1   # Pentamidine inhibition of streptopain attenuates *Streptococcus*

2   # *pyogenes* virulence

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4   Running title: Small molecule SpeB inhibitors

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19   screening

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22   **ABSTRACT**

23   The obligate human pathogen Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) carries high

24   morbidity and mortality, primarily in impoverished or resource-poor regions. The failure rate of

25   monotherapy with conventional antibiotics is high, and invasive infections by this bacterium

26   frequently require extensive supportive care and surgical intervention. Thus, it is important to find

27   new compounds with adjunctive therapeutic benefits. The conserved secreted protease streptopain

28   (Streptococcal pyrogenic exotoxin B; SpeB) directly contributes to disease pathogenesis by

29   inducing pathological inflammation, degrading tissue, and promoting the evasion of antimicrobial

30   host defense proteins. This study screened 400 diverse off-patent drug and drug-like compounds

31   for inhibitors of streptopain proteolysis. Lead compounds were tested for activity at lower

32   concentrations and anti-virulence activities during *in vitro* infection. Significant inhibition of

33   streptopain was seen for pentamidine, an anti-protozoal drug approved for the treatment of

34   pneumocystis pneumonia, leishmaniasis, and trypanosomiasis. Streptopain inhibition rendered

35   GAS susceptible to killing by human innate immune cells. These studies identify unexploited

36   molecules as new starting points for drug discovery and a potential for repurposing existing drugs

37   for the treatment of infections by GAS.

38

39   **IMPORTANCE**

40   *Streptococcus pyogenes* (group A strep; GAS) is a common cause of severe invasive infections.

41   Repeated infections can trigger autoimmune diseases such as acute rheumatic fever and rheumatic

42   heart disease. This study examines how targeting a specific, highly conserved virulence factor of

43   the secreted cysteine protease streptopain, can sensitize a serious pathogen to killing by the

44   immune system. Manipulating the host-pathogen interaction, rather than attempting to directly kill

45   a microbe, is a promising therapeutic strategy. Notably, its benefits include limiting off-target

46   effects on the microbiota and potentially limiting the development and spread of resistance.

47   Streptopain inhibitors, including the antifungal and antiparasitic drug pentamidine as identified in

48   this work, may therefore be useful in the treatment of *S. pyogenes* infection.

49 **INTRODUCTION**

50 Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) is a top cause of infectious mortality and

51 is responsible for over half a million annual deaths worldwide (1). Humans are colonized

52 throughout childhood, leading to bouts of pharyngitis or pyoderma, but often without overt

53 symptoms of disease (2). Rheumatic heart disease can follow repeated infections, while any

54 infection can become more invasive, leading to cellulitis, necrotizing fasciitis, scarlet fever, or

55 sepsis. GAS remains sensitive to penicillin antibiotics, which are a mainstay of pharyngitis ("strep

56 throat"). However, this monotherapy is typically insufficient during invasive infections, which is

57 highly pro-inflammatory (3) and can require adjunctive antibiotics, surgical removal of infected

58 tissue, and extensive supportive care (4). The case fatality of these severe infections is high in the

59 United States and worse in resource-limited environments where infection is common.

60

61 The secreted cysteine protease SpeB is a primary virulence factor of GAS and is an attractive drug

62 target for several reasons (5). Among these, SpeB is unique to GAS, but highly conserved within

63 the species, is abundant during infection (6–8), and correlates with the severity of disease in

64 humans and in model infections (9–13). *In vitro* and *in vivo* experiments show that SpeB degrades

65 tissue (14, 15), inactivates immune antimicrobials (13, 16–24), and activates pathological

66 proinflammatory pathways through direct and indirect mechanisms (25–30). These activities

67 cumulatively contribute to both GAS growth and injury to the host (31). Consequently, SpeB

68 promotes pathogenesis in skin (32–34) and upper respiratory (26, 35) infection models in mice.

69

70 The aim of this study was to identify small molecule inhibitors of SpeB that would have therapeutic

71 potential for treating infections by GAS. The catalytic cysteine of SpeB, like most other cysteine

72   proteases, is highly reactive and can be inhibited by most common inhibitors of this family (5, 36).

