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Dioxane-Linked Amide Derivatives as Novel Bacterial Topoisomerase Inhibitors against Gram-Positive *Staphylococcus aureus*

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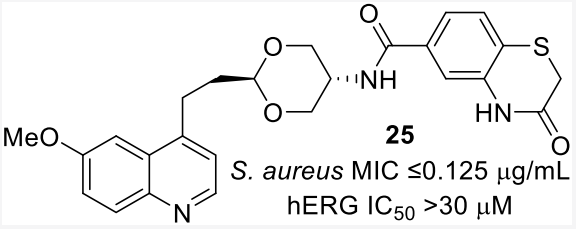
ABSTRACT: In recent years, novel bacterial topoisomerase inhibitors (NBTIs) have been developed as future antibacterials for treating multidrug-resistant bacterial infections. A series of dioxane-linked NBTIs with an amide moiety has been synthesized and evaluated. Compound **3** inhibits DNA gyrase, induces the formation of single strand breaks to bacterial DNA, and achieves potent antibacterial activity against a variety of Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA). Optimization of this series of analogues led to the discovery of a subseries of compounds (**22**−**25**) with more potent anti-MRSA activity, dual inhibition of DNA gyrase and topoisomerase IV, and the ability to induce double strand breaks through inhibition of *S. aureus* DNA gyrase.



KEYWORDS: *MRSA, antibacterial, gyrase, TopoIV, cleavage*

Infections from multidrug-resistant (MDR) bacteria are widely recognized as a threat to both human health and the continued success of modern medicine.[1] In recent years, however, progress has been made in reducing the incidence of infections by some key pathogens, including the Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus spp.* (VRE) as well as Gram-negative organisms such as MDR *Pseudomonas aeruginosa* and carbapenem-resistant *Acinetobacter baumannii*.[2] A resurgence of the early discovery and development pipeline, promoted in part by novel funding mechanisms,[3] likewise provides grounds for some optimism. Nevertheless, overall mortality and economic costs associated with MDR bacterial infections remain unacceptably high. According to the United States Centers for Disease Control and Prevention, for example, MRSA and VRE together caused >15,000 deaths in the US in 2017, alongside >$2 billion of attributable healthcare costs.[4] Against this backdrop, ongoing efforts to discover innovative therapies are sorely needed.

The development of new therapies benefits from multiple strategies with orthogonal risks. New classes of antibacterial agents with novel mechanisms/targets may circumvent pre-existing target-based resistance mechanisms. Advances in the treatment of tuberculosis have been particularly notable, most recently with the nitroimidazooxazine pretomanid[5] gaining approval from the US Food and Drug Administration. The optimization of well-established classes to expand coverage to MDR bacteria offers an alternative strategy; delafloxacin[6] achieved approval for certain MRSA infections previously untreatable by the fluoroquinolone class. Resistance to agents from well-established mechanisms is expected, however, and has been reported for delafloxacin.[7] The advancement of chemically innovative classes inhibiting well-validated biological targets offers a hybrid of these approaches. New inhibitors of β-lactamases (a key bacterial resistance mechanism) represent notable successes, most recently exemplified by the diazabicyclooctane relebactam.[8] The novel bacterial topoisomerase inhibitor (NBTI) gepotidacin,[9] currently in Phase 3 clinical trials, is another promising example. Like the fluoroquinolones, gepotidacin targets DNA gyrase and topoisomerase IV (TopoIV), but its novel binding mode[10,11] confers potent activity against fluoroquinolone-resistant bacteria.[12] Indeed, the NBTIs have seen substantial research investment in recent years.[13−15]

The majority of NBTIs possess either a secondary or tertiary amine. Analysis of ternary X-ray crystal structures[10,11,16] with NBTIs, DNA, and *S. aureus* gyrase reveal a key interaction between this amine nitrogen and an aspartate (D83) located at the entrance to a small hydrophobic binding pocket formed at the interface of two GyrA domains. Studies on acquired

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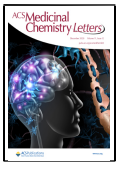

resistance to NBTIs support the relevance of this interaction; D83N and D83G substitutions are among the most commonly reported NBTI resistance determinants in *S. aureus*.[17−20] Unfortunately, the basicity of the amine moiety of the NBTIs has been correlated with hERG inhibition,[21−24] an adverse *in vitro* safety finding. Consequently, significant effort has been devoted to reducing the basicity of the amine moiety to improve safety.

