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Triaminotriazine DNA helicase inhibitors with antibacterial activity

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Abstract—Screening of a chemical library in a DNA helicase assay involving the *Pseudomonas aeruginosa* DnaB helicase provided a triaminotriazine inhibitor with good antibacterial activity but associated cytotoxicity toward mammalian cells. Synthesis of analogs provided a few inhibitors that retained antibacterial activity and demonstrated a significant reduction in cytotoxicity. The impact of serum and initial investigations toward a mode of action highlight several features of this class of compounds as antibacterials.

Resistance to antibacterials is a public health issue of increasing concern that is compounded by the inability of the pharmaceutical industry to generate new classes of antibiotics to combat infections.[1] To tackle this problem, researchers have in recent years increased their focus on the identification of new targets and on the exploitation of targets beyond those against which marketed antibiotics have been rendered ineffective by the development of resistance.[2] From this perspective, DNA helicases, enzymes involved in the nucleoside triphosphate dependent unwinding of duplex DNA into single strands,[3] an essential activity for replication,[4] appear to be attractive targets. Whereas replicative helicases require assistance from helicase loaders to assemble at chromosomal origins of replication,[5] some helicases[6] including the replicative helicase DnaB of *Pseudomonas aeruginosa*[7] are able to unwind model oligonucleotide substrates in their absence, providing a convenient in vitro assay to study helicase mechanism and inhibition.

The increased recognition of *P. aeruginosa* as a key factor in nosocomial infections[8] as well as a major pathogen in cystic fibrosis patients[9] is compounded by the very robust nature of this microbe. To discover new antiinfectives against this bacterium, a library of 230,000 commercially available compounds was screened against the DnaB helicase in a functional assay measuring the unwinding of a short DNA partial duplex.[10] Amongst the resulting active compounds, the triaminotriazine **1** (Fig. 1) was identified as a low micromolar inhibitor of the enzyme.

Compound **1** does not display antibacterial activity against Gram-negative organisms (*P. aeruginosa* or *Escherichia coli*), but has moderate activity against Gram-positive organisms (*Staphylococcus aureus*, *Streptococcus pneumoniae*) (Table 1) and even *Candida albicans* (data not shown). This could be a result of the high degree of homology between Gram-positive and Gram-negative helicases (41% identity and 62% similarity between the *P. aeruginosa* and the *S. aureus* helicases). It does however also result in significant cytotoxicity against HeLa cells as determined by an MTS assay (Table 2).[11]

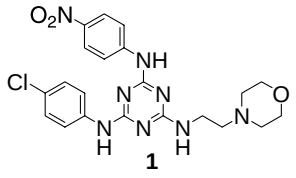

Figure 1.

Keywords: Antibacterial; Cytotoxic; Helicase.
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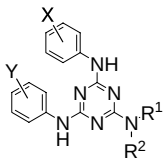
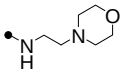
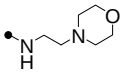
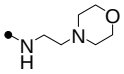
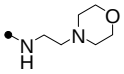
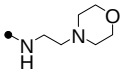
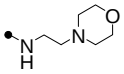
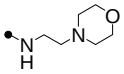
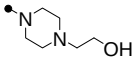
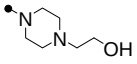
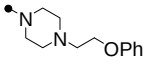
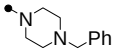
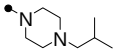
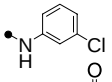
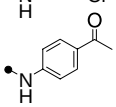
Table 1. Inhibitory and antibacterial activities of triaminotriazines **1**–**15**



Compound	$N^{R_1}_{R_2}$	X-	Y-	IC_{50} (μM)	MIC (μg/mL)		
					S. aureus RN4220	*E. coli* LBB925	*P. aeruginosa* Z61
1		4-NO_2	4-Cl	5	4	>128	>128
2		H-	H-	>100	nd	nd	nd
3		4-Cl	4-Cl	20	4	>128	>128
4		4-Cl	H-	65	64	16	>128
5		4-OMe	4-OMe	>100	nd	nd	nd
6		4-F	4-F	>100	nd	nd	nd
7		3,4-Di F	3,4-Di F	19	16	16	>128
8		4-F	4-F	22	32	16	>128
9		4-F	4-F	23	64	32	>128
10		3,4-Di-F	3,4-Di-F	18	16	16	>128
11		3,4-Di-F	3,4-Di-F	5	>128	>128	>128
12		3,4-Di-F	3,4-Di-F	6	>128	>128	>128
13		3,4-Di-F	3,4-Di-F	5	4	>128	>128
14		3-Cl	3-Cl	31	>128	>128	>128
15		4-Br	4-Br	6	>64	>64	>64

MIC: minimum inhibitory concentration.
nd: not determined.

