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# Efficacy Evaluation of Iclaprim in a Neutropenic Rat Lung Infection Model with Methicillin-Resistant *Staphylococcus aureus* Entrapped in Alginate Microspheres

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## Abstract

**Purpose**: The objective of this study was to demonstrate the efficacy of iclaprim in a neutropenic rat lung infection model with methicillin-resistant *Staphylococcus aureus* (MRSA) entrapped in alginate beads.

**Methods**: An inoculum of $5.25 \times 10^4$ colony-forming units (CFU)/ml of *S. aureus* strain AH1252 was administered intratracheally to rats with prepared alginate bacteria suspensions. Beginning 2 hours post infection, rats received: (1) iclaprim 80 mg/kg (n=17); (2) iclaprim 60 mg/kg (n=16) or (3) vancomycin 50 mg/kg (n=24), for 3 days via subcutaneous (SC) injection every 12 hours. Twelve hours after the last treatment, rats were euthanized and lungs collected for CFU determination.

**Results**: Iclaprim administered at 80 mg/kg or 60 mg/kg or vancomycin 50 mg/kg SC twice a day for 3 days resulted in a 6.05 $\log_{10}$ CFU reduction (iclaprim 80mg/kg compared with control, $p < 0.0001$), 5.11 $\log_{10}$ CFU reduction (iclaprim 60 mg/kg compared with control, $p < 0.0001$), and 3.42 $\log_{10}$ CFU reduction, respectively, from the controls ($p < 0.0001$). Iclaprim 80 mg/kg and 60mg/kg resulted in a 2.59 and 1.69 $\log_{10}$ CFU reduction, respectively, from vancomycin treated animals (80mg/kg iclaprim vs. vancomycin, $p=0.0005$; 60 mg/kg iclaprim vs. vancomycin, $p = 0.07$). Animals receiving iclaprim, vancomycin and controls demonstrated 100%, 91.7%, 48.3% survival, respectively.

**Conclusions**: In this neutropenic rat *S. aureus* lung infection model, rats receiving iclaprim demonstrated a greater CFU reduction than the controls or those receiving vancomycin.



Word Count: 228

Keywords: iclaprim, vancomycin, pneumonia, alginate beads, *in vivo*

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) [1,2]. The annual incidence rate of healthcare-related MRSA pneumonia has increased from 11.3 cases per 100,000 patient-days in 2008 to 15.5 cases per 100,000 patient days in 2012 [3]. MRSA causes invasive infections, like HAP and VAP, that are associated with organ dysfunction, poor outcomes with excess morbidity and mortality and high costs to the healthcare system [4,5].

Iclaprim represents a novel diaminopyrimidine, which inhibits bacterial dihydrofolate reductase (DHFR) and is active against drug-resistant pathogens [6,7]. Iclaprim exhibits potent *in vitro* activity against *S*. *aureus* including MRSA [6], linezolid-resistant and vancomycin-resistant *S*. aureus [8] that cause pneumonia. Iclaprim demonstrates rapid *in vitro* bactericidal activity in time-kill studies in human plasma [9]. Because of these findings, iclaprim is potentially well suited for treating patients with pneumonia caused by susceptible and multidrug-resistant pathogens and the hypothesis of this study is that iclaprim would be effective and reducing colony forming units and prolonging survival among animals infected with MRSA compared to both vancomycin and controls.

The present study employed an experimental model of methicillin resistant *S*. *aureus* (MRSA) pulmonary infections as previously [10]. Notably, by encapsulating the bacteria within alginate, the infection model allowed for a lower inoculum to be utilized and a biofilm type environment established within the lung.

## Materials and Methods

*Collection of bacterial strains*

*S. aureus* AH1252 and AW6 were provided by IHMA and were gifts from Jose Entenza, Lausanne [11]. Strain AH1252 is a thymidine kinase-deficient mutant of the MRSA isolate AW6. *S. aureus* ATCC 29213 was used as CLSI quality control (QC) isolate for MIC determinations.

