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Elucidating a Specific Minimal Concentration of Taurine Chloramine that Inhibits Growth of Skin Commensal *Staphylococcus epidermidis*

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Abstract

Staphylococcus epidermidis (*S. epidermidis*) and *Propionibacterium acnes (P. acnes*) are skin commensal bacteria. Both organisms have been implicated in causing skin conditions; acne for the former and rosacea for the latter. Taurine chloramine (TauCl) is an anti-inflammatory molecule that has been found to have antimicrobial properties. Previous research in our laboratory has found that treatment of *P. acnes* with TauCl at varying concentrations had inhibitory effects on growth. Because *S. epidermidis* and *P. acnes* live together in the same environment, it was also questioned how TauCl would affect *S. epidermidis*. A previous screening found the variable method of adding TauCl (in suspension or after plating) to *S. epidermidis* was statistically insignificant ($p = 0.1819$). This screening also found a [TauCl] range that may inhibit growth of *S. epidermidis*. The next central hypothesis will investigate the minimum [TauCl] that will exhibit a ten-fold decrease in the growth of *S. epidermidis*. This research is warranted, since minimum concentrations of TauCl for therapeutic use have not yet been quantified, and a therapeutic range for topical use without adverse effects is not known. Elucidating this minimum concentration range of TauCl is the goal of the research because of its possible applications in the production of antimicrobials for either skin conditions or systemic infections. Clinical trials in animal models will eventually be required to determine the LD_{50} and therapeutic index for TauCl *in vivo* once a minimum concentration for decreasing bacterial growth is found.

Keywords: Staphylococcus, Taurine, Rosacea

1. Introduction

The surface of the human skin is one of the largest organs and has been characterized similarly to a niche within the human ecosystem. As with any environment, there are organisms that consistently colonize the skin, called normal flora. These organisms are usually beneficial by protecting the host while receiving nutrients and an ecological niche from the host, a relationship known as commensalism[3]. The two main skin commensals discussed here are *S. epidermidis* and *Propionibacterium acnes* (*P. acnes*). Previous clinical trials have found *S. epidermidis* to be a possible major etiological agent in the skin condition rosacea as well as possibly having a smaller role in acne vulgaris compared to the major etiologic agent, *P. acnes*. Rosacea is very similar to acne with noticeable outbreaks of pustules and flushing on the face as well as thickening of the skin and higher temperatures around the flushed areas. While rosacea has not been seen to be harmful or infectious, 45 million individuals are affected worldwide by this condition and suffer from the same social, psychological and self-esteem damage that acne vulgaris patients suffer[7].

Taurine is a sulfonated amino acid derivative that is produced by activated neutrophils in abundance and reacts spontaneously with hypochlorite (HOCl) *in vivo* to form the anti-inflammatory molecule taurine chloramine (TauCl) while forming water as a by-product[4]. Previous research revealed that while TauCl is not a large component of

antimicrobial activity in the reduction of infection by bacteria, it may have a role among the other major mechanisms of the innate immune system. TauCl has been seen to be antimicrobial[5] and was found to inhibit growth of *P. acnes in vitro*[6].

Because *S. epidermidis* and *P. acnes* inhabit the same environment, if the innate immune system is using TauCl as an antimicrobial component against *P. acnes* during an acne vulgaris infection, it is likely that *S. epidermidis* will also be affected by the molecule. Also the inflammation-like characteristics of rosacea could be brought on by *S. epidermidis* becoming pathogenic, in which case *S. epidermidis* could also be a direct target for the antimicrobial effects of TauCl.

Previous research revealed that inhibition of growth of *S. epidermidis* by TauCl is concentration dependent but the exact minimal concentration at which growth is inhibited effectively (at least 10-fold) was not known. The objective of this project was to find the range of inhibition within an experimental window of TauCl concentrations within defined limits. These concentration limits (150 mM, 300 mM, 450 mM and 600 mM of TauCl) encompass the central composite design of the experiment.

