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Correlation of carboxylic acid pK_a to protein binding and antibacterial activity of a novel class of bacterial translation inhibitors

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Abstract—Previously we reported the discovery and initial optimization of a novel anthranilic acid derived class of antibacterial agents which suffered from extensive protein binding. This report describes efforts directed toward understanding the relationship of the acidity of the carboxylic acid with the extent of protein binding. The pK_a of the acid was modified via the synthesis of a number of anthranilic acid analogs which vary the aromatic ring substituent at the 4-position. The pK_a and HSA binding constants have been determined for each of the analogs. Our results indicate a correlation between pK_a and HSA K_d. The physical properties and antibacterial activities will be discussed as well as how these results help address the protein binding issue with this series of compounds.

Emergence of bacterial resistance continues to be a growing problem in the treatment of bacterial infections.[1–3] Thus, Pharmacia invested significant resources in an attempt to discover antibiotics that targeted novel mechanisms of action, with the thought that this would be the most expedient way to overcome resistance. We recently reported the discovery of a novel class of protein transcription/translation inhibitors[4] through a combination of high throughput screening and medicinal chemistry optimization (compounds **1** and **2**). While compound **2** exhibited good in vitro antibacterial activity, there was no in vivo activity in a standard mouse bacteremia model of infection. Since antibacterial activity is greatly reduced in the presence of human serum, the lack of in vivo activity was attributed to the high protein binding of these compounds. As a consequence, subsequent optimization efforts focused on further improving the potency and reducing the protein binding of these leads.[5,6]

By focusing on reducing lipophilicity and molecular weight, several analogs with improved antibacterial activity and/or reduced protein binding, relative to initial leads (e.g., **1** and **2**), were designed and synthesized (Fig. 1, compound **3**; Fig. 2, compounds **4**–**6**). Protein binding was routinely assessed by adding human serum to the in vitro antibacterial assay. Compounds **3**–**6** have MICs of 0.125–1 μg/mL against *Staphylococcus aureus*, but the addition of 10% serum raises the MICs to the 4–8 μg/mL range; a 4- to 64-fold difference. Even the level of reduced protein binding afforded by compound **6** did not translate into in vivo activity (ED_{50}s >200 mg/kg in a mouse bacteremia model). Since human serum albumin (HSA) represents 60% of the total mass of plasma proteins and is known to bind aromatic carboxylic acids (e.g., salicylates, ibuprofen),[7] we hypothesized that further reduction of protein binding was required in order to attain in vivo activity.

Keywords: Translation inhibitors; Protein binding; Antibacterial; Capillary electrophoresis; Acidity.
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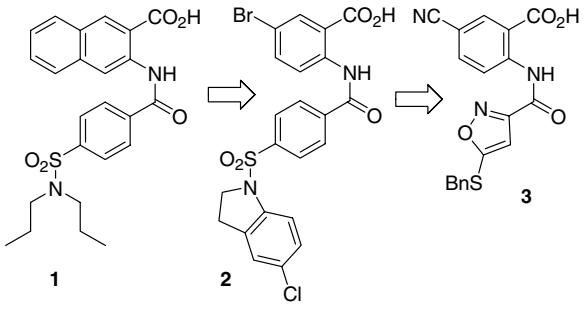
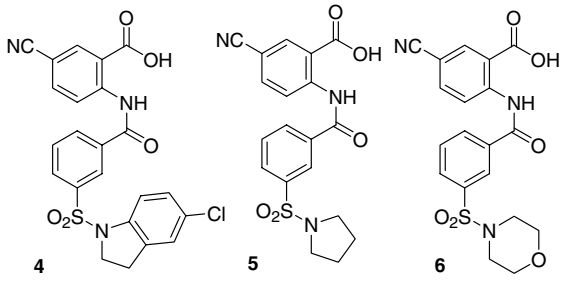


	MIC SAUR 9218	MIC SAUR 9218 + 10% serum	MW	ClogP
1	32 μg/mL	>64 μg/mL	454	6.0
2	0.125 μg/mL	64 μg/mL	536	6.2
3	0.125 μg/mL	4 μg/mL	380	3.8

Figure 1. Discovery and optimization of translation inhibitors.



	MIC SAUR 9218	MIC SAUR 9218 +10% serum	MW	ClogP
4	0.125 μg/mL	8 μg/mL	482	5.1
5	1μg/mL	8 μg/mL	399	3.1
6	1μg/mL	4 μg/mL	415	2.4

Figure 2. Meta-sulfonamide-substituted translation inhibitors with good antibacterial activity, but no in vivo activity.

