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Anthranilic Acid Derivatives as Novel Antibiotics against MRSA and other Gram Positive Microorganisms: Combating Antibiotic Resistance

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ABSTRACT

Implementation of antibiotics to treat bacterial infections began during World War II. Since then, a number of antibiotic resistance microorganisms have emerged, one of these being Methicillin Resistant *Staphylococcus aureus* (MRSA). This resistance can be accredited to multiple factors, but the greatest contributors are the similarity in the chemical composition of the commonly prescribed antibiotics used to treat MRSA and the improper use and disposal of these agents. MRSA is the most frequent health acquired infection in the United States and to combat this growing problem, we have developed a novel class of antibiotics derived from anthranilic acids that show antibacterial activity against MRSA. Our derivatives record a minimum inhibitory concentration (MIC) ranging from 2-64 μg/ml, however when in the presence of human serum protein (HSP) this value increases, decreasing their effectiveness. We have identified a component of HSP, albumin, that causes the increase in MIC, and have determined that intermolecular hydrogen bonding is the cause of this increase. Using this information we are currently synthesizing new derivatives with a low binding affinity for albumin, or when bound do not lose antibacterial activity.

INTRODUCTION

In 1929 a microbiologist by the name of Alexander Flemming accidentally contaminated a microbial sample with the fungus *Penicillium*. After incubating the media for growth, he found that in areas where fungal growth was observed, bacterial growth was inhibited. By the year 1940 the antimicrobial properties of the fungus were investigated and then made available for medical use under the trade name penicillin (1). Penicillin functions as an exogenous metabolite belonging to the antibiotic family known as β-Lactams. Implemented into the health care system in the latter years of WWII, β-Lactams are still the most widely prescribed antibiotics in the world today. The effectiveness of the medications derived from this chemical class is attributed to its ability to inhibit the bacterial enzyme transpeptidiase. This enzyme is required to form crosslinks between the sugars of the peptidoglycan layers, a mechanism that inevitably leads to the formation of a viable bacterial cell wall (2).

As penicillin became widely used, a strain of *Staphylococcus aureus* was found to have conferred resistance to the antibiotic, and in 1959, methicillin, a synthetic variation of penicillin was introduced (as a member of the β-Lactam class) to ward off these infections. The longevity of its effectiveness only lasted two years, and in 1961 the first case of Methicillin-Resistant *Staphylococcus aureus* (MRSA) was described in the United Kingdom (3), with the first case reported in the United States in 1968 (4). In the years following its observance in the United States, studies involved with the isolation of MRSA in hospitals found that between 1975 and 1980 there was an increase in the number of isolates amounting to 467% (4).

Although infectious diseases, particularly those caused by bacterial microorganisms, are still among the top causes of mortality in the world, pharmaceutical support for the development of novel antibiotics against them continue to decline. Furthermore, when novel antibiotics are developed, they are introduced as modified derivatives of compounds already known to be effective (5). This practice has inadvertently become problematic; the lack of novel antibiotics to reduce natural selection and beneficial mutations in bacterial microorganisms has led to the emergence of antibiotic resistant organisms including MRSA, Vancomycin Resistant Enterococci (VRE), and Extreme Drug Resistant Tuberculosis (XDR-TB). Of these microorganisms, MRSA accounts for 1.9 million infections, 99,000 deaths, and health care costs amounting to over 5 billion dollars annually, and is the number one health acquired infection in the United States (6). It is for these societal concerns that novel antibiotics are generated, but this is a trivial task.

In addition to the inherent costs associated with drug development, there are multiple intermolecular interactions that occur *in vivo* between drug and host that must be taken into account including drug toxicity, dosage, and excretion. A major contributor to these interactions is the well-studied protein component of human serum, albumin. Accounting for 65% of serum composition, albumin functions as a transporter for fatty acids and endogenous and exogenous metabolites (7). Research has shown that albumin drug binding occurs primarily at two "Sudlow" sites located in subdomains IIA (site 1) and IIIA (site II) of the protein; as a result, binding sequesters the compounds deeming them inactive (8)which is yet another deterrent in developing novel drugs. However, as the advent of MRSA and other resistant microorganisms have emerged, the demand for new antibacterial reagents has caught the interest of pharmaceutical companies once again.

