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The Identification and Characterization of Hydrazinyl Urea-Based Antibacterial Agents through Combinatorial Chemistry

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Abstract—An effort to identify novel inhibitors of peptidoglycan synthesis with antibacterial activity resulted in the discovery of a series of biaryl urea-based antibacterial agents through isolation of a by-product from a mixture-based combinatorial library of semi-carbazones and subsequent parallel synthesis efforts. The compounds were shown to possess broad spectrum antibacterial activity against gram-positive drug resistant pathogens, and showed apparent specificity for disruption of the bacterial cell wall biosynthesis pathway.

The rapid emergence of drug resistant bacterial pathogens emphasizes the need for new classes of antibacterial agents.[1] Historically, new types of compounds have been identified from the diverse pool of natural products, for which there are many proven clinically efficacious agents. Over the past few decades, only a few new classes have been identified (quinolones, oxazolidinones, etc.), indicating the great degree of technical difficulty in such an effort.[1,2] The chemical diversity of compounds being generated using novel methods, especially combinatorial chemistry, coupled with rapid screening programs offers the opportunity to identify new classes of antibacterials. Many existing antibacterial drugs act by direct inhibition of one or more key steps in the metabolic pathway for synthesis of bacterial cell wall peptidoglycan. These agents include β-lactams (penicillin and methicillin), glycopeptides (vancomycin), and a variety of other natural products (bacitracin, *D*-cycloserine, and fosfomycin).[1] In our program, we sought to identify cell wall active agents through screening and deconvolution of mixture-based combinatorial libraries, similar to the screening of natural products via plant and animal extracts. The cell wall assay we implemented was one designed to measure the final product of over 20 different interdependent enzyme reactions that create peptidoglycan. Also, part of the strategy was to simultaneously screen against bacterial growth inhibition to identify chemicals which act as both inhibitors of the cell wall biosynthesis and have antibacterial properties. This report describes the serendipitous discovery of aryl hydrazine urea-based antibacterial agents identified from a 10,000 member indexed library of semi-carbazones.

Solution-based combinatorial chemistry has been utilized to make mixture-based libraries of smaller proportions.[3] Identification of the active components can be addressed through synthesizing the library in a particular format (indexed, scanning, etc.) and subsequent guided deconvolution.[4] In our program, a broad variety of combinatorial libraries were screened in parallel for inhibition of growth, bacterial histidine kinases,[5] and peptidoglycan biosynthesis in whole cells of *Staphylococcus aureus*. Among the 20 plus libraries that were tested, only an indexed library of 10,000 semi-carbazones (**4**, Scheme 1), showed activity in both cell wall-peptidoglycan biosynthesis and growth inhibition assays that led to successful isolation and follow-up of an active component. Semi-carbazones (**4**) and their derivatives (i.e., acyl hydrazones) exhibit diverse pharmacology, and the aza linkage resident in this class has

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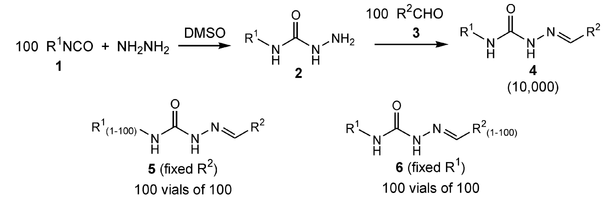
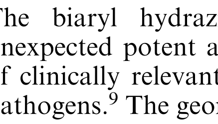


Scheme 1. Reaction sequence for the 10,000 member semi-carbazone indexed combinatorial library **4**.

been shown to be peptidomimetic in nature.[6] The indexed library was synthesized in solution in pools of 100 compounds by the following sequence: (a) reaction of hydrazine with an isocyanate (**1**) to form semi-carbazides (**2**); followed by (b) reaction of the resulting semi-carbazide with a carboxaldehyde (**3**, Scheme 1). The indexed library was synthesized in two sets of 100 vials.[7] The first set (**5**) contained 100 isocyanate variants per vial, the second (**6**) 100 aldehyde variants. Building blocks were chosen to match desirable properties (i.e., average molecular weight for **4** was 306 amu's). Several vials were assayed by LC–MS for completeness, and at least 80–90% of the 100 target compounds were found in these cases.[8] The vials were weighed and dissolved in DMSO at a range of 100–500 mg/mL.

