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Development of a novel expression, ZI_{MAX}/K_{ZI}, for determination of the counter-anion effect on the antimicrobial activity of tetrabutylammonium salts

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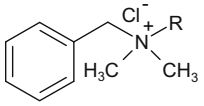
ABSTRACT

Due to the increasing number of strains of drug-resistant bacteria, the development of new antibiotics has become increasingly important. The antibacterial properties of quaternary amines and their derivatives on both Gram-positive and Gram-negative bacteria are well known. However, an encompassing study with specific emphasis on the role of the counter-anion has not been reported in the literature. By monitoring the Zone of Inhibition of various concentrations of tetrabutylammonium (TBA) salts, we observed that the counter anion plays a significant role in activity. We developed a novel method of reporting activity using zone of inhibition tests (ZI_{MAX}/K_{ZI}) and found it to be strongly correlated with the minimum inhibitory concentration (MIC).

Antibiotic resistance as a result of the adaptability of microorganisms has presented problems with the use of current antimicrobial agents.[1] It has been known for a long time that quaternary amine compounds have strong antimicrobial activity. These compounds act as detergents as they possess both polar hydrophilic and nonpolar hydrophobic ends.[2] Due to this amphipathic nature, they solubilize otherwise insoluble material and are very effective cleaning agents. Nearly permanent, non-leaching antimicrobial activity has already been demonstrated in other reports using quaternary amines.[3] One common example is benzalkonium chloride (BAC), shown in Figure 1. BAC is considered a hard antibacterial agent, meaning that it is a drug that is biologically active and cannot be metabolized in vivo.[4] The alkyl chain length of BAC strongly affects its bactericidal activity.[5,6]

Quaternary ammonium compounds (QACs) are classified as cationic antibacterial agents.[2] Their mode of action involves the initial binding to the negatively charged cell wall and membrane. The surface of these cells are generally stabilized by the presence of divalent cations, typically Ca^{+2} and Mg^{+2}, which are associated with lipopolysaccharides of Gram (−) bacteria and both the teichoic acid and polysaccharides of Gram (+) bacteria.[2] QACs displace these surface cations, and if a hydrophobic region is present, can integrate into the cytoplasmic membrane. With Gram (−) bacteria self-promoted uptake pathways can enhance the influx of more of the cationic antibacterial agent.[7] A parabolic relationship has been observed when examining the lipophilic chain length of these amphoteric surfactants.[5,6] The maximal activity towards Gram (+) bacteria is when the chain length is between 12 and 14 carbons, whereas with Gram (−) bacteria it is between 14 and 16 carbons.[5,6] It has been shown that at low concentrations of QACs, osmoregulatory capability is impaired, and leakage of K^+ and H^+ has been observed.[8] At higher levels, solute transport, cell wall biosynthesis, and respiration are adversely affected.[9] Ultimately, the membranes themselves are solubilized by the surfactant ability of the QACs which causes leakage of the cells contents.[10,11] The surfactant concentration required for complete solubilization was shown to be directly related to the QACs critical micelle concentration.[12]

As indicated earlier, QACs are generally classified as cationic antibacterials. However, in all cases a counter anion must be present, which is typically either chloride or bromide. In this study we examined a library of simple tetrabutylammonium (TBA) compounds with differing counter anions. TBA compounds are not the strongest of the quaternary ammonium antibacterial agents however they are commercially available in a large variety of forms with differing counter anions. Thus, they could be used to deter-



R= C_nH_{2n+1}, n=8,10,12,14,16,or 18

Figure 1. Chemical structure of benzalkonium chloride (BAC).

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mine what effect, if any, the counter anion has on the antimicrobial activity of that biocide.

The rational for choosing the counter-anion as the focus of this project is because several experiments have already been carried out which emphasize the role and function of the cation. For example, it has been shown that the TBA cation is a non-depolarizing muscle relaxant,[13] a potassium channel antagonist,[14] and is toxic in aquatic organisms.[15] There are many ways that an anion could affect the antibacterial efficacy of a compound, which include the size, dipole moment, lipophilicity, coulombic forces, and pK_b of the counter anion, or a combination of these factors. Studies have already shown that some antibacterial agents with high pK_a values have a stronger antimicrobial effect than those with lower pK_a values.[16] Since Gram (−) and Gram (+) bacteria have different susceptibilities to antimicrobials,[2] we tested *Escherichia coli* and *Staphylococcus epidermidis* as representative Gram (−) and Gram (+) bacteria, respectively.

