Bioorganic & Medicinal Chemistry Letters 21 (2011) 5697–5700

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Mannich reaction derivatives of novobiocin with modulated physiochemical properties and their antibacterial activities

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ARTICLE INFO

Article history:
Received 2 May 2011
Revised 5 August 2011
Accepted 5 August 2011
Available online 12 August 2011

Keywords:
Novobiocin
Gyrase B
Tuberculosis
Antibacterial
Aminocoumarin

ABSTRACT

Synthetic derivatives of the natural product antibiotic novobiocin were synthesized in order to improve their physiochemical properties. A Mannich reaction was used to introduce new side chains at a solvent-exposed position of the molecule, and a diverse panel of functional groups was evaluated at this position. Novobiocin and the new derivatives were tested for their binding to gyrase B and their antibacterial activities against *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Francisella tularensis* and *Escherichia coli*. While the new derivatives still bound the gyrase B protein potently (0.07–1.8 μM, IC_{50}), they had significantly less antibacterial activity. Two compounds were identified with increased antibacterial activity against *M. tuberculosis*, with a minimum inhibitory concentration of 2.5 μg/ml.

The therapeutic treatment of bacterial diseases with antibacterial drugs has had a remarkable and profound impact on human health around the world. Despite the tremendous success of such drugs, the increase in diseases attributed to drug-resistant and multi-drug resistant (MDR) bacterial pathogens indicates a growing need for new antibacterial drugs. New therapeutics are needed for bacterial pathogens that have become resistant to many of the currently approved drugs as well as bacterial strains that are naturally resistant to many antibacterial drugs and have become increasingly pathogenic. Some of the most difficult types of infections to treat are those involving Gram-negative bacteria and mycobacteria, since these classes of bacteria often possess multiple mechanisms that make them resistant to entire classes of antibacterial drugs.

The majority of the most effective new treatments that have been developed for treating drug-resistant strains of bacteria have come from modifications to existing classes of antibacterial drugs. Currently, new generations of cephalosporins, carbapenems and tetracyclines have proven to be some of the most valuable new drugs for treating drug-resistant infections. The newer generations of these drugs have been developed to maintain their potency at their molecular target while circumventing the drug-resistant mechanisms that have made the older generations of these drugs less effective. Additionally, some of the modifications to these drugs have increased the spectrum of activity to include some of the more difficult to treat pathogens, particularly the Gram-negative ones.

Since modifying existing classes of drugs to evade resistance mechanisms has proven to be an effective strategy for multiple classes of antibacterial drugs, this approach may also be effective for improving the activities of the antibacterial drug novobiocin (**1**). Novobiocin targets the bacterial topoisomerase II B subunit, referred to as gyrase B, and is the only approved drug against this target. A natural product isolated from *Streptomyces niveus*, novobiocin was licensed for clinical use in the 1960s and has proven to be an effective treatment for methicillin-resistant *Staphylococcus aureus* (MRSA) infections. The drug has since been withdrawn from the market, primarily due to the availability of superior drugs. Two related compounds, clorobiocin and coumermycin A1, are even more potent inhibitors than novobiocin, but they have not been approved for clinical use.

Although novobiocin is a highly potent inhibitor of gyrase B in vitro, it has a very high level of serum protein binding and therefore must be given at high doses for in vivo efficacy.[1,2] Also, the drug is only effective against Gram-positive bacteria, such as *S. aureus*.[3] Given these shortcomings, modifications to novobiocin that would reduce the drug's serum protein binding and perhaps increase its spectrum of activity to include Gram-negative bacteria and mycobacteria could produce a useful next-generation aminocoumarin antibiotic.

Many drugs that target Gram-positive bacteria are unable to penetrate the outer membrane of Gram-negative bacteria. This membrane is an orthogonal permeability barrier to the cellular membrane and keeps out many types of drugs that would otherwise be considered membrane-permeable. The structure of novobiocin is amphipathic, with a polar noviose sugar moiety and

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0960-894X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.08.035

a weakly acidic aminocoumarin core structure attached to a non-polar hydroxybenzoate group (Fig. 1).[4] A potential strategy for increasing the permeability of novobiocin is to introduce functional groups that can modulate its permeability properties, thereby potentially increasing its spectrum of activity.