73   However, broad-spectrum inhibitors would be unsuitable as anti-infectives due to targeting of

74   mammalian proteases leading to toxicity and immunosuppressive effects (37). Furthermore, in

75   addition to looking for strong activity against SpeB, we also considered potential accessibility to

76   the populations where it would be most needed. To these ends, we screened a diverse library of

77   off-patent drug and drug-like compounds that was developed by the Medicines for Malaria Venture

78   for the treatment of poverty-related diseases. All compounds of the library have been screened for

79   toxicity (38), a large fraction of them have been tested *in vivo*, and >10% are approved for use in

80   humans for at least one indication. Most potential drugs fail in pre-clinical or clinical studies;

81   repurposing of existing pharmaceuticals is an avenue to potentially bypass this bottleneck

82   inexpensively, quickly, and safely. We define several lead compounds with therapeutic potential.

83

84

85   **MATERIALS AND METHODS**

86   ***Bacterial strains***. GAS 5448, *S. aureus* F-182, and their growth are previously described (39–41).

87   Briefly, bacteria were routinely grown in Todd-Hewitt Yeast (THY; Difco) or on THY-agar at

88   37°C and 5% CO_2. Bacteria from overnight cultures were first washed and diluted in PBS in

89   preparation for infection experiments. Native SpeB was purified from culture supernatants by

90   ammonium sulfate (Sigma) precipitation followed by cleanup by size-exclusion chromatography

91   (ÄKTA FPLC, PE) and Centricon filtration (EMD Millipore), as previously described (24).

92

93   ***Chemical library.*** The Pathogen Box library compound was kindly provided by Medicines for

94   Malaria Venture (MMV, Switzerland) dissolved in DMSO and arrayed in 96-well format. Stocks

95   were duplicated in DMSO and stored at -80°C, and dilutions were made fresh for each experiment.

96   Test compounds in this library were selected as having a minimum of 5-fold selectivity over

97   mammalian cells for either *Plasmodium*, *Mycobacterium tuberculosis*, or kinetoplastid protists. 26

98   additional drugs, including conventional antibiotics (eg. doxycycline, rifampin, streptomycin,

99   linezolid), anti-infectives against non-bacterial pathogens (eg. praziquantel, nifurtimox,

100   miltefosine), and other common drugs (eg. fluoxetine, auranofin) were included as an additional

101   reference set. The lead hit pentamidine was validated with additional high purity compounded

102   purchased from commercial supplier (Sigma).

103

104   *Molecular docking*. The interaction of mature SpeB sequence of PDB:2uzj , and the chemical

105   structures of each antibiotic from PubChem was examined using the Chai-1 Model (42). Modeling

106   was agnostic with no input restraints specified, and the best scoring model was selected for analysis

107   in ChimeraX. Each molecule was oriented to the same position using Matchmaker to displaying

108   antibiotic occupancy in the substrate cleft of the enzyme, and red and blue used to indicate

109   computed surface electrostatics, as previously described (43).

110

111   *SpeB inhibitor screen.* In the initial screen, SpeB activity was measured in the presence of test

112   compound in individual wells using the substrate azocasein (Sigma) essentially as previously

113   described (32). Briefly, test compounds were added to achieve a 100 µM final concentration with

114   10 nM SpeB and 2% (w/v) casein labeled with azo dye (Sigma), all in PBS pH 7.4 with 2 mM

115   dithiothreitol (Sigma). Reactions were incubated 18 h at 37°C. Inhibition was observed if the

116   opacity of the solution remained comparable to controls to which no SpeB was added.

117

118   *SpeB inhibitor validation*. Kinetics of SpeB inhibition by compounds passing the first screen were

119   measured using the internally-quenched peptide sub103, Mca-IFFDTWK-Dnp (CPC Scientific),

120   as previously (32). In triplicate, dilutions of peptides were incubated in PBS with 5 mM

121   dithiothreitol and 0.01% Tween 20 with 10 nM SpeB and dilutions of test compound. The reaction

122   was incubated at 37°C and continuously monitored with fluorophore excitation at 323 nm and

123   emission at 398 nm on a Nivo plate reader (PerkinElmer).

124

125   **Neutrophil infection assays.** Whole human blood was collected from healthy adult donors with

126   informed consent and approval from the Institutional Review Board at Emory University. Blood

127   was collected into heparinized Vacutainer tubes, and primary neutrophils were isolated using

128   PolymorphPrep (Axis-Shield). Neutrophils were diluted to 10^5 cells/mL in RPMI containing 10%

129   FBS with no antibiotic, infected with 1×10^6 CFU of GAS for 60 minutes, then plated on THY

130   agar, essentially as previously (44). CFU were enumerated after overnight incubation of THY agar

131   plates at 37°C.

