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Adenosine A₁ receptor antagonist, L-97-1, improves survival and protects the kidney in a rat model of cecal ligation and puncture induced sepsis[☆]

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Abstract

Previously it was reported that combining antibiotics with L-97-1, an adenosine A₁ receptor antagonist, significantly improves survival and blocks acute lung injury induced by *Yersinia pestis* CO 99 in a rat model of pneumonic plague. In the current studies using a conscious rat model of cecal ligation and puncture (CLP) sepsis, L-97-1 was administered in daily intravenous infusions in combination with antibiotics to simulate the use of L-97-1 as an anti-sepsis therapeutic in the clinical setting. In these studies, when administered at 12 hours (h) following CLP, in combination with broad spectrum antibiotics, ceftriaxone and clindamycin, L-97-1 improves 7 day (d) survival [25%, 35%, and 75%, respectively for L-97-1 (10 mg/kg/h, 12.5 mg/kg/h, and 15 mg/kg/h) versus (vs.) 25% for antibiotics alone] in a dose-dependent manner. The addition of L-97-1, 15 mg/kg/h to antibiotics significantly increased 7 d survival following CLP compared to therapy with either antibiotics alone ($P = 0.002$) or L-97-1 at 15 mg/kg/h alone ($P < 0.001$) and was not significantly different than survival in sham CLP animals (Log-rank (Mantel-Cox) test with Bonferroni's correction for multiple comparisons). Moreover, in these studies, in combination with antibiotics L-97-1 dose-dependently protects the kidney, significantly improving renal function at 24 h post CLP at 10 mg/kg/h ($P < 0.001$), 12.5 mg/kg/h ($P < 0.0001$), and 15 mg/kg/h ($P < 0.0001$) vs. antibiotics alone (ANOVA followed by Tukey's post-hoc test for pair-wise comparisons). The results of these studies support efficacy for L-97-1 as an anti-sepsis therapeutic.

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Keywords

Sepsis; adenosine A₁ receptors; adenosine A₁ receptor antagonists; L-97-1; cecal ligation and puncture; renal protection

1. Introduction

Sepsis, a medical syndrome characterized by an overwhelming systemic response to infection that can rapidly lead to shock, organ failure, and death, is one of the largest unmet medical needs. In the U.S., sepsis is the 10th leading cause of death overall and, annually, accounts for over 750,000 cases, 215,000 deaths, and \$17 billion in health care expenditures (National Vital Statistics Report, 2002; Angus et al., 2001). Moreover, the incidence of sepsis may be rising due to the increasing age of the population, growing numbers of immunocompromised patients, use of life-sustaining technologies, and increased resistance of bacteria to antimicrobial agents. Furthermore, despite advances in critical care medicine, new antibiotics, and FDA approval and use of an anti-sepsis therapeutic, (drotrecogin alfa [activated], recombinant human activated protein C), Xigris®, the mortality rate for severe sepsis and septic shock of 30–60% remains high and the long term outcome is poor for patients that survive sepsis (Angus, 2010; Banta, et al., 2012; Iwashyna, et al., 2010; Suffredini and Munford, 2011). These discouraging statistics suggest a need for additional therapies to the conventional approach to sepsis, adjunctive therapies that enhance the host's immune response, and interrupt the complex cascade of events in sepsis leading to shock, multisystem organ dysfunction, and death. In view of the withdrawal of Xigris due to lack of efficacy, as well as other failures by the pharmaceutical industry to develop a safe and effective anti-sepsis therapeutic, including the TLR4 inhibitor, Eritoran, there is a compelling need for new, effective, and safe drugs that target novel molecular targets for the treatment of sepsis (Angus, 2011; Suffredini and Munford, 2011).

Previously it was reported that L-97-1, an adenosine A₁ receptor antagonist, significantly improves six day (d) survival, blocks acute lung injury, and prevents the decline in mean arterial pressure in a rat model of pneumonic plague and *Yersinia pestis* (*Y. pestis*) induced sepsis when administered 72 hours (h) post intratracheal administration of *Y. pestis* CO 99 (Wilson, et al., 2011). Moreover, previously it was reported in proof of concept studies in a rat model of cecal ligation and puncture (CLP) induced polymicrobial intraabdominal sepsis, when administered alone or in combination with antibiotics, that a single intravenous (i.v.) dose of L-97-1 immediately post CLP significantly improves 7 d survival in a dose-dependent manner versus (vs.) antibiotics alone (Wilson et al., 2006). It was the objective of the current studies to expand these earlier studies in the rat CLP model of sepsis to determine efficacy for L-97-1 as a delayed treatment 12 h post CLP when administered as multiple daily i.v. infusions in combination with broad spectrum antibiotics, a treatment regimen that more closely simulates the clinical approach to sepsis.

2. Materials and Methods

2.1 Rat model of cecal ligation and puncture sepsis

All surgical procedures and animal care were in accordance with National Institutes of Health (NIH) guidelines and are approved by the Animal Care Committee of Saint Louis University (SLU). Male pathogen-free S-D rats (8–12 weeks old, 275–325 g, Harlan,) were housed in positive-pressure isolation carrels with free access to food and water before experiments and in a biosafety laboratory (BSL)-2 lab thereafter. Under 3% isoflurane, 97% O₂ and using aseptic technique throughout, a 2-cm incision was made in the midventral neck to expose and catheterize the left carotid artery and right jugular vein (PE-50; Clay Adams). The surgical incision was closed, anesthesia was withdrawn, and the animal was monitored until conscious (typically 3–5 min) and then returned to its cage. Catheters were shielded within stainless steel springs and mounted to swivels (Instech) fastened to the cage lid to allow free movement and access to food and water. The next morning (18–24 h later), animals were re-anesthetized and a sterile 3 cm midventral abdominal incision was made to expose the intestines. The intestines were gently retracted below the ileocecal valve with 3-0 silk and two perforations were then made in the cecum by passing a 19-gauge needle “through and through” starting on its anti-mesenteric surface. To ensure consistent cecal injuries among all animals, the perforated cecum was gently squeezed until 40 – 80 µL of feces extruded onto both surfaces, and then the bowel was gently reinserted into the abdomen and the incision closed. A single post-operative normal saline (NS) bolus was provided (50 ml/kg subcutaneous) for fluid support.