Our initial approach was to reduce amine basicity with dioxygenated linkers derived either from 1,3-dioxane[23,25] or isomannide.[24] Matched pair comparisons of dioxane-linked (e.g., **2**) and cyclohexane-linked NBTIs (e.g., **1**) demonstrated consistent reductions in hERG inhibition,[23] illustrated in Figure 1. Nevertheless, achieving a lead-like hERG profile, with

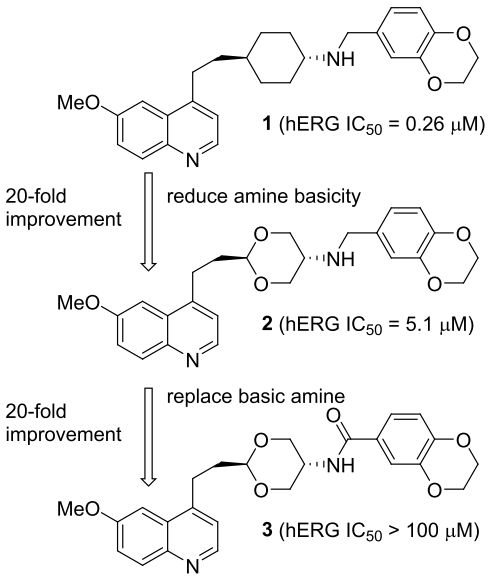

Figure 1. Evolution of strategy for reduced hERG inhibition.

an IC_{50} > 100 μM, has proved challenging. We reported that compound **3**,[25] in which the secondary amine of **2** has been replaced with a secondary carboxamide, achieved lead-like hERG criteria while maintaining potent antistaphylococcal activity. Limited additional amides with potent antibacterial activity have been reported in the peer-reviewed literature,[26−31] suggesting that amides represent an attractive avenue of further investigation. Herein, we disclose our efforts in pursuit of this strategy.

In order to better understand the binding mode of amide NBTIs such as **3** with gyrase, we used molecular docking to determine potential binding modes for our compounds. Ombrato and co-workers disclosed a series of piperazine-linked NBTIs, including the amide derivative ID-130.[31] The ternary X-ray crystal structure of ID-130 in complex with DNA and gyrase was reported at 2.7 Å resolution (PDB code 6FM4). The authors suggested that the amide N−H interacts with the gyrase D83 residue through hydrogen bonding, in much the same way as amine derivatives bind. In the first docking study, we used Glide (SP scoring function)[32] to dock **3** into the binding pocket of 6FM4 as specified by the location of ID-130 in the crystal structure. The top-scoring docked pose for **3** (magenta) is shown (Figure 2, left) overlaid with ID-130 from the crystal structure (black). The DNA- and enzyme-binding motifs of **3** nicely match those of ID-130 and position the benzodioxine ring of **3** to reach deep into the GyrA dimer pocket. The positioning of the linker domain of **3** diverges substantially from ID-130, but this region makes no key contacts with either DNA or the enzyme and has been shown to tolerate considerable structural diversity.[13−15] The amide N−H of **3**, while not directly overlapping that of ID-130, is nevertheless positioned to hydrogen bond with D83; the hydrogen bonding distance to the oxygen of D83 is 2.5 Å. We employed a similar approach beginning with the breakthrough 2.1 Å structure of GSK299423 reported by Bax et al. (PDB code 2XCS).[10] Again, we used Glide (SP) to dock **3** into the GSK299423 binding pocket of 2XCS. The top-scoring pose is also shown in Figure 2 (right) overlaid with GSK299423 from the crystal structure. In this case, the amide N−H of **3** clearly overlays with the amine N−H of GSK299423, supporting the involvement of a hydrogen-bond with D83 in facilitating target binding for these amide NBTIs. Here, the hydrogen bonding distance to the D83 oxygen is 1.9 Å.

A broader determination of the antibacterial activity of **3** was conducted (Laboratory Specialists, Inc., Westlake, OH). An MIC of 0.25 μg/mL was observed with quality control strain *S. aureus* ATCC 29213, similar to our reported[25] value of 1 μg/mL. Potent activity was observed against a levofloxacin-resistant MRSA strain: MIC of 0.5 μg/mL for **3** as compared to >16 μg/mL for levofloxacin. Using a small panel of fluoroquinolone-resistant MRSA strains (n = 11), **3** yielded an

