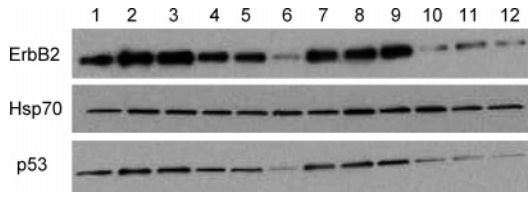


Figure 5. Western blot analyses of DHN1 and DHN2 after 24 h incubation in SKBr3 breast cancer cells. Lane 1 = 1% DMSO (control); lane 2 = 0.01 μM DHN1; lane 3 = 0.1 μM DHN1; lane 4 = 1.0 μM DHN1; lane 5 = 5 μM DHN1; lane 6 = 10 μM DHN1; lane 7 = DMSO (control); lane 8 = 0.01 μM DHN2; lane 9 = 0.1 μM DHN2; lane 10 = 1.0 μM DHN2; lane 11 = 5 μM DHN2; lane 12 = 10 μM DHN2.

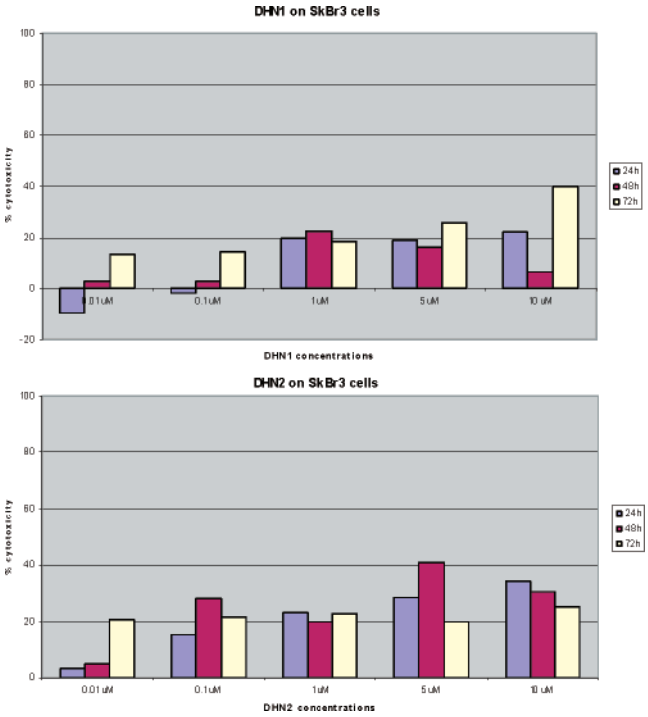


Figure 6. % Cytotoxicity of DHN1 and DHN2 on SKBr3 cells at varying concentrations and time.

to elucidate substrates for the independent N- and C-terminal client protein binding domains.

DHN1 and DHN2 Are Selective for Hsp90 and Not DNA Gyrase. To determine whether the modifications we introduced onto the novobiocin scaffold that resulted in increased Hsp90 inhibition could also produce compounds devoid of DNA gyrase inhibitory activity, both DHN1 and DHN2 were evaluated in a DNA gyrase assay and their activities relative to novobiocin compared (Figure 7).[65,66] At 0.9 μM, novobiocin inhibited the supercoiling of relaxed DNA by 50%, consistent with previous data[67] and in stark contrast to DHN1 which manifested 50% inhibition at ~250 μM. These data clearly demonstrate that the 4-hydroxyl moiety plays a critical role in DNA gyrase inhibition and that the loss of this group retards activity ~200 fold. Recall that in the Hsp90 assays, removal of this moiety improved inhibition ~70 fold. Based on the previously reported cocrystal