Upon screening the various mixtures in both assays, two particular vial sets showed up as having dual activity in the cell wall biosynthesis and the growth inihibition assays at a very high level (99% inhibition at <1 μg/mL per component).[9] One had been derived from 3,5-bis(trifluoromethyl)phenyl isocyanate (**6**), the other from indole-3-carboxaldehyde (**5**). The cross product semicarbazone from these two building blocks (**7**) was made and tested. Unfortunately, it lacked the potency seen in the mixture vials (MIC and IC_{50} for **7** >300 μM). It was noteworthy that none of the fixed aldehyde mixture sets (**5**) exhibited significant antibacterial properties, unlike the fixed position isocyanate mixture libraries (**6**). This supported the observation that the dual properties seen in the mixtures were only isocyanate dependent. Careful scrutiny of the reaction conditions and further analysis revealed the presence of two potentially active compounds resulting from the combination of hydrazine and 3,5-bis(trifluoromethyl)phenyl isocyanate. The two compounds, semicarbazide **8** and the bis-urea **9** (an aza-Gly peptidomimetic[10]) were synthesized by combining hydrazine and 3,5-bis(trifluoromethyl)phenyl isocyanate (ratio of **8**:**9** = 81:17) and one (**9**) was found to have the activity seen in the original mixtures (for **8**; $IC_{50} > 300$ μM; MIC (G+6) = 101 μM).[11]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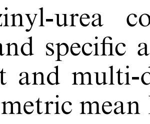
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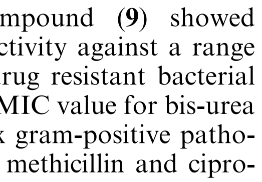
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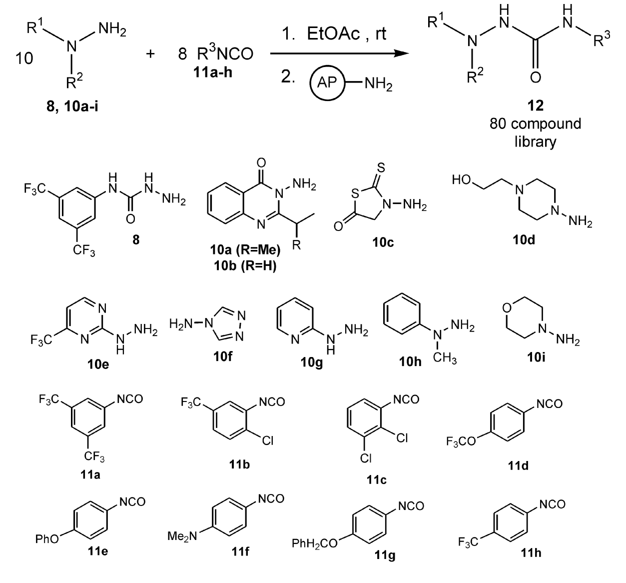
The biaryl hydrazinyl-urea compound (**9**) showed unexpected potent and specific activity against a range of clinically relevant and multi-drug resistant bacterial pathogens.[9] The geometric mean MIC value for bis-urea **9** was 3 μM against a panel of six gram-positive pathogens (MIC G+6). This included methicillin and ciprofloxacin-resistant *S. aureus* (MI339, 4.7 μM), vancomycin-resistant *Enterococcus faecium* (EF12, 4.9 μM), and penicillin-resistant *Streptococcus pneumoniae* (STP51, 1.7 μM). It also showed inhibition of cell wall biosynthesis ($IC_{50} = 17$ μM) at a level similar to the MIC values. The compound also showed growth inhibition against a number of fastidious microbes from different families including gram-negative strains (*Legionella* sp., 1.1 μM) and some anaerobes (*Bacteroides fragilis*, MIC = 0.004 μM). It did not show activity against typical gram-negative *Enterobacteriaceae* (*Escherichia coli*, *Klebsiella*, *Proteus*, and *Enterobacter*, MIC > 300 μM), suggesting that the compound did not penetrate the gram-negative envelope and/or was actively excluded. To further assess its biological specificity, compound **9** was tested against eucaryotic cells, and found to be non-inhibitory to *Saccharomyces cerevisiae* (MIC > 300 μM) as well as non-hemolytic to sheep red blood cells ($LC_{50} > 300$ μM). Further observations showed that this compound was bactericidal at a level only slightly above the MIC.