2. Materials And Methods

2.1 Bacterial Strains

The *Staphylococcus epidermidis* strains used in this study was a subculture that has been grown from previous experiments. The original organisms were lyophilized strains ordered from Carolina Biological Supply (Burlington, NC).

2.2 Media

Overnight cultures were grown before the experimental trials in Bacto® Brain Heart Infusion (BHI) broth (Becton-Dickinson) at 35-37 ºC on a rotary shaker. All experiments for growing the treated organism used blood agar plates (BAP), Columbia Nutrient Agar containing 5% sheep's erythrocytes from BD Diagnostics (Franklin Lakes, NJ).

2.3 Chemical Supplies

Certified Laboratory Grade 6% sodium hypochlorite solution (NaOCl) was ordered from Fisher Scientific (Pittsburgh, PA) and 99% taurine ($H_2NCH_2CH_2SO_3H$) in purified solid form was ordered from VWR, (Radnor PA) chemical supplier. All dilutions were performed with phosphate buffered saline (PBS), which is made up using a common lab recipe and deionized H_2O.

2.4 Stock Solution Preparation

Synthesis of TauCl involved two chemical components; stock solutions of 20mM sodium hypochlorite and 24mM taurine were prepared and stored at room temperature as 100mL stock solutions to be used at the time of synthesis.

2.5 Preparation of Phosphate Buffered Saline (PBS)

This solution was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of distilled H_2O. The pH to 7.4 was adjusted to 7.4 with HCl, and 100mL aliquots were sterilized with an autoclave for 20 minutes at 15 lb/sq. in. on liquid cycle[1].

2.6 TauCl Synthesis

The synthesis was carried out by placing 5 mL of 20mM NaOCl into a burette to allow dropwise addition of NaOCl to 24mM taurine in a beaker under constant agitation via a stir-bar. At room temperature, this drop-wise synthesis usually yields TauCl but can also yield $TauCl_2$. In order to ensure that the species in the flask is the desired mono chlorinated compound, it was necessary to visualize solutions immediately after synthesis using a Cary-UV Vis spectrophotometer, which gives an absorbance value of the analyte based on the Beer-Lambert Law. The λ max is

used to identify the species; TauCl will have a λ max at ~252 nm and $TauCl_2$, if it is present, will have its λ max at ~300nm[2].

2.7 Calibration Curve

Due to the project being largely dependent on concentration points of TauCl, a calibration curve was performed for accuracy. TauCl was synthesized using taurine and NaOCl in the method above at an approximate 1:1 molar and volume ratio. A freshly synthesized TauCl solution was visualized and found to have an absorbance of ~2.48. Using Beer-Lambert Law (A= Σbc) and the known extinction coefficient (Σ) for TauCl (reported to be $429m^{-1}/mol)^{2}$, the concentration was found to be 599mM. This 599mM TauCl was diluted using PBS to a 1/3 diluted solution (199mM) as this is the upper limit at which absorbance stays below 1.0; subsequent dilutions were ¼, 1/6, 1/12 and 1/24. These dilution factors are multiplied by undiluted TauCl concentration 599mM to get their respective concentrations. The trend line given can be used to extrapolate concentrations where the absorbance value would be greater than 1. All dilution points were visualized using Cary UV-Vis and the absorbance points were plotted to give the following curve.