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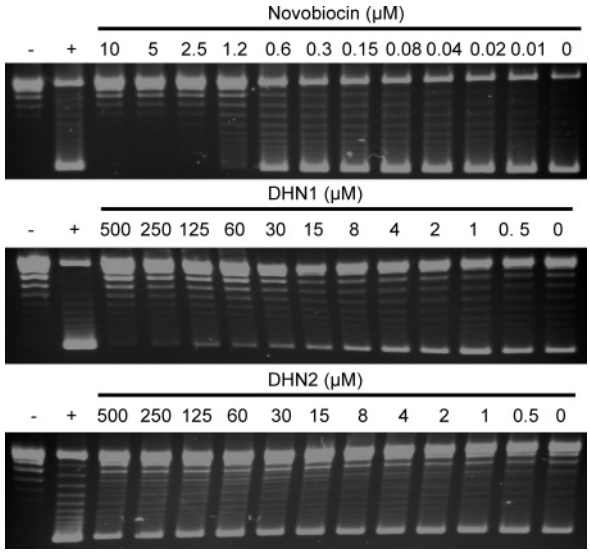

Figure 7. Novobiocin, DHN1, and DHN2 in DNA gyrase-catalyzed DNA supercoiling assays. Concentrations of inhibitors are noted above each lane in micromolar; − = no gyrase control; + = no drug control.

structure of novobiocin bound to DNA gyrase,[33,34] which clearly shows key interactions between the 3′-carbamate of novobiocin with Asp73, we expected removal of this moiety to have a significant impact on its ability to inhibit DNA gyrase. Therefore, we were not surprised to see that DHN2 possessed no activity in the supercoiling assay even at the highest concentration tested, 500 μM. Once again, this is a significant contrast to Hsp90 inhibition where removal of this moiety provided an additional ~10-fold increase in Hsp90 inhibitory activity, clearly indicating that the diol is preferred over the 3′-carbamate.

Conclusion

Novobiocin has been used clinically for the treatment of bacterial infections, and its mechanism of action has been verified to result from the inhibition of DNA gyrase. However, recent studies have shown that this compound also possesses poor Hsp90 inhibitory activity, albeit at a newly discovered C-terminal ATP-binding site. By the preparation of a library of novobiocin analogues, we discovered key elements that appeared to be essential for Hsp90 inhibition and validated their essential nature by the construction of natural product analogues. The natural product derivatives, DHN1 and DHN2, were shown to mimic the structure−activity relationships observed for members of the library, supporting our hypothesis that the 4-hydroxyl and the 3′-carbamate are detrimental for Hsp90 inhibitory activity. In contrast, these moieties proved to be critical for DNA gyrase inhibitory activity. Consequently, we have converted a clinically used DNA gyrase inhibitor into a relatively selective inhibitor (>500 fold preference for Hsp90 vs DNA gyrase) of Hsp90 and provided confirmation of the first set of structure−activity relationships for this class of molecules and Hsp90.

Experimental Section

Methyl 3-Allyl-4-hydroxybenzoate (5). A mixture of methyl-4-allyloxy-benzoate (4.74 g, 24.7 mmol) in *N*,*N*-diethylaniline (10 mL) was heated at reflux for 48 h and cooled to room temperature. The mixture was diluted with diethyl ether (50 mL), washed with aqueous HCl (10% v/v, 3 × 20 mL), dried (Na_2SO_4), filtered, and concentrated. The residue was purified by chromatography (10:1 → 5:1, hexanes:EtOAc) to afford **5** (3.55 g, 75%) as an off-white solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.86−7.84 (m, 2H), 6.85 (dd, J = 2.8, 7.7 Hz, 1H), 6.07−5.95 (m, 1H), 5.68 (s, 1H), 5.21−5.15 (m, 2H), 3.88 (s, 3H), 3.45 (d, J = 6.3 Hz, 2H).