Animals were monitored daily for vital signs (mean arterial blood pressure, carotid pulse rate, and respiratory frequency) as well as for clinical signs (degree of piloerection and presence of periorbital bleeding or nasal discharge). Arterial blood samples were obtained (1.0 ml) for hematology, blood gases, and serum samples. Each ml of withdrawn blood was replaced with 3 ml sterile NS. Monitoring of animals for hemodynamics was recorded at least at 0, 1, 3, 6, 24, and every 24 h after 24 h up to 7 d after CLP.

Rats were prepared for time-sacrifice necropsies at one of three time intervals (24 h, 72 h, or 7 d) by induction of deep isoflurane anesthesia. Aseptic laparotomy was then rapidly performed to facilitate exsanguination of each rat via the abdominal aorta to collect a final large aseptic, arterial bleed. This bleed was followed by penetration of the diaphragm immediately beneath the xiphoid process to create an observed pneumothorax and the animal was then necropsied. Rats dying overnight were recorded as having survived to the previous 24 h observation period but were not necropsied.

In 24 h and 72 h time killed studies serum samples were obtained and analyzed for levels of blood urea nitrogen (BUN) and cytokines, interleukin (IL)-6, IL-1 β , and tumor necrosis factor (TNF)- α . Serum BUN levels were determined by colorimetric method with an automatic analyzer (TBA-120FR, Toshiba, Japan). Serum levels for IL-1 β , IL-6, and TNF- α were measured with the use of commercially available ELISA kits (R&D Systems, Minneapolis MN).

2.2 Treatment Groups

Following CLP in separate 24 h, 72 h, and 7 d time killed studies, separate groups of rats ($n =$ a minimum of 10/group) were randomized to receive one of the following treatments: Group 1 (sham CLP, with laparotomy and bowel manipulation but no CLP, i.v. infusion of sterile water, diluent for L-97-1, $n = 11$); Group 2 (CLP control, i.v. infusion of sterile water but no antibiotics *immediately post* CLP, $n = 21$); Group 3 (CLP, i.v. infusion of sterile water plus antibiotics *immediately post* CLP, $n = 16$); Group 4 (CLP, i.v. infusion of sterile water plus i.v. antibiotics begun at 6 h *post* CLP, $n = 16$); Group 5 (CLP, i.v. antibiotics plus i.v. infusion of L-97-1 (10 mg/kg/h) begun at 6 h *post* CLP, $n = 16$); Group 6 (CLP, i.v. infusion of L-97-1 (15 mg/kg/h) but no antibiotics starting at 12 h *post* CLP, $n = 15$); Group 7 (CLP, infusion of sterile water plus i.v. antibiotics starting at 12 h *post* CLP, $n = 16$); Groups 8 – 10 (CLP, i.v. antibiotics plus i.v. infusion of L-97-1 (10, 12.5 or 15 mg/kg/h) at 12 h *post* CLP, $n = 16, 23, 20$, respectively). All i.v. infusions of either sterile water or L-97-1 (10, 12.5 or 15 mg/kg/h) were prepared in the appropriate volumes that permitted their being administered via the indwelling jugular venous catheter at a consistent rate of 0.5 ml/h for 8 h/d for three consecutive days. Antibiotics [a broad spectrum third-generation cephalosporin, ceftriaxone (80 mg/kg/d) and clindamycin (40 mg/kg/d)] or 2 ml of NS were administered i.v. through the same jugular venous catheter as a slow bolus in 2 minutes daily for 3 d. With these regimens, all animals received equivalent amounts of fluid volume administered as i.v. treatments, *i.e.* administered as an i.v. infusion of sterile water or L-97-1 at 0.5 ml/h $\times$ 8h/d $\times$ 3 d plus small boluses of either i.v. antibiotics or NS.

2.3 Materials

The adenosine A₁ receptor antagonist, L-97-1 (3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-[2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl]-1-propyl-3,7-dihydro-purine-2,6-dione), was synthesized by Scynexis, Inc. (Research Triangle Park, NC) for Endacea Inc. and stored as anhydrous powder in the dark at 26°C until reconstituted daily in USP-grade water for use as described above. This sterile USP-grade water as well as USP-grade normal saline (NS), isoflurane, and sterile sodium-heparin stock solution (1000 U/ml) were from Baxter Healthcare (Deerfield IL). Intermedic[®] polyethylene (PE-50) tubing for catheterizations and serum separation tubes were obtained from BD Biosciences (San Jose CA). USP-grade ceftriaxone and clindamycin were obtained as a 10 mg/ml i.v. solution (Hospira Inc., Lake Forest IL). Sterile 1 X PBS (pH 7.4) was from Lonza (Switzerland). Unless otherwise noted, other chemicals were from Sigma-Aldrich (St. Louis MO).

2.4 Statistical Analysis

Differences in survival among treated and untreated groups were evaluated by Kaplan-Meier curves and tested for significant differences by Log-rank (Mantel-Cox) test with subsequent pair-wise comparisons using Bonferroni's correction for multiple comparisons.