Initially, we began our experiments by measuring the zone of inhibition produced by each compound at a multitude of concentrations.[17] What we discovered was that it was impossible to choose a concentration to accurately compare compounds. Varying the concentrations of compounds gave an antimicrobial profile that was non-linear in nature. For example, TBA bisulfate reaches near maximal activity at relatively low concentrations, whereas TBA sulfamate produced zones of inhibition with a broader range of sizes as a function of concentration (Fig. 2). Thus, to quantitatively measure the antimicrobial activity of a bactericidal agent using the halo test, the entire concentration range must be taken into account, so we developed an equation to explain this relationship.

Herein, we report a novel method to express antimicrobial activity using the halo test. Similar to the Lineweaver–Burke equation for determining enzyme kinetics, we graphed the reciprocal of the Zone of Inhibition versus the reciprocal of the antimicrobial concentration (8–12 different concentrations used depending on strength of agent), which produces a linear relationship (Fig. 3). We then defined the reciprocal of the *y*-intercept as the maximum zone of inhibition (ZI_{MAX}) and the negative reciprocal of the *x*-intercept as the concentration to one-half the maximum zone of inhibition (K_{ZI}). An ideal antimicrobial agent would possess a large ZI_{MAX} and a small K_{ZI}. By dividing ZI_{MAX} by K_{ZI} we have a new equation to express antimicrobial activity using the halo test. Thus, activity = ZI_{MAX}/K_{ZI}.

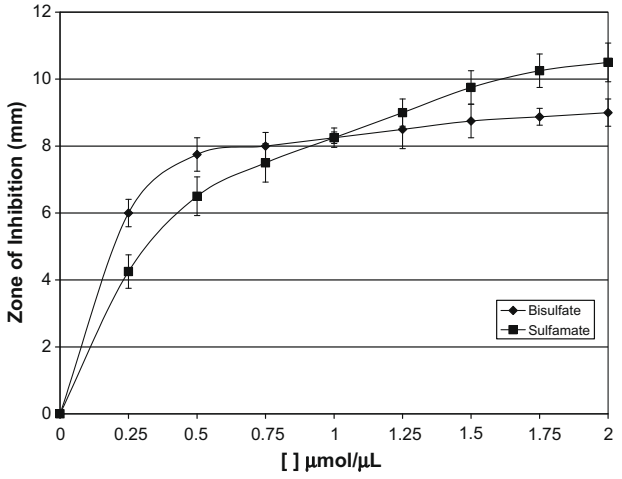

Figure 2. Zone of inhibition versus concentration on select TBA compounds against *Staphylococcus epidermidis*. Error bars represent one standard deviation.

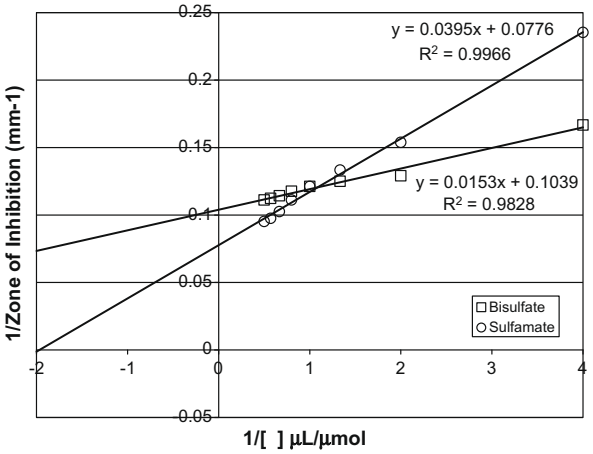

Figure 3. Reciprocal of zone of inhibition versus reciprocal of concentration on select TBA compounds against *Staphylococcus epidermidis*.

