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N-Hydroxybenzimidazole inhibitors of ExsA MAR transcription factor in *Pseudomonas aeruginosa*: In vitro anti-virulence activity and metabolic stability

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ABSTRACT

ExsA is a multiple adaptational response (MAR) transcription factor, regulating the expression of a virulence determinant, the type III secretion system (T3SS) in *Pseudomonas aeruginosa*. Non-cytotoxic, non-antibacterial *N*-hydroxybenzimidazoles were identified as effective inhibitors of ExsA-DNA binding, and their potential utility as anti-virulence agents for *P. aeruginosa* was demonstrated in a whole cell assay. Select *N*-hydroxybenzimidazole inhibitors were stable in an in vitro human liver microsomal assay.
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Life-threatening infections caused by multi-drug resistant (MDR) pathogens continue to present serious public health issues worldwide. About half of MDR bacteria are Gram-negative, including *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter* spp. *P. aeruginosa*, in particular, are associated with hospital-acquired pneumonia (HAP) as well as the chronic lung infections in cystic fibrosis patients.[1,2] Ventilator-associated pneumonia (VAP) especially poses great challenges in antimicrobial chemotherapy, because of the high frequency of MDR strains including *P. aeruginosa*.

Currently marketed antibiotics are becoming increasingly inactive against MDR *P. aeruginosa* mainly because of resistance.[3,4] The next generation of antibiotic derivatives (e.g., carbapenems and cephalosporins), demonstrating good activities against clinical isolates of resistant *P. aeruginosa,* are in the late stage of clinical development.[5] However, given the same mechanism of action and the similar chemical structures, these newer antibiotics would also be subject to already existing resistance mechanisms. This situation has fostered interest in pursuing novel approaches as alternatives to traditional antimicrobial chemotherapy.

Previously we reported MAR (multiple adaptational response) technology in which we developed small molecule inhibitors for bacterial transcription factors SoxS, MarA and Rob in *E. coli*, and LcrF in *Yersinia pseudotuberculosis*.[6,7] MAR proteins, a subgroup of AraC family proteins, regulate the virulence gene expression in many clinically important bacterial species. This novel strategy of targeting bacterial virulence, as compared to traditional antibiotics targeting bacterial growth or survival, may offer an opportunity to circumvent resistance development issues. Given that MAR proteins are not required for bacterial survival outside of a host, inhibitors of MAR proteins are less likely to apply the selection pressure for resistance development.

ExsA is a MAR transcription factor in *P. aeruginosa* which regulates the expression of a virulence determinant, the type III secretion system (T3SS).[8] The *exsA* null mutants do not express the T3SS and exhibit substantially attenuated virulence in whole cell assays and animal models of *P. aeruginosa* infection.[9,10] Furthermore, many clinically important *P. aeruginosa* strains express the T3SS, which is correlated with the increased severity of clinical pneumonia cases such as HAP/VAP.[11,12] Therefore, developing anti-virulence agents by targeting ExsA would be a viable approach to prevent infections caused by *P. aeruginosa*, especially among patients in a high-risk environment such as receiving mechanical ventilation.

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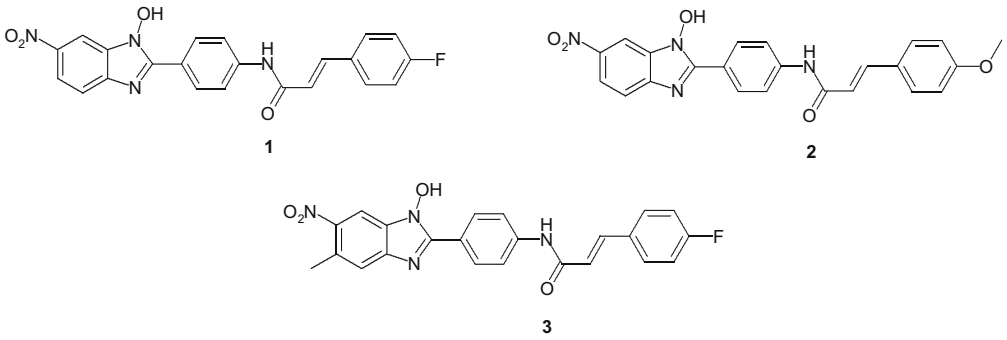


Figure 1. The initial *N*-hydroxybenzimidazole inhibitors for ExsA-DNA binding.