*Antimicrobial Susceptibility Testing*

MICs were determined for iclaprim and vancomycin by broth microdilution according to CLSI criteria [12,13].

*Preparation of bacteria*

Test isolates were grown overnight on trypicase soy agar (TSA) from frozen stock cultures. After overnight incubation, colonies were resuspended in saline and adjusted to an optical density of 0.1 at 625 nm. The adjusted suspensions were further diluted in a 2% alginate buffer, which was added dropwise in a ratio of 1:5 into 50 mM $CaCl_2$ to form alginate beads. The alginate beads were stirred during the dropwise addition and then for an additional 30 minutes to ensure that the beads were fully formed. The bacterial preparation in alginate beads allowed for the establishment of a prolonged infection due to reduced efficiency of bacterial clearance with a low inoculum input. Serial dilutions of the inoculum preparations were performed to determine inoculum size (colony forming units (CFU) per mL).

*Preparation of compounds*

Cyclophosphamide was prepared in sterile deionized water, and the mixture was vortexed and sonicated in a water bath sonicator until fully dissolved. Iclaprim was prepared by weighing

91 out the appropriate amount and dissolving in 30% propylene glycol vehicle. The preparation was
92 sonicated in a water bath sonicator until dissolved. Vancomycin was prepared in sterile deionized
93 water and vortexed to dissolve.
94
95 *Animal Acquisition and Acclimatization*
96 All procedures in this protocol were in compliance with the Animal Welfare Act, the
97 Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal
98 Welfare. Upon receipt at NeoSome Life Sciences, Sprague Dawley male rats (Charles River
99 Laboratories, Wilmington MA) were examined by personnel to ensure acceptable health status.
100 Veterinary care was provided by the veterinarians and staff employed by NeoSome Life Sciences.
101 Rats were acclimated for at least 5 days prior to testing. Rats were housed 3 per cage.
102 Cage size met or exceeded the requirements set forth by the ILAR Guide for the Care and Use of
103 Laboratory Animals. The animals were kept in a room maintained at 68 to 79°F (20-26°C) with
104 humidity set at 30 to 70%. The room was illuminated with fluorescent lights timed to give a 12-
105 hour light, 12-hour dark cycle. Rodent diet (Purina 5001) and water were available for all rats.
106 The feed was analyzed by the supplier detailing nutritional information and levels of specified
107 contaminants.
108
109 *Pre-treatment, Inoculation Procedure, and Treatment*
110 Rats were pretreated with cyclophosphamide monohydrate to render them neutropenic.
111 Based on literature review and previous experience with this model, rats were dosed
112 intraperitoneally (IP) on day -4 with 100 mg/kg. On day -1 rats received a second IP dose at 75
113 mg/kg [14, 15]. This regimen of neutropenia has been found to be effective in suppressing the

114 immune system of the rat for this model.

115 Rats were infected with prepared alginate bacteria suspensions while under isoflurane
116 anesthesia (4.5% isoflurane; 2.5 L/min $O_2$). Utilizing a sterile 20G, 3-inch stainless steel feeding
117 needle, a 0.5 mL volume was delivered via intratracheal inoculation (IT). Holding the
118 anesthetized rat in a vertical plane, the feeding needle was advanced into the trachea and the
119 volume was instilled. The rat was returned to its cage and allowed to recover from the anesthesia.