132

133   *Statistical analysis*. Statistical analyses were performed using Prism 10 (GraphPad). Values are

134   expressed as means ± standard error unless otherwise specified. Differences were determined using

135   the Mann-Whitney U test (paired) or the Kruskal-Wallis test with Dunn's post-analysis (multiple

136   groups) unless otherwise specified.

137

138   **RESULTS**

139   ***Initial screen***. The complex regulation of streptopain/SpeB (45–48) can lead to identification of

140   compounds whose impact on SpeB is indirect if tested in culture with live bacteria (49–51).

141   Therefore, we conducted a screen using purified SpeB to focus only on those compounds that

142   directly impact on the enzyme's ability to hydrolyze substrates. Azo dye-labeled casein was

143   incubated with purified SpeB in the presence of 400 compounds, each at 100 µM, and conversion

144   of the chromogenic substrate measured after 18 h incubation at 37°C. 9 test compounds inhibited

145   SpeB activity >50% across 2 independent runs (**Figure 1A**), for a hit rate of ~2% (**Figure 1B**). No

146   compound significantly increased apparent proteolysis of the azocasein substrate by SpeB.

147   Compounds were selected for this library based on having known bioactivity toward one or more

148   pathogens, possibly contributing to this enriched hit rate relative to similar screens (52).

149

150   ***Inhibition of SpeB by library compounds.*** Conversion of azocasein is a measurement of

151   cumulative substrate degradation over a prolonged period of time (32). To more sensitively

152   monitor the kinetics of proteolysis, we used the internally-quenched Förster resonance energy

153   transfer (FRET) peptide substrate sub103, which is highly selective and only contains a single

154   cleavage site for SpeB (32). Under these more stringent conditions, all compounds retained

155   inhibitory activity (**Figure 2**), but not to sub-micromolar concentrations, a typical target for

156   antiinfective drugs (37, 38). Nonetheless, these can be used to inform lead compounds for further

157   chemical refinement.

158

159   ***Structural similarity analysis.*** To determine whether there was any commonality between SpeB

160   inhibitors, with the expectation that compounds with similar chemical structures could be used to

161   identify additional compounds for testing, used molecular structure prediction modeling to

162 examine their predicted interaction with SpeB. By unguided analysis, each compound was

163 predicted to occupy the same region in the substrate pocket of SpeB (**Figure 3**). There was limited

164 structural homology between compounds, but this modality was consistent with the kinetic

165 analysis (**Figure 2**) that these compounds are competitive inhibitors for substrate binding.

166

167 ***Inhibition of SpeB by pentamidine.*** Since this library was targeted for neglected tropical diseases,

168 it additionally contained pentamidine as a positive control. While it is effective against several

169 eukaryotic pathogens, efficacy against bacteria and their toxins, and SpeB of GAS in particular,

170 has not been established. Pentamidine was predicted to occupy the catalytic site of SpeB similarly

171 to the established inhibitor E64, in its cocrystal structure (53) and by prediction (**Figure 4A, 4B,**

172 **4C**). Further testing pentamidine's efficacy at SpeB inhibition with titrations of drug, we find an

173 IC_{50} of 9.671e-09 (**Figure 4D**). This was suggestive that pentamidine could be effective at

174 inhibiting SpeB.

175

176 ***In vitro* activity of pentamidine against *S. pyogenes.*** SpeB is important for GAS resistance to

177 innate immune antimicrobials, including those produced by neutrophils (46, 54). To examine

178 whether SpeB inhibition would be useful for sensitizing GAS to neutrophil killing, we modeled

179 this important host-pathogen interaction *in vivo*. 10 µM had no impact on the growth of GAS

180 (**Figure 5A**) or *Staphylococcus aureus*, a bacteria included as a control for species-specific effects

181 (**Figure 5B**). Incubation with neutrophils led to significant decreases in the viability of GAS

182 (**Figure 5A**) and *S. aureus* (**Figure 5B**), and the killing of GAS was significantly increased by the

183 addition of pentamidine. This suggests the potential for pentamidine for sensitizing GAS to killing

184 by the innate immune system.