In contribution to this increased interest, our research group has developed a novel class of antibiotics derived from anthranilic acids. Our class varies in effectiveness with many compounds showing antibacterial activity *in vitro* at a minimum inhibitory concentration (MIC) ranging between 2-64 µg/ml; however, when in the presence of human serum protein (HSP) this value increases, decreasing their antibacterial activity. We have targeted albumin as the probable protein target responsible for this inactivating interaction. Our research group hopes to verify the role of albumin in the sequestration and inactivation of our novel antibiotics using computational biology software. By deducing if and how our compounds bind to albumin, we hope to use this information to develop derivatives with a lower binding affinity for HSP, or when bound do not lose antibacterial activity. If successful, the engenderment of such derivatives will add to the treatment options available to combat bacterial infections including MRSA, reducing antibiotic resistance and the costs associated with treatments currently administered.

METHODS

Antibacterial activity determination. Twenty two novel antibiotics were generated to test for antibacterial activity. The anthranilic acid derivatives were dissolved at .01g each in 0.2 ml DMSO and 0.8 ml of 0.1 molar KOH equating to a final concentration of 10µg/ml. Using *Staphylococcus aureus* and *Escherichia coli*, the effectiveness of the compounds were analyzed for antimicrobial activity by employing the Kirby-Bauer disk diffusion method (CLSI; 9, 10). Ten microliters of each test compound was added to the 6 mm sterile disks, followed by incubation at 35.7°C for 18-24 hours to stimulate bacterial growth. Our standard derivative, GV-2 (Fig. 1) was used as a positive control and was added at the same volume and concentration. After incubation, the agar plates were inspected and the zones of inhibition were measured. For each active compound, the minimum inhibitory concentration (MIC) was determined using standard microdilution protocol as prescribed by the Clinical and Laboratory Standards Institute (CLSI; 9, 10).

Albumin binding. Any compound with an MIC ≤ 64µg/ml was tested in a second MIC assay with the addition of 10% HSP to determine if serum inactivation was observed. To assess albumin binding, bovine serum albumin (BSA) was prepared at 20%, 10%, and 5% concentrations (final concentrations 2%, 1%, .05%) in autoclaved 18 M Ohms dH_2O solvent and was substituted for HSP in the dilution series using our standard test compound GV-2. BSA was used for the analysis based on a 76.36% sequence alignment between the BSA protein structure and Human Serum Albumin (HSA).

In order to determine whether our compounds were binding to albumin, the online docking server at http://swissdock.ch was used. SwissDock is a free web service that determines the most probable binding interactions taking place between a target protein and ligand based on the EADock DSS algorithm (11). Only three steps are required to start a docking assay: 1) users define a target protein, 2) a ligand is selected from the comprehensive list supplied or from one that has been uploaded by the user, and 3) the docking parameters are set (11). The target protein for our assay, albumin (PDB: 2BXN), was obtained from the RCSB Protein Data Bank.

To ensure a representative simulation, SwissDock runs a thorough check on the target protein and ligand(s) before running an assay (11). During this investigation, if any aspect of the protein structure is incomplete or defective and if the topology and tautomeric form of the ligand is incorrect, the assay is rejected (4, **11**). The albumin structure we selected was found to have 39 missing residues (http://www.rcsb.or/pdb/files/2BXN.pdb). To correct this issue, the structure was patched using the Visual Molecular Dynamic software package. We generated our ligand (GV-2) using the Avogadro interface to ensure that the ligand met the criteria for the simulation. After completing these manipulations, albumin and GV-2 were uploaded into SwissDock and was accepted for the assay. The docking parameters were set to an accurate screening of the entire protein with a flexibility range within 3Å of any atom of the ligand in its reference binding mode.

RESULTS

Of the 22 compounds that were synthesized, 13.6% showed antibacterial activity against *Staphylococcus aureus* (Table 1). Of those that showed activity, compound KU1-1-A had the greatest efficiency with a MIC equating to 16µg/ml. A secondary screening for antibacterial activity completed in 10% human serum protein on compounds with a MIC ≤ 64µg/ml showed an increase in the initial MIC recorded (Table 1). The results from the BSA MIC assay demonstrated that GV-2 did bind to albumin and its inactivation was concentration dependent upon the protein (Table 2). Thereafter, HSA was obtained from Sigma Aldrich and a MIC assay conducted using HSA demonstrated results that were consistent with that of BSA's MIC results (Table 2).