Differences in mean serum cytokine or serum BUN concentrations at 24 h were compared among control sham, untreated CLP, and treated groups by ANOVA followed by Tukey's post-hoc test for pair-wise comparisons.

Logistic regression analysis was performed to evaluate L-97-1 and antibiotic treatment as independent predictors of outcome in this conscious rat model of sepsis. The outcome measure was defined dichotomously as either survival or death within 72 h after CLP; antibiotic administrations and L-97-1 treatments were coded as binary independent variables. A second logistic regression analysis was performed using the above parameters, but L-97-1 treatment was coded as an ordinal variable across dosages. Additionally, antibiotic therapy, L-97-1 therapy, IL-6, IL-1 β , TNF- α , and BUN levels at 24 h were evaluated as predictors of time of death within 72 h after CLP by multiple regression analysis.

Statistical analyses were performed using Prism 6.0 (GraphPad) and SPSS Statistics 21.0 (IBM). For all analyses, the threshold for significance was $P < 0.05$. To assure the adequacy of sample sizes needed to achieve statistical significance among groups, group sizes of at least $n = 10$ rats per group, provided the power to detect a 20% difference in the proposed criteria at the $P < 0.05$ level.

3. Results

3.1 L-97-1 plus Antibiotics Improve Seven Day Survival Rates in CLP Sepsis

In this rat model of CLP sepsis, in 7 d time killed survival studies, administration immediately after CLP of broad spectrum antibiotics, ceftriaxone (80 mg/kg/d) plus clindamycin (40 mg/kg/d), plus an infusion of sterile water (diluent for L-97-1) ($0.5 \text{ ml/h} \times 8 \text{ h/d} \times 3 \text{ d}$) produced a 75% 7 d survival rate with a mean time of death (TOD) of 157 h (Table 1). Of note, this model of CLP sepsis in the rat was universally fatal within 48 h in the absence of such antibiotic intervention (Figure 1). When administered at 6 h post CLP, treatment with these same antibiotics plus only an infusion of sterile water produced a 7 d survival rate of 69% with a mean TOD of 148 h. The addition of an infusion of L-97-1 [10 mg/kg/h ($0.5 \text{ ml/h} \times 8 \text{ h/d} \times 3 \text{ d}$)] to antibiotics starting at 6 h post CLP did not significantly alter the 7 d survival rate or TOD vs. antibiotics alone (Table 1). It is possible that this dose of L-97-1 was too low to affect the 7 d survival rate in these 6 h delay treatment studies.

In 12 h delayed treatment studies, when L-97-1 was administered with or without antibiotic therapy starting at 12 h after CLP, median survival at 7 d (168 h) in CLP animals that received either treatment alone or in combination was significantly improved compared to CLP animals that received vehicle only treatment ($P = 0.002$ for all) (Figure 1). Specifically, when L-97-1 was administered starting at 12 h post CLP as a continuous i.v. infusion [at doses of 10, 12.5, or 15 mg/kg/h (each at $0.5 \text{ ml/h} \times 8 \text{ h/d}$)] in combination with antibiotics (ceftriaxone plus clindamycin) over the subsequent 72 h time period, L-97-1 significantly improved 7 d survival in a dose-dependent manner to as much as 75% vs. just 25% with antibiotics alone [$P = 0.002$, Log-rank (Mantel-Cox) test] (Table 1, Figure 1). Furthermore, combined treatment with L-97-1 at 15 mg/kg/h plus antibiotics produced significantly greater mean survival following CLP compared to L-97-1 at 15 mg/kg/h alone ($P < 0.001$). Notably, median survival after CLP with the combination L-97-1 at 15 mg/kg/h and antibiotic therapy also was significantly better than combination therapy with L-97-1 at either 10 or 12.5 mg/kg/h ($P = 0.001$ and $P = 0.018$, respectively) and was not significantly different from the median survival for the sham, CLP group. The addition of L-97-1 therapy

at 10 or 12.5 mg/kg/h did not produce statistically significant increases in median survival compared to antibiotics alone.

In the 72 h time killed studies, median survival at 72 h among untreated, control CLP and 12 h delay treatment groups was significantly different ($P < 0.0001$, Log-rank Mantel-Cox test) (Figure 2). Post-test pair-wise analyses showed that median survival for rats receiving L-97-1 treatment at 10, 12.5, or 15 mg/kg/h in combination with antibiotics starting at 12 h post CLP was significantly better than that for untreated, CLP rats ($P = 0.013$, $P = 0.001$, and $P < 0.001$, respectively). Additionally, median survival in rats treated with L-97-1 at 15 mg/kg/h plus antibiotics was significantly greater than survival in groups receiving either antibiotics alone ($P = 0.02$) or L-97-1 therapy alone ($P = 0.005$). Further, median survival with L-97-1 at 15 mg/kg/h plus antibiotics was not significantly different from median survival in the sham, CLP group. For other L-97-1 doses, the difference in median survival between combined therapy with L-97-1 plus antibiotics vs. antibiotic therapy alone did not reach statistical significance.

A logistic regression analysis for the 72 h time killed studies was conducted to determine the contribution of antibiotic treatment and L-97-1 treatment as predictors of mortality from CLP at 72 h. A test of the full model against a constant only model was statistically significant, indicating that the predictors as a set reliably distinguished between animals that died or survived to 72 h (Chi square = 17.82, $P < 0.001$ with $df = 2$). Furthermore, the Wald criterion demonstrated that both antibiotics and L-97-1 treatment individually were significant contributors to the prediction of survival. The odds ratio of antibiotic treatment in surviving vs. dead animals was 7.1 (95% CI 2.0 – 25.0, $P = 0.002$) and the odds ratio of L-97-1 treatment was 4.3 (95% CI 1.4 – 13.0, $P = 0.011$). Similar evaluation of L-97-1 treatment by logistic regression analysis with stratification across different doses demonstrated that L-97-1 treatment at 15 mg/kg/h, but not at 10 or 12.5 mg/kg/h, was an independent predictor of 72 h survival. The odds ratio of L-97-1 at 15 mg/kg/h treatment in surviving vs. dead animals at 72 h was 7.6 (95% CI 1.5 – 40.0, $P < 0.016$).