Due to the symmetrical nature of this compound and the lack of ionic functionality, the aqueous solubility was low (≤ 0.5 μg/mL). In order to improve physical properties and gain more understanding into the mechanism as well as develop structure–activity relationships (SARs), we embarked on a program to expand around this potential lead structure (**9**) through parallel synthesis. As we were interested in improving solubility, minimizing symmetry, and elucidating the minimum pharmacophore, an 80 compound set was designed to quickly scan both portions of compound **9** through urea chemistry (**12**, Scheme 2). We chose substituted hydrazine molecules (**8** and **10a**–**i**) as one component (columns 1–10), and isocyanates (**11a**–**h**) as the other (rows A–H). We employed macro porous polystyrene amino methyl resin to sequester remaining isocyanate.[12] We synthesized the compounds in a 96-well reaction plate using EtOAc as solvent, added the resin via a slurry, and then filtered the compounds into a deep well 96-well



Scheme 2. Synthesis route and list of synthetic inputs for parallel urea library **12**.

plate.[13] The compounds were analyzed by flow injection mass spectroscopy. The active wells were further analyzed by HPLC–mass spectroscopy to identify the target purity. The solvent was removed and the compounds were re-dissolved from dry films into DMSO for screening in the two assays.

According to our previously outlined goals, the set was then screened for both cell wall synthesis and bacterial growth inhibition (Table 1).[9] An IC_{50} and MIC value of 125 µM was chosen as a cut-off point to assign actives for follow-up on compounds from the plate. The compounds were screened at an effective concentration based on amount of starting materials, and assumed theoretical yields and purity. Compounds having both growth and cell wall inhibition values below this point with purity above 50% were deemed promising (boxes in Table 1). A definite trend is seen in terms of structure. It was clear from this limited SAR that one side of the molecule needs to contain at least one trifluoromethyl group (rows A and H, column 1), and that the relative placement of these groups was important (row B vs row H). The plate was also assayed for adverse effects on eucaryotic cells. Only a few wells showed yeast growth inhibition and red blood cell lysis (rows D and E), and these did not have any of the desired antibacterial properties we saw in the other wells. Well A1 contained our benchmark (**9**), which served as an internal standard for both the synthesis and screening of this plate.

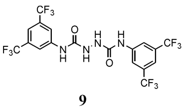
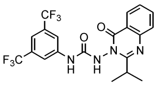
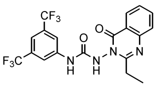
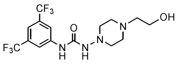
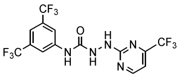
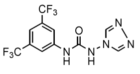
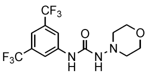
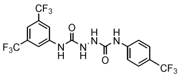
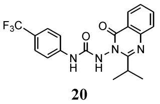
We then picked a few compounds from the plate to follow up on. We were especially interested in the structures that eliminated the second urea functionality on the hydrazine of the parent structure. Several compounds were re-synthesized and purified,[11] and then tested in the cell wall synthesis assay and against a panel of gram-positive pathogens (Table 2).[9] The 3-amino-4(3*H*)-quinazolinone based ureas (**13**, **14**, and **20**) were an interesting subclass because of their similar activity and improved solubility and ClogP values. The 4(3*H*)-quinazolinone nucleus is a strong departure from the urea structure of **9**, and represents a promising class for further optimization. For example, compounds **13**, **14**, and **20** showed similar profiles to the lead structure (**9**, Table 2), with low MIC values (≤10 µM) and similar cell wall inhibition values (IC_{50} = 36–66 µM).

