210 Characterization of a Stereo-Specific Class of Inhibitors of Type III Secretion

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ABSTRACT

The type III secretion system (T3SS) is a clinically important virulence mechanism in *Pseudomonas aeruginosa* (*Paer*) that secretes and translocates up to four protein toxin effectors into human cells, facilitating the establishment and dissemination of infections. We describe a new phenoxyacetamide class of inhibitors of *Paer* T3SS with the ability to rapidly and selectively block T3SS-mediated secretion and translocation of effectors.

Preliminary studies of structure-activity relationships in this phenoxyacetamide series demonstrated a strict requirement for the *R*-enantiomer at the single stereocenter, suggesting that this series targets a specific component required for type III secretion. Three of the most potent members of the class were shown to block T3SS-mediated translocation, as demonstrated by rescue of cultured CHO cells from intoxication by *Paer* producing the effector ExoU. These phenoxyacetamides also inhibited T3SS-mediated effects in two additional species of bacteria, (a) blocking secretion of an effector-β-lactamase (YopE-βLA) fusion protein from an attenuated strain of *Yersinia pestis*, and (b) arresting the growth of *Chlamydia trachomatis* L2 in Hep-2 cells in culture.

Kinetic studies revealed that the phenoxyacetamide inhibitor MBX 1641 at 50 μM arrested the T3SS-mediated secretion of an ExoS'-βLA fusion protein from *P. aeruginosa* cells by 50% within 15 min. and 100% within 45 min. of addition to the culture. Such rapid kinetics rule out effects on gene expression as the primary mechanism and support the hypothesis that these compounds inhibit T3SS directly.

BACKGROUND

T3SS secretes protein cytotoxins and translocates them into host eukaryotic cells[1]

Four T3SS "effectors" have been identified in *P. aeruginosa* strains[2]

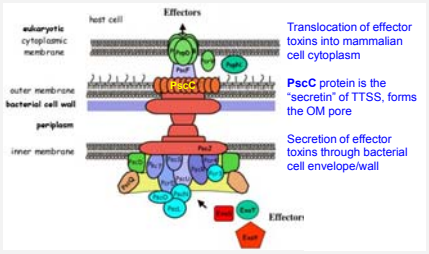
- ExoS and ExoT are bifunctional, consisting of an N-terminal small G-protein activating protein (GAP) domain + a C-terminal ADP ribosylation domain
- ExoY is an adenylate cyclase
- ExoU is a phospholipase

These cytotoxins are not essential for *P. aeruginosa* viability but play major roles in virulence[2]

The presence of a functional T3SS is significantly associated with poor clinical outcomes and death in patients[3,4]

We have developed and applied cellular reporter screens to identify and characterize inhibitors of *P. aeruginosa* T3SS.

Schematic of the type III secretion system



Translocation of effector toxins into mammalian cell cytoplasm

PscC protein is the "secretin" of TTSS, forms the OM pore

Secretion of effector toxins through bacterial cell envelope/wall

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2. Hauser, A.R., 2009. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat.Rev.Microbiol. 7:654-65.
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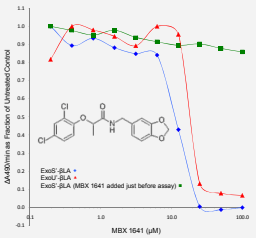
RESULTS

Concentration-Dependent Inhibition of T3SS-Mediated Secretion of Effector-β-Lactamase Fusion Proteins



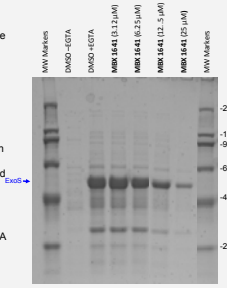
Results. MBX 1641 inhibits secretion of both effector ExoS- and ExoU-βLA fusion protein reporters with similar potency (IC_{50} ~15 μM), but does not inhibit βLA activity when added after secretion has occurred.

Method. Concentration-dependence of inhibition of T3S-mediated secretion of effector toxin reporter fusion proteins ExoS'(234aa)-βLA and ExoU'(354aa)-βLA from *P. aeruginosa*. The rate of hydrolysis of nitrocefin by culture supernatants (ΔA_{490} per min slopes) are plotted vs. the concentration of inhibitor MBX 1641 added during EGTA-induction of T3S or added directly prior to assay, as noted in the legend.