120 Beginning at 2 hours post infection, rats were randomized to treatment with iclaprim,
121 vancomycin or 30% propylene glycol vehicle (controls). Test articles were prepared fresh for
122 each day of dosing and formulated material was stored at $4^{O}C$, protected from light between the
123 two daily doses. Animals were dosed by body weight in a volume of 5 mL/kg subcutaneously.
124 These series of studies were designed to evaluate efficacy in a step-wise fashion. An initial dose
125 of iclaprim 80 mg/kg per dose was selected based on a previous studies in animal models of
126 infection, including a model of bacteremia and abscess (unpublished data). Two different
127 dosages of iclaprim (80 mg/kg and 60 mg/kg) were used to show comparability with other
128 iclaprim animal infection models and to establish which dose works best in this alginate bead
129 model. Vancomycin was selected as a comparator based on activity observed in other rats
130 models with difficult to treat infections [16, 17] and given its clinical use for staphylococcal
131 infection. Non-treatment infection control animals received vehicle (30% propylene glycol).
132
133 *Sample processing*
134 No samples were collected before euthanasia. At 74 hours post infection, rats were euthanized
135 by $CO_2$ inhalation. One group of rats was euthanized at 2 hours post-infection to determine
136 bioload at initiation of therapy. Rat lungs were aseptically removed, weighed, and homogenized

to a uniform consistency using a Polytron PT2100 with a 12 mm dispersing homogenizer (Bohemia, NY). The homogenized samples were serially diluted (10 fold dilutions) in sterile 0.9% saline and plated on TSA plates. The plates were incubated overnight at 37$^{o}$C and CFUs were enumerated by counting the plated colonies, adjusting for dilution factor and lung weight to obtain CFUs/ gram of lung. The recovered bacteria were MRSA. While not conducted routinely, spot checking of bacteria recovered from the lung homogenates were also plated on oxacillin containing media with the same results (same CFU count). This suggests the recovered organisms were MRSA. Some minor contamination is expected with this model, though it is at low levels and most often a fungus or mold, both of these are easily identified and can be discounted when quantifying the CFUs. This model has been previously validated and confirmed that the bacterial input and recovery are consistent. Additionally, the rats are maintained in a clean environment and are received from the vendor in good health (with health reports provided) which significantly limits the potential for contamination.

*Statistical analyses*

Group sizes of nine animals each were determined to be adequate through power analysis assuming 80% probablility and a standard deviation 0.5 $\log_{10}$ CFU. These numbers allowed for the detection of 0.7 $\log_{10}$ CFUs between groups. The average, standard deviation, and standard error of the mean (SEM) CFUs were calculated for each group of animals. One-way analysis of variance (1way ANOVA) with multiple comparison post test (Bonferroni) was used to compare the means of CFUs/ gram of lung between experimental groups at two time points, 2 and 74 hours post-infection. Specifically, comparisons were made of the 74 hour post-infection CFU / gram of lung and survival > 60 hours between both iclaprim dosing regimens (80 mg/kg and 60

mg/kg) compared to infection controls (vehicle alone), vancomycin compared to infection controls, and both iclaprim dosing regimens compared to vancomycin. A p-value $\leq 0.05$ was considered to be significant.

**Results**

Against isolates AW6 and AH1252, MIC values for iclaprim were 0.015 μg/ml for both and 0.5 μg/ml and 0.25 μg/ml for vancomycin, respectively.

Table 1 and Figure 1 show the CFU reduction and mortality by treatment. Rats infected with *S. aureus* AH1252 demonstrated an average bioload of 3.53 $\log_{10}$ CFU per gram of lung at the 2 hour initiation of therapy. Besides MRSA, no other microorganisms were identified in the lungs of any animals. Untreated infected rat lungs demonstrated an average bioload of 8.70 $\log_{10}$ CFU/gram of lung at 74 hours post infection, a 5.17 $\log_{10}$ CFU increase in bioburden over 72 hours. Iclaprim administered at 80 mg/kg subcutaneously twice a day for 3 days resulted in a 6.05 $\log_{10}$ CFU reduction from the 74 hour infection controls ($p$ <0.0001). Additionally, a 0.88 $\log_{10}$ CFU reduction was observed for iclaprim dosed at 80 mg/kg when compared with the bioload at initiation of therapy. This reduction suggests bacterial killing is occurring which was not observed with vancomycin under these study conditions. Iclaprim administered 60 mg/kg subcutaneously twice per day demonstrated activity in the rat lung infection model with a 5.11 $\log_{10}$ CFU reduction from the 74 hour infection controls ($p < 0.0001$). In comparison, vancomycin administered at 50 mg/kg subcutaneously twice a day for 3 days demonstrated a 5.28 $\log_{10}$ CFU/gram of lung bioburden, a 3.42 $\log_{10}$ CFU reduction from the 74 hour infection controls ($p < 0.0001$). Iclaprim 80 mg/kg and 60mg/kg resulted in a 2.59 and 1.69 $\log_{10}$ CFU reduction, respectively, from vancomycin treated animals (80mg/kg iclaprim vs. vancomycin,

