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Original Research

Alpha-1 Acid Glycoprotein as a Biomarker for Subclinical Illness and Altered Drug Binding in Rats

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Alpha-1 acid glycoprotein (AGP) is a significant drug binding acute phase protein that is present in rats. AGP levels are known to increase during tissue injury, cancer and infection. Accordingly, when determining effective drug ranges and toxicity limits, consideration of drug binding to AGP is essential. However, AGP levels have not been well established during subclinical infections. The goal of this study was to establish a subclinical infection model in rats using AGP as a biomarker. This information could enhance health surveillance, aid in outlier identification, and provide more informed characterization of drug candidates. An initial study (*n* = 57) was conducted to evaluate AGP in response to various concentrations of *Staphylococcus aureus (S. aureus)* in Sprague–Dawley rats with or without implants of catheter material. A model validation study (*n* = 16) was then conducted using propranolol. Rats received vehicle control or *S. aureus* and when indicated, received oral propranolol (10 mg/kg). Health assessment and blood collection for measurement of plasma AGP or propranolol were performed over time (days). A dose response study showed that plasma AGP was elevated on day 2 in rats inoculated with *S. aureus* at 10^6, 10^7 or, 10^8 CFU regardless of implant status. Furthermore, AGP levels remained elevated on day 4 in rats inoculated with 10^7 or 10^8 CFUs of *S. aureus*. In contrast, significant increases in AGP were not detected in rats treated with vehicle or 10^3 CFU *S. aureus*. In the validation study, robust elevations in plasma AGP were detected on days 2 and 4 in *S. aureus* infected rats with or without propranolol. The AUC levels for propranolol on days 2 and 4 were 493 ± 44 h × ng/mL and 334 ± 54 h × ng/mL, respectively), whereas in noninfected rats that received only propranolol, levels were 38 ± 11 h × ng/mL and 76 ± 16.h × ng/mL, respectively. The high correlation between plasma propranolol and AGP demonstrated a direct impact of AGP on drug pharmacokinetics and pharmacodynamics. The results indicate that AGP is a reliable biomarker in this model of subclinical infection and should be considered for accurate data interpretation.

Abbreviations: AGP, Alpha-1 acid glycoprotein; APP, acute phase protein; PK, pharmacokinetic; PK/PD, pharmacokinetic/pharmacodynamic; *S. aureus*, *Staphylococcus aureus*; SCM, sterile catheter material

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Protein binding is an important component of pharmacokinetic/pharmacodynamic (PK/PD) research. In vitro measurement of protein drug binding is an essential component of the research and development of novel drugs. However, in vitro studies often poorly mirror the in vivo condition.[9,42] Pharmacokinetic studies early in drug development provide a means to assess the time course of drug effects in the body and drug distribution and availability.[42] From a PK/PD modeling perspective, protein binding is an important factor in the kinetics and dynamics of drug availability in vivo.[21,35,36,40] These complex relationships are used to project efficacious doses in humans and take into consideration differences in plasma protein binding between preclinical species and humans.[8,44]

A variety of acute phase proteins (APP) exist across all species and increase in response to inflammatory, infectious and traumatic events.[5,9,12,13,19,21,22,29,45,53] APPs are potential biomarkers for detection and monitoring of various disease states including cancer.[2,18,24,34,39,40,47,50,52] Because of this, enhanced understanding of drug binding characteristics to APPs early in the development phase will promote the design of more efficacious therapeutics. Alpha-1 acid glycoprotein (AGP), a ubiquitous major APP that is present in rats,[9,46] has significant drug binding properties and binds to many basic and neutral compounds. Normal AGP levels in plasma of naïve rats range from 0.1 to 0.32 mg/mL.[44] The importance of AGP as related to drug discovery and development will be bolstered by greater understanding of the sources of AGP stimulation in established animal models. For example, AGP modulates the immune response in a rodent shock model in which it is thought to maintain normal capillary permeability to ensure perfusion of vital organs.[30,33] In addition, elevated AGP levels are present in animal models of infection and inflammation.[11,20,27,32,41,48]

In surgically modified animals, AGP levels may be elevated after surgical manipulation, which unavoidably induces local transient inflammatory responses.[8,25,51] In addition, infections may develop postoperatively leading to increased AGP levels. Chronic catheterization has been linked to increased incidence

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of infection.[3,8,37] Surgically modified animals should not be placed on study if aseptic technique was not adhered to during surgical preparation and instrumentation.[6,37] Contamination may occur within or at the external portion of a catheter, usually resulting in more obvious signs of infection. Routine PK studies in rats involve implantation of vascular catheters through which drugs are administered and blood samples are taken over time. Catheterized animals are typically perceived as being healthy and thus are enrolled in and remain on study unless they develop obvious clinical signs of infection or illness. However, an occult infection may be present even with a patent catheter. As such, understanding the direct effect of subclinical infection in modulating AGP levels and drug binding is critical, as AGP levels may affect drug levels in study animals with persistent subclinical infection. In this event, the PK data generated may be altered due to selective binding to AGP, thus confounding data interpretation.

A possible application of AGP is its potential utility as a biomarker for evaluating health status animals in drug development. The use of AGP as a select biomarker for monitoring and identifying sick animals and/or predicting the potential impact of subclinical infection on drug PK/PD is highly desirable. A screening tool such as this could help to optimize animal selection by reliably identifying healthy animals. Improved intrastudy health monitoring would promote confidence in PK/PD data and its predictive value.

The focus of this research was to develop a sensitive, reliable and reproducible model of subclinical infection in the rat using the ubiquitous skin contaminant, *S. aureus*. We selected AGP as a biomarker that would promote health status screening and enhance PK/PD characterization of AGP binding drugs (that is basic and neutral) in the presence or absence of subclinical infection. The model was validated by evaluating the impact of increased AGP levels on propranolol, a drug known to have high binding affinity to AGP.[4,7,10,26,28,31,49] Ultimately, establishing this model will provide heightened visibility of the protein binding characteristics of drugs and yield more informed data interpretation.

Materials and Methods

All animal care and procedures were conducted in accordance with the GlaxoSmithKline Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by GlaxoSmithKline's Institutional Animal Care and Use Committee (Collegeville, PA). The facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animals were cared for according to the *Guide for the Care and Use of Laboratory Animals*[23] and institutional standard operating procedures.