3.2 L-97-1 Protects the Kidney in CLP Sepsis

In this rat model of CLP sepsis, in 24 h and 72 h time killed studies, serum BUN levels were increased at 24 h post CLP in the untreated CLP, animals and animals treated with antibiotics alone vs. the sham, CLP animals (20.9 ± 1.4 mg/dL, $n = 8$, sham, CLP; 46.5 ± 7.2 mg/dL, $n = 11$, untreated, CLP; and 59.4 ± 8.4 mg/dL $n = 11$, antibiotic treatment alone) (trend for CLP, $P < 0.001$ for antibiotics) (Figure 3). Importantly, when only antibiotics were administered starting at 12 h post CLP, such treatment alone did not protect the kidney. Serum BUN levels measured at 24 h following CLP in animals treated with antibiotics alone were higher than those for CLP control animals (59.4 ± 8.4 mg/dL, antibiotic controls, $n = 11$ vs 46.5 ± 7.2 mg/dL CLP controls, $n = 11$ (Figure 3).

When L-97-1 was administered as a delayed treatment starting at 12 h post CLP as a continuous i.v. infusion (10, 12.5, or 15 mg/kg/h $\times$ 8 h/d) in combination with antibiotics over a 72 h time period, L-97-1 significantly protected the kidney in a dose-dependent manner (Figure 3). The addition of L-97-1 to antibiotic treatment significantly reduced serum BUN levels at drug dosages of 10 mg/kg/h (24.6 ± 5.6 mg/dL, $n = 9$, $P < 0.001$), 12.5

mg/kg/h (21.3 ± 4.8 mg/dL, $n = 11$, $P < 0.0001$), and 15 mg/kg/h (17 ± 5.7 mg/dL, $n = 11$, $P < 0.0001$) vs. antibiotic treatment alone (59.4 ± 8.4 mg/dL $n = 11$). Furthermore, the combined therapy of antibiotics plus L-97-1 at 15 mg/kg/h produced a significant difference in the serum BUN level vs. untreated, CLP (17 ± 5.7 mg/dL, $n = 11$, L-97-1 15 mg/kg/h + antibiotics vs. 46.5 ± 7.2 mg/dL, $n = 11$, untreated, CLP controls, $P < 0.01$). Finally, and importantly, the serum BUN levels for the treatment groups for L-97-1 with and without antibiotics were not significantly different from the sham, CLP group (Figure 3).

3.3 L-97-1 plus Antibiotics and Antibiotics Alone Reduce Serum Cytokine Levels in CLP Sepsis

In this rat model of CLP sepsis, in 24 h and 72 h time killed studies, serum levels for the cytokines IL-6, TNF- α , and IL-1 β , all increased in a time-dependent manner over 24 h following CLP (Table 2). At 24 h post CLP mean serum IL-6, TNF- α , and IL-1 β levels were significantly different among experimental groups ($P < 0.009$) (Figure 4). Pair-wise comparisons revealed significant changes in serum TNF- α and IL-1 β levels with antibiotics alone, with L-97-1 alone, and with all dosages of L-97-1 in combination with antibiotics compared with untreated CLP animals. Pair-wise comparisons revealed significant changes in serum IL-6 levels with antibiotics alone and with all dosages of L-97-1 in combination with antibiotics vs. untreated CLP animals. L-97-1 therapy alone did not produce a significant change in the serum IL-6 level vs. CLP animals, perhaps limited by sample size ($n = 4$). Differences in serum cytokine levels among treatment groups (antibiotics alone or L-97-1 with and without antibiotics) were not significant.

A multiple regression analysis of the data from the 72 h time killed studies was performed to evaluate antibiotic (ceftriaxone plus clindamycin) therapy, L-97-1 therapy, and serum IL-6, TNF- α , IL-1 β , and BUN levels as predictors of time of death. These variables together significantly predicted time of death up to 72h, $F(6,32) = 4.79$, $P = 0.001$, $R^2 = 0.473$. In this analysis, antibiotic therapy and L-97-1 therapy were each significantly and positively correlated with time of death ($R^2 = 0.292$, $P = 0.012$, and $R^2 = 0.375$, $P = 0.002$, respectively). Serum TNF- α and BUN levels were independent predictors of time of death ($P = 0.014$ and $P = 0.009$, respectively), and correlation analyses showed that serum levels of BUN, IL-6, TNF- α , IL-1 β were all significantly and negatively correlated with survival.

4. Discussion

In a rat model of polymicrobial peritonitis that rapidly progresses to fatal sepsis within 48 h in the absence of intervention, when L-97-1 is administered as a delayed treatment 12 h post CLP in combination with broad spectrum antibiotics similar to those currently used clinically to treat sepsis, this novel adenosine A₁ receptor antagonist significantly and dose-dependently improves 72 h and 7 d survival rates vs. untreated CLP. At the highest dose tested when administered at 12 h post CLP in combination with antibiotics, L-97-1 significantly improves 72 h and 7 d survival rates vs. antibiotics alone or vs. L-97-1 therapy alone, and that are not significantly different from those vs. sham, CLP. Further, at this dose, L-97-1 independently and significantly predicts 72 h survival following CLP.