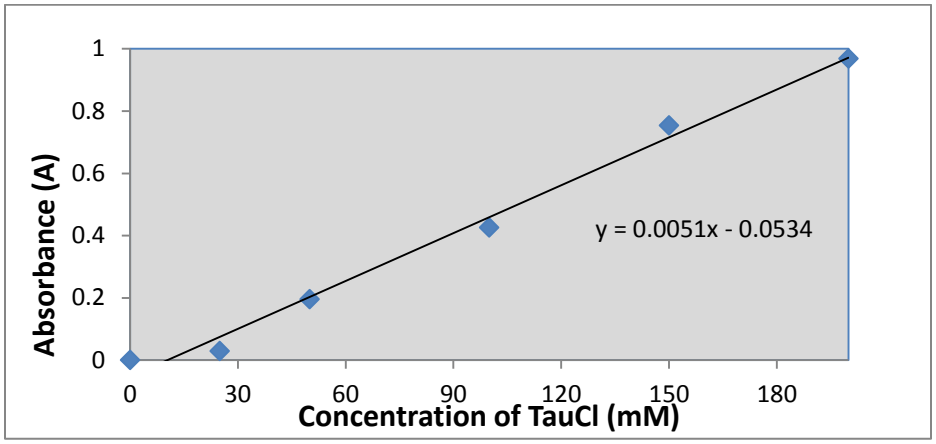

Figure 1. Shown is the corrected calibration curve of absorbance plotted against concentration of TauCl. The linear trend line is given to find absorbance (A) for any concentration of TauCl from 0mM to 199mM. Beer-Lambert Law: A= Σbc and Concentration based on $c = \frac{A}{\Sigma(1cm)}$. The trend line is given. Limit of detection was found to be less than 1/12 diluted solution.

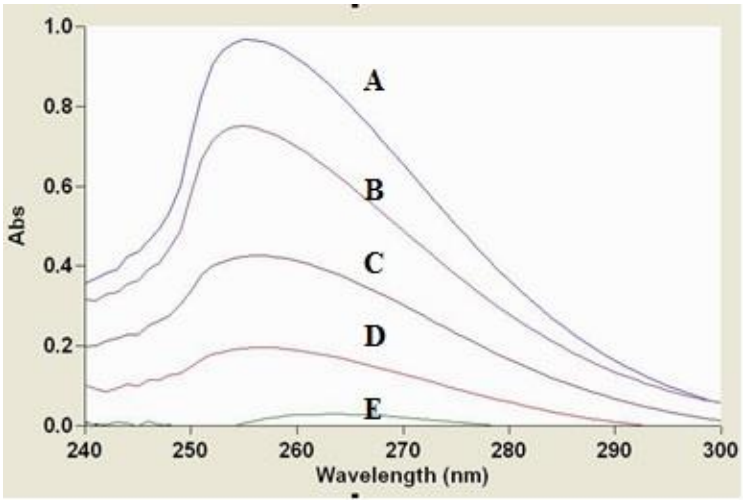

Figure 2. Spectral analysis of TauCl corrected calibration. The wave length max for all samples appears to optimize at approximately 252 nm. Absorbance values for traces A (1/3 TauCl), B (1/4 TauCl), C (1/6 TauCl), D (1/12 TauCl) and E (1/24 TauCl) are given as 0.968, 0.753, 0.425, 0.195, and 0.028 respectively. PBS was used as the

blanking solution. Spectral data was obtained using a Cary UV Spectrophotometer. All dilutions were performed using PBS.

3. Experimental Trials

3.1 Growing *S. epidermidis*

S. epidermidis was grown in an overnight (~8-12 hours) culture in BHI broth using a rotary shaker. These organisms were used in the experimental trials immediately after the tubes reached saturation.

3.2 Dilution of *S. epidermidis*

Serial dilutions were performed of the overnight cultures in 1mL total volume out of 1.5mL tubes. The organism taken was centrifuged twice at 14,000 x g for three minutes each and the pellet was resuspended in 500µL of PBS. These samples were then diluted in succession as follows: from undiluted to 10^{-2} using a 1/100 dilution, taking 10µL of culture and placing into 990µL of PBS for a total volume of 1mL. Dilutions of 10^{-2} to 10^{-4} and to 10^{-6} were also performed and 10^{-7} and 10^{-8} were done by performing a 1/10 dilution, taking 100µL of organism and placing into 900µL of PBS. These dilutions were performed five times and the experimental dilutions (10^{-6} – 10^{-8}) were then divided into four groups. Each group corresponds to the dilution of TauCl the organism would be receiving: 150 mM, 300 mM, 450 mM, 600 mM TauCl and a control group which received PBS (expressed as 0 TauCl) giving a total of five groups.