Animals. Male Sprague–Dawley rats (n = 73) weighing greater than 250 gm were purchased from the virus-antigen-free barrier at Charles River Laboratories (Kingston, NY). Rats were maintained under Biosafety Level 2 (BSL2) conditions in a temperature (standard room range 68 to 77 °F, room set point 72 °F) and humidity (range 30% to 70%, room set point 50%) controlled room with a 12:12 h light:dark photoperiod as per the *Guide for the Care and Use of Animals*.[23] Standard commercial diet (Rat Chow 5001, Purina Mills, Gray Summit, MO) and water were provided ad libitum. Microisolation caging was used exclusively and animals were pair-housed prior to the study. Animals were also given a variety of tactile and nesting enrichment. Animals were acclimated for a minimum of 1 wk prior to being placed on study and underwent a routine physical examination to determine study eligibility. Disease status monitoring was performed quarterly by dirty-bedding sentinel screening.

Test Agents and Preparation. ***Bacterial Test Agent.*** *Staphylococcus aureus* (*S. aureus;* ATCC#12598), was purchased from American Type Cell Collection (Manassas, VA). Single use stock concentrations of S. *aureus* for inoculations were prepared using sterile 0.9% saline (Baxter Healthcare, Deerfield, IL). Briefly, fresh inoculum was prepared by suspending 1 colony of *S. aureus* into 50 mL trypsin soy broth (Gibco Life Sciences, Grand Island, NY) and incubating at 37 °C for 18 h. The bacterial suspension was centrifuged using a Beckman Coulter Allegra 6R Centrifuge, (Beckman Coulter Life Sciences, Indianapolis, IN) at 3200 x g, 4 °C for 15 min. The bacterial pellet was washed once with phosphate buffered saline (Gibco Life Sciences, Grand Island, NY) with subsequent quantification of bacteria. Bacterial quantification was performed by using bacterial enumeration and working stocks formulated in 0.9% sterile saline (Baxter Healthcare, Deerfield, IL) to yield dosing concentrations of either 1×10^3, 1×10^6, 1×10^7, or 1×10^8 colony forming units (CFU) suspended in 0.1 mL of 0.9% sterile saline per inoculation dose as per study design.

Sterile saline (0.9%) was purchased from Baxter Healthcare (Deerfield, IL) for use as the vehicle control and was stored at room temperature prior to use.

Propranolol Test Agent. Propranolol (Sigma Aldrich Corporation, St Louis, MO) dosing solution (2.0 mg/mL) was freshly formulated in sterile water for oral delivery of 10 mg/kg to selected rats on each day of dosing.

Catheter Implant Preparation. Sterile, size 2 French, coated polyurethane catheters (Access Technologies, Skokie, IL) were prepared for implantation inside a BSL2 cabinet. For this, sterile catheter material (SCM) was aseptically cut into 2cm lengths and loaded into sterile 18g needles (Becton Dickinson, Franklin Lakes, NJ). The SCM needle complex was then packaged and resterilized using a hydrogen peroxide vapor sterilizer (Steris Corporation, Mentor, OH) and held in reserve for use in the study.

General In-Vivo Study Procedures. Depending on study design, and when indicated, a rat received either one injection subcutaneous (SC) of vehicle control (0.9% sterile saline) or one SC injection of a select concentration of *S. aureus* with or without concurrent subcutaneous implantation of SCM in the region overlying the scapulae. In preparation for this, the rat was anesthetized and maintained on isoflurane (approximately 2%) inhalation anesthetic (Henry Schein Animal Health, Dublin, OH) and given thermal support for the duration of the procedure and anesthetic recovery period. The rat was placed in ventral recumbency, the hair clipped from the skin overlying the scapulae, and the site aseptically prepared for subcutaneous injection of the test agent (propranolol) or for SCM implantation. The skin was then aseptically marked with an "X" to define the site of inoculation and/or implant and to serve as a reference mark for follow-up clinical surveillance over the course of the study. The rat was immediately transferred to a BSL2 cabinet and remained under inhalational anesthesia for the remainder of the procedure to ensure maintenance of asepsis during inoculation and/or implantation, as described below. For inoculation or implantation, a sterile 18-gauge needle was placed transcutaneously for use as a trocar and was gently advanced through subcutaneous tissue to the site intended for propranolol injection or SCM implantation. A 1 mL syringe (Becton Dickinson, Franklin Lakes, NJ) was preloaded to deliver 0.1 mL of either vehicle control or a select concentration of *S. aureus* for injection into the site. If a rat was slated for a SCM implant, a sterile preloaded 18g needle containing the implant was inserted and advanced just caudal to the reference mark, and a sterile stylet was used to advance the implant into the subcutaneous space. Once the SCM was implanted, the stylet was removed with the needle remining

in place for final delivery of a select inoculum. As previously described, a preloaded syringe containing a specific dose was then attached to the needle, and the contents injected into the subcutaneous space. The needle was then carefully retracted, and the skin gently compressed around the needle upon exit to prevent inoculum efflux and to secure the catheter placement.

In-vivo Study Designs. First, a study was conducted to determine the most appropriate dose of S. *aureus* for inducing a reliable subclinical infection in rats. Next, the model was validated using propranolol, which is known to strongly bind to AGP.

The first study (Study 1) measured the AGP response to varying concentrations of *S. aureus*. For this, rats were assigned to one of 5 groups (n = 5 per group) and received a single subcutaneous inoculation on day 0 of either vehicle control or a select concentration of a *S. aureus* (that is, 10^3, 10^6 10^7, or 10^8 CFU). Body weight measurement, health score monitoring and blood collection (that is, tail vein collection) for plasma AGP level measurement was performed on day 0 (that is, prior to treatment) and on days 2, 4, 7 and 10 after inoculation.