In order to validate this new measure of antimicrobial activity, as well as to insure that the variation in activity obtained was not due to solubility or diffusion rate differences, we compared our ZI_{MAX}/K_{ZI} values to a minimum inhibition concentration (MIC) study for several of the compounds tested.[18] We randomly chose eight compounds and obtained MIC values for both *S. epidermidis* and *E. coli* (Table 1). There is a good correlation ($R^2 = 0.90$) of these two independent terms when graphing the $\ln(ZI_{MAX}/K_{ZI})$ versus MIC (Fig. 4), which is not observed when graphing MIC versus $\ln(K_{ZI})$ or $\ln(ZI_{MAX})$ alone ($R^2 = 0.36$ and 0.07, respectively) (Fig. 5). This confirmed that our novel expression of antimicrobial activity was valid for this class of compounds. Thus, we used ZI_{MAX}/K_{ZI} values to determine the antimicrobial activity for the remaining compounds in our library. We used up to 12 different concentrations for stronger antibacterial agents (typically with an increased number of low concentration data points) to insure more accurate data.

From Table 2, it is apparent that with all of the TBA compounds tested the ZI_{MAX}/K_{ZI} values for *S. epidermidis* are larger than those for *E. coli*. For *S. epidermidis*, TBA iodide and TBA *l*-lactate have ZI_{MAX}/K_{ZI} values above 100. These are vastly different anions. The pK_a of iodide is −9 whereas the pK_a of *l*-lactate is 3.8. On the other end, TBA tribromide was by far the strongest antibacterial agent for *E. coli* followed by TBA azide and tetrachlorogallate. Towards *S. epidermidis*, these had moderate to good activity (ZI_{MAX}/K_{ZI} = 14–32). What is surprising is the great diversity of activity values. The activity of TBA cyanide is surprisingly low towards *S. epidermidis* compared to TBA iodide, considering the antibacterial efficacy of other cyanide salts. Likewise, the high activity of TBA *l*-lactate is unexpected. Sodium lactate has been reported to have antimicrobial activity towards Gram (+) bacteria, however only at concentrations much greater (50×) then reported here.[19] This high activity is not observed with the other two carboxylates tested (i.e., TBA acetate and benzoate). The TBA halides all showed comparable activity (ZI_{MAX}/K_{ZI} = 14.77–25.91) towards *S. epidermidis* with the exception of TBA iodide. Towards *E. coli* there was a great diversity of activity (ZI_{MAX}/K_{ZI} = 0–37.59). With the sulfate, sulfonate, and sulfamate series there were drastic differences. TBA bisulfate was the most potent in this series for both *S. epidermidis* and *E. coli*, with TBA *p*-toluenesulfonate being the least active. The phospho, nitro, and cyano series all demonstrate similar activity, with TBA thiocyanate being the least active towards *S. epidermidis* but most active towards *E. coli*. The non-Brønsted-Lowry bases were of moderate to good activity towards *S. epidermidis* (i.e., ZI_{MAX}/K_{ZI} = 25.71 for TBA tetrafluoroborate; to ZI_{MAX}/K_{ZI} = 50.76 for TBA triphenyldifluorosi-

Table 1
Comparison of ZI_{MAX}/K_{ZI} to MIC of eight TBA compounds

Name	*S. epidermidis* (ATCC12228) ZI_{MAX}/K_{ZI} (mm μL/μmol)	*S. epidermidis* (ATCC12228) MIC (μmol/μL)	*E. coli* (K-12 (C600)) ZI_{MAX}/K_{ZI} (mm μL/μmol)	*E. coli* (K-12 (C600)) MIC (μmol/μL)
Chloride	23.58	0.08	0.382	0.35
Acetate	37.88	0.06	4.20	0.11
Sulfamate	25.91	0.08	2.34	0.2
Thiocyanate	11.59	0.09	6.28	0.1
Bisulfate	84.03	0.04	4.98	0.11
Hexacyanoferrate	37.74	0.06	0.705	0.3
Iodide	140.85	0.02	0.381	0.35
l-Lactate	103.09	0.02	0	0.5