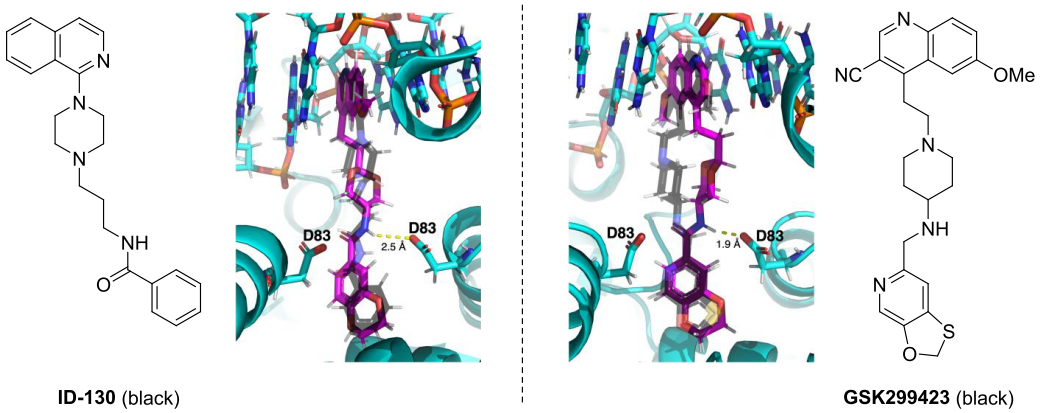

Figure 2. Binding poses of **3** (magenta) overlaid with ligands (black) from PDB 6FM4 (left) and 2XCS (right). Bacterial DNA and gyrase shown (blue), with gyrase in cartoon form.

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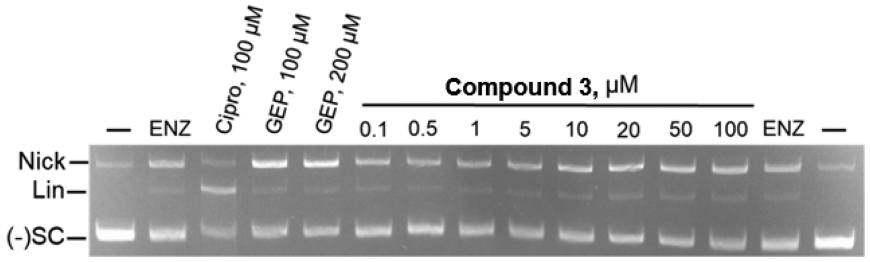

Figure 3. Compound **3** enhances only single-strand DNA breaks in the presence of gyrase. The ethidium-stained gel shows positions of DNA after incubation with enzyme in the absence or presence of **3** (0.1−100 μM). The various forms of DNA are indicated as negatively supercoiled ((−)SC), nicked open-circular (Nick), and linearized (Lin). Formation of single-stranded (Nick) and double-stranded (Lin) DNA breaks were observed by fluorescence band intensity during conversion of (−)SC to Nick and Lin reaction products.

MIC_{90} of 2 μg/mL, compared to 8 μg/mL for levofloxacin and 32 μg/mL for ciprofloxacin. Potent MICs were also observed for other Gram-positive pathogens, including *Streptococcus pyogenes* (0.5 μg/mL), penicillin-susceptible and nonsusceptible *Streptococcus pneumoniae* (0.25 μg/mL), and vancomycin-resistant *Enterococcus faecium* (VRE, 0.5 μg/mL). MICs for *Enterococcus faecalis* (ATCC 29212) and vancomycin-susceptible *E. faecium* were slightly higher (1 and 2 μg/mL, respectively). On the whole, amide **3** shares a similar profile to our earlier reported dioxane-linked amine NBTIs, with promising activity against Gram-positive bacteria.[23,25]

The promising antibacterial activity of **3** prompted us to characterize its mode of action in greater detail. NBTIs have generally been described to enhance gyrase-mediated single strand breaks (SSBs) to DNA without induction of double strand breaks (DSBs).[10,11,17,33−35] However, detailed pharmacological studies have been reported in only a few instances, including the recent study of gepotidacin by Osheroff and colleagues.[11] As shown in Figure 3, ciprofloxacin (100 μM) induced DSBs, evidenced by conversion of negatively supercoiled pBR322 DNA to linearized DNA. In contrast, consistent with the results reported by Osheroff and colleagues,[11] gepotidacin (100, 200 μM) enhanced DNA SSBs evidenced by formation of nicked open circular DNA, without substantially enhancing DSBs. Compound **3** behaved similarly to gepotidacin, demonstrating a concentration-dependent increase in SSBs without an appreciable increase in DSBs (*vide infra*, Figure 5A and 5B).

In addition to **3**, we reported[25] that the more lipophilic 3,4-dichlorobenzamide **4** (Table 1, below) displayed superior antibacterial activity at the cost of more potent (less desirable) hERG inhibition (IC_{50} = 3.2 μM). Given the wide diversity of available benzoic acid starting materials, we initially constructed a small set of benzamide derivatives to evaluate structure−activity and structure-toxicity relationships (**5**−**15**, Table 1). Amide derivatives were synthesized from our previously reported primary amine and the corresponding carboxylic acids (see Supporting Information).

Minimum inhibitory concentrations (MICs) were determined using *S. aureus* ATCC 29213 and a ciprofloxacin-resistant USA300 MRSA isolate,[36] as reported previously.[23−25] Select compounds, including all analogues with MICs ≤ 2 μg/mL, were tested using biochemical gel-based assays for inhibition of *S. aureus* gyrase supercoiling and TopoIV decatenation activity. We also measured MICs using a previously reported[19,37] NBTI-resistant strain of *S. aureus* with the gyrase D83N substitution alongside the otherwise isogenic parent strain bearing the canonical aspartate (strain 3527).