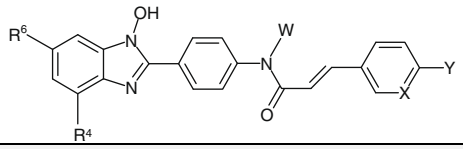
We have identified, and developed small molecule inhibitors of LcrF, a MAR protein of *Yersinia* spp., namely *N*-hydroxybenzimidazoles as exemplified in Figure 1.[7] Given the high degree of homology between LcrF and ExsA in their DNA binding domain (85% identity and 92% similarity), the inhibitors of LcrF (**1, 2** and **3**) were considered potential inhibitors of ExsA. In fact, ExsA IC_{50} values of compounds **1**–**3** were very similar to those of LcrF.[7] However, surprisingly, compounds **1**, **2** and **3** were inactive in the T3SS dependent *P. aeruginosa* macrophage whole cell cytotoxicity assay, whereas they were highly active in the corresponding *Y. pseudotuberculosis* based whole cell assay.[7] For this reason, we further expanded our investigation in the acrylic amide series of *N*-hydroxybenzimidazoles (Table 1) in order to identify effective inhibitors of ExsA in the whole cell assay.

Compounds listed in Table 1 were synthesized by following previously described synthetic routes with modifications (Schemes 1 and 2). For compounds **12**–**22**, acid chlorides **5** were prepared by the reaction of oxalyl chloride with the corresponding acid derivatives that were obtained from a palladium(II)-catalyzed Heck-type olefination of aryl halides **4** with an acrylic acid (Scheme 1). In Scheme 1, the substitutions at the amide nitrogen were introduced to intermediate **7** which underwent subsequent reactions to provide compounds **23**–**25**. For R^4 substituted derivatives, 2-chlorophenylacetic acid **9** was nitrated to provide intermediate **10** which was then subjected to the established reaction conditions to produce compounds **26** and **27** (Scheme 2).

The inhibitory activity of *N*-hydroxybenzimidazole derivatives in Table 1 was measured using an in vitro cell-free ExsA-DNA binding assay, and is reported as IC_{50} values. Compounds **12**–**19**, possessing Y = $COCH_3$, SCH_3, and CF_3 (X = C or N; R^6 = NO_2 or CN), exhibited reasonably good inhibitory activities in the range of 3.0–13.0 μM. Compounds possessing basic amino groups, **20** and **21**, showed weak to no-measurable inhibitory activity. An attempt to introduce a $COCF_3$ group at Y resulted in compound **22**, possessing a $C(OH)_2CF_3$ group under the reaction conditions in Scheme 1, which proved to be devoid of any measurable activity. For the substitution effect of W at the amide nitrogen, a carbamoyl methyl group in **23** and **24**, or an acetic acid group in **25** was unfavorable for activity. On the other hand, substitution of an acetic acid group at R^4 resulted in two active compounds **26** and **27** (IC_{50} = 3.7 and 4.6 μM, respectively).

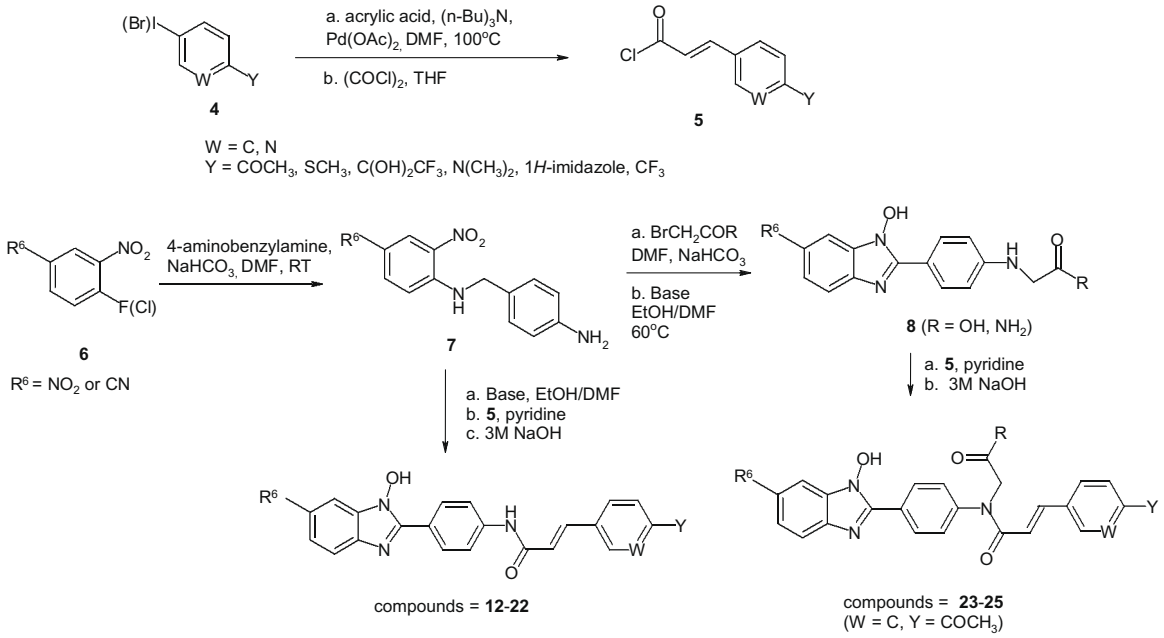
Compounds exhibiting IC_{50} values of less than 20 μM (**12**–**19**, **26** and **27**) in an ExsA-DNA binding assay were further tested in a whole cell assay (Table 1). As control experiments, the inhibitors were tested for intrinsic cell cytotoxicity toward mammalian cells as well as antibacterial activity, and they were non-cytotoxic and