In the current study, delaying treatment with antibiotics alone for 12 h does not protect the kidney; however, when added to antibiotic treatment at 12 h post CLP, L-97-1 significantly improves renal function reducing serum levels of BUN in a dose-dependent manner *vs.* antibiotics alone. This effect of L-97-1 on renal function is particularly important given that antibiotic treatment alone does not significantly improve renal function in this rat model of CLP sepsis. Further, in the current studies, the serum levels of BUN in groups treated with L-97-1 alone, as well as different doses of L-97-1 in combination with antibiotics, are not significantly different from those of the sham, CLP group, suggesting that L-97-1 has a direct beneficial effect on renal function.

Further in the current study there are no significant differences between different doses of L-97-1 in combination with antibiotics or L-97-1 therapy alone versus antibiotic therapy alone on circulating levels of canonical pro-inflammatory cytokines that have been consistently linked to organ injury and fatal septic shock. This is in contrast to the beneficial effect of highest dose of L-97-1 in combination with antibiotics *vs.* antibiotics alone on 7 d survival rates and different doses of L-97-1 in combination with antibiotics, as well as L-97-1 alone, on renal function. Taken together, these results suggest that as an anti-sepsis therapeutic, L-97-1 would provide additional benefit for the outcome of sepsis *vs.* the use of antibiotics alone. The results of these studies support the further development of L-97-1 as an anti-sepsis therapeutic that has now shown the ability to extend the “treatment window” for successful interventions in two very different rodent sepsis models. Indeed, this goal has been underscored throughout the long and frustrating search for such sepsis therapies (Angus, 2011; Suffredini and Munford, 2011).

Previously it was reported that when administered in combination with antibiotics as a 72 h delayed treatment, L-97-1 improved survival and acute lung injury in a rat model of *Y. pestis* induced pneumonic plague and sepsis (Wilson, et al., 2011). Moreover, it was reported that adenosine A₁ receptor antagonists block endotoxin induced acute lung injury and the release of thromboxane and IL-6 from human pulmonary artery endothelial cells (Neely et al., 1997; Wilson and Batra, 2002). Furthermore, recently it was reported that an adenosine A₁ receptor antagonist reduces the severity of acute lung injury in influenza-infected mice (Aeffner, et al., 2014). Taken together with the results of the current study in an animal model of polymicrobial sepsis, these data support an expansive immunomodulatory role for adenosine A₁ receptors in multiple types of clinically important sepsis.

Adenosine receptors are members of the superfamily of G-protein-coupled receptors and the four subtypes (A₁, A_{2a}, A_{2b}, and A₃) are well recognized as novel molecular therapeutic targets (Wilson and Mustafa, 2009). Adenosine A₁ receptors are coupled via a pertussis toxin-sensitive inhibitory G protein to a number of different intracellular signaling pathways and effector systems, including adenylate cyclase, phospholipase A₂, phospholipase C, potassium channels, calcium channels, and protein kinase C (Wilson et al., 2009). Adenosine A₁ receptors are upregulated by hypoxia, stress, ischemia/reperfusion, lipopolysaccharide, and inflammation, including in the heart, brain, and kidney (Blackburn et al., 2009; Funakoshi et al., 2007; Jhaveri et al., 2007; Nie et al., 1998).

Plasma adenosine levels are increased and predict negative outcomes in humans with sepsis (Martin et al., 2000). *In vitro* studies in humans and *in vivo* studies in animals suggest that activation of adenosine A₁ receptors may contribute to the pathophysiology of acute lung injury and renal failure of sepsis (Knight, et al., 1993; Neely et al., 1997; Wilson and Batra, 2002; Wilson et al., 2011). In the lung, activation of adenosine A₁ receptors on endothelial cells increases their permeability, while their activation on alveolar type 2 cells decreases lung fluid clearance, actions on both cell types that promote pulmonary edema (Factor et al., 2007; Neely and Keith, 1995; Neely et al., 1997; Satoh et al., 2000). Further, in the lung, adenosine A₁ receptor antagonists, 1,3 dipropyl, 8-cyclopentylxanthine (DPCPX) and bamiphylline, significantly reduced endotoxin induced acute lung injury (Neely et al., 1997). The results of these studies taken together with the beneficial effect on acute lung injury for L-97-1 in a rat model of *Y. pestis* induced sepsis and for DPCPX in a mouse model of influenza induced sepsis, suggest an important role for adenosine A₁ receptors in the acute lung injury of sepsis.

In the kidney, by acting on adenosine A₁ receptors on preglomerular afferent arterioles, adenosine produces vasoconstriction and decreases glomerular filtration rate (Vallon and Osswald, 2009). In a rat model of endotoxemic acute renal failure, an adenosine A₁ receptor antagonist, DPCPX, produced a significant attenuation of the endotoxin induced decline in inulin clearance, renal blood flow, urine output, and electrolyte excretion (Knight et al., 1993). In the current study of polymicrobial sepsis, antibiotics alone did not protect the kidney; however, when administered alone or in different doses in combination with antibiotics, L-97-1 produces a beneficial effect on renal function that is not significantly different from the sham, CLP group. These findings suggest that L-97-1 has a direct, beneficial effect on renal function in this rat CLP sepsis model most likely by improving renal blood flow and glomerular filtration rate, and perhaps by reducing inflammation in the kidney.