New novobiocin analogs were designed using the X-ray crystal structure of novobiocin bound to a 35-kD fragment of gyrase B[5] as well as other available structure–activity relationship (SAR) information (Fig. 1).[6–8] From this information, it was clear that the noviose sugar and aminocoumarin components of novobiocin bind deep into the ATP-binding pocket of the protein and make most of the key binding interactions. The hydrozybenzoate group also makes hydrophobic interactions with the protein, but this group is partially solvent-exposed and more amenable to substitution from prior SAR data (Fig. 1).[7] This structural information suggests that the position *ortho* to the phenol group would be ideal to append an additional solvent-exposed side chain that could modulate the overall physiochemical properties of the molecule. The two closest amino acids to this position are Asp80 and Glu136 (1KIJ structure numbering), two negatively charged residues, but Arg135 is also adjacent to this position so it was reasoned that a neutral functional group should be used as an attachment point off of the hydroxybenzoate ring.

Previous work on analogs of novobiocin using either synthetic or chemoenzymatic methods have established that the noviose group is essential for binding to gyrase B. Compounds such as clorobiocin, which have an extended group off of the 3-position of the noviose ring, more deeply penetrate the binding pocket and generally have greater potency.[7,9] A previous effort to improve the physiochemical properties of novobiocin was done by removing the entire hydroxybenzoate group and replacing the coumarin hydroxyl group with amines.[10,11] Modifications of the hydroxybenzoate group have been done primarily by chemoenzymatic methods and have produced analogs with modifications to the isoprenyl functional group.[12,13] Most of the modifications that shorten or significantly alter the isoprenyl group result in losses of both binding and antimicrobial activities.

Developing chemistry that could be used to make semi-synthetic analogs of novobiocin is challenging because its structure is not stable in strongly acidic or strongly basic conditions. Additionally, since the structure of the drug includes many different functional groups, it was important that the synthetic transformations be highly regioselective. Based on these criteria, it was deduced that a Mannich reaction would be ideal for introducing substitutions *ortho* to the phenol group since the reaction can be carried out under neutral conditions and is compatible with all of the other functional groups on novobiocin.

In order to test the chemistry, novobiocin sodium was reacted with pyrrolidine (1.5 equiv) and paraformaldehyde (1.1 equiv) in ethanol in a sealed glass tube heated to 150 °C with microwave irradiation. The desired product was formed in low yields, but there was also a significant amount of hydrolysis of the carbamate group on the noviose group, and this side product was inseparable by chromatographic methods. This side product was minimized by the addition of acetic acid (1.5 equiv) to neutralize the slightly acidic sodium phenolate on the coumarin ring, and this modification eliminated the formation of this side product. Finally, it was found that switching the solvent from ethanol to tetrahydrofuran (THF) improved the isolated yield of pure product from 14% to 65% in this model system. Subsequently, these conditions were used for all of the Mannich derivatives, with THF as the solvent. The resulting products contained a basic amine group; with the acidic coumarin ring, the resulting compounds were zwitterionic and required reversed-phase C18 prep-HPLC purification.

In order to create a small set of derivatives functionalized at this position, we wanted to add the smallest possible amine group to the novobiocin structure, since the molecular weight of novobiocin (612 g/mol) was already quite high for a drug. The smallest amine that produced the desired Mannich product was methylamine, so this product (**2**) was scaled up to Gram scale in order to produce a key intermediate (Scheme 1). This secondary amine intermediate could then be coupled to a diverse set of side chains to produce a set of analogs with modulated physiological properties.