183 $p$=0.0005; 60 mg/kg iclaprim vs. vancomycin, $p$ = 0.07).

184 Control animals infected with *S. aureus* had 48.3% (14 of 29) survival. In contrast,
185 animals receiving iclaprim had 100% survival (33 out of 33), while vancomycin-treated animals
186 had 91.7% (22 out of 24) survival (both iclaprim and vancomycin treated animals showed
187 increased survival compared to control animals, chi-square test, $p < 0.01$).

188

189 **Discussion**

190 This report demonstrates that iclaprim produces significant and sustained efficacy in the
191 current pulmonary model of lung infection due to MRSA, compared with vancomycin. The data
192 support the potential use of iclaprim in the treatment of staphylococcal pulmonary infections.
193 The combination of the alginate encapsulated bacteria and ensuing biofilm formation established
194 a bacterial growth environment that was difficult to treat and eradicate, providing a useful model
195 to test the ability of antibiotics to treat challenging pulmonary bacterial infections. Patients with
196 CF can have *S. aureus* pulmonary infections. Therefore, it is important that antibiotics aimed to
197 treat such infections be able to distribute and concentrate in the lung compartments.

198 A Phase 1 study investigated the tissue distribution of a single IV dose of iclaprim in
199 relevant lung compartments [18]. Iclaprim concentrations found in epithelial ling fluid (ELF)
200 and alveolar macrophages (AM) were up to 20- and 40-fold higher, respectively, than in plasma.
201 In addition, iclaprim concentrations in plasma, ELF and AM after a single IV dose of 1.6 mg/kg
202 exceeded iclaprim MICs for penicillin- susceptible *S. pneumoniae* ($MIC_{90}$ 0.06 mg/L) and
203 methicillin-resistant *S. aureus* ($MIC_{90}$ 0.12 mg/L) for up to 7 hours; mean iclaprim
204 concentrations in ELF exceeded the iclaprim MICs observed for *S. pneumoniae* with
205 intermediate penicillin resistance ($MIC_{90}$ 2 mg/L) and full resistance ($MIC_{90}$ 4 mg/L) for up to 7

206 and 4 hours, respectively, after a single dose.

207 *S. aureus* AH1252 strain, which is deficient in thymidine kinase, was used in this study
208 because it has been reported that the uptake of exogenous thymidine and it conversion into
209 thymidylate by thymidine kinase in certain bacteria, including *S. aureus*, antagonize with the
210 antimicrobial activity of the DHFR inhibitor trimethoprim *in vitro* [19]. It also is known that the
211 serum of rodents contains large concentrations of thymidine compared to human serum, i.e., ≥1
212 and ≤0.01 μg/ml, respectively [19]. This is the most plausible reason to explain why testing
213 trimethoprim or trimethoprim-sulfamethoxazole (TMP-SMX) can sometimes be ineffective in
214 staphylococcal infection models in rodents [12,17]. A previous study by Entenza *et al* [11]
215 described the use of *S. aureus* thymidine kinase-deficient mutants (unable to utilize exogenous
216 thymidine) in a*n in vitro* fibrin clot model employing iclaprim in the presence of rat and human
217 clots. The utility of the thymidine kinase mutants was evident, as thymidine no longer
218 antagonized the action of iclaprim in the rat. Thus, iclaprim demonstrated high efficacy in rat
219 (high thymidine) containing clots generated in the presence of these thymidine kinase deficient *S.*
220 *aureus* strains, but not, as expected, with the thymidine kinase producing wild type strains [11].