Results are presented in Table 1. Our previous studies[25] of dioxane-linked amine-containing NBTIs suggested that 4-substitution was advantageous in delivering potent antistaphylococcal agents. The 4-methoxybenzamide (**5**) had modest MICs of 4−16 μg/mL against the ATCC and USA300 strains. Derivatives with more lipophilic substituents such as 4-chloro (**6**), 4-methyl (**7**), 4-trifluoromethyl (**8**), 4-trifluoromethoxy (**9**), and 4-*tert*-butyl (**10**) all demonstrated potent activity against the ATCC strain (MICs from 0.25 to 1 μg/mL), on par with or superior to that of the previously reported amines. MICs for the USA300 strain were similar but slightly higher (range of ≤0.5 to 4 μg/mL). Amides with smaller and/or less lipophilic 4-substituents such as fluoro (**11**) and cyano (**12**) were devoid of activity. Surprisingly, the 3-chlorobenzamide (**13**) was essentially equipotent to 4-Cl derivative **6**. This finding represents a departure from SAR in the amine series, where lipophilic substitution of the 4-position consistently outperformed 3-substitution. The 2-chlorobenzamide **14** was wholly inactive. The unsubstituted benzamide **15** proved poorly soluble at high concentrations and afforded highly variable results, with MICs ranging from 8 to >128 μg/mL in four separate assays. The more polar picolinamide (**16**) and nicotinamide (**17**) were inactive.

Our attention turned next to biochemical evaluations of target inhibition. The 4-substituted benzamides with MICs ≤ 1 μg/mL (**6**−**10**) demonstrated potent inhibition of gyrase supercoiling activity, with IC_{50} values ranging from 0.41 to 1.4 μM. The 3-Cl benzamide **13** likewise potently inhibited gyrase (IC_{50} = 0.32 μM). The 4-methoxybenzamide **5** afforded a somewhat higher IC_{50} of 3.9 μM, in line with its reduced antibacterial activity (4−8 μg/mL). Notably, the bicyclic amide **3** yielded a similar IC_{50} (3.7 μM) but a more potent MIC (1 μg/mL), possibly reflecting superior cellular penetration/accumulation. Unfortunately, none of these compounds potently inhibited decatenation mediated by TopoIV. Thus, these benzamide analogues appear to preferentially inhibit gyrase in *S. aureus*.

Understanding the potential implications of the selective targeting of gyrase in a cellular context is an important objective with regard to resistance. To probe this question, we determined MICs using a parent multidrug-resistant *S. aureus* strain (3527, known previously as 1095) and an otherwise isogenic first-step mutant with a D83N amino acid substitution in gyrase.[19] Previous studies on NBTIs have demonstrated that the D83N mutation is associated with a loss of gyrase inhibition[17−19] and that whole cell killing of the mutant strain generally improves with more potent inhibition of TopoIV.[18,19,37,39] Whereas MICs for the parent strain were similar to the USA300 strain, the compounds generally lacked whole

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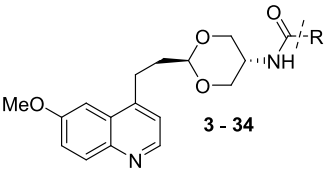
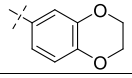
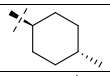
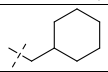
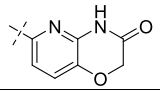
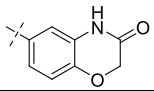
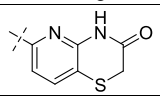
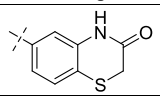
Table 1. Chemical Structures and Assay Data for Amide NBTIs