Using intermediate **2**, a set of carboxylic acid chlorides, symmetric anhydrides and acids were selected to explore the effects of hydrophobic, polar neutral, acidic, and basic functionalities at this position (Scheme 1). In all cases we attempted to select groups that were as small as possible, given the molecular weight of novobiocin. The coupling reactions of intermediate **2** and the acid chlorides and anhydrides were carried out by a reaction in pyridine using standard amide formation conditions to give products **3a**, **3b**, and **3f** in 10–35% isolated yields following prep-HPLC purifications. The reaction of intermediate **2** with acids was performed with HBTU/EDCI coupling conditions to give products **3c–e** and **3g–j** in 10–55% yields following purification. The synthesis of some of the basic side-chain derivatives was performed by directly coupling novobiocin sodium to the appropriate amine using our standard Mannich conditions to give products **4k–n** in 15–55% yields. This set of 14 new analogs was characterized for purity and identity by LC/MS, HPLC and ^{1}H NMR analysis and shown to be of >95% purity by HPLC.

The introduction of non-polar, polar neutral, acidic, and basic groups at the solvent-exposed position enabled us to survey the effects of such modifications on the physiological properties, binding

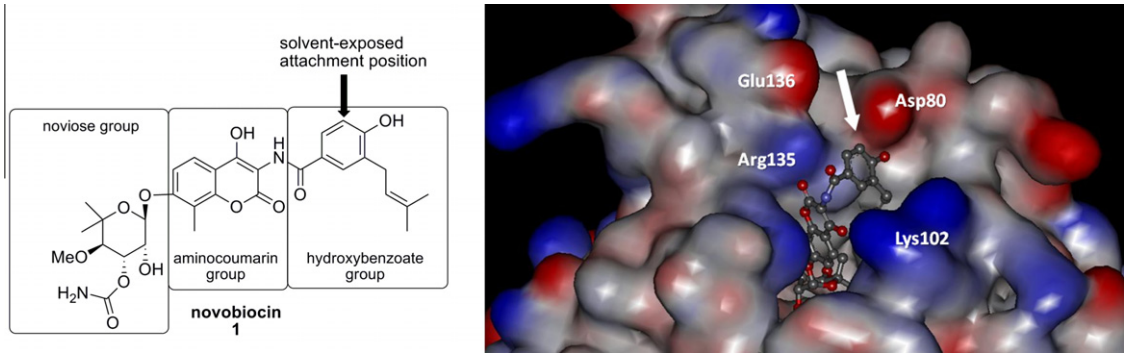
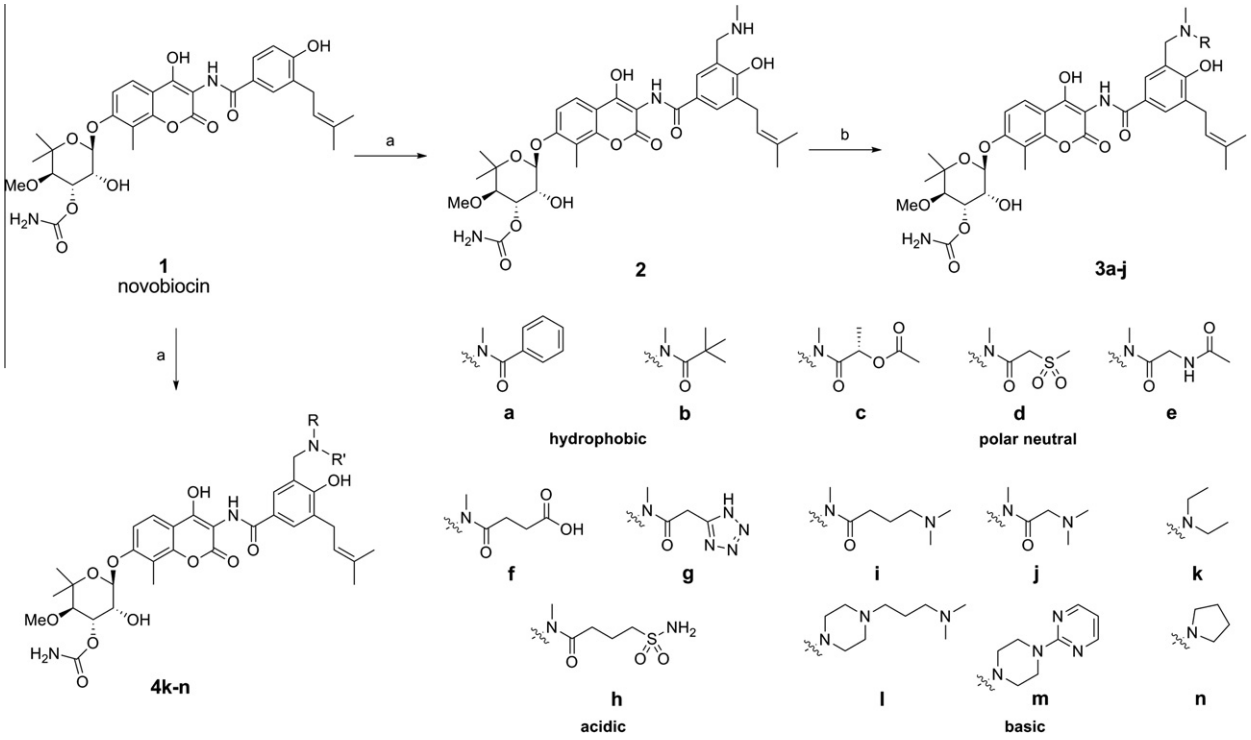