Methyl 4-Acetoxy-3-allylbenzoate (6). Acetic anhydride (200 μL, 218 mg, 2.13 mmol) was added dropwise to a solution of phenol **5** (315 mg, 1.64 mmol) in pyridine (1.5 mL) at room temperature. The mixture was stirred for 14 h before the solvent was removed. The residue was purified by chromatography (10:1, hexanes:EtOAc) to afford **6** (337 mg, 88%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.95−7.90 (m, 2H), 7.11 (d, J = 8.2 Hz, 1H), 5.92−5.83 (m, 1H), 5.12−5.03 (m, 2H), 3.88 (s, 3H), 3.33 (d, J = 6.5 Hz, 2H), 2.29 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.2, 166.8, 153.0, 135.6, 132.7, 132.4, 129.4, 128.4, 123.0, 117.2, 52.6, 35.0, 21.3. IR (neat): ν_{max} 3080, 3005, 2980, 2953, 2916, 2845, 1765, 1722, 1639, 1609, 1589, 1493, 1437, 1418, 1369, 1285, 1263, 1190, 1163, 1121 cm^{-1}. HRMS (ESI+) m/z 235.1076 (M + H$^+$, $C_{13}H_{15}O_4$ requires m/z 235.0970).

Methyl 4-Acetoxy-3-(3-methylbut-2-enyl)benzoate (7). Grubbs' second generation catalyst (11 mg, 0.0130 mmol, 1 mol %) was added to a solution of acetate **6** (305 mg, 1.30 mmol) in a 1/10 solution of DCM/2-methyl-2-butene (5.5 mL). The mixture was stirred 14 h and was concentrated. The residue was purified by chromatography (10:1, hexanes:EtOAc) to afford **7** (339 mg, 99%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.92 (d, J = 1.9 Hz, 1H), 7.88 (dd, J = 1.9, 8.4 Hz, 1H), 7.07 (d, J = 8.4 Hz, 1H), 5.22 (td, J = 1.3, 7.1 Hz, 1H), 3.87 (s, 3H), 3.25 (d, J = 7.1 Hz, 2H), 2.24 (s, 3H), 1.72 (s, 3H), 1.68 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.2, 166.9, 153.0, 134.3, 134.1, 132.3, 129.0, 128.3, 122.9, 121.4, 52.5, 29.2, 26.1, 21.2, 18.2. IR (neat): ν_{max} 2970, 2953, 2916, 2856, 1765, 1724, 1609, 1589, 1493, 1437, 1369, 1285, 1263, 1204, 1192, 1165, 1111 cm^{-1}. HRMS (ESI+) m/z 263.1296 (M + H$^+$, $C_{15}H_{19}O_4$ requires m/z 263.1283).

4-Hydroxy-3-(3-methylbut-2-enyl)benzoic Acid (8). Lithium hydroxide (85 mg, 2.02 mmol) was added to a mixture of methyl ester **7** (106 mg, 0.405 mmol) in 0.5 mL of a 3/1/1 THF/MeOH/H_2O solution. The reaction mixture was stirred at reflux for 14 h, cooled to room temperature, and diluted with THF (1 mL). The solution was acidified to pH = 3 by the dropwise addition of 6 M HCl. The layers were separated, and the organic phase was dried (Na_2SO_4), filtered, and concentrated to afford acid **8** (63 mg, 75%) as a red oil that was suitable for use without further purification.

4-Acetoxy-3-(3-methylbut-2-enyl)benzoic Acid (9). Acetic anhydride (1 mL) was added dropwise to a solution of acid **8** (178 mg, 2.00 mmol) in pyridine (3 mL) at room temperature. After being stirred for 48 h, the mixture was poured into water (6 mL) and acidified to pH = 2 by the dropwise addition of 6 M HCl. The suspension was extracted with EtOAc (2 × 10 mL), and the combined organic fractions were dried (Na_2SO_4), filtered, and concentrated. The residue was purified by chromatography (5:1, hexanes:EtOAc) to afford acetate **9** (223 mg, 51%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.01−7.96 (m, 2H), 7.15 (dd, J = 3.5, 8.2 Hz, 1H), 5.24 (tt, J = 1.3, 7.2 Hz, 1H), 3.30 (d, J = 7.2 Hz, 2H), 2.34 (s, 3H), 1.97 (s, 3H), 1.76 (s, 3H).