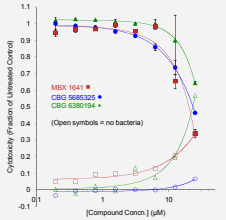
Concentration-Dependent Inhibition of T3SS-Mediated Secretion of Native ExoS

Results. MBX 1641 inhibits the appearance of native ExoS in *P. aeruginosa* culture supernatants in a concentration-dependent manner, with a half-maximal inhibition value similar to that observed in the effector-βLA assays above

Method. Evaluation of inhibition of type III secretion of native ExoS from *P. aeruginosa*. *P. aeruginosa* ExoS-secreting strain PAKΔTY was grown under T3SS inducing conditions (LB+5 mM EGTA) for 3h in the presence of the indicated concentrations of compounds, culture medium (1 ml) was concentrated in SDS-PAGE sample buffer, separated by 12.5% SDS-PAGE, and stained with Coomassie Blue. The positive control, DMSO+EGTA was treated with 5 mM EGTA but not inhibitors, and the negative control, DMSO-EGTA, was treated with neither EGTA nor inhibitors. The band corresponding to ExoS is marked.



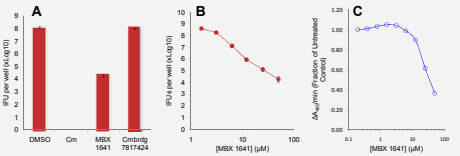
Concentration-Dependent Inhibition of T3SS-Mediated Translocation of ExoU



Results. MBX 1641 and analogs 5685325 and 6380194 (Chembridge, Inc., see SAR Table) not only block T3SS-mediated secretion of effectors from *P. aeruginosa* into culture medium, but also block translocation of effectors into mammalian cells. Compounds in the absence of *P. aeruginosa* display various degrees of inherent cytotoxicity as shown.

Method. Concentration-dependent rescue of CHO cells from ExoU cytotoxicity by T3SS inhibitors. Inhibitors were added to CHO cells at 80-90% confluency in 1% FCS, followed by infection with mid-log-phase *P. aeruginosa* strain PAKΔSTYexoU at MOI ≈5. After incubation at 37°C in 5 % CO_2 for 2 h, culture supernatants were collected, and LDH released by dead cells was measured. Cytotoxicity is calculated as the fraction of LDH released from cells intoxicated with *P. aeruginosa* +/- inhibitor as compared to LDH released from intoxicated cells that were not treated with inhibitor.

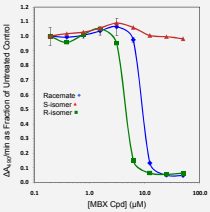
Species Spectrum of Activity: MBX 1641 Inhibits T3SS of *Yersinia pestis* and *Chlamydia trachomatis*

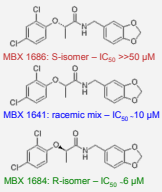


Results. MBX 1641 inhibits, in a concentration-dependent manner, both the growth of *C. trachomatis* L2 cells in Hep-2 cells in culture and T3SS-mediated secretion of YopE-βLA from *Y. pestis*.

Methods. **A**. Confluent monolayer Hep-2 cells were infected with L2 at an MOI of 0.5 and treated with compounds at indicated concentrations, followed by sonication and measurement of IFUs on HeLa monolayers. Experiments were done in triplicate, and averages +/-standard deviation are shown. Chloramphenicol (Cm) was used as a control. **B**. Concentration-dependence of the inhibition of *C. trachomatis* L2 growth in Hep-2 cells by MBX 1641. **C**. Concentration-dependence of inhibition of T3SS-mediated secretion of YopE-βLA fusion protein from attentuated *Y. pestis* by MBX 1641.

SAR: Dependence of T3SS Inhibition by MBX 1641 on Stereochemistry

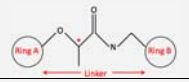




Results. Only the R-isomer is active, and it is almost twice as potent as the racemic mixture.