221 There are limitations to this study. First, only two doses and two timepoints with a single
222 inoculum challenge were used in this study. No pharmacokinetics (i.e., no blood or lung levels
223 of iclaprim or vancomycin) were performed because the two doses were based on previous
224 studies of iclaprim in animal infection models and mimicking exposures in patients (unpublished
225 data). Second, the starting inoculum in rodents was low compared to other models because by
226 encapsulating bacteria within alginate, the infection model allowed for a lower inoculum to be
227 utilized and a biofilm type environment established within the lung. Although this may lower
228 the therapeutic hurdle, all groups, control, iclaprim and vancomycin treated rats, received the

same starting inoculum. Furthermore, this starting inoculum is consistent with other published alginate bead pneumonia models [10]. Third, no microbiological samples or counts were collected or measured before euthanasia of the animals therefore the initial challenge might not be the same in the lungs.

This current pulmonary model of lung infection due to MRSA is consistent with results from a Phase 2 study showing activity of iclaprim in patients with nosocomial pneumonia. In the Phase 2 study, the clinical cure rates of two iclaprim dosages were compared with that of vancomycin in the treatment of patients with nosocomial pneumonia suspected or confirmed to be caused by Gram-positive pathogens; this study showed iclaprim and vancomycin to have comparable clinical cure rates and safety profiles in these patients [20]. The cure rates in the intent-to-treat population were 73.9% (17 of 23), 62.5% (15 of 24), and 52.2% (12 of 23) at the post-treatment test of cure visit in the iclaprim 0.8 mg/kg intravenous (IV) q12h, iclaprim 1.2 mg/kg IV q8h, and vancomycin 1 g IV q12h groups, respectively (iclaprim q12h versus vancomycin $p = 0.13$; and iclaprim q8h versus vancomycin $p = 0.47$). The death rates within 28 days of the start of treatment were 8.7% (2 of 23), 12.5% (3 of 24), and 21.7% (5 of 23) for the iclaprim q12h, iclaprim q8h, and vancomycin groups, respectively (no statistically significant differences).

Collectively, the current *in vivo* study, and previous Phase 1 and 2 clinical studies support the use of iclaprim development as a potential treatment for *S. aureus* pneumonia, including possibly among patients with biofilm mediated infection, as seen for example in CF.

Table 1 Colony forming unit change at 74 hours and survival at >60 hours by treatment groups

