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NOVEL INHIBITORS OF BACTERIAL TWO-COMPONENT SYSTEMS WITH GRAM POSITIVE ANTIBACTERIAL ACTIVITY: PHARMACOPHORE IDENTIFICATION BASED ON THE SCREENING HIT CLOSANTEL

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Abstract: This SAR study has shown that the salicylanilide is the pharmacophore for inhibition of the bacterial two-component system. Hydrophobic substituents improve the potency of inhibitors in this series; however, hydrophobicity is not the sole determinant for inhibition; structural and electronic requirements also exist. Closantel (**1**) was found to inhibit a two-component system and to have antibacterial activity against drug resistant *S. aureus* and *E. faecium.* © 1998 Elsevier Science Ltd. All rights reserved.

New classes of antibacterials are needed to combat emerging drug-resistant bacteria. Since the 1960's a wide range of antimicrobials have become available that are structurally diverse and highly effective agents. Bacterial resistance to all existing classes of antibacterials/antibiotics has escalated in the last decade, and in the last year, intermediate level resistance to vancomycin was detected in a strain of *Staphylococcus aureus*,[3] a common pathogen that is responsible for many types of infections including pneumonia, endocarditis, soft skin and tissue. Vancomycin is the last uniformly effective antibacterial for treating serious *S. aureus* infections. A critical need now exists for antibacterials with new mechanisms of killing bacteria.[4]

Two-component systems (TCS) are pervasive among bacteria, and this signal transduction pathway is used by bacteria to sense and respond to environmental changes. In pathogenic bacteria, the TCS often regulate the expression of virulence factors that are required for survival inside the host. A two-component system is comprised of a histidine protein kinase (HPK) and a response regulator (RR). In all bacterial species there are multiple two-component systems, each controlling transcription of several genes. Among the TCS proteins of different species of bacteria, significant homology is shared. The TCS are not found in animal cells, making this an important new target that could lead to a novel class of antibacterial agents.[5,6] Compounds that inhibit the TCS could be expected to block important bacterial signaling pathways that may lead to bacterial cell death.

We sought to identify potent inhibitors of the bacterial TCS and determine if such compounds had antibacterial activity. In vitro screening of compounds in our chemical library resulted in the identification of a new class of TCS inhibitors that inhibited the KinA/Spo0F system: a model two-component system from *B. subtilis*. Closantel (**1**)[7] was found to inhibit the KinA/Spo0F system (IC_{50} = 3.8 μM) and to have antibacterial

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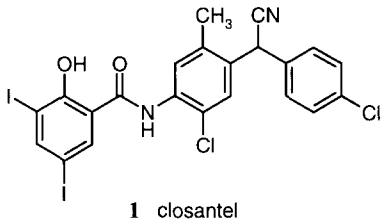
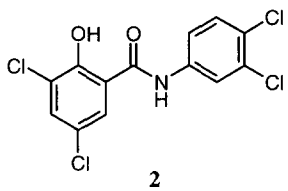
activity against drug resistant *S. aureus* and *E. faecium* (MICs of 1–2 μg/mL). The topical antibacterial tetrachlorosalicylanilide **2**,[8] an analog of closantel, was less potent than closantel, but had similar antibacterial activity. Salicylanilides are known to uncouple oxidative phosphorylation in mitochondria by dissipating the proton-motive force necessary for ATP synthesis.[9] This activity could lead to serious adverse effects in mammalian cells. We embarked on a structure–activity study to determine the pharmacophore for TCS inhibition, attempting to find analogs without the *o*-hydroxybenzamide core, which appears to be responsible for the uncoupling of oxidative phosphorylation.[9]



1 closantel



2

Chemistry:

The TCS inhibitors **1–7**, **9–11**, and **14** were prepared by acylation of an amine with the corresponding acid chloride in dioxane at 50 °C in isolated yields of 45–91%.[10] Treatment of closantel (**1**) with sodium hydride in DMF followed by methyl iodide gave the *O*-methyl derivative **8** in 50% yield. 2,4-Dichloro-6-nitrophenol was reduced to the aniline with stannous chloride in ethanol (97% yield).[11] Acylation with 3,4-dichlorophenyl isocyanate in ether at room temperature gave the urea **12** in quantitative yield. Reaction of 3,4-dichlorobenzyl chloride with triphenylphosphine in DMF at 100 °C for 3 h gave the phosphorane in 76% yield.[12] The ylide of the phosphorane was formed with potassium *t*-butoxide (2.5 equiv.) in THF and reaction with 3,5-dichlorosalicylaldehyde at 60 °C for 19 h gave the *trans*-olefin **13** in 85% yield.

Results and Discussion:

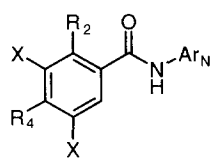
We found that inhibitors with hydrophobic/electron-withdrawing substituents on either aromatic ring of **1** or **2** afforded inhibitors with increased potency. Thus, replacing the salicyloyl chlorine atoms in **2** and **3** with iodine showed a trend toward increased potency as shown by derivatives **4** and **1** (Table 1). The same result was found in the aniline substitution pattern (compare inhibitors **2** and **10** in Table 2). However, hydrophobicity is not the sole determinate for potent inhibitors. Structural and electronic requirements exist. For example, removal of the 2-OH resulted in loss of activity as in analog **5**, but a hydroxyl at the 4-position (**6** and **7**) gave inhibitors—although with decreased potency compared to **2** and **1**. The importance of the salicylanilide pharmacophore was noted in compounds **8** and **9** where methylation of the phenol or the amide gave inactive compounds.

To test the need for the salicylanilide pharmacophore, a series of derivatives were prepared (Table 2). In every case, the analogs with a modified anilide were inactive or weakly active TCS inhibitors. Two compounds were prepared that have similar hydrophobicity to **2** as measured by ClogP values.[13] The reverse amide **11** and the aniline homolog **14** were inactive, yet had ClogP values of 4.8 and 4.6 compared to 4.8 for **2**. The urea **12**

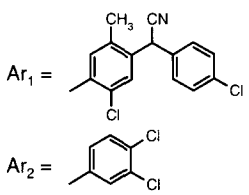
and *trans*-olefin **13** were prepared as bioisosteres of the amide **2**; however, these compounds were inactive, demonstrating that the salicylanilide is the pharmacophore for TCS inhibition.

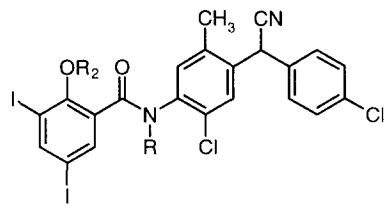
The TCS salicylanilide inhibitors **1** and **2** were potent antimicrobial agents against gram-positive bacteria and also had good activity against methicillin-resistant *S. aureus* (MRSA) and against vancomycin-resistant *E. faecium* (VRE) (Table 3). Activity against these organisms, which are clinically difficult to treat, is very important in the next generation of antibacterial agents.[4] When compared to the clinically important agents, oxacillin and vancomycin, the salicylanilides had superior activity against MRSA and VRE. Inhibitors **1** and **2** were inactive (MICs ≥ 64 μg/mL) against gram-negative bacteria (*E. coli*, *K. pneumoniae*, and *P. aeruginosa*) possibly due to poor permeability of the additional outer membrane. The reverse amide **11** was inactive as a TCS inhibitor, but had similar antimicrobial activity to **2**. This activity is possibly due to the uncoupling of oxidative phosphorylation in mitochondria, a known action of the salicylanilides.[9] However, we have reported that salicylanilides also inhibit a TCS in a reporter-gene cell-based assay at concentrations that have little effect on bacterial growth.[14] Studies are in progress clarify the mechanism of action of these salicylanilides that inhibit bacterial growth.