Table 1
In vitro activities of *N*-hydroxybenzimidazole inhibitors: cell-free ExsA-DNA binding assay and whole cell assay



Compd	R^6	R^4	W	X	Y	ExsA[c] IC_{50} (μM)	% WT cytotoxicity[d] (at 100 μM)
12	NO_2	H	H	C	$COCH_3$	3.0[a]	22.4 ± 3.6
13	CN	H	H	C	$COCH_3$	13.0[a]	19.2 ± 3.1
14	NO_2	H	H	C	SCH_3	10.4[b]	>100
15	CN	H	H	C	SCH_3	7.3[b]	>100
16	NO_2	H	H	N	$COCH_3$	7.5[a]	12.1 ± 9.5
17	NO_2	H	H	N	SCH_3	6.9[b]	52.7 ± 6.3
18	CN	H	H	N	SCH_3	3.5[b]	46.6 ± 7.3
19	NO_2	H	H	N	CF_3	6.7[b]	78.5 ± 5.0
20	NO_2	H	H	N	1*H*-Imidazole	31.2[b]	—
21	NO_2	H	H	N	$N(CH_3)_2$	>100[b]	—
22	NO_2	H	H	C	$C(OH)_2CF_3$	>100[b]	—
23	NO_2	H	CH_2CONH_2	C	$COCH_3$	>47.5[b]	—
24	CN	H	CH_2CONH_2	C	$COCH_3$	>49.0[b]	—
25	CN	H	CH_2COOH	C	$COCH_3$	>51.0[b]	—
26	NO_2	CH_2COOH	H	C	$COCH_3$	3.7[b]	65.4 ± 7.5
27	NO_2	CH_2COOH	H	N	$COCH_3$	4.6[b]	33.1 ± 6.7

[a] An ExsA protein of 298 amino acids from *P. aeruginosa* strain 388.
[b] An ExsA protein of 278 amino acids from *P. aeruginosa* strain PAO1.
[c] Most IC_{50} data are mean or average values of at least two independent experiments (see supplementary data for individual values).
[d] Data are mean values ± standard deviation from at least four replicates.



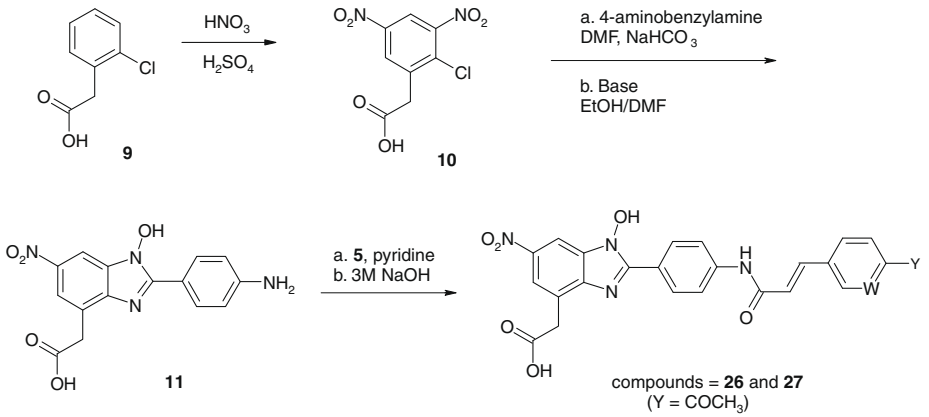
Scheme 1. Preparation of substituted *N*-hydroxybenzimidazoles.

non-antibacterial, respectively (data not shown). In the whole cell assay, *P. aeruginosa* strains cause lysis of infected macrophages through the ExsA-regulated delivery of type 3 secreted toxins (e.g., ExoU, ExoT, and ExoY).[8,9] Therefore, effective inhibitors of ExsA are expected to protect macrophage cells from the cytotoxicity induced by wild-type (WT) *P. aeruginosa*. In Table 1, the in vitro whole cell activities of inhibitors are expressed as % cytotoxicity relative to that of WT *P. aeruginosa* (100% cytotoxicity).[13] While the Δ*exsA* null mutant typically induced almost no cytotoxicity to macrophage cells, compounds **12**, **13**, and **16** effectively attenuated the cytotoxicity of WT *P. aeruginosa*. Compound **16**, in particular, inhibited cytotoxicity (12.1% of WT cytotoxicity) nearly reaching the level of Δ*exsA* null mutant (<10% of WT cytotoxicity).