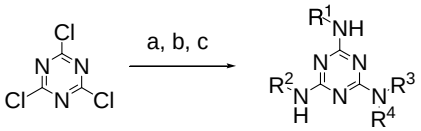
There is no inhibition of mammalian DNA replication in the presence of up to 100 μM of **1**. Given the impact of DNA binders on the activity of helicases,[12] binding to DNA was measured in a dye displacement assay.[13] DNA binding by triaminotriazine **1** was found to be weak relative to distamycin A (minor groove binder) and *m*-AMSA (intercalator) controls, suggesting an alternate mechanism for its inhibitory activity on DnaB.

In an effort to establish whether the scaffold presented in **1** can be used to generate helicase inhibitors with antibacterial activity, but with reduced cytotoxicity, a number of analogs were prepared. This can be done from cyanuryl chloride by the selective stepwise displacement of each of the three chlorine atoms by an amine (Scheme 1).

The inhibitory activities of these compounds toward the *P. aeruginosa* DnaB helicase were measured and compared to their antibacterial activities[14] against a wild-type strain of *S. aureus* (RN4220) as well as against hypersusceptible strains of *E. coli* (LBB925, *tolC*

Table 2. C log *D* (calculated log *P* value adjusted to pH 7.40), antibacterial activities, and cytotoxicities (measured by the MTS method over three concentrations) for selected compounds

Compound	C log *D* (pH 7.40)	MIC (μg/mL) *S. aureus*	Cytotoxicity, μM (% survival)		
			12.5	25	50
1	3.36	4	78	19	10
3	4.46	4	103	90	6
4	3.25	64	98	74	14
7	3.53	16	101	91	47
8	4.66	64	100	64	11
9	2.88	64	101	104	51
10	2.88	16	80	91	47
11	5.58	>128	97	100	99
12	5.65	>128	96	99	97
13	3.95	4	99	99	86
14	6.76	>128	74	83	64



1-15

Scheme 1. Reagents and conditions: (a) R^1NH_2, DIPEA, THF, 60 °C; (b) R^2NH_2, DIPEA, THF, 60 °C; (c) R^3R^4NH, acetone, room temperature, for aliphatic amines, R^3R^4NH DIPEA, THF, 60 °C, for aromatic amines.

deficient) and *P. aeruginosa* (Z61, permeability barrier deficient) (Table 1).

These data establish the correlation between the electron-withdrawing substituents on the aryl rings of **1** and its inhibitory activity. Indeed, compounds **2** (lacking substituents) and **5** (bearing electron-rich substituents) are completely inactive, whereas the replacement of the nitro group in **1** by a chlorine atom (compound **3**) results in a reasonable inhibitory activity. The replacement of the same nitro group with a hydrogen atom (compound **4**) brings about an intermediate activity in a consistent manner. Surprisingly, substitution of each of the aryl groups with a fluorine atom (compound **6**) results in a completely inactive compound, whereas the parent molecule bearing four fluorine atoms (compound **7**) remains capable of inhibition. Substituting the (2-morpholinoethyl)amino group in **6** by a piperazine restores the inhibitory activity (compound **8**). The use of four fluorine atoms and substituted piperazines results in respectable inhibitory activities and the more hydrophobic substituents on the piperazine result in a slightly improved inhibition. In terms of antibacterial activities, the MIC values against *S. aureus* seem to match the inhibitory activity against the helicase with the exception of the more hydrophobic compounds **11** and **12**. The activity against *E. coli* responds positively to the introduction of the fluorine atoms and the piperazine moiety. Yet, no activity is detected against *P. aeruginosa*.

The role of the electron withdrawing substituents in bringing about inhibitory activity is further highlighted in compounds **14** and **15** which lack the aliphatic side chains of **1**–**13**, yet maintain respectable levels of inhibition. They do not display antibacterial activity, a measure of the role of an aliphatic chain with a tertiary amino group for cellular activity.

A comparison of the MICs against *S. aureus*, the cytotoxicities as established through the MTS assay, and the C log *D* (calculated log *P* adjusted for pH)[15] is presented in Table 2. MICs and cytotoxicities mirror each other fairly consistently, with compounds resulting in both antibacterial activity and cytotoxicity, or not displaying either. The exceptions are compounds **7**, **10**, and **13** all of which bear the 3,4-difluorophenyl moieties as the two aryls on the triazine core and whose antibacterial activities are accompanied by a noticeable decrease in cytotoxicity.

It is clear that the four fluorine substituents impart selective activity toward the bacteria. The more hydrophobic compounds **11** and **12** are neither antibacterial nor cytotoxic, a matter that could result from their inability to penetrate the cells efficiently either as a consequence of reduced diffusion across the membrane and/or cell wall, increased efflux or the inability to sustain a sufficient intracellular concentration.[16] This is mirrored by compound **14** both in terms of hydrophobicity and bioactivities. The role of the hydrophobic character of the molecules must not however be overstated, as the comparison between compounds **9** and **10** reveals that two compounds of similar levels of inhibition and hydrophobicities result in different antibacterial activities. This also points to the favorable role of the 3,4-difluorophenyl substituents.