In a rat model of renal impairment induced in salt-depleted rats treated with a high dose of furosemide, an animal model used to induce a sodium retentive state and low creatinine clearance (ca. 50% that of normal rats), oral administration of L-97-1 produced a strong renoprotective effect, significantly increasing creatinine clearance at a low fractional sodium excretion (Wilson, unpublished results). These results of these studies suggest that L-97-1 may provide protection to the kidney by increasing renal blood flow. Furthermore, adenosine A₁ receptor antagonists block cisplatin induce nephrotoxicity (Gill et al., 2009; Knight et al., 1991; Nagashima et al., 1995). In one study, the adenosine A₁ receptor antagonist, tonapofylline (BG 9928), significantly reduced elevated levels of serum BUN and creatinine and improved renal pathology scores, including reducing interstitial inflammation (Gill et al., 2009). Thus in sepsis, by blocking activation of adenosine A₁ receptors in the lung and the kidney, an adenosine A₁ receptor antagonist may improve outcome by attenuating acute lung injury and maintaining renal blood flow and function.

In contrast to the results from the current study with L-97-1 that support the use of an adenosine A₁ receptor antagonist in sepsis, utilization of a structurally dissimilar adenosine A₁ receptor antagonist, DPCPX, in a mouse model of CLP sepsis did not provide a beneficial effect on survival (Gallos et al., 2005). Moreover, in that study, the use of

adenosine A₁ receptor knockout mice suggested a protective, anti-inflammatory role for the adenosine A₁ receptor in this mouse model of CLP sepsis. However, as noted by others, caution should be exercised when interpreting data in knockout models, since altered expression of other proteins may affect the phenotypes of cells, cell signaling, receptor crosstalk, organ function, and systemic outcomes (Fernandez et al., 2013).

It is important to note that in this mouse CLP sepsis study, the adenosine A₁ receptor antagonist, DPCPX, was administered as a subcutaneous injection, with no pharmacokinetic data substantiating its presence in the plasma. Therefore, it is very difficult to assess the value of any systemic exposure following this route of administration for this adenosine A₁ receptor antagonist. In the current study L-97-1 is administered as a continuous i.v. infusion, a route for which pharmacokinetic data for L-97-1 in normal rats has been documented, and for which correlative *in vitro* pharmacological data for the rat adenosine A₁ receptor, have been previously reported (Wilson et al., 2011). Although plasma levels of L-97-1 were not measured in the current study, the highly significant dose-dependent effects for L-97-1 on 72 h and 7 d survival *vs.* untreated CLP and on renal function *vs.* antibiotics alone suggest pharmacological inhibition of adenosine A₁ receptors.

5. Conclusions

In a rat model of CLP-induced polymicrobial peritonitis that invariably progresses to fatal sepsis, the adenosine A₁ receptor antagonist, L-97-1, significantly and dose-dependently improves 7 d survival rates when administered at 12 h post CLP as multiple daily i.v. infusions in combination with broad spectrum antibiotics, a treatment regimen that closely resembles the clinical approach to sepsis. Moreover, in this animal model of sepsis, in combination with antibiotics, L-97-1 significantly improves renal function in a dose-dependent manner *vs.* antibiotic therapy alone which does not protect the kidney. These findings taken together with the improvement in renal function produced by L-97-1 alone suggest a direct effect of L-97-1 on renal function independent of antibiotics. The results of these studies support efficacy for L-97-1 as a delayed treatment in this animal model of polymicrobial sepsis and are particularly significant given the unmet medical need for new effective anti-sepsis therapeutics.

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References

- Aeffner F, Woods PS, Davis IC. Activation of A1-adenosine receptors promotes leukocyte recruitment to the lung and attenuates acute lung injury in mice infected with influenza A/WSN/33 (H1N1) virus. *J Virol.* Jun 25.2014 (Epub ahead of print).
- Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med.* 2001; 29:1303–1310. [PubMed: 11445675]
- Angus DC. The lingering consequences of sepsis: a hidden public health disaster. *JAMA.* 2010; 304:1833–1834. [PubMed: 20978262]