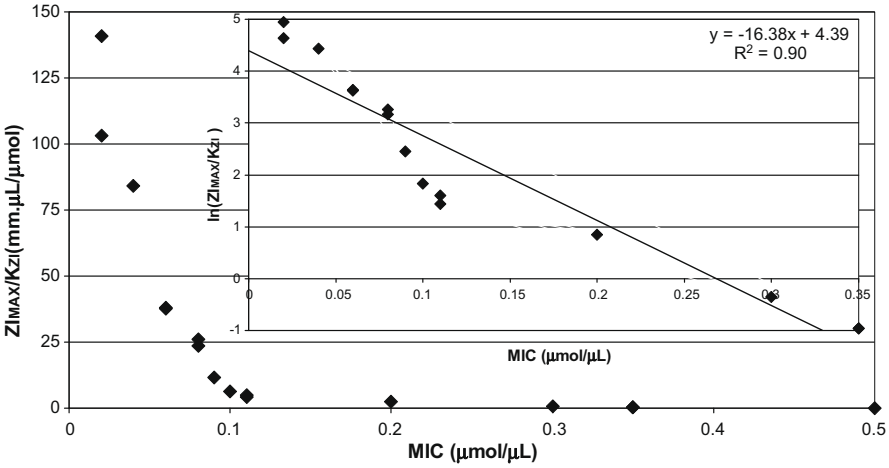

Figure 4. Comparison of ZI_{MAX}/K_{ZI} and MIC on select group of TBA compounds. An approximately linear relationship is observed when comparing $\ln(ZI_{MAX}/K_{ZI})$ with MIC.

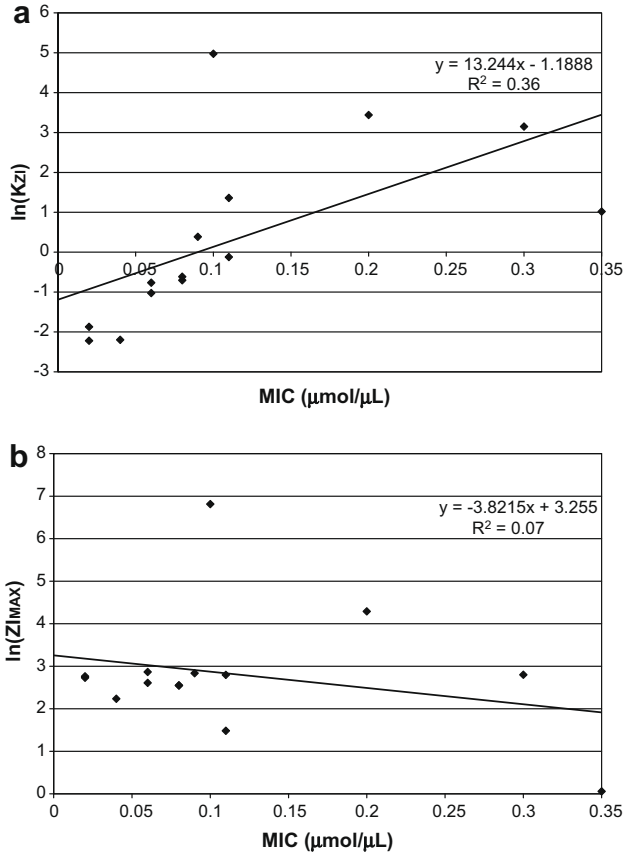

Figure 5. Comparison of (a) MIC versus $\ln(K_{ZI})$ and (b) MIC versus $\ln(ZI_{MAX})$ for eight compounds tested against *S. epidermidis* and *E. coli*.

Table 2
ZI_{MAX}/K_{ZI} values for library of TBA compounds against both *S. epidermidis* and *E. coli*