The antibacterial activities of these compounds were further characterized through the ability of compounds **1** and **3** to inhibit macromolecular biosynthesis in *S. aureus* (Fig. 2).[17] The expected inhibition of DNA synthesis, given the role of the DnaB helicase in replication, is accompanied by a decrease in transcription and translation as well. This contrasts with other inhibitors of DNA replication such as DNA gyrase inhibitors like the fluoroquinolone antibiotics, which selectively impact the biosynthesis of DNA. Given the lack of precedence of well-defined helicase inhibitors,[18] the meaning of this unselective behavior is difficult to gauge. The lack of selectivity toward the inhibition of DNA synthesis may suggest an impact on the uptake or retention of the labeled macromolecule precursors, as has been noted for inhibitors of histidine protein kinases that were found to act via membrane disruption.[19] Alternatively, since the shallow slope of the concentration-dependent decrease in synthesis of DNA, RNA, and protein by compounds **1** and **3** contrasts somewhat from the sigmoidal curves generally associated with well-defined inhibitors, antibacterial activity could be the result of the conjunction of weak inhibitory activities over more than one cellular target. Such a phenomenon has been reported to complicate the predictions of mechanisms of action of novel antibacterial agents by gene expression profiling.[20]

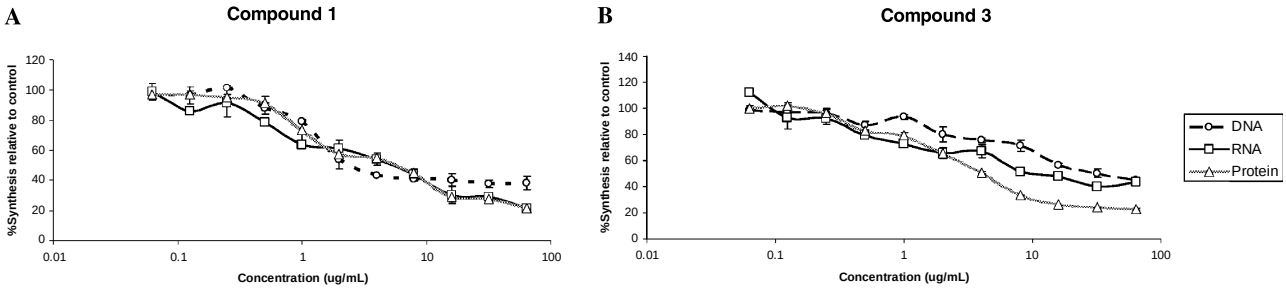


Figure 2. Inhibition of macromolecular biosynthesis by compounds (A) **1** and (B) **3**.

The promiscuity of these inhibitors is further supported by the impact of serum on the MIC against *S. aureus* which shifts to >128 μg/mL in the presence of 50% human or mouse serum in the growth medium.[21,22] A similar shift is obtained with 4% human serum albumin (HSA). A gradual increase in albumin concentration results in a gradual linear shift in MIC, the slope of which points to a stoichiometry of 0.47:1 for the binding of **1** to HSA and 0.23:1 for that of compound **3** and HSA.[21] Although these low stoichiometries of serum protein binding by compounds **1** and **3** may leave some room for attempts to retain target-specific activity while reducing serum binding, the steeper slope of the MIC:serum protein curve when using whole serum in place of HSA is evidence that these compounds bind several components of serum. This renders the possibility of avoiding serum shift by design very small.

Because of this somewhat unselective character, it is not surprising that when evaluated for potential as topical antibacterial agents in vivo in the mouse wound model of *S. aureus* infection,[23] compounds **1** and **3** (0.2 mL of compound formulated as 2% by weight in PEG400, applied over the wound three times per day) were found to be completely inactive (data not shown).

The present study has allowed the identification of 1,3,5-triaminotriazines as inhibitors of the replicative helicase DnaB from *P. aeruginosa*. The compounds lack antibacterial activity against this organism, but are active against *S. aureus* and sporadically against a *tolC* mutant of *E. coli*. Additional attributes of a lack of detectable inhibition of mammalian DNA replication and little affinity for DNA in vitro must be countered with the demonstrable HeLa cell cytotoxicity of the original hit. The development of brief structure–activity relationships around the initial structure has allowed the discovery of compounds with comparable antibacterial activity but with a highly reduced cytotoxicity. Further investigations into the activity associated with this class however show an unselective mode of action. This is additionally supported by the detectable binding to a number of serum components. While this sort of behavior leaves little room to develop these compounds into useable therapeutics, it serves to highlight the necessity of stringent assessment of specificity and selectivity as early as possible in the characterization of inhibitors of in vitro enzyme assays.

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