- Angus DC. The search for effective therapy for sepsis: back to the drawing board. *JAMA*. 2011; 306:2614–2615. [PubMed: 22187284]
- Banta JE, Kamlesh PJ, Beeson L, Nguyen HB. Patient and hospital characteristics associated with inpatient severe sepsis mortality in California, 2005–2010. *Crit Care Med*. 2012; 40:2960–2966. [PubMed: 22926330]
- Blackburn, MR.; Vance, CO.; Morschl, E.; Wilson, CN. Adenosine receptors and inflammation. In: Wilson, CN.; Mustafa, SJ., editors. *Adenosine Receptors in Health and Disease, Handbook of Experimental Pharmacology*. 1. Berlin Heidelberg: Springer-Verlag; 2009. p. 215-269.
- Factor P, Mutlu GM, Chen L, Mohameed J, Akhmedov AT, Meng FJ, et al. Adenosine regulation of alveolar fluid clearance. *Proc Natl Acad Sci*. 2007; 104:4083–4088. [PubMed: 17360481]
- Fernandez LG, Sharma AK, LaPar DJ, Kron IL, Laubach VE. Adenosine A₁ receptor activation attenuates lung ischemia-reperfusion injury. *J Thorac Cardiovas Surg*. 2013; 145:1654–1659.
- Funakoshi H, Zacharia LC, Zhonghua T, Zhang J, Lee LL, Good JC, Herrmann DE, Yoshihiro H, Koch WJ, Jackson EK, Chan TO, Feldman AM. A₁ adenosine receptor upregulation accompanies decreasing myocardial adenosine levels in mice with left ventricular dysfunction. *Circulation*. 2007; 115:2307–2315. [PubMed: 17438146]
- Gallos G, Ruyle TD, Emala CW, Lee HT. A₁ adenosine receptor knockout mice exhibit increased mortality, renal dysfunction, and hepatic injury in murine septic peritonitis. *Am J Physiol Renal Physiol*. 2005; 289:F369–F376. [PubMed: 15784841]
- Gill A, Wortham K, Costa D, Davis W, Ticho B, Whalley E. Protective effect of tonapofylline (BG 9928), an adenosine A₁ receptor antagonist, against cisplatin-induced acute kidney injury in rats. *Am J Nephrol*. 2009; 30:521–526. [PubMed: 19828940]
- Iwashyna TJ, Ely EW, Smith DM, Langa KM. Long term cognitive impairment and functional disability among survivors of severe sepsis. *JAMA*. 2010; 304:1787–1794. [PubMed: 20978258]
- Jhaveri KA, Reichensperger J, Toth LA, Sekino Y, Ramkumar V. Reduced basal and lipopolysaccharide-stimulated adenosine A₁ receptor expression in the brain of nuclear factor- κ B p50^{-/-} mice. *Neurosci*. 2007; 146:415–426.
- Knight RJ, Collis MG, Yates MS, Bowmer CJ. Amelioration of cisplatin-induced acute renal failure with 8-cyclopentyl-1,3-dipropylxanthine. *Br J Pharmacol*. 1991; 104:1062–1068. [PubMed: 1810593]
- Knight RJ, Bowmer CJ, Yates MS. Effect of the selective A₁ adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine on acute renal dysfunction induced by *Escherichia coli* endotoxin in rats. *J Pharm Pharmacol*. 1993; 45:979–84. [PubMed: 7908041]
- Martin C, Leone M, Viviani X, Ayem M-L, Guieu R. High adenosine plasma concentration as a prognostic index for outcome in patients with septic shock. *Crit Care Med*. 2000; 28:3198–3202. [PubMed: 11008982]
- Nagashima K, Kusaka H, Karasawa A. Protective effects of KW-3902, an adenosine A₁-receptor antagonist, against cisplatin-induced acute renal failure in rats. *Jpn J Pharmacol*. 1995; 67:349–357. [PubMed: 7650867]
- National Vital Statistics Report. Sep 16.2002
- Neely CF, Keith I. A₁ adenosine receptor antagonists block ischemia-reperfusion injury of the lung. *Am J Physiol*. 1995; 268 (Lung Cell Mol Physiol 12):L1036–L1046. [PubMed: 7611426]
- Neely CF, Jin J, Keith IM. A₁ adenosine receptor antagonists block endotoxin-induced lung injury. *Am J Physiol Lung Cellular Mol Physiol*. 1997; 272:L353–L361.
- Nie Z, Mei Y, Ford M, Rybak L, Marcuzzi A, Ren H, Stiles GL, Ramkumar V. Oxidative stress increases A₁ adenosine receptor expression by activating nuclear factor kappa B. *Mol Pharmacol*. 1998; 53:663–669. [PubMed: 9547356]
- Satoh A, Shimosegawa T, Satoh K, Ito H, Kohno Y, Masamune A, et al. activation of adenosine A₁-receptor pathway induces edema formation in the pancreas of rats. *Gastroenterol*. 2000; 119:829–836.
- Suffredini AF, Munford RS. Novel therapies for septic shock over the past 4 decades. *JAMA*. 2011; 306:194–199. [PubMed: 21750297]

- Vallon, V.; Osswald, H. Adenosine receptors and the kidney. In: Wilson, CN.; Mustafa, SJ., editors. Adenosine receptors in health and disease, Handbook of Experimental Pharmacology. Vol. 193. Springer-Verlag; Berlin Heidelberg: 2009. p. 443-470.
- Wilson CN, Batra VK. Lipopolysaccharide binds to and activates A₁ adenosine receptors on human pulmonary artery endothelial cells. J Endotoxin Res. 2002; 8:263–271. [PubMed: 12230916]
- Wilson CN, Vance CO, Goto MA. ₁ adenosine receptor antagonist, L-97-1, improves survival and acute lung injury following cecal ligation and perforation. J Leukocyte Biol. 2006e; (Supp 2006): 62.
- Wilson, CN.; Mustafa, SJ., editors. Handbook of Experimental Pharmacology. Berlin Heidelberg: Springer-Verlag; 2009. Adenosine Receptors in Health and Disease.
- Wilson, CN.; Nadeem, A.; Spina, D.; Brown, R.; Page, CP.; Mustafa, SJ. Adenosine receptors and asthma. In: Wilson, CN.; Mustafa, SJ., editors. Adenosine Receptors in Health and Disease. Handbook of Experimental Pharmacology. Vol. 193. Springer-Verlag; Berlin Heidelberg: 2009. p. 329-362.
- Wilson CN, Vance C, Doyle TM, Brink DS, Matuschak GM, Lechner AJ. A novel post-exposure medical countermeasure L-97-1 improves survival and acute lung injury following intratracheal infection with *Yersinia pestis*. Innate Immunity. 2011; 18:373–389. [PubMed: 21862597]

Figure 1. Effect of Delayed Treatment at 12 Hours for L-97-1 With and Without Antibiotics on Seven Day Survival Rates in Rat CLP Sepsis

In 7 d survival studies separate groups of animals were treated with Sham (sham, CLP) ($n = 11$) or CLP (untreated, CLP control) ($n = 21$). At 12 hours (h) post CLP separate groups of animals were treated with, ABX (antibiotics) ($n = 16$), L-97-1 15 mg/kg/h ($n = 15$), ABX + L-97-1 10 mg/kg/h ($n = 16$), ABX + L-97-1 12.5 mg/kg/h ($n = 23$), or ABX + L-97-1 15 mg/kg/h ($n = 20$). All animals received either an intravenous infusion of L-97-1 (10, 12.5 or 15 mg/kg/h) or sterile water (diluent for L-97-1) at 0.5 ml/h $\times$ 8 h/day $\times$ 3 days. All animals received either antibiotics [ceftriaxone (80 mg) plus clindamycin (40 mg)/day or normal saline (2 ml/day) $\times$ 3 days. Differences in survival among treated and untreated groups were evaluated by Kaplan-Meier curves and tested for significant differences by Log-rank (Mantel-Cox) test with subsequent pair-wise comparisons using Bonferroni's correction for multiple comparisons.