185

186 **DISCUSSION**

187 The Pathogen Box drug library has previously identified new inhibitors against a diverse set of

188 pathogens including *Toxoplasma gondii* (55), *Candida albicans* (56), *Giardia lamblia,*

189 *Cryptosporidium parvum* (57), *Vibrio cholerae* (58), *Acinetobacter baumannii* (59) and *E. coli*

190 (60). In our study examining GAS, ~2% of the compounds had significant protease inhibitor

191 activity toward SpeB. A previous screen of 16,000 compounds of the Maybridge HitFinder HTS

192 library had a hit rate of 0.018%, and identified a competitive inhibitor (52). Of our top 9 hits,

193 activity against *Plasmodium falciparum* has been shown by MMV689758 and MMV676603 (61,

194 62), *Mycobacterium tuberculosis* by MMV687254 (63), *Candida auris, Trypanosoma evansi*, and

195 *Schistosoma mansoni* by MMV022478 (64–66), and none could be found for MMV676588,

196 MMV676512, MMV637953, MMV023953, and MMV024101. Altogether insufficient support

197 could be found for any commonality in activity or mechanism between these compounds.

198

199 Pentamidine is a cationic aromatic diamine, which enters is known to interferes with polyamine

200 synthesis and RNA polymerase activity in protozoal cells. It is typically delivered by oral,

201 intravenous, and intramuscular routes, by topical delivery for skin disease and aerosolized delivery

202 for pulmonary diseases have seen some success (67, 68). These results argue another potential

203 utility for its use. Consistent with our findings, pentamidine has been observed to be an inhibitor

204 of the gingipains of *Porphyromonas gingivalis* (69). These secreted virulence factors are, like

205 streptopain, abundant and highly active cysteine proteases that target multiple host proteins to

206 allow the bacterium to resist killing by neutrophils and other immune effector mechanisms (70).

207 Additional pathogens encode cysteine protease virulence factors, leaving the possibility that

208 pentamidine may be more broadly useful as an inhibitor of bacterial virulence.

209

210 While other inhibitors of SpeB exist, such as the epoxide E-64 (N-[N-(L-3-trans-carboxyirane-2-

211 carbonyl)-L-leucyl]-agmatine), their broad-spectrum activities can also inhibit essential host

212 proteases (32). That pentamidine is already in clinical use argues that it can be tolerated. It has

213 additionally been seen to prevent IL-1 cleavage, suggesting it can inhibit the host cysteine protease

214 caspase-1 (71), without other obvious effects on the inflammasome (72). However, if this does

215 occur in physiologically relevant conditions, it is likely to still not impair the immune responses

216 because GAS is known to activate IL-1β and related cytokines independent of caspase-1 and other

217 inflammasome-associated proteases (27, 32), and in some infection modes may even be protective

218 (26). Lastly, there is significant overlap in the burden of the tropical diseases for which

219 pentamidine is used and the burden of GAS. The long duration of pentamidine administration as a

220 therapeutic or prophylactic typically required for these diseases has the possibility to impact

221 whether an individual is co-infected by GAS. No existent clinical data could be found to support

222 a correlation, which can be addressed in further studies examining disease incidence in these

223 populations.

224

225

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239

240   **Author contributions:** K.T. and C.L., conducted the studies, wrote the manuscript, and approved

241   the final manuscript.