Figure 1. The structure of novobiocin is comprised of three primary chemical groups: A noviose group, an aminocoumarin group and a hydroxybenzoate group. Previous SAR data and the crystal structure of novobiocin bound to a 43-kD fragment of gyrase B (1KIJ) indicates that the position ortho to the phenol on the hydroxybenzoate group (white arrow) is well positioned for appending an additional solvent-exposed side chain.



Scheme 1. Reagents and conditions: (a) Amine (1.1 equiv), paraformaldehyde (1.1 equiv), CH_3COOH (2.0 equiv), THF, 120 °C (2 h); (b) acid chlorides and anhydrides (1.2 equiv), pyridine, rt (1–4 h); (c) acids (1.2 equiv), HBTU (1.2 equiv), DIPEA (3.0 equiv), MeCN, rt (1–4 h).

Table 1
Gyrase B inhibitory and antimicrobial activities of novobiocin derivatives

Compound	*c* Log *D*[a] pH 7.4	Gyrase B IC_{50} (μM)	Minimum inhibitory concentration (MIC) (μg/mL)			
			S. aureus ATCC 29213	*M. tuberculosis* H37Rv	*F. tularensis* Schu S4	*E. coli* ATCC 25922
1	2.65	0.008	0.125	4-8	2	4–16
3a	3.63	1.8	>64	>10	>64	>64
3b	3.58	0.12	16	>10	64	>64
3c	1.97	0.09	16	2.5	>64	>64
3d	0.60	0.46	>64	>10	>64	>64
3e	0.67	0.07	16	2.5	>64	>64
3f	−1.70	0.18	64	>10	>64	>64
3g	−0.35	0.14	>64	>10	>64	>64
3h	0.62	0.07	16	10	>64	>64
3i	−0.02	0.23	>64	>10	>64	>64
3j	1.32	0.37	>64	>10	>64	>64
4k	1.53	0.81	>64	>10	>64	>64
4l	−0.59	0.63	>64	>10	>64	>64
4m	2.48	0.80	>64	>10	>64	>64
4n	1.39	0.68	>64	10	>64	>64

[a] *c* Log *D* values were calculated using Chem Axon software.

affinities, and antibacterial activities of the new analogs (Table 1). The calculated Log *D* values (at pH 7.4) demonstrated a wide range of partition coefficients ranging from −1.70 to 3.63. The partition coefficients alone proved to have no correlation with the binding affinities or the antibacterial activities, suggesting that this single property of the compounds is not sufficient to significantly increase their permeability to bacterial membranes.