Benzyl 7-Hydroxy-8-methyl-2-oxo-2*H*-chromen-3-yl Carbamate (13). 2-Methyl rescorcinol (1.20 g, 9.71 mmol) was added to a solution of vinyl carbamate **12** (2.7 g, 9.71 mmol) in acetic acid (50 mL). The mixture was stirred at reflux for 48 h, cooled to room temperature, and filtered. The solid was recrystallized from methanol and H_2O to afford **13** (1.30 g, 41%) as a yellow solid. ^{1}H NMR (DMSO, 400 MHz): δ 10.30 (s, 1H), 9.10 (s, 1H), 8.12 (s, 1H), 7.46−7.30 (m, 6H), 6.85 (d, J = 8.4 Hz, 1H), 5.17 (s, 2H), 2.16 (s, 3H).

Benzyl-7-((3a*R*,4*R*,7*R*,7a*R*)-7-methoxy-6,6-dimethyl-2-oxotetrahydro-3a*H*-[1.3]dioxolo[4,5-*c*]pyran-4-yloxy)-8-methyl-2-oxo-2*H*-chromen-3-ylcarbamate (15). Boron trifluoride etherate (61 μL, 69 mg, 0.49 mmol, 30 mol %) was added dropwise to a solution of (3a*R*,4*S*,7*R*,7a*R*)-7-methoxy-6,6-dimethyl-2-oxo-tetrahydro-3a*H*-[1,3]-

dioxolo[4,5-*c*]pyran-4-yl 2,2,2-trichloroacetimidate (**14**, 588 mg, 1.62 mmol) and benzyl-7-hydroxy-8-methyl-2-oxo-2*H*-chromen-3-yl carbamate[6] (**13**, 527 mg, 1.62 mmol) in DCM (16 mL). After the mixture was stirred for 14 h, three drops of Et_3N were added and the mixture was concentrated. The residue was purified by chromatography (DCM → 100:1, CH_2Cl_2:acetone) to afford **15** (670 mg, 81%) as a yellow foam: $[\alpha]^{22}_D = -19.7°$ ($c = 1.54$, 20% MeOH in DCM). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.27 (s, 1H), 7.85 (s, 1H), 7.55−7.35 (m, 5H), 7.29 (d, $J = 2.9$ Hz, 1H), 7.11 (d, $J = 8.7$ Hz, 1H), 5.77 (d, $J = 1.9$ Hz, 1H), 5.23 (s, 2H), 5.05 (d, $J = 1.9$ Hz, 1H), 4.95 (t, $J = 7.7$ Hz, 1H), 3.59 (s, 3H), 3.30 (d, $J = 7.6$ Hz, 1H), 2.27 (s, 3H), 1.34 (s, 3H), 1.19 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 159.0, 155.2, 153.6, 153.6, 149.2, 136.0, 129.1 (2C), 129.0, 128.7 (2C), 125.8, 122.6, 122.1, 115.2, 115.1, 111.6, 94.8, 83.3, 78.4, 77.6, 77.0, 67.9, 61.0, 27.9, 22.6, 8.8. IR (film): ν_{max} 3402, 3319, 3063, 3034, 2984, 2939, 2839, 1817, 1709, 1634, 1609, 1587, 1522, 1456, 1383, 1366, 1331, 1296, 1263, 1229, 1205, 1175 cm^{-1}. HRMS (ESI+) *m*/*z* 526.1688 (M + H$^+$, $C_{27}H_{28}NO_{10}$ requires *m*/*z* 526.1713).