242

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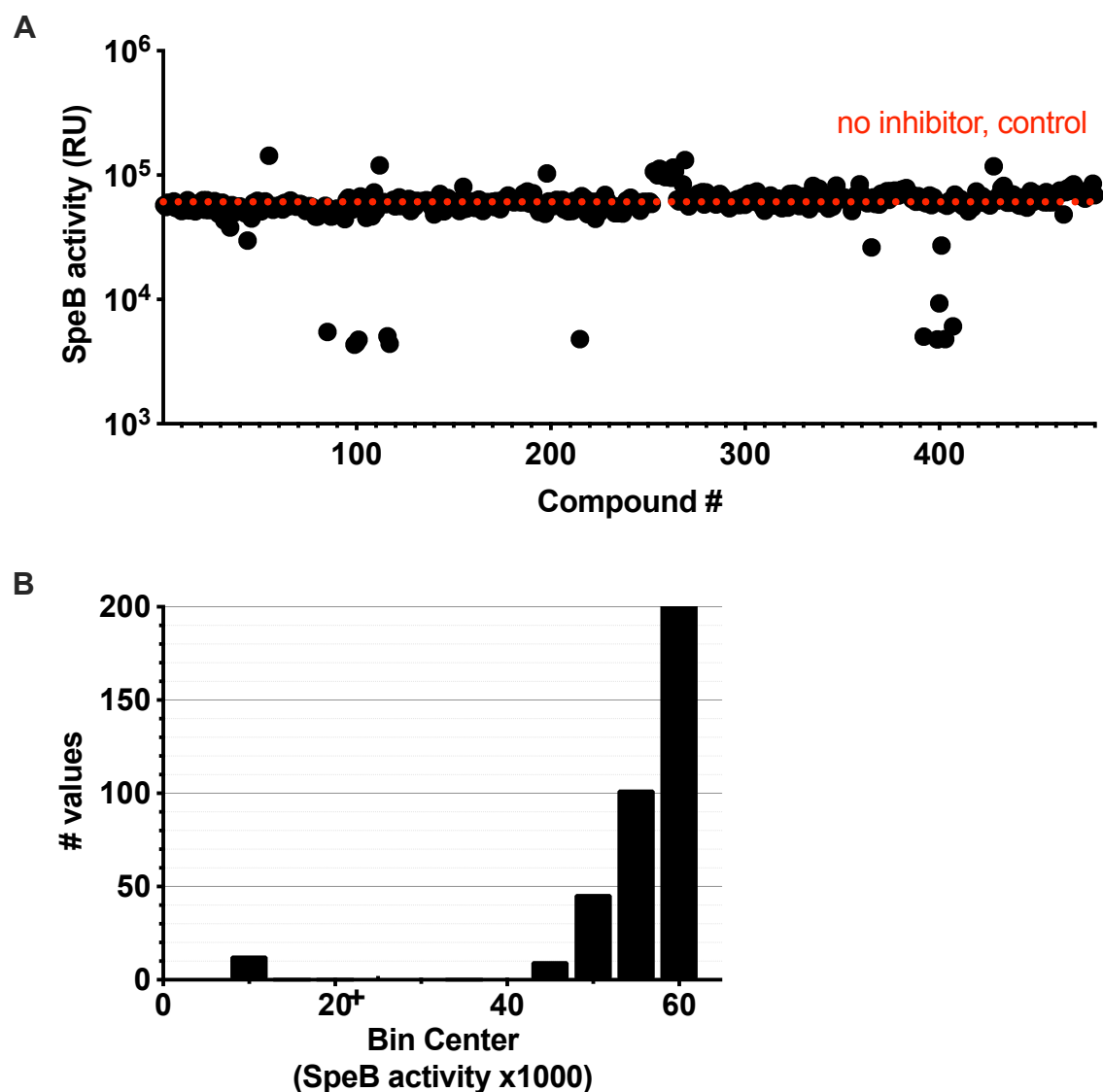
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FIG 1. Graphical representation of results from the primary screen for each of the 400 Pathogen compounds. (A) Each individual compound was tested at a concentration of 100 μM for hydrolysis of the substrate azo-casein. (B) Distribution of results.
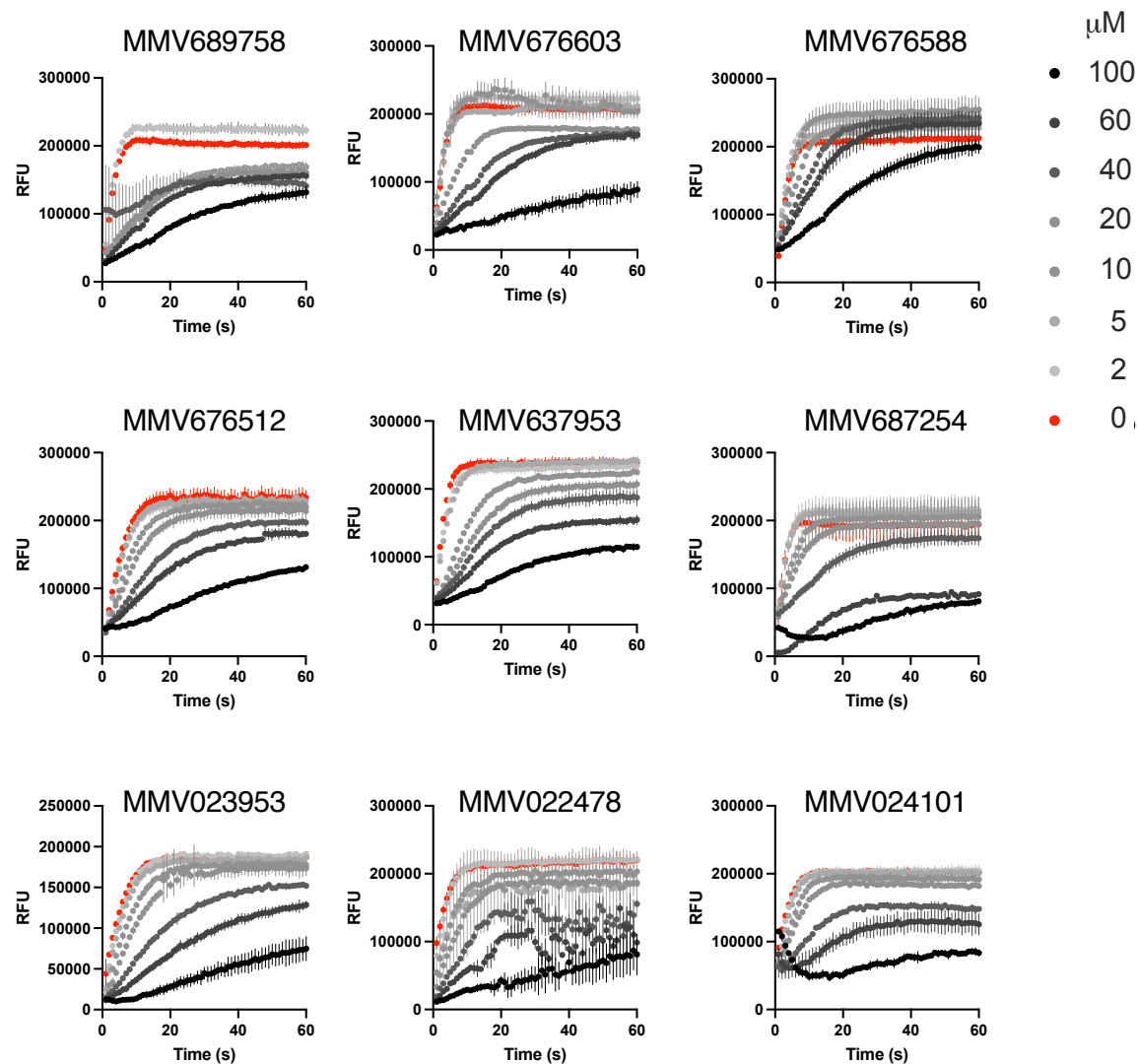