Table 1. [a] LC–MS purity, effective growth (MIC), and cell wall inhibition values for urea library **12** in Scheme 2 in 80-well plate format

%	8	10a	10b	10c	10d	10e	10f	10g	10h	10i
11a	>50	>66	94		85	>60				>52
11b	>61									
11c	>67				79			⊕		
11d		>53	>85	⊕	⊕		⊕		>57	
11e	>66			⊕	⊕		⊕			
11f	>79									
11g	>52									
11h	>59	>53	80		>91	>64	56		62	60

[a] Wells are described according to standard plate formatting, with columns 1–10 from left to right, and rows A–H from top to bottom. The data represent LC–MS purity via evaporative light scattering detection (ELSD), and a > sign indicates a saturated detector response. Data in boxes indicate a cell wall IC_{50} and an MIC value ≤125 µM both against *Staphylococcus aureus* (MI246), and an observed purity of the target compound of ≥50%. Values are based on theoretical reaction yield, and are not adjusted for actual yield and purity of the target compound. The ⊕ symbol designates wells that were hemolytic to red blood cells and/or inhibitory to yeast organisms with LC_{50} or MIC values <125 µM.

Table 2. Biological and physical data for selected re-synthesized hydrazinyl-urea compounds from library **12**

Compound	Well no.	Cell wall IC_{50} (µM)[a]	MIC G+6 (µM)[b]	Solubility at pH 7.4 (µg/mL)[c]	ClogP[d]
 **9**	A1	17	3	<0.5	7.7
 **13**	A2	66	1.5	3	5.3
 **14**	A3	36	7	59	4.9
 **15**	A5	167	>300	—	2.9
 **16**	A6	47	4	2	4.0
 **17**	A7	236	198	53	3.1
 **18**	A10	135	68	5	2.8
 **19**	H1	145	2	—	5.8
 **20**	H2	44	10	6	3.4
Vancomycin		0.4	0.3	—	—

[a] Cell wall IC_{50} values determined in duplicate in *Staphylococcus aureus* (MI246).

[b] MIC G+6 represents the geometric mean MIC determined in a panel of the following six gram-positive organisms: *Staphylococcus aureus*, MI273 and MI339 (methicillin and ciprofloxacin resistant); *Enterococcus faecium*, EF12 (vancomycin resistant); *Enterococcus faecalis*, STD44; and *Streptococcus pneumoniae*, STP51 and STP6301 (penicillin-resistant).

[c] Determined in phosphate buffered saline.

[d] Calculated using Advanced Chemistry Development Labs, Inc. (ACD) software.

Furthermore, we were encouraged to find that these compounds exhibited increased solubility profiles while not compromising the biological activity (3, 59, and 6 μg/mL for **13**, **14**, and **20**), possibly due in part to their lower ClogP values (7.7 for **9** vs 5.3, 4.9, and 3.4 for **13**, **14**, and **20**). There were also several other heterocyclic hydrazine based products which showed various degrees of activity in both assays (**16**, **17**, and **18**). These compounds exhibited better solubility and ClogP values than the parent compound **9** (Table 2). Further progress in this area will be the subject of future reports.