The second study (Study 2) did not use bacterial dosing with 10^8 CFU of *S. aureus* because this concentration has been well documented for inducing infection, including clinical signs, in rats.[3,25] Thus, Study 2 focused on evaluating the effect of a SCM implant on plasma AGP levels in animals inoculated with *S. aureus* at 10^6 CFU or 10^7 CFU. Select rats received a combination of SCM implant and either vehicle control or S. *aureus* inoculum or did not receive an implant but were only inoculated with vehicle control or *S. aureus.* Rats were assigned to the following treatment groups: vehicle control with (n = 3) or without (n = 5) SCM implants; *S. aureus* inoculation of 10^6 CFU with (n = 7) or without (n = 5) SCM implants; *S. aureus* inoculation of 10^7 CFU with (n = 7) or without (n = 5) SCM implants. Body weight measurement, health score monitoring, and blood (tail vein) collection for plasma AGP measurement was performed on day 0 (prior to inoculation ± SCM implantation) and on days 2, 4, 7 and 10.

Based on Studies 1 and 2, the highest bacterial dose of *S. aureus* (10^8 CFU) that did not cause overt clinical illness was used for the validation study to obtain the most robust signal for eliciting subclinical infection, based on increased AGP above the vehicle level). Rats in all cohorts received SCM implants and when indicated, were inoculated with vehicle control or *S. aureus* on day 0. Rats were randomly selected for placement in one of 4 groups (n = 4 per group) as follows; Cohort 1 (vehicle control) rats received a single inoculation of 0.9% saline and did not receive oral dosing with propranolol; Cohort 2 (propranolol control) rats received a single inoculation of vehicle control and were orally dosed over time with propranolol; Cohort 3 (subclinical infection control) rats were inoculated with 10^8 CFU *S. aureus* but did not receive oral dosing with propranolol; Cohort 4 (propranolol treatment in the presence of subclinical infection) rats were inoculated with 10^8 CFU *S. aureus* and orally dosed with propranolol, with repeat dosing over time. Body weight measurement and blood collection for plasma AGP measurement were performed on day -5 and day 0 (prior to treatment) and on days 2, 4, 7 and 14 after vehicle control or *S. aureus* inoculation. For measurements of plasma propranolol, rats in cohorts 2 and 4 received a single orogastric dose of propranolol (10 mg/kg) on day -5, 2, 4, 7 and 14. On these days, blood was collected from the tail vein for plasma propranolol measurement before and at 0.25, 0.5, 1, 2, 4 and 6 h after dosing

Clinical Assessment and Observation Parameters. Routine daily clinical observation of animals consisted of blind animal health cage-side assessments that were performed every morning for up to 14 d during the study period. Cage-side health assessments were scored using a scale of 1 to 5 for each clinical parameter assessed. The 9 parameters assessed included activity, species-specific behavior, hair coat condition, posture, respiratory signs, presence of porphyrin staining, injection site swelling, erythema and discharge. The health scoring system comprised subjective assignment of a numeric value in assessing the severity of each clinical sign observed as follows; 1 = none (normal), 2 = slight, 3 = mild, 4 = moderate, and 5 = severe. For a given day, the cumulative score for an animal reflected the sum of all clinical parameters assessed. Any individual animal presenting with a cumulative score of 10 or greater was considered to have clinically relevant, overt signs of illness that required veterinary intervention. In addition, body weights were obtained just prior to the start of a study and routinely thereafter as per study design. The dermal injection sites were also evaluated throughout the study period and again at necropsy, which included culturing of suspect tissues to confirm *S. aureus* infection. Any rat showing signs of pain or distress was removed from study and treated or euthanized as deemed necessary by veterinarian. Euthanasia was performed by CO_2 overdose, consistent with AVMA guidelines for euthanasia[1].

AGP Quantification. All plasma AGP data were analyzed using the Acute Phase Protein Panel 1 (rat) Kit and on the MESO QuickPlex SQ 120 instrument (Meso Scale Diagnostics, Rockville, MD). Whole blood samples were collected in EDTA and centrifuged for 5 min at 8165 x g (Eppendorf 5415 D, Eppendorf North America, NY) and plasma was transferred to -80 °C pending analysis as per manufacturer's instructions.

Plasma Propranolol Level Analysis. Plasma samples were analyzed in house using protein precipitation extraction, followed by UHPLC/MS/MS analysis using a Waters Acquity UPLC (Waters, USA) paired with a SCIEX API-4000 triple-quadrupole mass spectrometer (SCIEX, North America). The lower and higher limits of quantification for propranolol in 25 µL of plasma were 0.2 ng/mL and 200 ng/mL, respectively. The computer systems used to acquire and quantify data included Analyst Version 1.4.2/1.6.1 and SMS2000 version 2.3.

Statistical Analyses. Statistical analyses were performed on AGP and propranolol data via a 2-way ANOVA followed by Dunnett multiple comparisons test using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA). Summary data are expressed as mean ± SD and a P value less than 0.05 was considered statistically significant.

Results

During model development, the utility and dependability of using S. *aureus* as the bacterial agent for inducing a reliable and reproducible state of subclinical infection in rats was optimized. In model development studies, animals were subjected to inoculation with either vehicle control or various concentrations of S. *aureus* in the presence or absence of SCM. Bodyweight, cage-side health scoring, gross tissue evaluation, and plasma AGP levels were evaluated throughout the course of these studies. To characterize the extent and relationship of propranolol binding to AGP in control and infected animals this minimally invasive model was validated using 1×10^8 CFU S. *aureus*. In the validation study, parameters monitored included; bodyweight, AGP and propranolol drug levels.

Body weights. Animal body weights remained stable and increased as expected over time in all treatment groups across all model development studies from baseline to study completion. No significant differences were observed for body weight measurements across all groups throughout the studies (Table 1).

Table 1. Model development studies 1 and 2: Body Weight Summary

Treatment group	Saline control (n = 5)	Saline control + catheter (n = 3)	1×10^3 CFU *S. aureus* (n = 5)	1×10^6 CFU *S. aureus* (n = 5)	1×10^6 CFU *S. aureus* + Catheter (n = 7)	1×10^7 CFU *S. aureus* (n = 5)	1×10^7 CFU *S. aureus* + Catheter (n = 7)	1×10^8 CFU *S. aureus* (n = 5)
Day 0	306 ± 58	466 ± 54	289 ± 23	334 ± 109	387 ± 95	410 ± 59	422 ± 38	328 ± 63
Day 2	323 ± 60 (5%)	473 ± 55 (2%)	308 ± 13 (2%)	354 ± 99 (6%)	402 ± 92 (4%)	421 ± 53 (3%)	427 ± 37 (1%)	343 ± 58 (4%)
Day 7	355 ± 52 (16%)	490 ± 56 (5%)	330 ± 17 (14%)	383 ± 82 (15%)	423 ± 81 (9%)	433 ± 51 (6%)	438 ± 38 (4%)	367 ± 43 (12%)

Independent of treatment type, animal body weight (gm) remained stable with expected increases over time. Day 0 body weights were obtained prior to treatment. Data reflects the mean and standard deviation for each treatment group. The percent change from baseline (%) is shown below the mean and standard deviation data.