FIG 2. Activity of candidate compounds against SpeB. Cleavage of sub103 peptide after 30 min incubation with purified SpeB with each compound at the concentration indicated. N=4, error bars represent standard deviations.
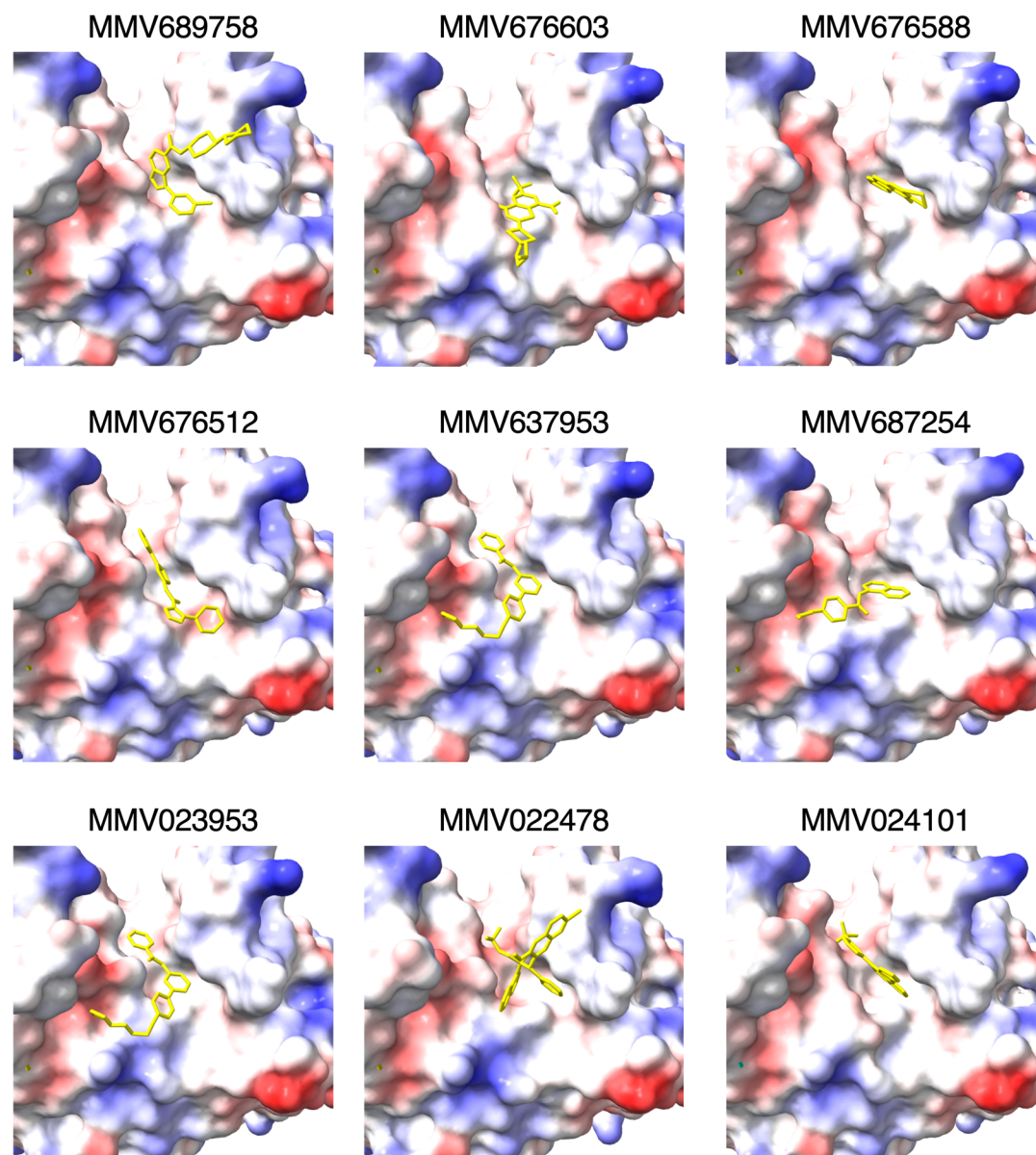
FIG 3. SpeB-inhibitor interactions. Models of the substrate pocket of SpeB and each indicated compound (yellow). Protein surface electrostatics are colored red and blue for negative and positive charges, respectively, and white color represents neutral residues.