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6. Limal, D.; Grand, V.; Vanderesse, R.; Marraud, M. *Tetrahedron Lett.* **1994**, *35*, 3711.
7. Pirrung, M. C.; Chen, J. *J. Am. Chem. Soc.* **1995**, *117*, 1240.
8. LC–MS data for **5** and **6** were obtained on a Fisons Platform-II mass spectrometer with electrospray ionization equipped with a Hewlett–Packard 1050 HPLC. A Waters Symmetry C-18 column was utilized with MeOH–water as the mobile phase. Chromatography was monitored via UV detection at 252 nm. LC–MS data for **5** ($R^2 = 2,4\text{-Me}_2\text{-Ph}$): 82 of 100 had two observed ions, with 14 having isobar co-elution = 82–98 of 100 molecular ions identified; LC–MS for **6** ($R^1 = n\text{-C}_6H_{13}$): 80 of 100 had two observed ions, with seven having isobar co-evolution = 80–90 of 100 molecular ions identified.
9. Materials and methods: Microbial growth inhibition tests: *Saccharomyces cerevisiae* strain INVSc1 was from Invitrogen (Carlsbad, CA). All other strains were either clinical isolates or from the American Type Culture Collection (Rockville, MD). Minimum inhibitory concentrations (MICs) for bacterial and yeast growth were determined by standard NCCLS microbroth dilution methods in Mueller Hinton broth (MHB) or brain heart infusion (BHI) for bacteria, and in yeast potato dextrose for *S. cerevisiae*. Inhibition of bacterial cell wall synthesis: Determined by monitoring incorporation of [^{3}H]-glycine into peptidoglycan by intact cells of *S. aureus* strain MI246. Cells at 37 °C were pretreated 40 min with test agents, then radiolabeled for 30 min prior to radiometric measurement. Data expressed as the concentration inhibiting synthesis by 50% relative to control. Sheep red blood cell lysis: Membrane disruption was assessed by colormetric measurement of hemoglobin release from erythrocytes treated with test agents.
10. Hyunsoo, H.; Janda, K. D. *J. Am. Chem. Soc.* **1996**, *118*, 2539.
11. Physical data for selected compounds: **9**: MS (DCI): 543 (M + H), 523, 288, 236; ^{1}H NMR (CD_3OD): δ 7.55 (s, 2H), 8.2 (s, 4H); ^{13}C NMR (CD_3OD): δ 115.3, 118.9, 123.6 (quart, *J* = 270 Hz), 131.8 (quart, *J* = 33 Hz), 141.3, 157. Anal. calcd for $C_{14}H_{10}N_4O_2F_6$: C, 39.87%, H, 1.86%, N, 10.33%; found: C, 39.50%, H, 1.99%, N, 10.51%; **13**: MS (APCI): 459 (M + H), 204; ^{1}H NMR (CD_3OD): δ 1.4 (d, 3H, *J* = 3 Hz), 1.42 (d, 3H, *J* = 3 Hz), 3.5 (quin, 1H, *J* = 3 Hz), 7.5 (t, 1H, *J* = 5 Hz), 7.6 (s, 1H), 7.7 (d, 1H, *J* = 5 Hz), 7.9 (t, 1H, *J* = 5 Hz), 8.15 (s, 2H), 8.25 (d, 1H, *J* = 5 Hz); ^{13}C NMR (CD_3OD): δ 18, 18.65, 29.62, 114.23, 117.28, 118.6, 119.8, 124.1, 124.91, 125.1, 125.7, 130.6 (quart, *J* = 25 Hz), 133.39, 139.1, 145.8, 153.9, 159.9, 162.1; ^{19}F NMR (CD_3OD): δ 102.1; **20**: MS (APCI): 391 (M + H), 230, 204, 189; ^{1}H NMR (CD_3OD): δ 1.37 (d, 3H, *J* = 3 Hz), 1.39 (d, 3H, *J* = 3 Hz), 3.5 (quin, 1H, *J* = 7 Hz), 7.5 (t, 1H, *J* = 8 Hz), 7.6 (d, 2H, *J* = 9 Hz), 7.7 (d, 2H, *J* = 9 Hz), 7.8 (d, 1H, *J* = 8 Hz), 7.9 (t, 1H, *J* = 9 Hz), 8.2 (dd, 1H, *J* = 1 Hz, *J* = 8 Hz); ^{19}F NMR (CD_3OD): δ 99.3; HPLC: 5.2 min (100% peak area).
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13. Preparation of urea library **12**. Hydrazines (**8** and **10a**–**i**) and isocyanates (**11a**–**e**) were purchased from various vendors. A 0.5 M solution in EtOAc of each reagent was made and the appropriate amount was added to a 96-well reaction block (Robbins Scientific Flexchem™ system). For the hydrazines, 1 mmol of reagent was dissolved in 2 mL of EtOAc. Then 200 μL of each solution (0.1 mmol) was added to each well in columns 1–10 according to the grid assigned in Table 1. For the isocyanates, 1.5 mmol of reagent was dissolved in 3 mL of EtOAc. Then 240 μL of each solution (0.12 mmol) was added to each well in rows A–H according to the grid assigned in Table 1. The reactions were shaken for 4 days. Then a slurry of ArgoPore aminomethyl polystyrene (loading = 1.1 mmol/g) was added to each well (about 100–150 mg of resin per well). The reaction block was resealed and agitated for an additional 4 days. Filtration into a 96-well collection plate, solvent removal, and subsequent MeOH washing and solvent removal gave a plate of 80 compounds as dry films. Flow injection data on library **12** showed positive molecular ions for 74 out of 80 compounds (92%).