3-Amino-7-((3a*R*,4*R*,7*R*,7a*R*)-7-methoxy-6,6-dimethyl-2-oxotetrahydro-3a*H*-[1.3]dioxolo[4,5-*c*]pyran-4-yloxy)-8-methyl-2*H*-chromen-2-one (16). Palladium on carbon (10%, 67 mg) was added to a solution of carbamate **15** (670 mg, 1.31 mmol) in THF (13 mL). The suspension was stirred for 6 h under a hydrogen atmosphere and was filtered through a plug of silica gel. The solvent was removed and the residue purified by chromatography (100:1 → 50:1, CH_2Cl_2:acetone) to afford **16** (425 mg, 83%) as a pale yellow foam: $[\alpha]^{23}_D = -26.4°$ ($c = 0.780$, 20% MeOH in DCM). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.10 (d, $J = 8.6$ Hz, 1H), 7.05 (d, $J = 8.6$ Hz, 1H), 6.68 (s, 1H), 5.73 (d, $J = 2.0$ Hz, 1H), 5.04 (dd, $J = 2.0$, 7.9 Hz, 1H), 4.95 (t, $J = 7.7$ Hz, 1H), 4.11 (s, 2H), 3.54 (s, 3H), 3.29 (d, $J = 7.6$ Hz, 1H), 2.28 (s, 3H), 1.34 (s, 3H), 1.21 (s, 3H). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 159.6, 153.3, 153.0, 148.1, 130.2, 122.7, 116.1, 114.8, 111.9, 111.0, 94.5, 83.0, 78.0, 77.3, 76.4, 60.6, 27.5, 22.2, 8.6. IR (film): ν_{max} 3462, 3362, 2984, 2937, 2839, 1807, 1707, 1636, 1595, 1497, 1387, 1371, 1331, 1263, 1169, 1109, 1078, 1036 cm^{-1}. HRMS (ESI+) *m*/*z* 392.1357 (M + H$^+$, $C_{19}H_{22}NO_8$ requires *m*/*z* 392.1346).

4-((7-((3a*R*,4*R*,7*R*,7a*R*)-7-Methoxy-6,6-dimethyl-2-oxotetrahydro-3a*H*-[1.3]dioxolo[4,5-*c*]pyran-4-yloxy)-8-methyl-2-oxo-2*H*-chromen-3-ylcarbamoyl)-2-(3-methylbut-2-enyl) Phenyl Acetate (17). Oxalyl chloride (15 mg, 119 μmol) was added to a solution of benzoic acid **9** (28 mg, 113 μmol) in CH_2Cl_2 (0.5 mL), followed by the addition of catalytic DMF. After the mixture was stirred for 2.5 h, the acid chloride (**10**) was concentrated. The yellow solid was redissolved in CH_2Cl_2 (0.5 mL) and added dropwise over 3 min to a stirred solution of aniline **16** (34 mg, 87 μmol) in pyridine (0.5 mL) at 0 °C. The resulting solution was stirred at room temperature for 3.5 h and concentrated. The residue was purified by preparative TLC (SiO_2, 40:1, CH_2Cl_2:acetone) to afford **17** (31 mg, 57%) as a colorless solid: $[\alpha]^{22}_D = -21.7°$ ($c = 0.840$, 20% MeOH in CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.72 (s, 1H), 8.64 (s, 1H), 7.73 (d, $J = 2.5$ Hz, 1H), 7.69 (dd, $J = 2.5$, 8.0 Hz, 1H), 7.29 (d, $J = 6.8$ Hz, 1H) 7.11 (d, $J = 8.0$ Hz, 1H), 7.07 (d, $J = 9.0$ Hz, 1H), 5.72 (d, $J = 2.0$ Hz, 1H), 5.18−5.14 (m, 1H), 4.99 (dd, $J = 1.5$, 7.5 Hz, 1H), 4.89 (t, $J = 8.0$ Hz, 1H), 3.53 (s, 3H), 3.26−3.22 (m, 3H), 2.27 (s, 3H), 2.23 (s, 3H), 1.70 (s, 3H), 1.66 (s, 3H), 1.29 (s, 3H), 1.13 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 167.9, 164.4, 158.1, 154.1, 152.2, 151.1, 148.1, 133.6, 133.3, 131.0, 128.6, 124.9, 123.1, 122.0 (2C), 121.2, 119.6, 113.8, 113.7, 110.2, 93.3, 81.9, 76.9, 76.3, 75.6, 59.5, 27.8, 26.5, 24.7, 21.1, 19.9, 16.9, 7.4. IR (film): ν_{max} 3400, 2982, 2935, 2856, 1811, 1763, 1715, 1674, 1634, 1607, 1526, 1489, 1437, 1369, 1250, 1202, 1175, 1111, 1090 cm^{-1}. HRMS (ESI+) *m*/*z* 622.2277 (M + H$^+$, $C_{33}H_{36}NO_{11}$ requires *m*/*z* 622.2289).