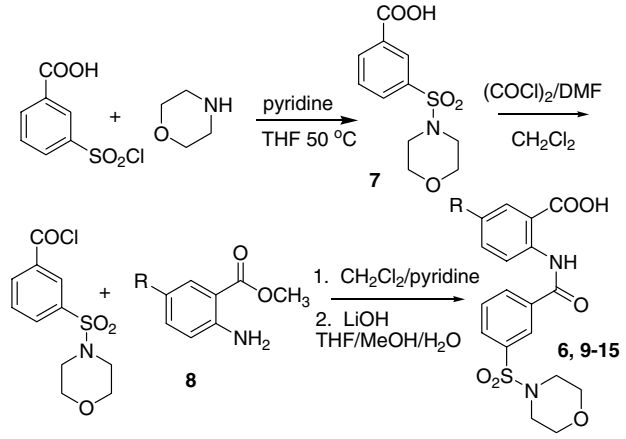
Prior SAR studies had demonstrated that the carboxylic acid was an essential part of the pharmacophore required for activity. Substituting the acid with other simple functional groups generally resulted in decreased activity, except in cases where the functional group also possessed an acidic proton. In such cases protein binding was still a problem. Since we were limited to acids or similar groups, it occurred to us that the acidity of the acid might impact the extent to which the compounds were protein bound. Therefore, we undertook an investigation into the impact of the acidity of the carboxylic acid on the protein binding, in order to gain additional insight into ways in which protein binding might be reduced.

Since previous studies had demonstrated that substitution on the 5-position of the aryl ring was detrimental and substitution on the 4-position of the aryl ring was beneficial[4] we targeted the synthesis of analogs in which the 4-aryl substituent was varied from electropositive substituents to electronegative substituents. Additionally, in the course of the exploration of the structure–activity relationships (SAR), it had been determined that sulfonamide substitution on the central aromatic ring either *meta* or *para* to the amide carbonyl was optimal.[4,6] In addition, the potency of compound **6** was one of the least impacted upon the addition of HSA. Therefore, for the purposes of this study, we elected to focus on the *meta*-substituted series of compounds (Scheme 1, compounds **6** and **9**–**15**).

The desired analogs were prepared following the route shown in Scheme 1. 3-Chlorosulfonylbenzoic acid was reacted with morpholine to provide the sulfonamide **7**, which upon conversion to its acid chloride was coupled to an anthranilic acid methyl ester (**8**). Basic hydrolysis of the methyl ester provided the desired carboxylic acids (**6** and **9**–**15**). In the few instances where the anthranilic esters were not commercially available, the desired methyl ester was obtained via esterification of the available acid or reduction of the corresponding nitro derivative.

The pK_a of each compound was determined using pressure-assisted capillary electrophoresis (PACE). The measurement of pK_a by PACE is based on the ionic mobility of the solute over a range of buffer pH values.[8] Mobility and pH data can then be fit to equilibrium expressions and the pK_a of the solute determined (Table 1, column 3). The accuracy of this method has been found to be ±0.2 pH units. Additionally, pK_as were predicted computationally using the computer program PALLAS (Table 1, column 4).

The binding of small molecules to HSA can be measured using a variety of methods. Previous experiments demonstrated that this class of translation inhibitors competitively displaces the fluorescent probe dansylsarcosine from site II on subdomain IIIA of HSA. While this result does not preclude the binding of the molecules to another site on HSA, it does indicate a high affinity for an area close to site II. Using a previously developed method,[9] the dissociation constant, K_d, for each compound was calculated from the percentage of dansylsarcosine displaced from HSA (Table 1, column 5). Finally, the MICs in the presence and absence of 5% human serum were determined to provide another measure of the impact of protein binding on activity.



Scheme 1. Synthesis of anthranilic acid analogs.

Table 1. Measured pK_a, calculated pK_a, measured protein binding, and antibacterial activity for the compounds with the 5-aryl ring substituent varied

Compound	R	*C*log *P*	pK_a PACE	pK_a Pallas	Protein binding K_d (μM)	MIC (μg/mL)[a]	
						SAUR[b]	SAUR 5% serum[c]
9	H	2.6	3.18	4.00	6.0 ± 1.2	>128	>128
10	CH_3	3.1	3.36	4.06	7.5 ± 1.6	>128	>128
11	OCH_3	2.6	3.12	3.89	8.1 ± 1.7	>128	>128
12	F	2.8	2.92	3.66	6.6 ± 1.9	128	128
13	Cl	3.4	2.81	3.63	6.5 ± 1.2	4	32
14	Br	3.6	2.79	3.61	4.8 ± 0.7	4	32
6	CN	2.4	2.56	3.38	5.3 ± 1.2	1	2
15	NO_2	2.7	3.44 (2.46)[d]	3.26	4.4 ± 0.3	4	16

[a] Minimum inhibitory concentration.
[b] *Staphylococcus aureus* UC9218.
[c] *Staphylococcus aureus* UC9218 + 5% pooled human serum. Human serum (male, from Sigma) was thawed at room temperature, then placed in a 56 °C water bath for 30 min. The serum was filtered using a 0.2-μm filtration system.
[d] Expected value of pK_a given 0.8 units difference between calculated and experimental values.[10]