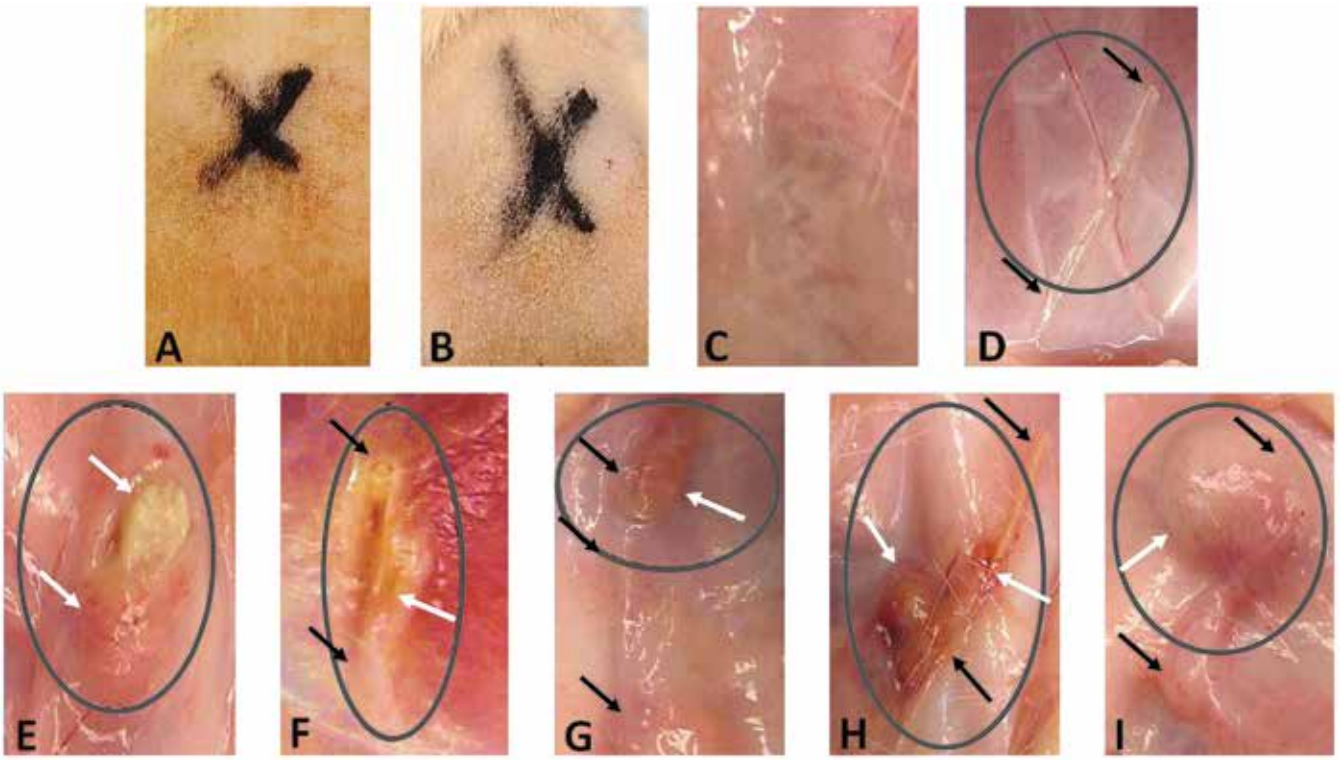

Figure 1. This series of representative pictures show the following typical gross dermal and subcutaneous findings in control and treated animals: Dermal site is referenced marked prior to injection and/or SCM implantation (A), Gross dermal presentation void of pathologic signs along with elliptical necropsy incision (B), Subcutaneous presentation in naïve and saline control animals (C), Subcutaneous presentation in naïve and saline control animals with SCM implants (D), Subcutaneous presentation in 1×10^8 CFU *S.* aureus treated animals (E), Subcutaneous presentation in 1×10^8 CFU *S.* aureus treated animals with SCM implants (F through I). White arrows indicate regions of infection and associated inflammation within the panniculus and black arrows indicate catheter tips.

Clinical assessment and observation parameters. Cage-side health assessments lacked sensitivity for confirming observable clinical abnormalities over the course of studies independent of treatment group. Despite rigorous surveillance of all animals (n = 73) throughout the study period, no overt clinical signs were noted in any treatment group. However, at necropsy, gross findings were observed in the panniculus associated with the injection site in some rats. Observations included inflammation or infection (Figure 1). In all cases, regions of interest were cultured, and results were consistent with the *S. aureus* strain used for inoculation, confirming infection. In contrast, increased levels of AGP, signifying subclinical infection and associated inflammation, were observed in various treatment groups in all studies.

Study 1. In this study, plasma AGP levels for the control animals ranged from 0.04 to 0.07 mg/ml for the duration of the study (Figure 2). However, fold change increases in plasma AGP levels were measured on day 2 in rats inoculated with 10^6 CFU *S. aureus* (3 fold; 0.14 ± 0.05 mg/mL) and on days 2 and 4 in rats inoculated with 10^7 CFU (6 fold; 0.27 ± 0.10 and 3 fold; 0.15 ± 0.06 respectively) and 10^8 CFU (10 fold; 0.46 ± 0.14 and 4 fold; 0.19 ± 0.06, respectively).The increases in plasma AGP were statistically significant on day 2 in the 10^6 CFU group ($P < 0.0001$) and on days 2 and 4 in the 10^7 CFU group ($P < 0.0001$ and $P = 0.0013$ respectively), and the 10^8 CFU group ($P < 0.0001$ and $P = 0.0063$ respectively) (Figure 2). These increases in plasma AGP had returned to baseline levels by day 7 and remained at baseline through study day 14. In contrast, AGP levels in both the vehicle control and lowest *S. aureus* dose groups remained within baseline range. An apparent dose response relationship was present on day 2, with the greatest increase seen in the 10^8 CFU *S. aureus* inoculated group followed by the 10^7 CFU *S. aureus* group and then the 10^6 CFU *S. aureus* group.