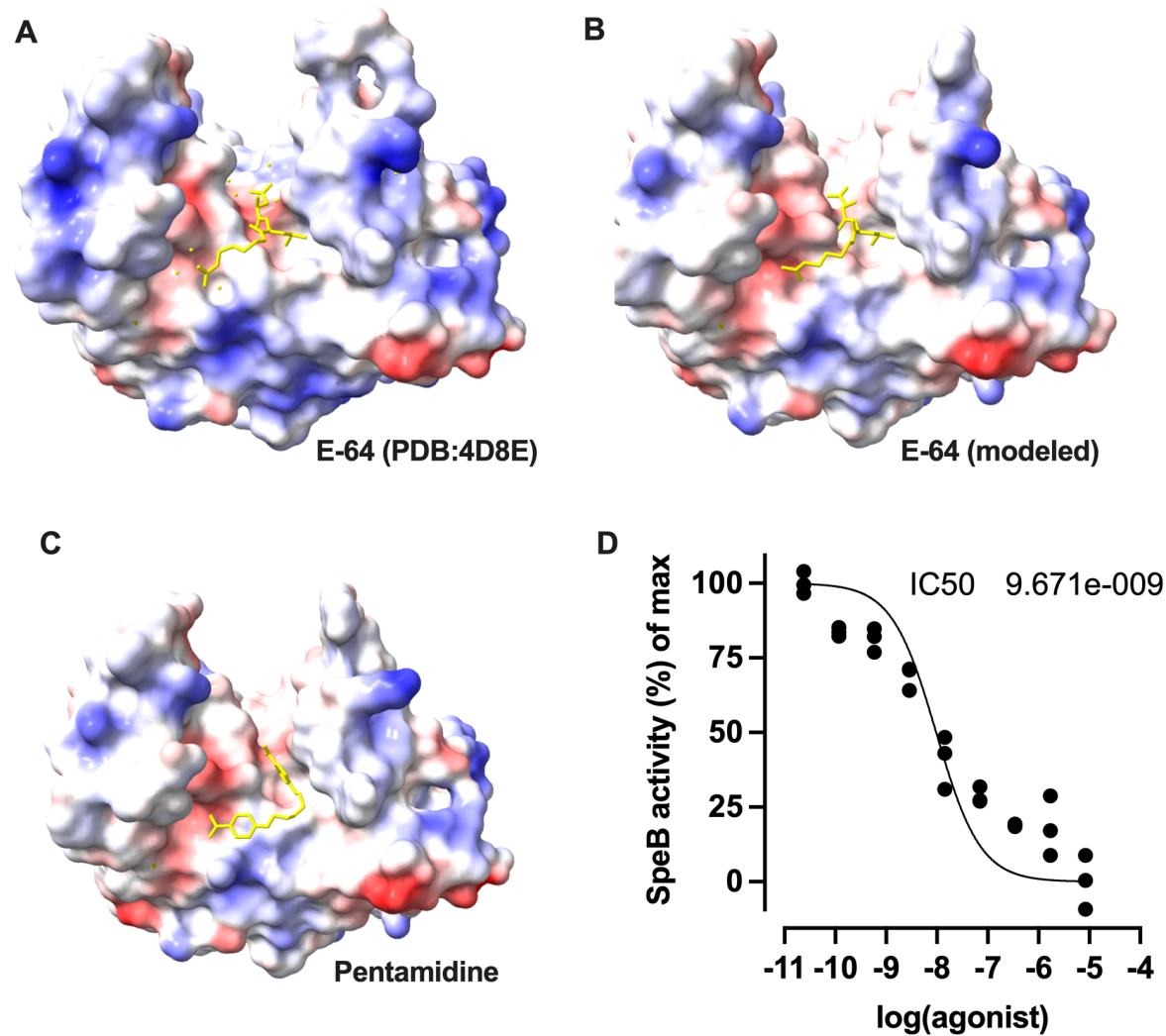
FIG 4. Pentamidine inhibits proteolysis by SpeB. (A-C) Models of the substrate pocket of SpeB and each indicated compound (yellow). Protein surface electrostatics are colored red and blue for negative and positive charges, respectively, and white color represents neutral residues. (D) Cleavage of sub103 peptide after 30 min incubation with purified SpeB was