Compounds in Table 1 are arranged from least acidic to most acidic. It is interesting to note that the computational predictions (PALLAS) of pK_a for this class of compounds were consistently 0.7–0.8 pH units less acidic than those determined experimentally by PACE. This difference can be attributed to an intramolecular hydrogen bond between the amide NH and the carbonyl of the carboxylic acid enhancing the acidity of the acid. Small molecule X-ray structures of compounds in this analog series confirm the presence of such a hydrogen bond.[11] The computational prediction is not able to take this interaction into account, and thus returns a less acidic pK_a.

The data collected in Table 1 indicate a correlation between the acidity of the acid (either calculated or measured) and the protein binding to HSA (K_d). Stated another way: generally, the more acidic the acid, the more highly protein bound the analog. A graphical representation of the data is shown in Figure. 3, which plots both the measured and calculated pK_a of the analogs versus the affinity for HSA (K_d). As the pK_as increase (either experimentally or calculated), the dissociation constant for the dansylsarcosine site increases.

The biological data in Table 1 also indicate that the antibacterial activity[12] is dependent on the pK_a of the acid.

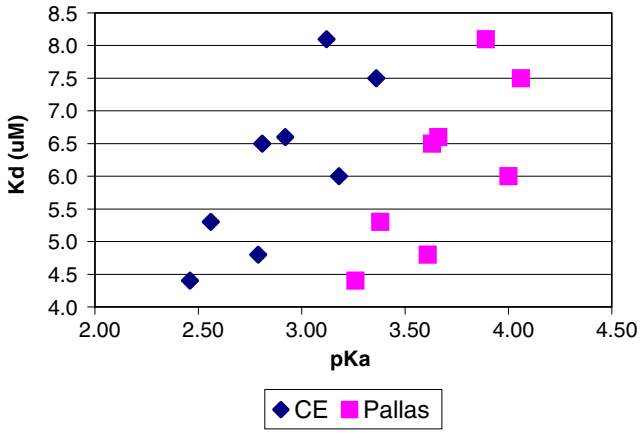


Figure 3. Anthranilic acid pK_a versus HSA K_d.

Compounds (**9**–**11**) containing a carboxylic acid with a pK_a ⩾ 2.92 (measured) show little or no antibacterial activity against *S. aureus*. For the compounds that exhibit antibacterial activity against *S. aureus*, the addition of just 5% serum reduces the potency. Calculating the ratio between potency with and without 5% serum also gives an indication of the extent of protein binding. For compounds **6** and **13**–**15** the ratio runs from 2- to 8-fold and generally trends with the K_d measurement.[13] Lipophilicity has also been shown to impact the degree of protein binding in this series of compounds.[6] Its impact is likely to be observed in the MIC assays with serum protein present as this system presents a more global view of the total extent of protein binding (i.e., in cases where the compounds bind to more than one site, or to other proteins in the media, vide infra). It is clear that a strategy built upon reducing pK_a to minimize protein binding will require balancing with the requirement to retain the degree of acidity needed to retain the potent antibacterial activity.

Compound **6** was selected for a more extensive evaluation of its protein binding characteristics. Isothermal titrating calorimetry (ITC) indicated that two or more molecules of compound **6** bound to fraction V of HSA, and that there is a 2 μM high affinity binding site and a 19-μM lower affinity site(s). K_ds of ~14 and 2 μM were measured using ultra filtration with human serum and stopped-flow fluorescence quenching, respectively.[14] Since the ITC measurement indicates more than one binding site, the K_ds in Table 1 determined by dansyl sarcosine are only representative of a portion of the affinity for HSA.

In conclusion, a study in which the pK_a of the carboxylic acid moiety was varied to determine impact on protein binding demonstrated that acidity of the carboxylic acid correlates with affinity to HSA as measured by dansyl sarcosine displacement. It also appears that there is a certain threshold of acidity required to retain the antibacterial activity. The fact that compound **6**, which is both highly acidic and highly protein bound, was one of the most potent analogs in the presence of human serum at the time of this study underscores the complexity of this issue. Correlation of antibacterial activity of

these compounds in the presence and absence of serum with the K_d measured via the dansylsarcosine displacement is complicated by experimental data that indicate binding at a second lower affinity site. Still, it appears that efforts to reduce acidity would be well placed.

In addition to protein binding, it is likely that a combination of factors such as unbound clearance and volume of distribution impacts the in vivo efficacy of this class of compounds. The correlation between protein binding and acid pK_a presented herein guided the strategy for further optimization of this series of compounds with the aim of identifying a novel antibacterial agent. Future reports will describe efforts to replace the carboxylic acid with bioisosteres.[11,15]

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