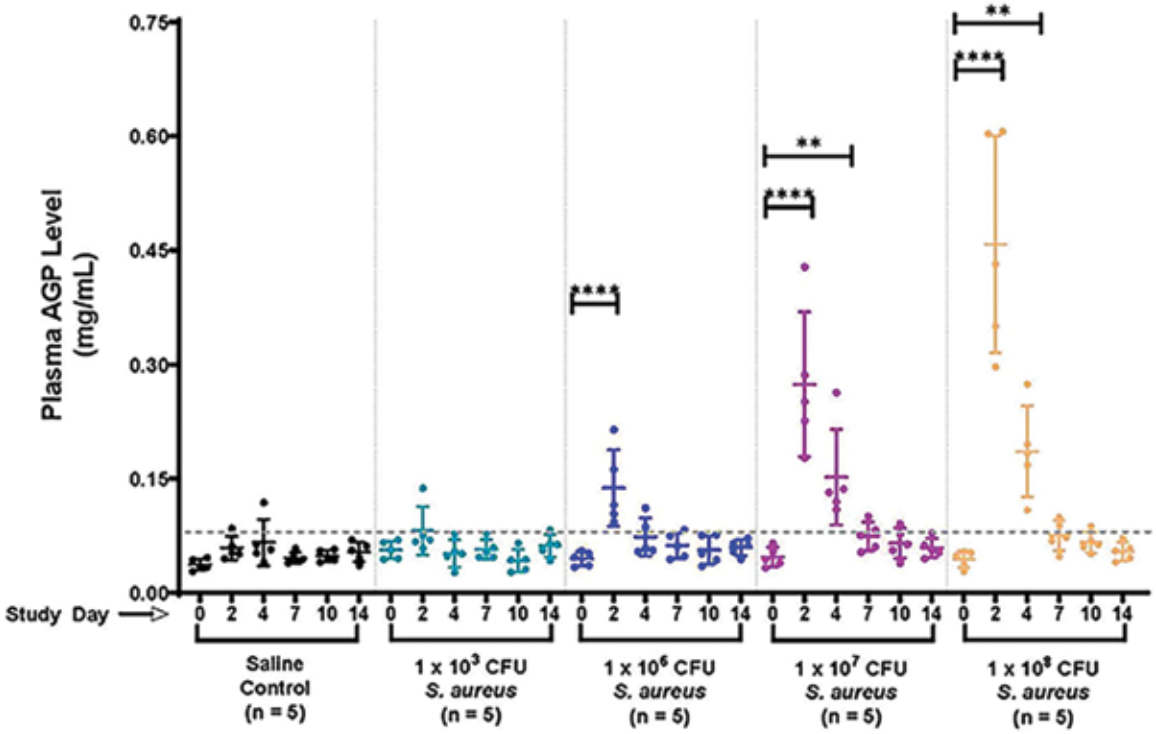

Figure 2. Model development study #1 plasma AGP level break-out result summary: Blood sampling for plasma AGP level (mg/mL) determination was conducted on day 0 prior to treatment and thereafter as indicated. The significant elevations in circulating plasma AGP levels were observed in all animals inoculated with *S.* aureus in the 1×10^6 CFU group on day 2 ($P < 0.0001$), the 1×107 CFU group on days 2 ($P < 0.0001$) and 4 ($P = 0.0013$) and the 1×10^8 CFU group on days 2 ($P < 0.0001$) and 4 ($P = 0.0063$). In addition, an apparent dose response elevation in AGP levels at day 2 was observed across these 3 groups with circulating plasma AGP levels returning to baseline by day 7. The graph reflects the individual breakout data for each group including the mean and standard deviation and the hash line indicating the normative plasma AGP level threshold.

Study 2. In this study, plasma AGP levels for the control animals without an implant ranged from 0.04 to 0.07 mg/mL for the duration of the study and levels for control animals with an implant ranged from 0.04 to 0.06 mg/ml for the duration of the study (Figure 3). However, rats subjected to subclinical infection with or without an implant showed fold changes in the level of AGP on day 2 in the 10^6 CFU treated group (3 fold; 0.14 ± 0.05) and the 10^6 CFU group with implants (4 fold; 0.17 ± 0.05) and on days 2 and 4 in both groups treated with 10^7 CFU without implants (6 fold; 0.27 ± 0.1 and 3 fold; 0.15 ± 0.06, respectively) and with implants (7 fold; 0.33 ± 0.06 and 4 fold; 0.18 ± 0.04, respectively). Significant ($P < 0.0001$) elevations in plasma AGP levels were detected for all groups inoculated with *S. aureus* independent of the presence or absence of an implant on days 2, 4 and 7 (Figure 3). These results indicate that *S. aureus* doses of both 10^6 CFU and 10^7 CFU effectively and reliability induced subclinical infection in rats based on the increased AGP levels observed in the absence of overt clinical signs, thus substantiating the utility of using AGP to detect subclinical *S. aureus* infection in rats.

Proof of concept, validation study. In study 3, rat body weights remained stable across all treatment groups for the duration of the study (Table 2). Although rats were observed daily for clinical signs, no abnormalities were visible throughout the study period. Plasma AGP levels in the vehicle and propranolol control groups (Cohorts 1 and 2 respectively) remained within normal range. In contrast, Cohort 3 (10^8 CFU) and Cohort 4 (10^8 CFU and propranolol) showed statistically significant increases in plasma AGP starting at day 2 and sustained through day 4 ($P < 0.0001$ and $P < 0.0001$, respectively) (Table 3), returning to baseline levels by day 14 (Figure 4). Large fold change increases in AGP levels were present at days 2 and 4 in both Cohorts 3 and 4. Rats in Cohort 4 showed significant elevations in propranolol levels ($P < 0.0001$) in parallel with increases in plasma AGP levels (Figure 5). This increase in propranolol concentration indicates prolongation of the overall clearance of propranolol as compared with Cohort 2 rats, in which propranolol levels remain stable over the course of the study. At day 2 after inoculation, a 16-fold increase in the AUC of plasma propranolol was observed for rats in Cohort 4 (493 ± 44 h × ng/mL) as compared with baseline and rats in Cohort 2 (38 ± 119 h × ng/mL). In addition, an 11-fold difference of AUC was observed at day 4 for rats in Cohort 4 (334 ± 54 h × ng/mL) as compared with baseline and contrasted to a 4-fold difference in rats from Cohort 2 (76 ± 16 h × ng/mL) (Table 4). In validating this model, the data clearly shows how subclinical infection altered propranolol's distribution due to AGP binding of propranolol, as reported previously.[4,7-10,27,49,51]