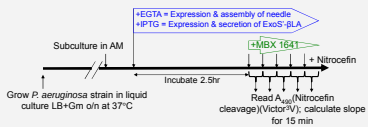
Method. Detection of ExoS'-βLA in culture supernatants by the rate of nitrocefin hydrolysis (ΔA_{490} per min)

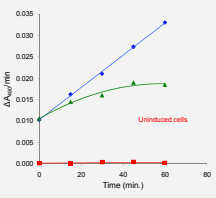
Preliminary SAR of TTSS Inhibitor MBX 1641



Vendor ID	IC_{50} (μM)(ExoS' -βLA assay)	Stereo-center(*)	Ring A	Linker Modification	Ring B
MBX 1641	**10**	**racemic**	**2,4-dichlorophenyl**	**None**	**1,3-methylenedioxyphenyl**
MBX 1684	6	R-isomer	2,4-dichlorophenyl	None	1,3-methylenedioxyphenyl
MBX 1686	>100	S-isomer	2,4-dichlorophenyl	None	1,3-methylenedioxyphenyl
MBX 1668	>100	none	2,4-dichlorophenyl	des-methyl	1,3-methylenedioxyphenyl
MBX 1685	>100	none	2,4-dichlorophenyl	Dimethyl	1,3-methylenedioxyphenyl
6109233	5	racemic	2,4-dichlorophenyl	None	4-methylphenyl
6380194	**9**	**racemic**	**2,4-dichlorophenyl**	**None**	**4-fluorophenyl**
6375680	10	racemic	2,4-dichlorophenyl	None	4-methoxyphenyl
6374948	12	racemic	2,4-dichlorophenyl	None	2-methoxyphenyl
6468028	21	racemic	2,4-dichlorophenyl	*N*-methyl	phenyl
5685325	**25**	**racemic**	**2,4-dichlorophenyl**	**None**	**furan-2-yl**
6374984	45	racemic	2,4-dichlorophenyl	None	pyridine-2-yl
6372013	59	racemic	2,4-dichlorophenyl	None	pyridine-4-yl
8804126	61	racemic	2,4-dichlorophenyl	constrained	1,3-dimethylpyrazol-4-yl 1,2,3,4-
7229146	100	racemic	2,4-dichlorophenyl	tert-amine	tetrahydroisoquinoline
6467504	>100	racemic	2,4-dichlorophenyl	$+CH_2$	2-cyclohexen-1-ylmethyl
7271715	>100	racemic	2,4-dichlorophenyl	None	3,4-dichlorophenyl
7314595	>100	racemic	2,4-dichlorophenyl	$+CH_2$	2-chlorophenyl
9153915	23	racemic	2-chlorophenyl	None	1,3-methylenedioxyphenyl
6116488	98	racemic	2-methyl-4-chlorophenyl	None	1,3-methylenedioxyphenyl
7339628	>100	racemic	2-fluorophenyl	None	1,3-methylenedioxyphenyl
7303859	>100	racemic	3-chlorophenyl	None	1,3-methylenedioxyphenyl

Mechanism: MBX 1641 Acts Rapidly to Inhibit ExoS'-βLA Secretion





Results. MBX 1641 does not inhibit βLA activity (see "0" timepoint), but inhibits secretion of additional ExoS'-βLA ~50% within15 min & completely by 45 min

Method. *P. aeruginosa* cells carrying the ExoS'-βLA fusion protein were grown under T3SS inducing conditions (+). As a control, a separate culture of the same cells was grown without EGTA induction of T3SS (■). After 2.5 h, MBX 1641 was added at 50 μM to one portion of the T3SS induced cells (+). Simultaneously, nitrocefin was added to all three cultures, and the A_{490} was recorded. The slope of A_{490}/min is proportional to the amount of ExoS'-βLA secreted into and accumulating in the culture medium.

ACKNOWLEDGEMENTS

We thank Dr. Herbert Schweizer (Colorado State U.) for advice and for plasmid pUCP24, Dr. Jon Goguen (U. Massachusetts Medical School) for advice and for the *Y. pestis* reporter strain.
This research was funded by an SBIR grant from the NIAID (AI068185)