3 - 34

Cmpd	R group	*S. aur.* 29213 MIC[a] (μg/mL)	*S. aur.* USA300[36] MIC[a] (μg/mL)	*S. aur.* 3527[19,37] MIC[a] (μg/mL)	*S. aur.* D83N[19,37] MIC[a] (μg/mL)	*S. aur.* Gyrase IC_{50}[b] (μM)	*S. aur.* TopoIV IC_{50}[c] (μM)
cipro	Not applicable	0.25-0.5	16-32[25]	>64	64	13.3[25]	4.0[25]
3[25]		1	1	0.5	>32	3.7	>200
4[25]	3,4-di-Cl-Ph	0.125-0.5	0.125-0.5	0.5	>32	0.59	>200
5	4-OMe-Ph	4-8	8-16	16	32	3.9	106
6	4-Cl-Ph	1	1-4	2	>32	0.61	No activity
7	4-Me-Ph	1	1-2	2	>32	0.41	No activity
8	4-CF_3-Ph	0.25-0.5	≤0.5-1	≤0.5-1	>64	1.1	>200
9	4-OCF_3-Ph	0.5-1	1-2	2-32	>32	1.4	No activity
10	4-*t*Bu-Ph	0.25-0.5	1-2	1-2	2-8	1.3[d]	>200
11	4-F-Ph	>256	>256	>256	>256	NT[e]	NT
12	4-CN-Ph	>8	>8	NT	NT	NT	NT
13	3-Cl-Ph	2	1	1	>32	0.32	No activity
14	2-Cl-Ph	>256	>256	>256	>256	NT	NT
15[f]	Ph	8->128	8-128	4-64	>256	NT	NT
16		>256	>256	>256	>256	NT	NT
17		256	128	128	128	NT	NT
18		8	16	8	32	NT	NT
19		4	8	8	>32	NT	NT
20		>64	>64	>64	>64	NT	NT
21		>64	>64	>64	>64	NT	NT
22		0.125-0.25	0.125-0.25	0.125-0.25	>64	0.040[d]	0.19[g]
23		0.25-0.5	0.25-0.5	0.5	>64	0.067	0.89
24		0.03	0.03-0.06	0.03-0.06	>64	0.17[h]	0.30
25		0.06	≤0.125	≤0.125	>64	0.13	0.77
26-34	See supporting information	>64	>64	>64	>64	NT	NT

[a]MICs determined according to CLSI guidelines,[38] minimally $n = 3$. [b]DNA gyrase supercoiling inhibition assay; $n = 2$ unless otherwise noted. [c]TopoIV decatenation inhibition assay; $n = 1$ unless otherwise noted. [d]$N = 3$. [e]Not tested. [f]Solubility issues led to poor reproducibility between replicate assays. [g]$N = 2$. [h]$N = 1$.

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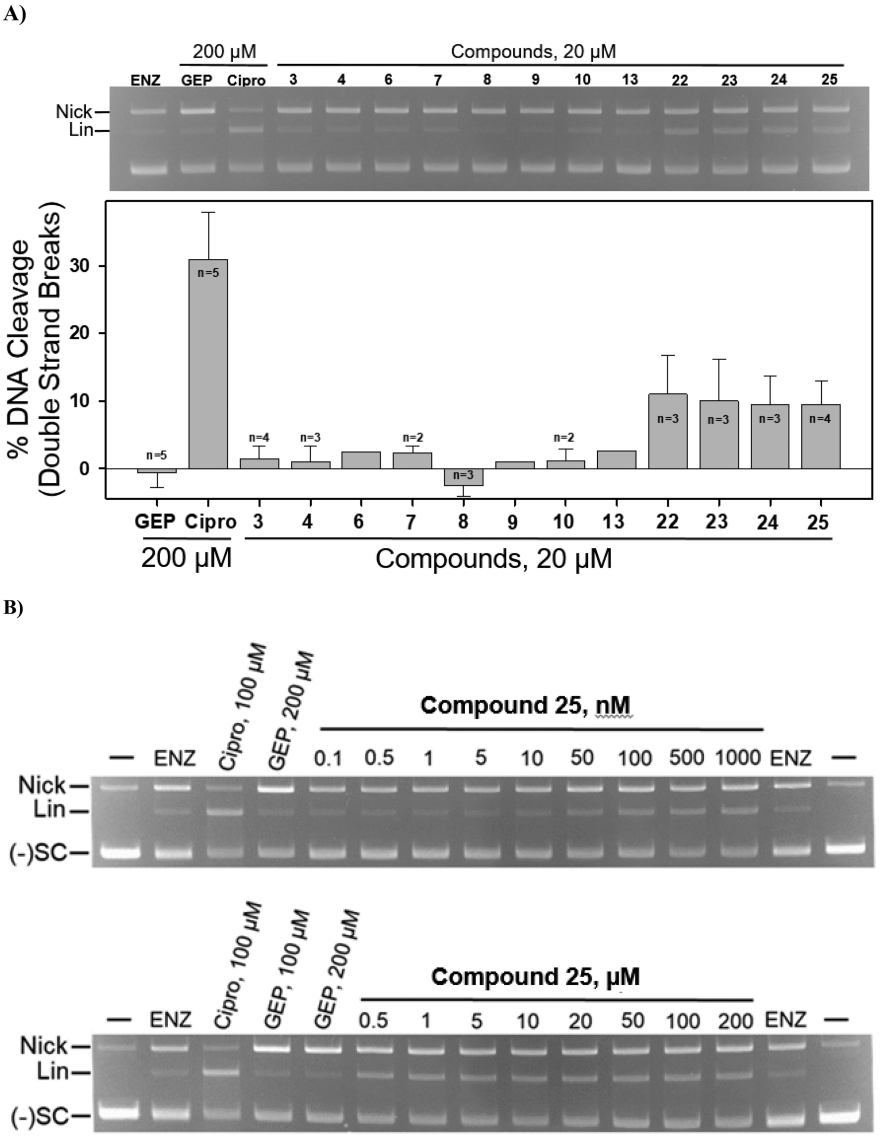