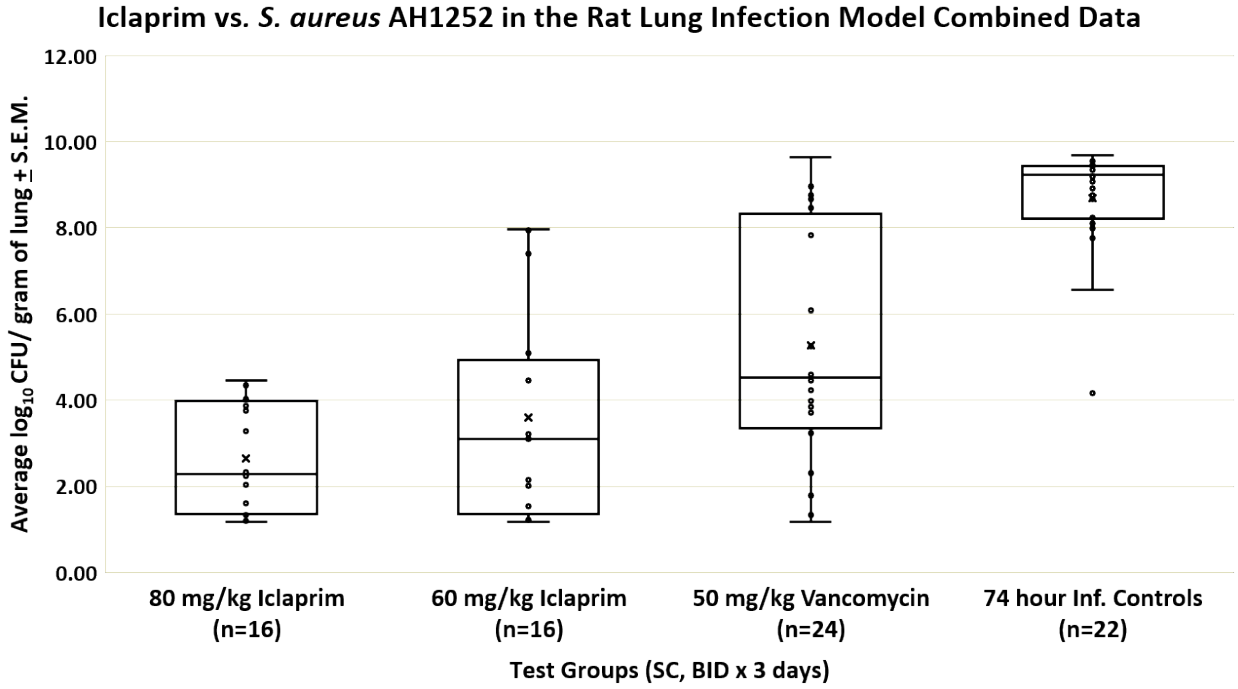
| **Group (number of rats)** | **Dose (mg/kg/dose)** | **Route / regimen** | **Survival > 60h** | **$Log_{10}$ Change in CFU at 74 hr.** | ***P*-value compared to control / vancomycin** |
|---|---|---|---|---|---|
| **Control (n=22)** | vehicle | SC/BID | 48.3% | NA | NA |
| **Iclaprim (n=16)** | 80 | SC/BID | 100% | -6.05 | <0.0001 / 0.0005 |
| **Iclaprim (n=16)** | 60 | SC/BID | 100% | -5.11 | <0.0001 / 0.0732 |
| **Vancomycin (n=24)** | 50 | SC/BID | 91.7% | -3.42 | <0.0001 / NA |

Abbreviations: NA, not applicable; SC, subcutanoues; BID, twice a day; CFU, colony forming unit; hr, hour

Figure Legend

Figure 1 The x and o indicate the geometric mean and individual rat log10 CFU/gram of lung, respectively. The box plot and whiskers indicate 25/50/75th percentile and <25/>75$^{th}$ percentile, respectively.

Figure 1 Average $log_{10}$ colony forming unit / gram of lung tissue at 74 hours among treatment groups




Abbreviations: CFU, colony forming unit; SC, subcutaneous; BID, twice a day

## Compliance with Ethical Standards

*Funding*

This study was funded by Motif BioSciences Inc., New York, USA.

*Conflicts of Interest*

DBH is an employee of Motif BioSciences. IM and SH are employees of IHMA. TM is an employee of NeoSome Life Sciences. MW has received consulting fees from Abbott Laboratories, Actelion, Astellas, AstraZeneca, Bayer, Biomèrieux, Cerexa, Cubist, Durata, The EuropeanTissue Symposium, The Medicines Company, MedImmune, Merck, Motif Biosciences, Nabriva, Optimer, Paratek, Pfizer, Qiagen, Roche, Sanofi-Pasteur, Seres, Summit, and Synthetic Biologics; lecture fees from Abbott, Alere, Astellas, AstraZeneca, Merck, Pfizer, and Roche; and grant support from Abbott, Actelion, Astellas, Biomèrieux, Cubist, Da Volterra, Micro-Pharm, Morphochem AG, Sanofi-Pasteur, Seres, Summit and The European Tissue Symposium, and Merck.

*Ethical Approval*

This research involved animals. All procedures in this research were in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare.

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