After this determination had been made, and the GV-2/albumin targets were uploaded into the SwissDock web interface, the output file of the binding interactions between GV-2 and albumin was visualized using Chimera 1.8 (12), which verified binding was occurring (Fig. 2). Binding interactions were considered significant if the interactions between atoms of GV-2 and the molecules of albumin were within 2 Å. Based upon this criterion, analysis

showed that the binding mode for the interactions between GV-2 and albumin is primarily due to intermolecular hydrogen bonding (Fig. 2) between the carboxylic acid functional group of the aromatic ring and the side chain residues spanning albumin in the region associated with both drug site I and II, with more interactions occurring within drug site II (Table 3).

After analyzing the binding data, it was proposed that coupling GV-2 would lead to a compound that was not inactivated *in vivo*. The theory was that coupled derivative would bind albumin while leaving the other end free and active, assuming a second albumin protein molecule was unable to bond the free end due to unfavorable electrostatic interactions. Five compounds meeting this structural proposition were synthesized by varying the chain length in the aliphatic region of GV-2, which was then coupled to another molecule of the same makeup (Fig. 3). The results of the disk diffusion assay did not uphold the proposition in that no antibacterial activity was observed.

DISCUSSION

It is well documented that binding to HSA controls the free, active concentration of a drug, provides a repository for a long duration of action, and ultimately affects drug absorption, metabolism, distribution and excretion (8). At one time these interactions were a major deterrent in generating novel drugs because of the unknown role of albumin on drug binding; however, the advancements that have been made in bioinformatics has revealed the mechanisms of action for albumin binding and in effect has increased pharmaceutical interests. With the information obtained from such molecular investigations, drug developers have been able to exploit albumin's high binding affinity for anionic and hydrophobic fatty acid compounds. This has led to the anchoring of novel pharmaceuticals to albumin binding molecules utilizing the protein's carrier capabilities and also to effective dosage schedules empirically devised for highly albumin – bound drugs (8).

The bioinformatics approach we have added to our study has also provided our team with some insightful information. Research conducted in our lab in the years preceding this investigation has shown that removal of the carboxylic acid functional group on the anthranilic core deems the synthesized derivatives inactive - results aligning with the information obtained from the binding analysis conducted in this study. Further analysis of the drug site for which GV-2 binding was most favorable was also of importance; the hydrophobicity of the binding cavity and the positively charged pocket entrance creates an environment that is attractive to the anionic, fatty acid chemical makeup of GV-2 and most of our derivatives. This information was unknown and with its revelation our team is synthesizing derivatives by modifying the hydrophobicity and hydrophilicity of GV-2 in an effort to create derivatives with a decreased binding affinity for HSP or when bound that do not lose its antibacterial activity. Although the five compounds we have synthesized reflecting the change did not show any antibacterial activity, we believe the reason to be because they were not in concordance with the weight limit of antibacterial compounds prescribed by Lipinski's rule of five, and we are currently making modifications so that these criteria are met.

In contrast to the positive information provided by the protein docking assay the SwissDock interface has its limitations. Due to the complexities associated with predicting the binding affinity between ligands and targets, SwissDock does not provide this type of data; without having such information, alternative intermolecular interactions that may be contributing to GV-2 inactivation, such as, Van Der Wall forces or ionic bonding has not been accounted for in this study. Furthermore, the interface predicts binding for one ligand at a time. This may not be a true representation of what is occurring *in vivo* due to albumin's ability to bind more than one ligand simultaneously by exposing other putative binding sites within the protein. Although these areas have not been addressed in this study, these limitations do not negate the results obtained in that many drug developers have found success using SwissDock and similar docking servers.

The implications of our research endeavors are far reaching. The amount of infections attributed to MRSA has seen a steady increase worldwide and is not only a common HAI but has also become a common community acquired infection as well. The decreasing availability of antibiotics once effective in treating MRSA increases the potential for more deadly encounters with the superbug. Modifying our class of compounds so that they do not lose antibacterial activity *in vivo*, would allow their implementation into the health care system, which will effectively decrease morbidity and the amount of money spent on treating infections caused by MRSA and other antibiotic resistant organisms worldwide.