Figure 4. Gyrase-mediated DNA cleavage induced by benzamide and oxazinone/thiazinone NBTIs (**22**−**25**). (A) DNA products after cleavage reactions in the presence of 20 μM benzamide analog NBTIs (**3**, **4**, **6**−**10**, and **13**) and oxazinone/thiazinone (**22**−**25**) NBTIs. Ciprofloxacin and gepotidacin were included as controls at the indicated concentrations. Quantitation of compound induced DSBs was accomplished by first measuring total fluorescence in each lane with corrections for differential emission in negatively supercoiled [(−)SC] compared to linearized (Lin) and nicked (Nick) DNA bands[41] followed by calculation of percent total fluorescence in the linearized bands (DSBs). Percent cleavage in enzyme controls were subtracted to yield final results. The number of replicate observations performed in separate experiments is indicated. Data is presented as the mean ± SD or range. (B) DNA products after cleavage reactions in the presence of **25** (0.1−1000 nM, top gel; 0.5−200 μM, bottom gel). Ciprofloxacin and gepotidacin were included as controls at the indicated concentrations.

cell activity against the mutant D83N strain. While these data are consistent with poor TopoIV inhibition, results from bicyclic derivatives (**22**−**25**, *vide infra*) suggest that the explanation is likely more complex. The highly lipophilic 4-*tert*-butylbenzamide **10** afforded an MIC range of 2−8 μg/mL in the D83N strain despite its lack of TopoIV inhibition *in vitro*, a finding that may indicate an additional mechanism of action.

Given the facile synthesis of amides and the diversity of available carboxylic acids, we sought to expand our understanding of SAR with additional analogues. Our previous work on amine-containing NBTIs afforded cyclohexane- or 4-methylcyclohexane-analogues with limited antibacterial activity (MIC range of 2−8 μg/mL).[25] The amide derivatives were similar. Cyclohexane **18** and *trans*-4-methylcyclohexane **19** were modestly active, with MICs of 4−8 μg/mL against the ATCC strain. Homologation of **18** as in compound **20** resulted in loss of activity, as did the 1-methylcyclohexyl derivative **21** (see Supporting Information, Figure S1 and accompanying discussion for additional analogues **26**−**34**).

Singh and colleagues previously reported amide-type NBTIs employing a pyridooxazinone as an enzyme-binding moiety.[29,30] We synthesized a set of dioxane-linked oxazinone (**22** and **23**) and thiazinone (**24** and **25**) amides. These compounds demonstrated excellent MICs against *S. aureus* ATCC 29213 and USA300 strains as well as potent inhibition of gyrase supercoiling activity. In contrast to compound **3** and the benzamide analogues, these oxazinone/thiazinone-type NBTIs showed submicromolar inhibition of TopoIV decatenation (IC_{50} range from 0.19 to 0.89 μM). Surprisingly though,

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these potent dual-target inhibitors completely lacked activity against the first-step D83N gyrase mutant strain of *S. aureus*. We anticipated a lack of killing via gyrase inhibition due to the presumed loss of the hydrogen-bond acceptor capability of D83. Indeed, compound **22** lost all inhibitory activity using the D83N mutant gyrase (IC_{50} > 200 μM). However, since prior research had demonstrated a general correlation between improved TopoIV inhibition and antibacterial activity against such mutant strains (i.e., killing via inhibition of a second target, TopoIV),[18,19,37,39] the poor MICs against the first-step mutant strain were unexpected.

Several potential explanations can be offered for these surprising findings. We interrogated the possibility of efflux by adding 20 μg/mL of the efflux inhibitor reserpine to the USA300, 3527 (parent), and D83N (mutant daughter) strains (Supporting Information, Table S1).[17] MIC values for **22**−**25** were unaffected for the USA300 and 3527 strains. Compounds **22** and **23** still lacked activity against the D83N strain (MIC > 64 μg/mL), but MICs for **24** and **25** dropped from >64 μg/mL to 2−4 μg/mL in the presence of reserpine. Efflux thus contributes to reduced susceptibility in some cases. Osheroff and colleagues also recently demonstrated that NBTIs can have differential activity with regard to catalytic inhibition versus enhanced DNA cleavage and that the profile is both enzyme- and species-dependent.[35] Also, Khodursky and Cozzarelli, in their *E. coli* studies of norfloxacin, established that bacterial cell death occurred via the formation of toxic DNA cleavage complexes rather than catalytic inhibition of TopoIV.[40] If these amide NBTIs lack TopoIV DNA cleavage stabilization activity, catalytic inhibition of TopoIV decatenation activity may be insufficient to afford antibacterial activity. It is also noteworthy that the D83N mutant *S. aureus* strain also bears an S80F mutation in TopoIV, whereas the TopoIV enzyme used in our biochemical assays has the native S80 residue. The pioneering studies on NXL101 demonstrated ~10-fold loss of decatenation potency for NXL101 against the S80F mutant TopoIV versus the wild-type enzyme.[17] Similarly reduced inhibition of the mutant TopoIV enzyme by our amide NBTIs might thus contribute to the observed results. Detailed mechanistic studies using both wild-type and mutant enzymes will be required to understand these results more completely.