Counter anion		ZI_{MAX}/K_{ZI} (mm μL/μmol)	
Name	Symbol	*S. epidermidis* (ATCC12228)	*E. coli* (K-12 (C600))
Fluoride	F^-	15.82	0.842
Chloride	Cl^-	23.58	0.382
Bromide	Br^-	16.69	0.516
Iodide	I^-	140.85	0.381
Tribromide	Br_3^-	48.08	37.59
Perchlorate	ClO_4^-	14.77	0
Periodate	IO_4^-	25.91	3.79
Bisulfate	HSO_4^-	84.03	4.98
Sulfamate	$NH_2SO_3^-$	25.91	2.34
Methanesulfonate	$CH_3SO_3^-$	23.53	0.167
Trifluoromethane-sulfonate	$CF_3SO_3^-$	64.94	0.067
Perfluorobutane-sulfonate	$C_4F_9SO_3^-$	14.62	0
p-Toluenesulfonate	$C_7H_7SO_3^-$	8.55	0
Phosphate monobasic	$H_2PO_4^-$	23.58	0
Hypophosphite	$H_2PO_2^-$	52.08	0.408
Nitrite	NO_2^-	24.63	0.97
Nitrate	NO_3^-	30.67	0.333
Thiocyanate	SCN^-	11.59	6.28
Cyanide	CN^-	16.07	2.48
Cyanate	OCN^-	33.11	1.29
Azide	N_3^-	15.11	12.64
Hydroxide	OH^-	27.17	4.06
Acetate	$CH_3CO_2^-$	37.88	4.20
Benzoate	$C_6H_5CO_2^-$	39.81	0.333
l-Lactate	$C_3H_5O_3^-$	103.09	0
Tetrachlorogallate	$GaCl_4^-$	32.05	10.64
Tetrafluoroborate	BF_4^-	25.71	0.499
Triphenyldifluorosilicate	$C_{18}H_{15}F_2Si^-$	50.76	0
Hexacyanoferrate	$Fe(CN)_6^-$	37.74	0.705

licate); however towards *E. coli* all were poor with the exception of TBA tetrachlorogallate. Since the mode of action of these com-

pounds presumably involves the initial binding of the TBA cation to the cell wall and membrane, followed by displacement of Ca^{+2} and Mg^{+2}; if a strong coulombic force existed between the TBA cation and its counter anion, it will not be fully dissociated from its counter anion, thus, the displacement of the Ca^{+2} and Mg^{+2} will be hampered. This is one factor that may explain the range of activity.

In conclusion we have proposed a novel method of reporting antimicrobial efficacy using the halo test (ZI_{MAX}/K_{ZI}). This new approach, which was validated by MIC results, takes into consideration both the maximum zone of inhibition and the concentration required to achieve that zone. Using this approach we examined how the counter anion of TBA affected antimicrobial activity. From the extensive library tested it became clear that the anion plays a significant role in either increasing or decreasing the antimicrobial efficacy of the compound. This knowledge may assist in the development of future generation of antibacterial agents. In order to determine the explanation for the changes in antimicrobial efficacy, a quantitative structure activity relationship (QSAR) study will need to be performed. It is anticipated that this correlation will most likely involve more than one factor, such as coulombic strength, pK_a, etc. In addition, it would be informative to determine whether particular counter anions have similar effects when paired with other quaternary amines.

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17. *Escherichia coli* K-12 (C600) and *Staphylococcus epidermidis* (ATCC12228) were used as representative Gram (−) and Gram (+) bacteria, respectively, in these assays. A single colony of cells was spread evenly over the surface of a Nutrient agar plate (Difco), so that growth would occur as a uniform lawn. After streaking, the plates were divided into four even quadrants in order to test different concentrations of the desired antimicrobial compound on a single plate.Quaternary amine compounds were tested by adding 10 μl of the compound dissolved in DMSO to sterile 6 mm diameter Whatman filter paper disks and placement of the disks in the center of each quadrant. After 24 h of growth at 37 °C, zones of inhibition were measured with a standard ruler. Each concentration of a given compound was measured a minimum of four times to obtain an average value.
18. Minimum inhibitory concentrations (MICs) of TBA compounds were determined by culturing cells in Luria Bertani (LB) medium. From a freshly saturated culture of *E. coli* or *S. epidermidis*, 10 μL was used to inoculate a set of 5 ml cultures of LB medium containing the desired TBA compound dissolved to a maximum concentration of 1 molar and successive 10-fold dilutions. Cultures were incubated for 24 h at 37 °C with rotation on a culture wheel, and then assessed for growth by eye. After these initial experiments, minimum inhibitory concentrations were further refined by testing a range of concentrations between those which initially inhibited or permitted growth.
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