Discussion

This study provides a minimally invasive model of subclinical infection in the rat that was successfully developed using 10^8 CFU of *S. aureus*, with or without SCM implants, and was validated using propranolol. Plasma AGP levels confirmed the presence of subclinical infection in all *S. aureus* inoculated groups, as confirmed by necropsy findings and stable body weights. The data showed that cage-side clinical assessments did not detect or confirm subclinical infection, in contrast to

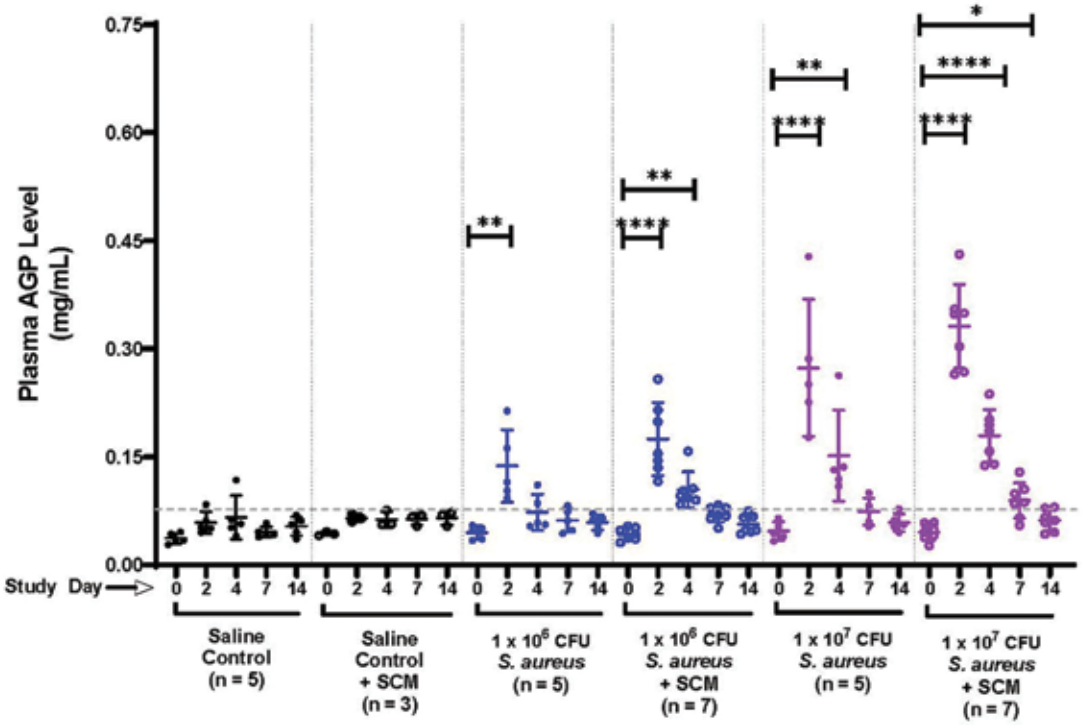

Figure 3. Model development study #2 plasma AGP level break-out result summary: Blood sampling for plasma AGP level (mg/mL) determination was conducted on day 0 prior to treatment and thereafter as shown on the graph. Significant ($P < 0.0001$) elevations in circulating plasma AGP levels were observed starting at day 2 in all group animals inoculated with *S.* aureus with or without SCM implants compared with control groups. Plasma AGP levels remained significantly elevated at day 4 in animals inoculated with 1×10^6 CFU *S. aureus* with SCM implants ($P = 0.0013$) and in the group animals inoculated with 1×10^7 CFU *S. aureus* without SCM implants ($P = 0.0031$). In addition, AGP levels remained significantly elevated on day 4 ($P < 0.0001$) and day 7 ($P = 0.0226$) in animals inoculated with 1×10^7 CFU *S.* aureus with SCM implants. The graph reflects the individual breakout data for each group including the mean and standard deviation and the hash line indicating the normative plasma AGP level threshold.

previously published work in which rats inoculated with 10^8 CFU *S. aureus* demonstrated obvious clinical signs during cageside assessment.[3] However, that model used a more invasive surgical procedure. All clinical assessments of rats in our study were conducted in a blind fashion, yet increases in plasma AGP were consistently present in infected rats. In addition, hematologic (white blood cell counts) and serological indices (total protein and albumin) were also evaluated and did not reflect subclinical infection (data not shown); increases in white blood cells and alterations in serum total protein were not detected in rats inoculated with *S. aureus*. A lack of correlation between increases in APP levels and relevant blood markers is possible, as reported for other species.[9,17] Consequently, as our hematologic and serological results were not informative, AGP stands out as a reliable biomarker for recognizing *S. aureus* subclinical infection in this rat model. AGP could potentially be used as a biomarker for other species in other disease states,[2,43,44] including those species in which AGP is characterized as a moderate APP.[8,13-15,36,45,489,14-16,38,47,50]

The finding that a subclinical infection leads to increased AGP can have profound effects on PK profiles and potentially confound decision making in drug discovery.[21] For example, an inaccurate relationship between bound and unbound drug availability could distort studies of dose efficacy and toxicity. If infected animals have lower drug availability, this could require additional studies to achieve efficacious target exposure levels. The concept of AGP altering plasma concentrations of drug due to binding is well established in the literature.[4,7,8,10,21,43] As demonstrated in the validation study and consistent with the literature, plasma propranolol levels are highly correlated to AGP levels, underscoring the important binding property of AGP. The normal level of plasma AGP was determined by using the means of AGP levels in naïve study animals (0.1 ± 0.01 mg/mL) as an indication of good health status. Because AGP levels above

Table 2. Model validation study body weight summary

Cohort	Baseline	Day 2	Day 4	Day 7	Day 14
1 Vehicle control	451 ± 7	454 ± 9 (1%)	457 ± 5 (1%)	465 ± 10 (3%)	486 ± 9 (8%)
2 Propranolol control	448 ± 14	463 ± 15 (3%)	458 ± 21 (2%)	459 ± 21 (3%)	479 ± 23 (7%)
3 Subclinical infection control	422 ± 27	432 ± 28 (2%)	433 ± 27 (3%)	436 ± 27 (3%)	453 ± 29 (7%)
4 Subclinical infection and propranolol	448 ± 10	461 ± 7 (3%)	456 ± 8 (2%)	457 ± 12 (2%)	485 ± 7 (8%)

All animals were implanted with SCM and independent of treatment, animal body weight (gm) remained stable with expected increases overtime. Baseline body weights were obtained prior to treatment. Data reflects the mean and standard deviation for each treatment group including the percent change from baseline (%).