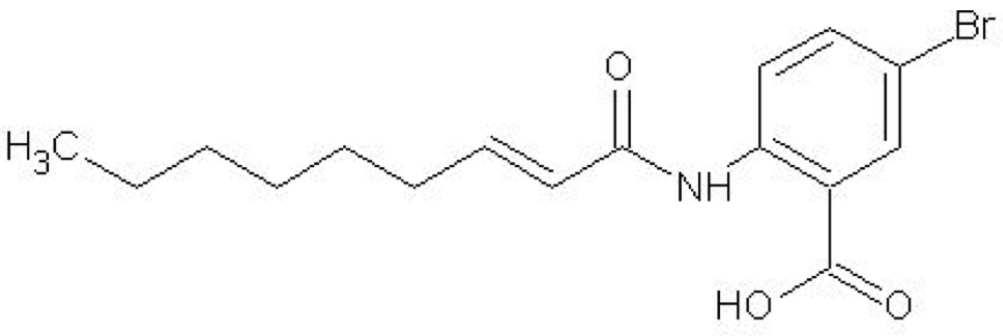

Fig. 1. Chemical structure of GV-2 (5-bromo-2-[(2E)-non-2-enoylamino] benzoic acid).

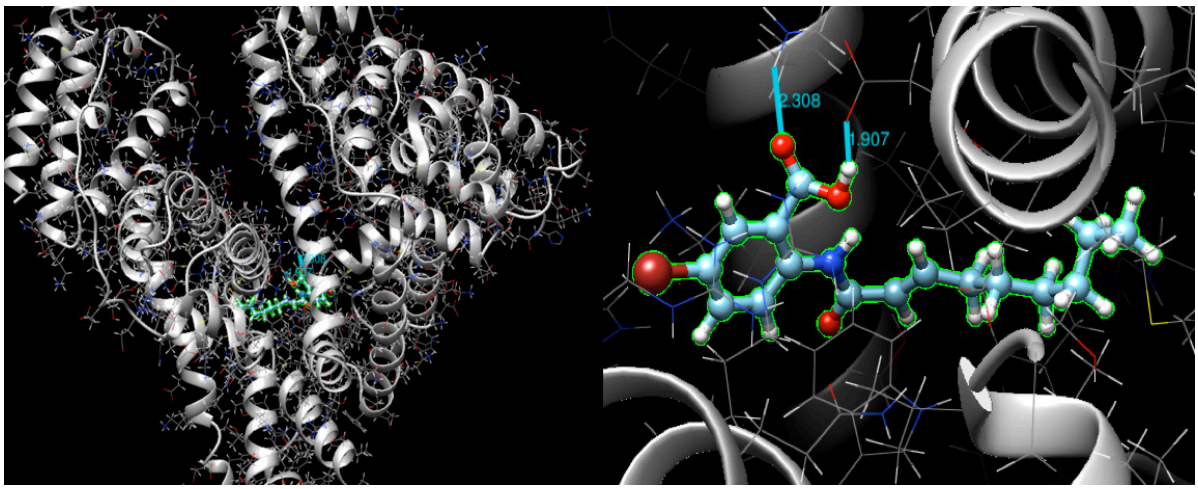

Fig. 2. A depiction of GV-2 bound to human serum albumin between the carboxylic acid functional group and albumin side chain residues by means of intermolecular hydrogen bonding.