The in vitro binding constants for all of the novobiocin derivatives were determined using a fluorescence polarization ligand displacement method with the full-length gyrase B protein from *Francisella tularensis* Schu S4 strain.[14] The IC_{50} values obtained for binding to gyrase B revealed that all of the derivatives were still potent binders to gyrase B but had some loss of binding activity relative to novobiocin (Table 1). Derivatives with sterically bulky groups, such as **3a**, **3b**, **3l**, and **3m**, had the greatest drop in binding potencies, suggesting that even though this position is solvent-exposed in the crystal structure, some obstruction from protein residues on the outside of the binding pocket might be clashing with these groups. The most potent compounds contained either small non-polar side chains (**3b**, 0.17 μM), polar neutral side chains (**3c**, 0.09 μM; and **3e**, 0.07 μM), or a weakly acidic sulfonamide side chain (**3h**, 0.07 μM). All of the derivatives with a basic side chain had greater losses in binding affinities, which was somewhat expected given that Arg135 is near these substitutions and could form unfavorable interactions with the positive charges on these basic side chains.

The antibacterial activities of these new derivatives were measured against a diverse panel of bacteria that included a

Gram-positive pathogen (*S. aureus*), two Gram-negative pathogens (*F. tularensis* and *Escherichia coli*) and a mycobacteria (*Mycobacterium tuberculosis*). Given that novobiocin has been used as a drug primarily for *S. aureus*, we expected the greatest activities against these bacteria. Novobiocin was quite potent against *S. aureus*, with an MIC of 0.125 μg/ml. The most potent new derivatives were all significantly less potent, with four compounds having MIC values of 16 μg/ml. These four compounds—**3b**, **3c**, **3e**, and **3h**—were the same four compounds that were the most potent in the gyrase B binding assay. Their binding affinities were approximately one order of magnitude weaker than novobiocin, but their antibacterial activities were closer to two orders of magnitude weaker than novobiocin. These data suggest that the modifications on these compounds may have actually decreased their ability to enter into the cytoplasm of *S. aureus*, even though these four compounds have calculated partition coefficients ranging from 0.62 to3.58, around that for novobiocin (2.65).

The antibacterial activities of the new derivatives against *M. tuberculosis* (Mtb) were slightly more encouraging, with two compounds, **3c** and **3e**, showing a significant increase in potency relative to novobiocin (Table 1). Interestingly, all of the compounds that showed any activity against *S. aureus* showed higher potencies against Mtb, which is surprising given that novobiocin itself is much more potent against *S. aureus* (the MIC value for novobiocin is 0.125 for *S. aureus* and 4–8 μg/ml for Mtb). Another unexpected discovery was that these two more active compounds were both more polar derivatives of novobiocin. The cell wall of mycobacteria is very lipid rich and thicker than that of many other bacteria. This waxy hydrophobic layer would be expected to have greater permeability to compounds with higher partition coefficients, but these two small polar neutral side chains both have lower calculated Log D values.

None of the new derivatives demonstrated any antibacterial activity against two types of Gram-negative bacteria, *F. tularensis* and *E. coli*, with the exception of **3b**, which had very weak activity (64 μg/ml). Novobiocin itself is also relatively weak against these bacteria, so it is possible that the reduction in binding affinity at the target makes these compounds unlikely to show antibacterial activity at the concentrations tested. Clearly, the modifications made to novobiocin did not cause any significant increase in permeability to the Gram-negative pathogens. The large size of novobiocin itself may make it extraordinarily difficult to get these compounds into the bacterial cytosol by adding additional molecular weight. An analysis of the physiochemical properties of antibiotics that target Gram-negative bacteria revealed that in general Gram-negative antibiotics have lower molecular weights and lower partition coefficients than antibiotics targeting Gram-positive bacteria.[15] In fact, it is believed that many of the Gram-negative antibiotics must enter the cytosol through porins rather than diffusion across the outer membrane.

Acknowledgments

The project described was supported by Award Number U01AI082070 from the National Institute of Allergy and Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

Supplementary data

Supplementary data (Synthetic and biological methods and analytical characterization of all compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.08.035.

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