Table 1. Modifications of the Salicylamide.



1-7



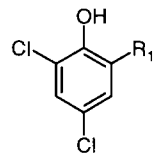


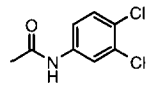
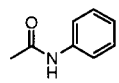
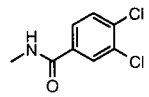
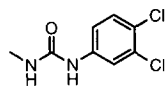
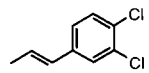
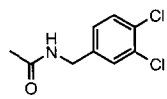
8 R = H $R_2 = CH_3$ $IC_{50} > 500$ μM
9 $R = CH_3$ $R_2 = H$ $IC_{50} > 500$ μM

Cmpd	R_2 =	R_4 =	X =	Ar_N =	KinA/Spo0F [a] IC_{50}, μM
3	OH	H	Cl	Ar_1	4.8
1	OH	H	I	Ar_1	3.8
2	OH	H	Cl	Ar_2	45
4	OH	H	I	Ar_2	22
5	H	H	Cl	Ar_2	>500
6	H	OH	Cl	Ar_2	98
7	H	OH	I	Ar_1	9

[a] This assay measures the ability of the test compounds to inhibit phosphorylation of the HPK, KinA. IC_{50}'s were repeated at least once (n ≥ 2) and the data reproducibility was within 50%.

Table 2. Modifications of the Salicylanilide Linker.



Cmpd	R_1 =	KinA/Spo0F [a] IC_{50}, μM
2		45
10		385
11		>500
12		365
13		>500
14		>500

[a] See footnote a in Table 1.

In conclusion, this SAR study has shown that the salicylanilide is the pharmacophore for inhibition of the bacterial two-component system and that hydrophobic substituents improve the potency of inhibitors in this series. Although the 4-OH analogs **6** and **7** were active, these compounds were less potent as TCS inhibitors and as inhibitors of bacterial growth. In this SAR study, we did not separate TCS inhibition activity from the second mechanism of uncoupling oxidative phosphorylation. All attempts to modify the salicylanilide pharmacophore did not give potent TCS inhibitors. We could not clearly demonstrate that selective TCS inhibitors in this series will inhibit bacterial growth; however, we have discovered the salicylanilides as a new class of inhibitors of the two-component system. Few TCS inhibitors have been reported[6] and this SAR study will be useful when related inhibitors are discovered in the future.

Table 3. Antimicrobial Activity.

Cmpd	KinA/Spo0F [a] IC_{50}, μM	MIC, μg/mL [b] *S. aureus* ATCC 29213	MRSA OC 2089	*E. faecalis* OC 3041	*E. faecium* (VRE) OC 3312
closantel (**1**)	3.8	1	2	1	1
2	45	0.5	0.25	0.5	0.5
6	98	16	16	64	64
11	>500	0.5	0.25	2	1
oxacillin	--	0.25	>64	16	>64
vancomycin	--	1	2	4	>128

[a] See footnote a in Table 1. [b] The variance in the determination of MIC values is twofold such that MIC differences of ≥2 dilutions are significant.

KinA/Spo0F Inhibition Assay: This assay detects the ability of test compounds to inhibit protein phosphorylation in a two-component system. Varying concentrations of the test compounds were incubated with 0.24 μM histidine protein kinase (KinA), 1.6 μM response regulator (Spo0F), and 37.5 μCi [^{32}P]ATP in 150 mM Tris buffer (pH 8.0). Phosphorylated products were separated by SDS-PAGE electrophoresis and quantitated by a phosphoimager. The intensity of each KinA band was converted to percent inhibition compared with the background and plotted as a function of inhibitor concentration. Results are reported as micromolar IC_{50} values.[15]

Minimum Inhibitory Concentration (MIC) Determinations: Antibacterial susceptibility testing was performed following the broth microdilution method of the National Committee for Clinical Laboratory Standards.[16]

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