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A new series of cinnamoyl analogs compound unveil both efflux pump inhibition and antibacterial activity

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Abstract:

Multidrug antibiotic resistance is a global public health crisis that leads to thousands of people's deaths every year. One of the primary causes of resistance in Gram-negative bacteria such as *Escherichia coli* is the overexpression of multicomponent molecular machines called multidrug efflux pumps. Efflux pump inhibitors (EPIs)are a promising alternative approach to combat antibiotic resistance. We previously identified a diaminoquinoline acrylamide, NSC-33353, as an active EPI of *E. coli* efflux pump.

This report will describe a series of cinnamoyl compounds that are analogs of NSC-33353 showing significant activity against the AcrAB-TolC efflux pump of *E. coli*. To determine the antibacterial properties and propensity of these analogs to act as efflux substrates, we analyzed bacterial growth inhibitory activities of the compounds using efflux proficient and efflux deficient *E.coli* cells. Surprisingly, the results show that a large number of the analogs possess an antibacterial activity, although their original hit was only a weak antibacterial agent. In the presence of antibiotics novobiocin and erythromycin compounds have a significant potentiation activity. The surface plasmon resonance data show that compounds bind with high affinities both AcrA, a membrane fusion protein, and AcrB, an efflux transporter. The fluorescence-based accumulation assay showed that these compounds inhibit the efflux of fluorescent probes. Taken together, these results show that this series of compounds are promising EPIs.

In summary, we have identified compounds that bind to AcrA, AcrB and potentiate the antibacterial properties of novobiocin and erythromycin in *E. coli.* We report a new series of EPIs that inhibit the activity of AcrAB-TolC efflux pump.

Background:

Multidrug antibiotic resistance is a global public health crisis that is leading to the deaths of thousands of people every year. One of the primary causes of resistance in Gram-negative bacteria such as *Escherichia coli* is the overexpression of multicomponent molecular machines referred to as efflux pumps.

In *E. coli,* the efflux pump is a tripartite assembly called AcrAB-TolC

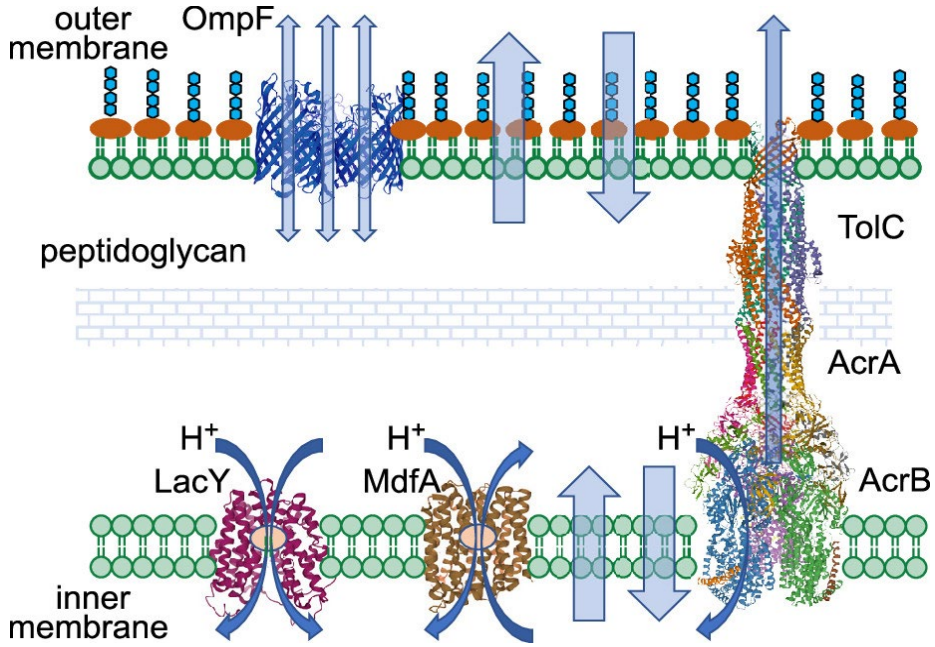


Figure 1: Organization of the cell envelope in Gram-negative bacteria on the example of *E. coli*. Key components of the cell envelope that define penetration of small molecules into bacteria include the inner and outer membranes, porins (such as OmpF), efflux transporters that act across the outer membrane (such as AcrAB-TolC), and substrate specific importers and exporters such as LacY and MdfA, respectively