3.3 Treatment of *S. epidermidis* with TauCl

S. epidermidis at the experimental dilution points was treated with TauCl such that each of the five groups received varying concentration of TauCl or PBS (the control group). One hundred microliters of TauCl at varying concentrations or PBS was placed into each of the experimental dilution tubes and lightly vortexed. Approximately 5 to 10 minutes was allowed for the organism to interact with TauCl in suspension. TauCl was freshly synthesized and the concentration was determined before every trial using the methods above (see TauCl synthesis) and stored at -4 ^{o}C. Dilution of TauCl to the experimental dilutions was performed directly before treatment.

3.4 Plating *S. epidermidis* and Plate counts

Treated *S. epidermidis* groups and the control group were plated onto BAP by taking 100µL aliquots of the organisms and placing it in the center of the plate. They were spread using aseptic technique and incubated for 24 hours at 37 ^{o}C. Individual colonies were then counted on each plate using a backlit Quebec colony counter. "TNTC" (too numerous to count) denotes a plate that had much more than 200 colonies – because of variation in counts, the counting range was moved up to accommodate plates that had less than 500 colonies but still more than 200.

Because the window of analysis for plate counts was too wide when plates with colonies greater than 500 were input, it was decided that the data should be ranked and expressed in a way that occurrences can be seen happening repeatedly within a certain range. The ranking system is discussed in further detail in the results section.

4. Results and Discussion

The results below express the growth of *S. epidermidis* at varying biological dilutions treated with the varying concentrations of TauCl. Plate counts performed had colonies within the ranges of 0-402 colonies per plate, per 100 µL aliquot of *S. epidermidis* and 100µL of TauCl. Colony counts above 500 were deemed too numerous to count (TNTC) The results altogether may indicate that there is an inverse relationship between the number of colonies of *S. epidermidis* observed on BAP and the concentrations of TauCl used to treat *S. epidermidis*. This relationship may be optimized within the concentration ranges of 300 mM TauCl and 450 mM TauCl.

Table 1. Plate counts at each dilution for each concentration of TauCl treatment

Plate Counts	0 TauCl	1 TauCl	0.75 TauCl	0.5 TauCl	0.25 TauCl
10^-6	TNTC	TNTC	TNTC	TNTC	TNTC
	TNTC	TNTC	TNTC	TNTC	TNTC
	TNTC	TNTC	TNTC	TNTC	TNTC
	TNTC	TNTC	TNTC	TNTC	TNTC
	TNTC	TNTC	TNTC	TNTC	TNTC
	TNTC	TNTC	TNTC	TNTC	TNTC
	TNTC	TNTC	TNTC	TNTC	TNTC
	TNTC	TNTC	TNTC	TNTC	TNTC
10^-7	TNTC	81	66	336	TNTC
	TNTC	34	58	228	TNTC
	TNTC	27	51	402	TNTC
	TNTC	33	78	105	TNTC
	TNTC	16	66	297	TNTC
	TNTC	15	89	237	311
	TNTC	72	84	224	366
	TNTC	45	71	194	337
10^-8	117	7	12	33	56
	210	3	23	38	45
	140	9	34	55	48
	108	2	26	6	89
	111	0	25	18	107
	127	2	31	40	64
	208	12	20	26	90
	134	0	25	37	69

Table 1. Each number represents the number of colonies counted on BAP. TNTC = >2000 colonies. The concentration of TauCl treatment is given at the head of each column and the dilutions of *S. epidermidis* for each plate count is given in the column to the left.