Figure 2. Effect of Delayed Treatment at 12 Hours for L-97-1 With and Without Antibiotics on 72 Hour Survival Rates in Rat CLP Sepsis

In 72 h time killed studies separate groups of animals were treated with Sham (sham, CLP) ($n = 9$) or CLP (untreated, CLP control) ($n = 7$). At 12 hours (h) post CLP separate groups of animals were treated with, ABX (antibiotics) ($n = 17$), L-97-1 15 mg/kg/h ($n = 10$), ABX + L-97-1 10 mg/kg/h ($n = 12$), ABX + L-97-1 12.5 mg/kg/h ($n = 15$), or ABX + L-97-1 15 mg/kg/h ($n = 14$). All animals received either an intravenous infusion of L-97-1 (10, 12.5 or 15 mg/kg/h) or sterile water (diluent for L-97-1) at 0.5 ml/h $\times$ 8 h/day $\times$ 3 days. All animals received either antibiotics [ceftriaxone (80 mg) plus clindamycin (40 mg)/day or normal saline (2 ml/day) $\times$ 3 days. Differences in survival among treated and untreated groups were evaluated by Kaplan-Meier curves and tested for significant differences by Log-rank (Mantel-Cox) test with subsequent pair-wise comparisons using Bonferroni's correction for multiple comparisons.

Figure 3. Effect of Delayed Treatment at 12 Hours for L-97-1 With and Without Antibiotics on Serum Levels of Blood Urea Nitrogen in Rat CLP Sepsis

In separate 24 and 72 h time killed studies serum levels of blood urea nitrogen (BUN) were measured at 24 hours (h) in rats with CLP sepsis. See Figure 1 for details about treatments. Data are expressed as mean $\pm$ S.E.M. Number of serum samples for each group is provided in parenthesis. Differences in mean serum levels of BUN at 24 h were compared among sham CLP, untreated CLP, and treatment groups by ANOVA followed by Tukey's post-hoc test for pair-wise comparisons. An asterisk (*) indicates a significant difference; * $P < 0.01$; ** $P < 0.001$; ***, **** $P < 0.0001$.

Figure 4. Effect of Delayed Treatment at 12 Hours for L-97-1 With and Without Antibiotics on Serum Levels of Cytokines in Rat CLP Sepsis

In separate 24 h and 72 h time killed studies serum levels of interleukin (IL)-6, tumor necrosis factor (TNF)- α , and IL-1 β were measured at 24 hours (h) in rats with CLP sepsis. See Figure 1 for details about treatments. Data are expressed as mean $\pm$ S.E.M. Number of serum samples for each group is provided in parenthesis. Differences in mean serum levels of cytokines at 24 h were compared among sham CLP, untreated CLP, and treatment groups by ANOVA followed by Tukey's post-hoc test for pair-wise comparisons. An asterisk (*) indicates a significant difference in mean serum cytokine concentration from the untreated CLP group; * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

Table 1

Summary Seven Day Survival Rat Model CLP Sepsis.

Group #: Treatment Summary	n	L-97-1 mg/kg/h	First Dose	7-d Survival	Mean TOD (h)
1: Sham CLP Control	11	NA	NA	100%	168
2: CLP Control	21	NA	NA	0%	21
3: CLP+CEF+CLIN	16	NA	0 h	75%	157
4: CLP+CEF+CLIN	16	NA	6 h	69%	148
5: CLP+CEF+CLIN+L-97-1	16	10	6 h	69%	144
6: CLP+L-97-1	15	15	12 h	7%	50
7: CLP+CEF+CLIN	16	NA	12 h	25%	92
8: CLP+CEF+CLIN+L-97-1	16	10	12 h	25%	72
9: CLP+CEF+CLIN+L-97-1	23	12.5	12 h	35%	140
10: CLP+CEF+CLIN+L-97-1	20	15	12 h	75%	145

All animals received either intravenous infusions of L-97-1 (10, 12.5 or 15 mg/kg/h) or of sterile water (diluent for L-97-1) at 0.5 ml/hour $\times$ 8 hour/day $\times$ 3 days. In addition, all animals received either antibiotics [ceftriaxone (CEF, 80 mg) plus clindamycin (CLIN, 40 mg)/day or normal saline (2 ml/day) $\times$ 3 days. TOD (time of death); n = number of animals/treatment group.

Table 2

Summary of Serum Cytokine Levels Following CLP

Cytokine	0 hours	3 hours	6 hours	24 hours
IL-6	10.41 ± 4.75 <i>n</i> = 15	411.21 ± 59.13 <i>n</i> = 15	512.63 ± 66.76 <i>n</i> = 13	1901.9 ± 920.6 <i>n</i> = 8
TNF-α	0.81 ± 0.50 <i>n</i> = 13	12.93 ± 2.62 <i>n</i> = 14	17.72 ± 2.89 <i>n</i> = 15	41.87 ± 12.25 <i>n</i> = 12
IL-1β	4.93 ± 1.46 <i>n</i> = 14	28.25 ± 5.21 <i>n</i> = 12	75.38 ± 9.71 <i>n</i> = 14	60.4 ± 15.9 <i>n</i> = 11

Data are expressed as mean ± S.E.M; n = number of samples per time period