(3*R*,4*S*,5*R*,6*R*)-5-Hydroxy-6-(3-(4-hydroxy-3-(3-methylbut-2-enyl) benzylamino)-8-methyl-2-oxo-2*H*-chromen-7-yloxy)-3-methoxy-2,2-dimethyltetrahydro-2*H*-pyran-4-yl Carbamate (DHN1). A solution of carbonate **17** (32 mg, 52 μmol) in 7 M methanolic ammonia (2 mL) was stirred for 14 h. The solvent was removed and the residue purified by preparative TLC (SiO_2, 25:1, CH_2Cl_2:methanol, developed seven times) to afford DHN2 (2.5 mg, 9%) and 4-deshydroxynovobiocin (DHN1, 17.5 mg, 57%) as colorless solids. DHN1: $[\alpha]^{31}_D = -20.3°$ ($c = 0.300$, 10% MeOH in CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.70 (s, 1H), 7.62 (d, $J = 2.3$ Hz, 1H), 7.56 (dd, $J = 2.3$, 8.4 Hz, 1H), 7.29 (d, $J = 8.8$ Hz, 1H), 7.13 (d, $J = 8.8$ Hz, 1H), 6.81 (d, $J = 8.4$ Hz, 1H), 5.50 (d, $J = 2.3$ Hz, 1H), 5.34−5.25 (m, 2H), 4.25 (t, $J = 2.6$ Hz, 1H), 3.53−3.51 (m, 1H), 3.50 (s, 3H), 3.34 (dd, $J = 3.2$, 8.9 Hz, 2H), 2.99 (s, 1H), 2.94 (s, 1H), 2.27 (s, 3H), 1.74 (s, 3H), 1.71 (s, 3H), 1.33 (s, 3H), 1.13 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 166.3, 159.6, 158.9, 156.8, 155.9, 149.0, 133.9, 129.0, 128.6, 126.4, 125.6, 124.6, 124.1, 121.8, 121.4, 114.9, 114.4, 114.1, 111.2, 98.3, 81.4, 78.9, 72.2, 69.6, 61.5, 29.7, 29.3, 27.2, 22.6, 17.9, 8.2. IR (film): ν_{max} 3400, 3379, 3360, 2978, 2928, 2853, 1709, 1659, 1632, 1605, 1528, 1504, 1367, 1254, 1136, 1117, 1086 cm^{-1}. HRMS (ESI+) *m*/*z* 597.2434 (M + H$^+$, $C_{31}H_{37}N_2O_{10}$ requires *m*/*z* 297.2448).