Having observed the striking divergence in TopoIV inhibition between the oxazinone/thiazinone-type NBTIs and the benzamide derivatives, we also conducted DNA cleavage assays using several of both types of amide analogues in the presence of *S. aureus* gyrase. As shown in Figure 4A, the benzamide analogues (**4**, **6**−**10**, and **13**) behaved similarly to compound **3** inducing SSBs (Nick bands) but not DSBs (Lin bands). In contrast, the oxazinone/thiazinone NBTIs (**22**−**25**) used at a single concentration (20 μM) clearly resulted in the accumulation of both SSBs and DSBs. In addition, compound **25** induced both SSBs and DSBs over a wide concentration range (0.1 nM−20 μM, Figures 4B and 5A, 5B).

Importantly, Figures 5A and 5B demonstrate the differential effects of compounds **3** and **25**. Both compounds induce concentration-dependent SSBs with **25** exhibiting at least two logs greater potency (Figure 5A). In addition, Figure 5B indicates the concentration-dependent induction of DSBs by **25** which is relatively unique for NBTIs (with the exception of the action of NXL 101 on *E. coli* TopoIV[17,42]). In contrast, even up to 100 μM, compound **3** does not induce DSBs. Previous reports have described the lack of DSB induction as a

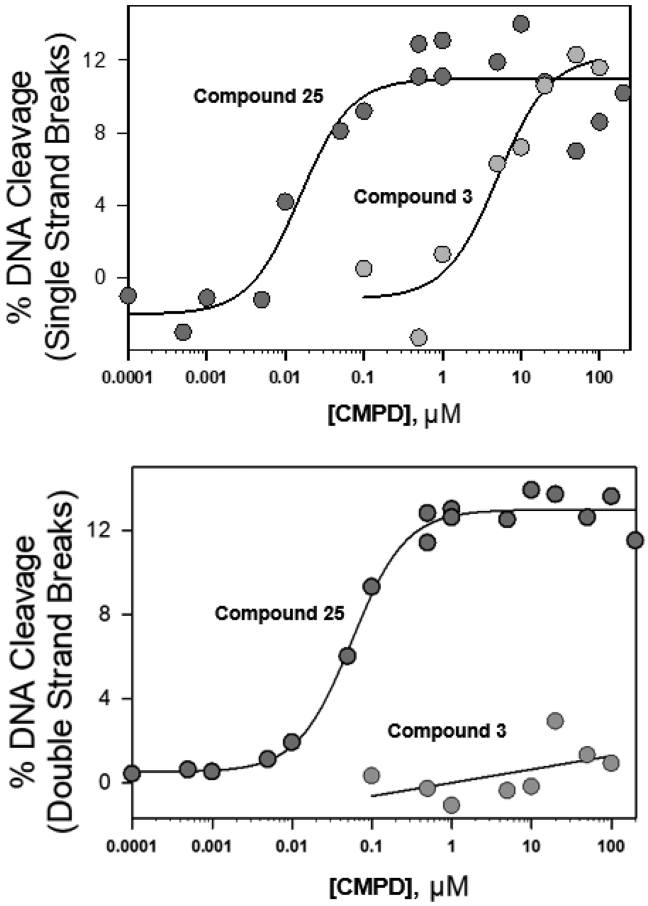

Figure 5. Concentration-dependent induction of Gyrase-mediated DNA single and double strand breaks by compounds **3** and **25**. (A) Percent DNA single strand breaks induced by **3** and **25** were quantified from experiments shown in Figures 3 and 4B, respectively, as described in the Supporting Information and in Figure 4A. (B) Percent DNA double strand breaks induced by **3** and **25** were quantified from experiments shown in Figures 3 and 4B, respectively.

distinctive feature of the pharmacology of the NBTIs.[10,11,16,17,34,35] Thus, the results with compound **25** and related analogues are especially noteworthy, and additional studies are underway to assess this phenomenon more broadly across several series of dioxane-linked NBTIs. It is worth commenting that NBTIs found to induce DSBs (**22**−**25**) have a hydrogen bond donor motif that distinguishes them from the remaining analogues. Computational studies have previously implicated this hydrogen bond donor in binding the pseudosymmetric D83 of the second GyrA subunit.[43]