Rybenkov, V. V., et al. (2021), Chemical Reviews

Aim of this research:

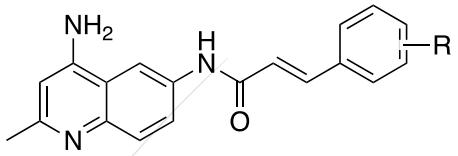
- **In this work, we aim to identify new inhibitors that can permeate the outer membrane of *E. coli*, inhibit efflux, and interact with AcrA, a membrane fusion protein, and AcrB, an efflux transporter of AcrAB-TolC efflux pump**

Approach (Methods) :

- Wild-type (WT) and ΔtolC strains of *E. coli* and their hyperporinated ("-Pore") variants used in this study are derivatives of BW 25113 and GD102, respectively.
- The *E. coli* BL21(DE3) strain was used for overexpression and purification of AcrA and AG100AX strain was used for overexpression and purification of AcrB .
- MICs were analyzed using a two-fold broth dilution method. For the checkerboard assay, an antibiotic and a test compound were serially diluted into 96-well plates as described previously.
- SPR experiments were carried out with purified AcrA and AcrB immobilized onto a CM5 chip (Biacore).
- The NR uptake assay was performed in a temperature-controlled micro-plate reader (Tecan Spark 10M) equipped with a sample injector, in fluorescence mode.

Results 1: Antibacterial and potentiation activities of the cinnamoyl compounds

Table 1: Cinnamoyl derived analogs



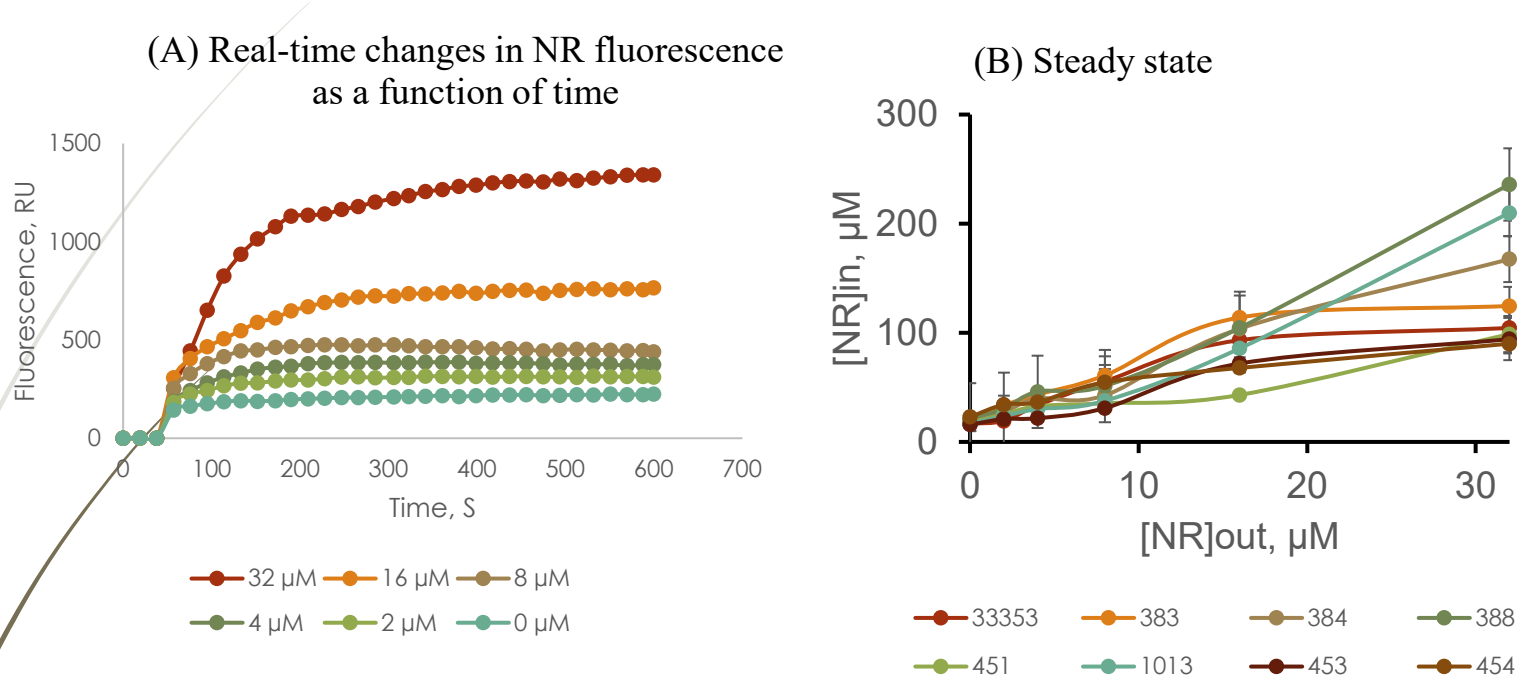
		MIC in efflux proficient strains		MIC in efflux deficient strains		Potentiation(MPC) in Wt-pore strain		MIC fold difference in (+/-) efflux
#	R	MCS-Wt (μM)[a]	WT-pore (μM)[a]	ΔTolC (μM)[a]	ΔTolC-pore (μM)[a]	MPC_4(N) (μM)[b]	MPC_4(E) (μM)[c]	WT-pore/ ΔTolC-pore
NSC-33353	**2-Cl**	**200**	**100**	**12.5**	**12.5**	**1.56**	**3.1**	**8**
383	2-Br	25	25	12.5	12.5	12.5	3.1	2
384	3-Cl	25	25	12.5	6.25	6.25	6.25	4
388	4-Cl	25	12.5	12.5	6.25	6.25	6.25	2
420	2-NO_2	>400	>400	>400	>400	>200	>200	1
451	4-iPr	50	25	25	25	12.5	12.5	1
1013	4-Br	>200	>200	25	12.5	200	200	>16
453	2-Me	50	25	25	50	6.25	12.5	0.5
454	2-F	100	100	50	50	12.5	12.5	2
994	2,3-diCl	>200	400	12.5	6.25	400	200	64