plotted against a range of pentamidine concentrations. N=4, error bars represent standard deviations.
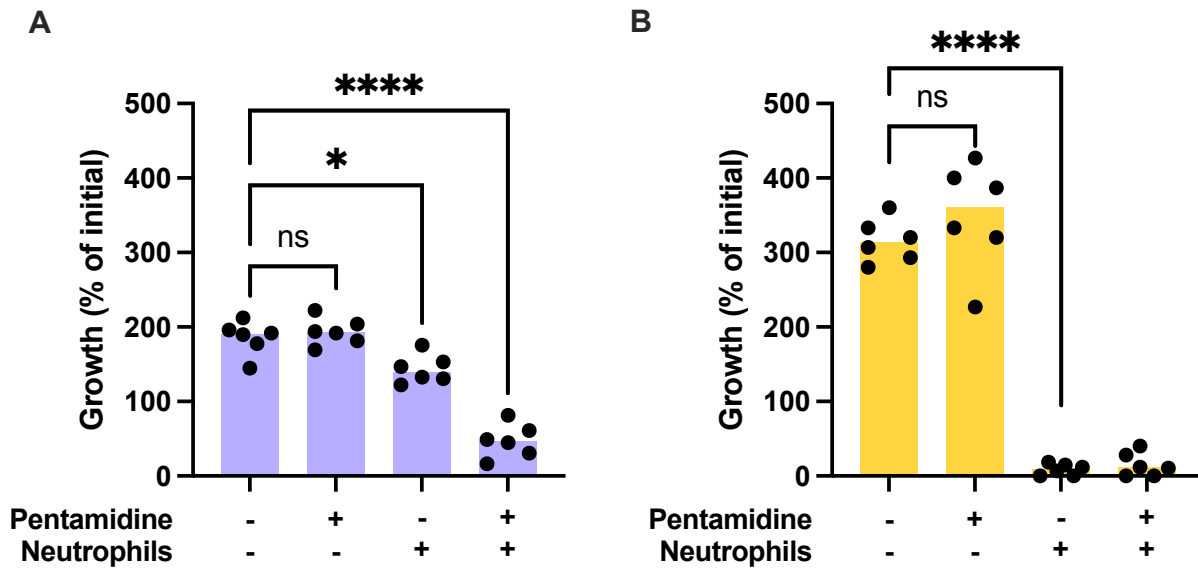
FIG 5. Pentamidine sensitized GAS to killing by neutrophils. Colony count of GAS (A) or *S. aureus* (B) after 2 hr infection of human primary neutrophils with 10 μM Pentamidine treatment. *P<0.05; ****P<0.00005 (ANOVA; Tukey post-test). Experiments were performed three times; error bars represent standard deviations.