Table 2. Ranking of Plate Counts

Ranking System:	0 - 5
0	20>
1	20-100
2	100-180
3	180-260
4	260-340
5	340-420

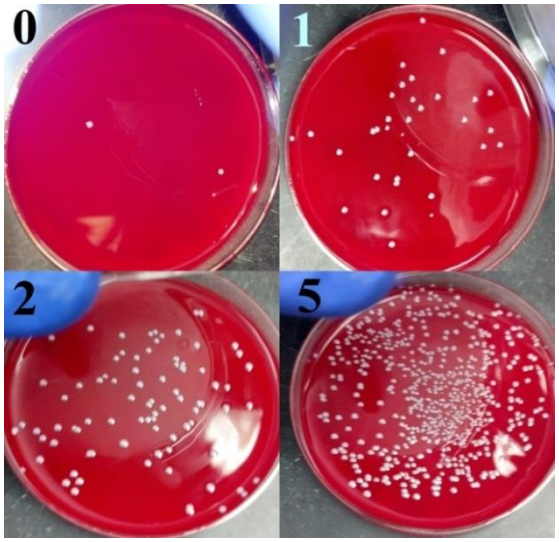

Table 2. The table shows the limits of colonies counted for each ranking type. Each plate was designated into a ranked group (0-5) based on the range in which the colony count fell. TNTC plates were not included in the distribution curve. To the right of the table is a visual aid for the some ranking system groups and how those plates presented in lab.

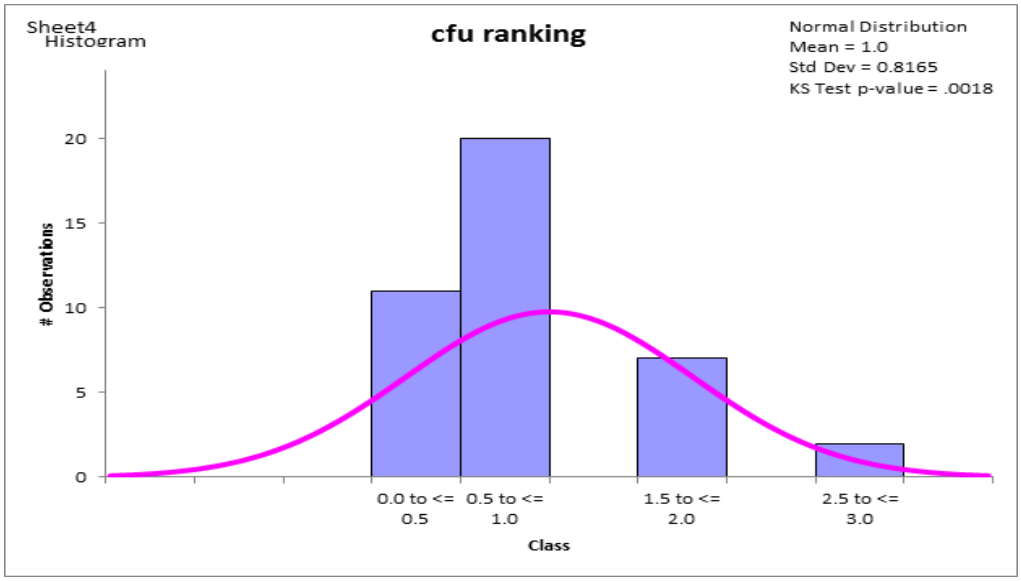

Figure 3. The colony counts per plate are given in Table 1 and the ranking system is given in Table 2. The ranking system allowed us to look at the number of similar occurrences within a particular range rather than individual plate counts which could skew results. The occurrences depicted give a bell-like distribution and a p-value was gained using KS test in order to analyze the data in a best-fit distribution.