***N*-(7-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2-yloxy)-8-methyl-2-oxo-2*H*-chromen-3-yl)-4-hydroxy-3-(3-methylbut-2-enyl)benzylamide (DHN2).** A solution of carbonate **17** (12 mg, 19.3 μmol) in 10/1 methanol/Et_3N (220 μL) was stirred for 14 h. The solvent was removed and the residue purified by preparative TLC (SiO_2, 10:1, CH_2Cl_2:methanol) to afford DHN2 (8 mg, 75%) as a colorless solid: $[\alpha]^{31}_D = -12.9°$ ($c = 0.310$, 10% MeOH in DCM). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.78 (s, 1H), 8.66 (s, 1H), 7.71 (d, $J = 2.2$ Hz, 1H), 7.68 (dd, $J = 2.2$, 8.3 Hz, 1H), 7.33 (d, $J = 8.8$ Hz, 1H), 7.19 (d, $J = 8.8$ Hz, 1H), 6.90 (d, $J = 8.3$ Hz, 1H), 6.05 (s, 1H), 5.61 (d, $J = 1.6$ Hz, 1H), 5.33 (t, $J = 7.1$ Hz, 1H), 4.27−4.23 (m, 2H), 3.61 (s, 3H), 3.45−3.35 (m, 3H), 2.77 (s, 1H), 2.67 (s, 1H), 2.05 (s, 3H), 1.80 (s, 3H), 1.79 (s, 3H), 1.38 (s, 3H), 1.14 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 165.9, 159.5, 158.3, 155.9, 149.0, 135.8, 129.5, 127.5, 126.9, 125.9, 125.8, 124.2, 122.0, 120.9, 115.9, 114.2, 114.1, 111.2, 97.7, 84.3, 78.6, 71.2, 68.6, 62.0, 29.6, 29.3, 25.8, 22.5, 18.0, 8.2. IR (film): ν_{max} 3402, 2974, 2928, 2854, 1717, 1701, 1645, 1605, 1526, 1506, 1367, 1254, 1088 cm^{-1}. HRMS (ESI+) *m*/*z* 554.2363 (M + H$^+$, $C_{30}H_{36}NO_9$ requires *m*/*z* 554.2390).

Cells and Reagents. SKBr3 and MCF7 human breast cancer cells were purchased from the American Type Culture Collection and grown as previously described.[64] Antibodies for α-tubulin and ErbB2 (Ab-2 and Ab-5) were from Calbiochem (La Jolla, CA).

Western Blotting. Cells were washed once with cold phosphate-buffered saline (pH 7.0) and lysed by scraping in TMNS (50 mM Tris-HCl, pH 7.5, 20 mM Na_2MoO_4, 0.1% NP-40, 150 mM NaCl) supplemented with 20 μg/mL aprotinin, 20 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride. Cell lysate was clarified by centrifugation at 14 000 rpm at 4 °C for 15 min, and protein concentration was determined by using the BCA method (Pierce, Rockford, IL). Twenty micrograms of total protein from cell lysates was separated by 4−20% gradient SDS-PAGE (Bio-Rad, Hercules, CA). Western blotting for ErbB2 was performed as described previously.[64] Blotting for α-tubulin was used to verify equal loading of lanes.

MTT Assay. Cell growth was monitored using methylthiazoltetrazolium (MTT). Briefly, cells (5×10^3) were plated in 96-well microtiter plates (Costar) in a volume of 0.1 mL of DMEM containing 0.1% FBS. After 12 h, cells were exposed to drugs (final volume 0.2 mL/well). At various times after drug addition, 20 μL of 5 mg/mL MTT solution in PBS was added to each well for 4 h. After removal of medium, 0.1 mL of DMSO was added to each well to dissolve the formazan crystals. Absorbance at 562 nm was determined using an ELx 808 microplate reader (Bio-Tek, Winooski, VT). Six wells were assayed at each concentration, and the mean absorbance was determined. Absorbance at 562 nm is directly proportional to viable cell number.

Protein and DNA. DNA gyrase subunits GyrA and GyrB were produced as previously described.[65] Relaxed pBR322 was purchased from John Innes Enterprises Ltd., Norwich, UK.

Enzyme Assays. DNA gyrase supercoiling assays were carried out as previously described,[66] using 5 nM gyrase, 3.5 nM relaxed pBR322 with 0−500 μM of inhibitor as indicated, and incubated at 37 °C for 1 h. DNA products were analyzed on 1% agarose gels.

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Supporting Information Available: Spectra for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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