Table 3. Model validation study plasma AGP level result summary

Cohort	Day - 5	Day 0	Day 2	Day 4	Day 7	Day 14
1. Vehicle control	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.03	0.09 ± 0.01	0.08 ± 0.01	0.06 ± 0.02
2. Propranolol (10.0 mg/kg) control	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.10 ± 0.02	0.08 ± 0.02	0.05 ± 0.00
3. Subclinical Infection control 1×10^8 CFU *S. aureus*	0.06 ± 0.01	0.08 ± 0.01	0.56 ± 0.06 (9 fold)	0.33 ± 0.06 (6 fold)	0.13 ± 0.01	0.06 ± 0.01
4. Subclinical infection 1×10^8 CFU *S. aureus* and Propranolol (10.0 mg/kg)	0.07 ± 0.01	0.07 ± 0.00	0.61 ± 0.09 (9 fold)	0.35 ± 0.06 (5 fold)	0.14 ± 0.05	0.06 ± 0.02

Plasma AGP levels (mg/mL) in the vehicle and propranolol control cohorts remained stable in contrast to the cohort infected with 1×10^8 CFU *S. aureus* as well as the cohort infected with 1×10^8 CFU *S. aureus* and treated with propranolol. The data reflects the mean and standard deviation for each treatment group and fold changes noted in parentheses.

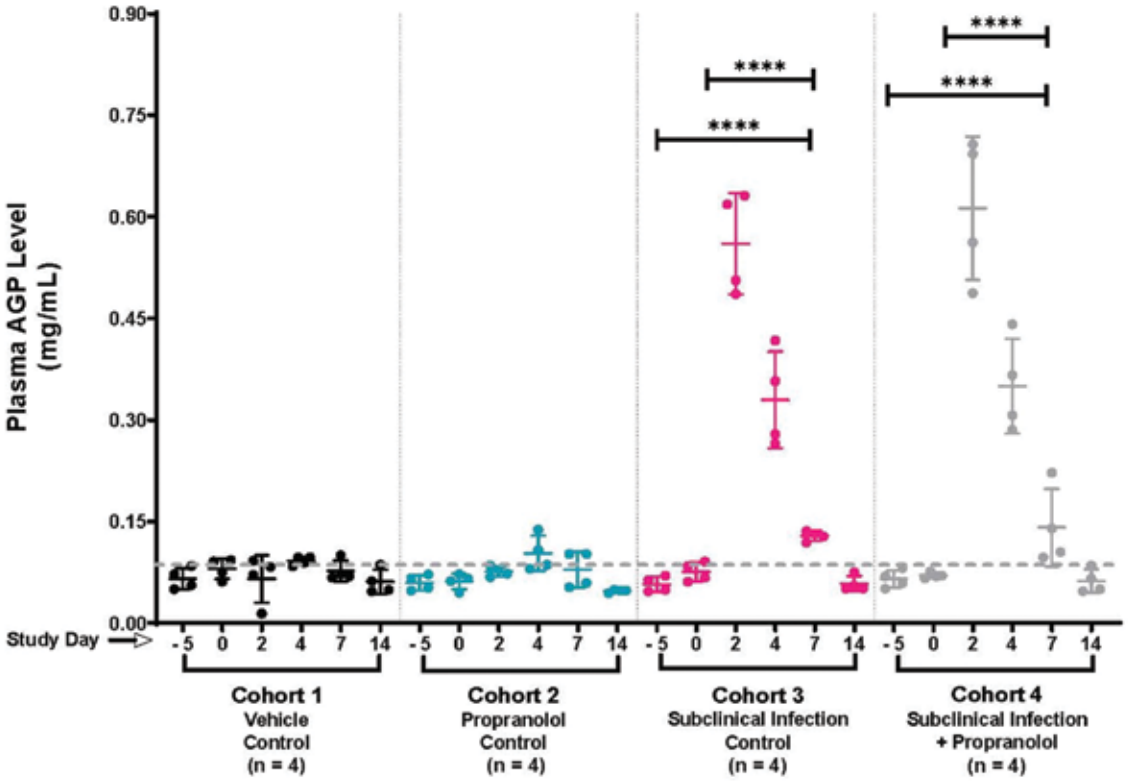

Figure 4. Model validation study plasma AGP level break-out result summary: All animals were implanted with SCM and significant elevation in circulating levels of plasma AGP were observed starting at day 2 and through day 4, in all animals in cohort 3 ($P < 0.0001$) and cohort 4 ($P < 0.0001$) contrasted to control cohorts 1 and 2. The graph reflects the individual breakout data for each group including the mean and standard deviation and the hash line indicating normative plasma AGP level threshold.

the normative threshold will potentially skew PK profiles, then greater scrutiny of AGP levels are necessary for determination of efficacious doses.

The research performed in this manuscript was intended as a proof of concept in terms of establishing a minimally invasive model of *S. aureus* subclinical infection with AGP as the biomarker for infection. The selective use of *S. aureus*, a ubiquitous skin contaminant, was chosen as a starting point for developing this model. In addition, the selection of AGP was based on its known response to infection and its ability to bind many basic drugs. Finally, propranolol, a drug known for its high binding affinity to AGP, was used to validate the model. Given the limitations of this purposeful selectivity, additional studies evaluating the complex relationships between other bacterial pathogens, APPs, and hematologic/serologic biomarkers in a subclinical infection may be useful.