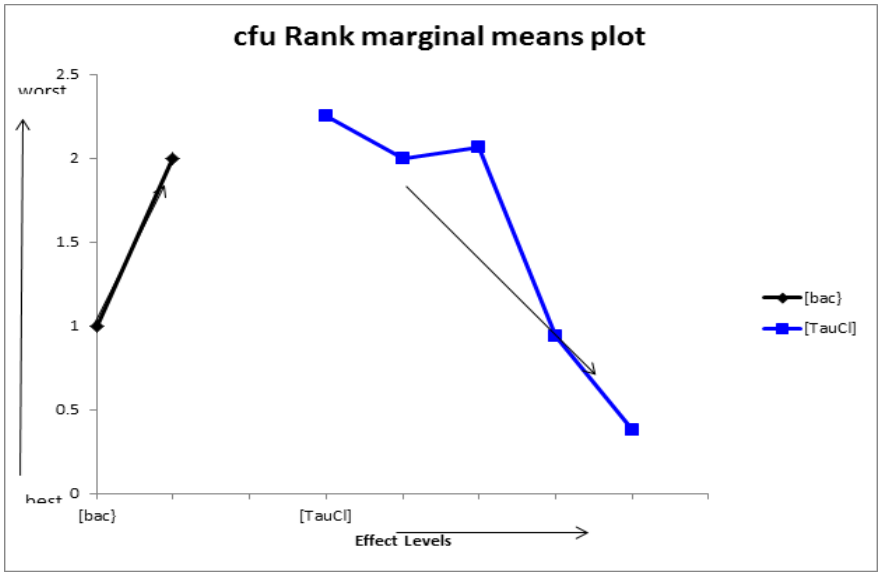

Figure 4. The figure depicts the plots of colony counts against bacterial dilutions against concentration of TauCl in a qualitative scale. The effectiveness of TauCl concentrations are relative.

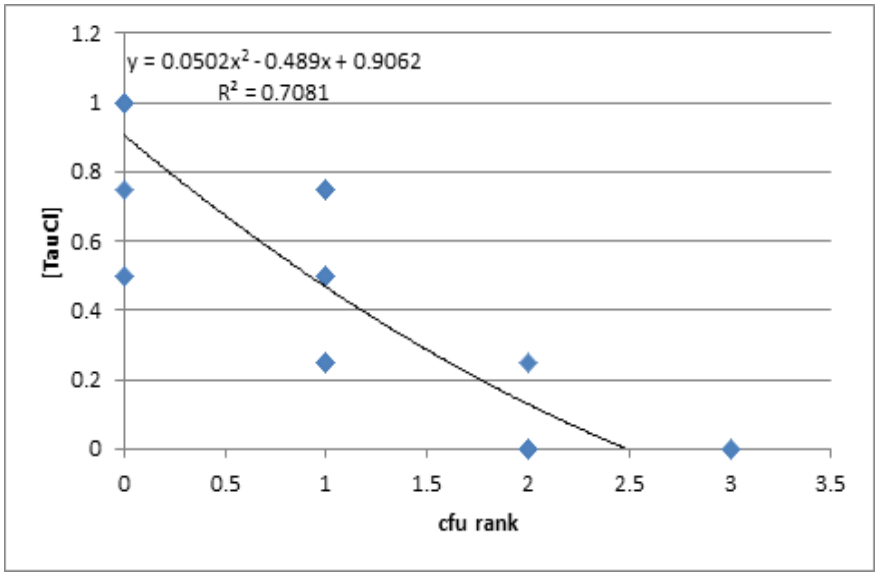

Figure 5. The plot shows the exponential curve derived from the ranked colony counts vs. concentrations of TauCl. The equation for the curve and R^2 are given above.

5. Discussion

It was found that TauCl did inhibit effectively the growth of *S. epidermidis in vitro*; however, the postulated experimental window was not within the postulated concentration range. The location of the range was found was from 0.5 TauCl to 0.75 TauCl for *S. epidermidis* at bacterial dilutions of 10^{-7} and 10^{-8}. The inhibition of growth was determined to be significant ($p = 0.0018$) using a Kolmogorov-Smirnov test. As concentration increases, there is a diminution in growth of *S. epidermidis*, although the decrease in growth is not as obvious. The CFU ranking system was developed to make data more manageable when analyzing. Because the method of getting colony counts was qualitative, the method of data analysis also had to be qualitative. Drawbacks of the project included bacterial

dilutions. The significant diminution of growth could be attributed to the dilutions performed and bactericidal activity of TauCl is not as significant.

Another possibility is that other halogenated taurine species may be reacting and inhibiting growth of *S. epidermidis* rather than TauCl (e.g. $TauCl_2$, reagent NaOCl, etc.). In order to evaluate this, it will be necessary for future research to purify TauCl which would allow TauCl to be analyzed with decreased possible interference from other factors. As of now, the claim that TauCl is the sole factor for diminution of growth observed cannot be claimed to be strictly due to TauCl, as dilution of organisms may have a larger role in the diminution of growth.

6. Acknowledgements

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7. References

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