The following structure–activity relationship (SAR) emerged from the whole cell assay: (i) Compounds with a nitrogen atom at X exhibited better whole cell activity than the corresponding carbon analogs (e.g., **16**, **17**, **18**, and **27** vs **12**, **14**, **15**, and **26**). (ii) Both $-NO_2$ group and –CN group at R^6 demonstrated similar whole cell activity (e.g., **12** vs **13**; **14** vs **15**; and **17** vs **18**). (iii) Acetic acid substitution of compounds at R^4 decreased the whole cell activity of their corresponding hydrogen analogs (e.g., **26** vs **12**; and **27** vs **16**).

Before evaluating in vivo efficacy of lead compounds, we were interested in investigating potential metabolic liabilities of pharmacophores in *N*-hydroxybenzimidazole inhibitors. For preliminary in vitro phase I metabolism, select compounds **1**, **12**, and **13** were incubated with pooled human liver microsomes. The microsomal stabilities of test compounds were determined quantitatively by monitoring the amount of parent compounds remaining in the microsomal supernatant at the end of each timed interval using LC/MS/MS analytical methods. Testosterone, a known substrate for in vitro metabolism, was tested as a positive control compound. In a separate control experiment, the test compounds were found to be chemically stable in the absence of microsomes (data not shown). In the in vitro microsomal assay, while testosterone was rapidly degraded as expected, compound **1** remained unchanged throughout the incubation period. Compounds **12** and **13** appeared to undergo enzymatic degradation at the beginning of incubation; however, they did not show significant changes after the first interval (0–10 min) (Fig. 2). This result implies that the $-NO_2$ group at R^6 as well as the acrylic amide linker may not be substrates for phase I metabolism in vitro.

In summary, we identified new *N*-hydroxybenzimidazole derivatives as inhibitors of the MAR transcription factor ExsA in *P. aeru-*



Scheme 2. Preparation of R^4 substituted *N*-hydroxybenzimidazoles.

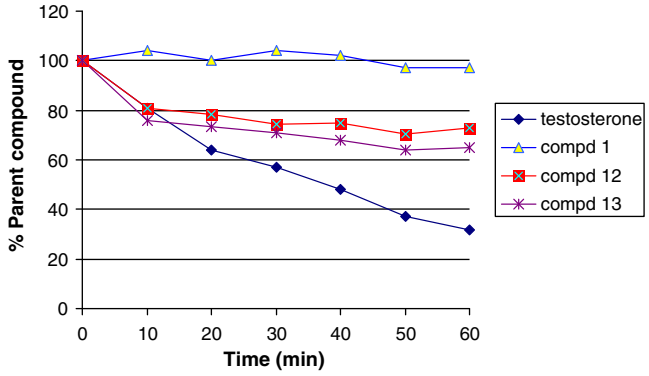


Figure 2. In vitro microsomal assay of select *N*-hydroxybenzimidazole inhibitors. All data points are average values of two independent experiments with less than 10% deviations.

ginosa from a cell-free ExsA-DNA binding assay. Among those, compounds **12**, **13**, and **16** effectively attenuated the cytotoxicity of *P. aeruginosa* in the T3SS dependent whole cell assay. Select compounds demonstrated good metabolic stability from in vitro human liver microsomal assay. This work suggests that these non-cytotoxic and non-antibacterial *N*-hydroxybenzimidazoles may be further developed as anti-virulence agents to prevent infections caused by *Pseudomonas* spp.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.014.

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13. In a control experiment, select *N*-hydroxybenzimidazoles (structures not shown) inactive in this whole cell assay were tested against *P. aeruginosa* strain K1119 (an efflux pump mutant Δ*mexAB-oprM*), or K767 strain (with efflux pump MexAB-OprM). Test compounds were also inactive against both the K1119 and K767 strains, indicating that lack of activity observed in the whole cell cytotoxicity assay was not likely to be caused by the constitutive efflux pump MexAB-OprM in *P. aeruginosa* (data not shown).