[a] MIC values, determined as the average of two replicates; [b] potentiation concentration for Novobiocin(N) measured in WT-pore cells; [c] potentiation concentration for Erythromycin(E) measured in WT-pore cells.

- Several cinnamoyl analogs (**383,384,388,453**) exhibited antibacterial activity in WT cells.
- Based on the WT-pore and ΔTolC data it appears this class of compounds permeated the OM, were only modestly impacted by efflux, and in general potentiated both NOV and ERY.
- Two analogs,**1013** and **994**, saw their antibacterial activities affected significantly by deletion of efflux, suggesting they are good substrates of the efflux pump and are unable to potentiate either NOV or ERY.
- The nitro analog (**420**) was the lone exception as it had no antibacterial activity, nor did it potentiate NOV or ERY.

Result 2 : Efflux inhibition in bacterial growth-independent assays

- To establish whether the potentiation of antibiotic activities is indeed due to inhibition of efflux, we next analyzed the ability of compounds to inhibit efflux in a bacterial growth-independent assay, using fluorescent probes that are substrates of AcrAB-TolC.

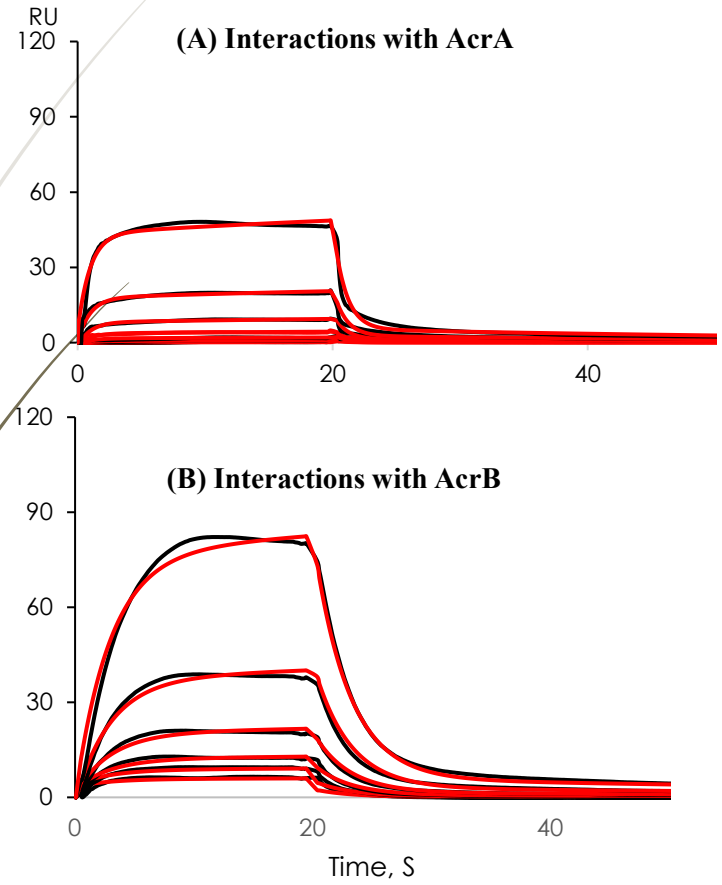


- Most of the tested cinnamoyl derivatives inhibited efflux of NR with efficiency similar to that of **NSC 33353.**
- Compounds such as **384, 388** and **1013** were more effective than **NSC 33353** at the highest (32 µM) concentration, whereas **420** and **994** failed to inhibit NR efflux

Figure 2. Activities of compounds in inhibition of efflux of the fluorescent probe Nile Red (NR) with wt-pore cells. Cells were mixed with NR in concentration 5 µM and then compounds were added at indicated increasing concentrations, change in NR fluorescence was monitored as a function of time and data were fitted into two-exponential equation to extract the intracellular steady-state accumulation levels of NR. The steady-state concentrations were plotted as a function of the compound concentration. Error bars are SD (n=2).

Result 3: Binding affinity was determined by Surface Plasmon Resonance (SPR)

- **To determine the binding affinity of these compounds with AcrA and AcrB we use SPR assay. SPR is a binding analysis method that usually used for small molecular interaction.**



(C) Binding affinity of AcrA and AcrB

#	K_D, mM	
	AcrA	AcrB
NSC-33353	+	ND
383	0.02	0.02
384	0.02	0.06
388	0.01	0.17
420	0.05	0.04
451	0.05	0.06
1013	0.08	0.01
453	0.26	ND
454	0.28	0.03
994	0.02	0.05

- Data shows that all the compounds are bind with AcrA and AcrB
- Compounds **453 and 454** which display high affinities for purified AcrA, on the other hand **388** showing high affinity for AcrB.
- However, SPR experiments suggest that compounds of the cinnamoyl series interact with AcrA and AcrB. These interactions apparently are not sensitive to chemical modifications and cannot explain the observed differences in the activities of the compounds.

Figure 4. Direct interactions of inhibitors with AcrA and AcrB as measured by surface plasmon resonance. The immobilized densities of both proteins (ligand) were 7167 and 7072 response units (RU), respectively, and compounds were injected in HEPES-NaCl buffer supplemented with 5% DMSO at concentrations 0.39, 0.78, 1.56, 3.12, 6.25, and 12.5 µM for the of the compounds. The SPR sensorgrams (black lines) were fitted into different kinetic models, and the best fits are shown as red lines.

Summary of this research

- The results presented here expand on our previous efforts to develop novel EPIs targeting the MFP AcrA of the AcrAB-TolC efflux pump of *E. coli*.
- We have modified the substituents of the cinnamoyl group of NSC-33353, giving rise to analogs that retain the ability to inhibit efflux, lost the features of efflux substrates, and gained antibacterial activity in wild-type cells.
- These results provide new insights into the duality of efflux substrate/inhibitor features in chemical scaffolds that will facilitate the development of future efflux pump inhibitors

Acknowledgements

Collaboration:

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Jerry M. Parks	Oak Ridge National Laboratory
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