Initial *in vitro* evaluations of safety and cellular assessments for selectivity in human cells were carried out using potent NBTI amides (Table 2). Paired results from drug-induced growth inhibition in human leukemia K562 cells and in an acquired etoposide-resistant clone, K/VP.5, with reduced levels of hTopoIIα protein, were used as an indirect assessment to determine potential hTopoIIα targeting of the novel NBTIs, as we have reported previously.[23,25]

Select benzamides (**5**−**10**) were assayed for inhibition of human topoisomerase IIα decatenation activity at 100 μM (Inspiralis, Ltd., Norwich, UK); none showed any inhibition (data not shown). The representative thiazinone **25** showed 37% inhibition at this concentration. The oxazinone/thiazinone-type NBTIs (**22**−**25**) showed no meaningful

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Table 2. *In Vitro* Safety Data, Lipophilicity, and Selectivity Data for Representative NBTIs

Cmpd	hERG IC_{50} (μM)[a]	cLogP[b]	K562 IC_{50} (μM)[c]	K/VP.5 IC_{50} (μM)[c]
6	11, 16	4.4	10	11
7	>30	4.0	18	17
8	>30	4.7	6.8	8.9
9	>10	4.8	11	9
10	5.2	5.3	8.4	8.8
13	>3	4.4	<200	50
22	>12	3.0	>200	>200
23	48	2.5	>200	>200
24	>3	3.3	>200	>200
25	>30	2.9	>200	>200

[a]Conducted at Charles River (Cleveland, OH) using previously reported methodology.[25] [b]Determined using Chemdraw Professional version 17.1. [c]Growth inhibition assay using previously reported methodology.[25]

inhibition of the growth of K562 or K/VP.5 cells (IC_{50} > 200 μM, Table 2), in striking contrast to their potent antistaphylococcal activity (Table 1). In contrast, the 4-substituted benzamide-type NBTIs (**6**–**10**) inhibited K562 growth at lower concentrations (IC_{50} range 6.8 to 18 μM). Importantly, however, IC_{50} values for inhibition of the growth of K/VP.5 cells were similar to values in K562 cells, suggesting a lack of hTopoIIα inhibition *in cellulo*.

hERG IC_{50} values were determined for representative analogues in Table 2. hERG inhibition is a key limitation to the development of NBTIs.[44] Although the benzamide derivatives lack a basic center, they are quite lipophilic, as indicated by their cLogP values, raising concern about potent hERG inhibition. However, no clear relationship between lipophilicity and hERG IC_{50} could be established in this small data set, particularly because solubility limitations in the assay frequently precluded the delineation of full concentration–response relationships: values reported as >X μM indicate compound insolubility in the hERG assay above that concentration. Generally speaking, the amides reported here do not exhibit potent hERG inhibition, but a more complete understanding will require the synthesis and evaluation of more soluble analogues, research which is ongoing in our laboratory.

In summary, a series of dioxane-linked NBTIs bearing an amide moiety has been synthesized and profiled. Broad anti-Gram-positive activity was shown for a representative compound (**3**), and gyrase-mediated cleavage assays demonstrated enhancement of single strand breaks to DNA, as previously seen with gepotidacin. Benzamide derivatives bearing lipophilic *para*-substituents showed potent antistaphylococcal activity through selective inhibition of gyrase. Bicyclic analogues with benzo- and pyrido-oxazinone/thiazinone moieties delivered even more potent antistaphlococcal MICs and inhibited both gyrase supercoiling and TopoIV decatenation activity. Surprisingly, potent TopoIV inhibition did not translate into whole cell antibacterial activity in a *S. aureus* strain bearing a gyrase mutation classically associated with NBTI resistance (D83N). The origins of these results require additional detailed study. Furthermore, oxazinone/thiazinone NBTIs induced both DNA single and DNA double strand breaks in the presence of *S. aureus* gyrase. These results have enhanced our understanding of the promising NBTI class of antibacterials and generated intriguing mechanistic questions worthy of additional study.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00428.

Synthesis and characterization of compounds, NMR spectra, structures and results from additional analogues, computational chemistry methods and docking of compounds **4** and **10**, assay details, and MICs in the presence of reserpine (PDF)

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Notes

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■ ABBREVIATIONS

NBTI, novel bacterial topoisomerase inhibitor; MRSA, methicillin-resistant *Staphylococcus aureus*; MDR, multidrug-resistant; VRE, vancomycin-resistant *Enterococcus spp.*; TopoIV, topoisomerase IV; hERG, human ether-related-a-go-go gene; MIC, minimum inhibitory concentration; SSB, single strand break; DSB, double strand break; Nick, nicked open-circular; Lin, linearized; (−)SC, negatively supercoiled; ATCC, American Type Culture Collection.

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