The advantages of this subclinical model are the ability to obtain pre- and intra-study AGP levels for establishing individual baselines and confirming health status and study worthiness of potential subjects. Consideration and periodic surveillance of AGP levels is particularly critical for surgically instrumented animals, whether it be an acute or chronic or an invasive or minimally invasive model. Overall, this novel approach permits better preclinical screening of subjects and test agents, yielding more comprehensive assessment with better informed data interpretation and greater predictive value.

In addition, improved clinical biomarker monitoring and evaluation of animals through AGP screening is consistent with the intent of the 3R's. In cases of animal reuse, establishing a

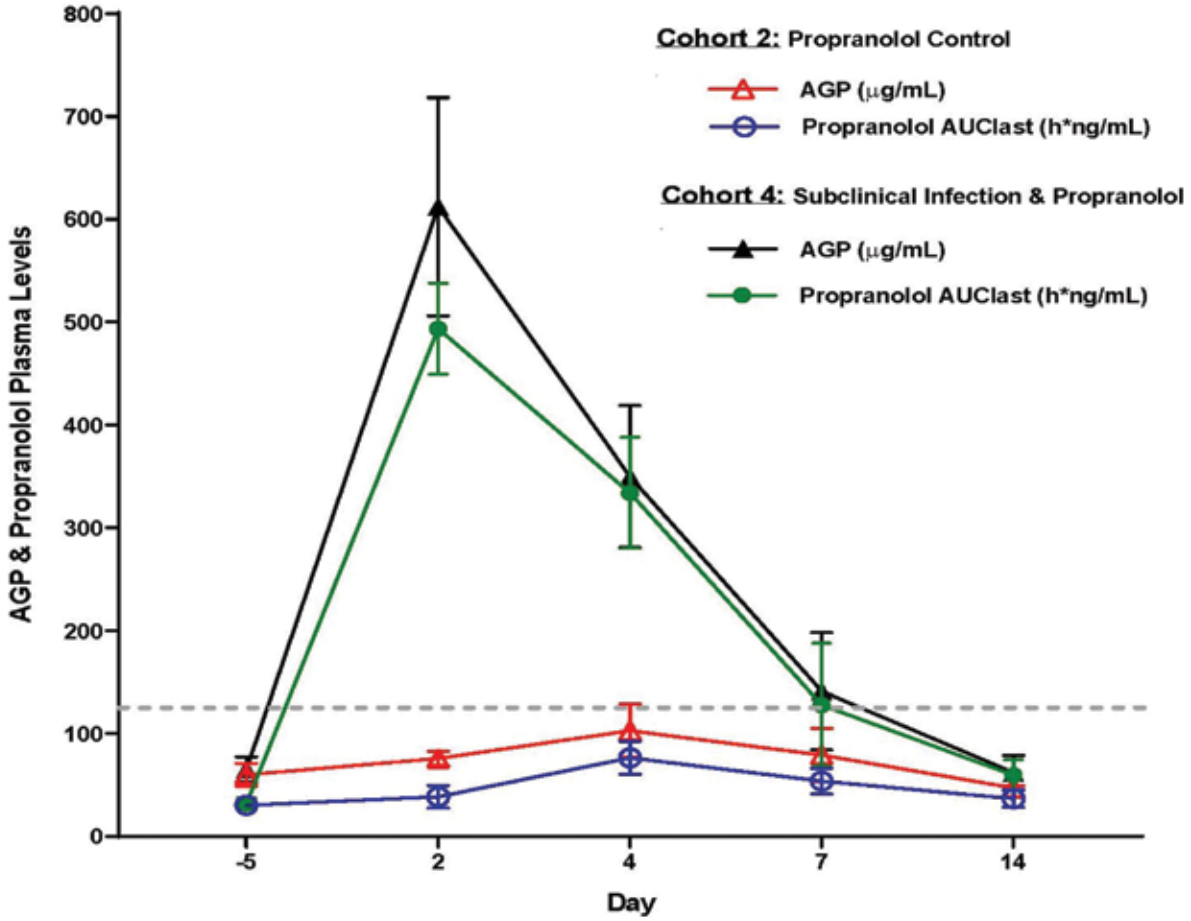

Figure 5. Model validation study plasma Propranolol and AGP level results: For animals in cohort 4, systemic propranolol exposure levels significantly increased ($P < 0.0001$) in parallel with those observed for circulating plasma AGP levels compared with animals in cohort 2. The clearance prolongation of propranolol is evident when comparing cohort 2 to cohort 4 animals. In addition, a tight correlation between AGP and propranolol AUC is clearly evident within each cohort. The data reflect the mean and standard deviation and the hash line indicating normative plasma AGP level threshold.

Table 4. Propranolol AUClast (h *ng/mL) results

Treatment group	Day - 5	Day 2	Day 4	Day 7	Day 14
Cohort 2 propranolol control	30 ± 3	38 ± 11	76 ± 16	54 ± 13	36 ± 8
Cohort 4 subclinical Infection+ Propranolol	32 ± 6	493 ± 44 (16 fold)	334 ± 54 (11 fold)	128 ± 60 (4 fold)	59 ± 15

Propranolol AUC levels (h*ng/mL) in Cohort 2 animals (SCM and propranolol) remained stable over the course of the study whereas 16, 11 and 4 fold changes were observed on days 2, 4 and 7 in Cohort 4 animals (SCM, *S. aureus*, and propranolol). The data reflects the mean and standard deviation for each treatment group and fold changes noted in parentheses.

longitudinal AGP baseline aids in characterizing the long-term and subclinical health status of an animal. This thereby facilitates the selection for inclusion or exclusion of an animal in studies over time. Early monitoring of health status and drug binding may reduce the need for follow-up studies and produce more accurate data that may bring important drugs to patients sooner.

In conclusion, our study has established AGP as a reliable biomarker for detection of subclinical infection in rats. Our model permits more informed decision making for the selection of study-worthy animals, better monitoring of health status during study participation, and more accurate characterization of drug PK in relation to protein binding. Overall, a marker that permits evaluation of these relationships in conjunction with routine parameters such as clinical observations and body weight will benefit data interpretation, particularly in the early stages of the development process for drugs that bind AGP. This approach will facilitate generation of unambiguous data and provide more robust characterization of therapeutic candidates.

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