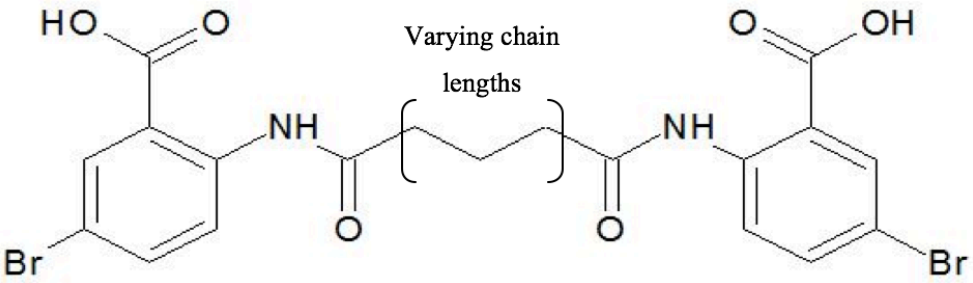

Fig. 3. Proposition for new chemical derivative based on the coupling of GV-2

Compound	Zone (mm)	MIC (µg/ml)	MIC W/10% HSP (µg/ml)
GV-2	28	16	256
AAB-1-10	24	128	N/A
AAB-1-15	0	N/A	N/A
KU1-1-A	25	16	128
KU1-4-A	0	N/A	N/A
KU1-5-A	0	N/A	N/A
AAB-1-19 (basic)	0	N/A	N/A
AAB-1-20	0	N/A	N/A
AAB-1-21	0	N/A	N/A
KU1-21 A	0	N/A	N/A
KU1-22 A	0	N/A	N/A
KU1-30 A	0	N/A	N/A
AAB-1-27	0	N/A	N/A
AAB-1-28	0	N/A	N/A
AAB-1-29	0	N/A	N/A
AAB-1-30	0	N/A	N/A
AAB-1-31	0	N/A	N/A
AAB-1-32	0	N/A	N/A
KU-1-36-C	20	64	256 (inconclusive)
KU-1-39-C	0	N/A	N/A
KU-2-7-A	0	N/A	N/A
KU-2-8-A	0	N/A	N/A
KU-2-5-B	0	N/A	N/A

Table 1. Antibacterial activity of GV-2 chemical derivatives against Staphylococcus aureus. The zone of inhibition was measured on MH agar plates and the minimum inhibitory concentration was determined in the absence or presence of 10% human serum protein.

Compound	Final Concentration	MIC W/O BSA (µg/ml)	MIC W/BSA (µg/ml)	MIC W/O HSA (µg/ml)	MIC W/HSA (µg/ml)
GV-2	2%	16	512<X	Not tested	Not tested
	1%	32	256<X<512	Not tested	Not tested
	0.05%	16	64<X<128	16	256

Table 2. MIC of GV-2 in the presence of bovine and human serum albumin at 2%, 1% and 0.05% concentrations. Human serum protein was not subjected to testing at 2% and 1% concentrations because of our interest in comparing the lowest concentration of bovine serum albumin that produced a MIC < 256 µg/ml.

INTERACTING GROUPS		AVERAGE DISTANCE INTERMOLECULAR BONDING (Å)					
GV-2 Functional Group	Residue	2BXN_TRAIL 2	2BXN_TRAIL 4	2BXN_TRAIL 5	2BXN_TRAIL 8	2BXN_TRAIL 9	ALL TRAILS
Hydroxyl	SER 202	N/A	N/A	1.838	N/A	N/A	1.838
	GLU 297	1.818	N/A	1.819	N/A	N/A	1.818
	ASP 451	1.931	N/A	1.907	1.828	1.793	1.863
	ASP 512	N/A	1.821	1.846	1.876	1.896	1.860
	GLU 520	N/A	N/A	1.892	N/A	N/A	1.892
	GLU 531	N/A	N/A	N/A	1.991	N/A	1.991
	MET 548	N/A	N/A	1.830	N/A	N/A	1.830
	ALA 552	N/A	N/A	1.927	N/A	N/A	1.927
Linear C=O	LYS 199	1.942	N/A	1.885	1.933	1.835	1.899
	TRP 214	1.963	N/A	1.916	1.884	N/A	1.921
	ASN 405	N/A	1.980	N/A	N/A	N/A	1.980
Aromatic C=O	LYS 199	1.932	N/A	N/A	N/A	N/A	1.932
	ASN 405	N/A	1.980	N/A	1.719	1.975	1.891
	LYS 525	1.946	1.916	N/A	N/A	N/A	1.931
Nitrogen	THR 508	N/A	1.969	N/A	N/A	N/A	1.969

Table 3. Binding interactions occurring within 2 Å of albumin side chain residues and atoms of GV-2 as determined using SwissDock interface and Chimera 1.8.

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