Title: The novel immunotherapeutic oligodeoxynucleotide IMT504 protects neutropenic animals from fatal Pseudomonas aeruginosa bacteremia and sepsis

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Running Title: Protection due to IMT504 in fatal Pseudomonas aeruginosa bacteremia and sepsis

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Abstract

**Background:** IMT504 is a novel immunomodulatory oligonucleotide that has immunotherapeutic properties in early preclinical and clinical studies. IMT504 was tested in a neutropenic rat model of *Pseudomonas aeruginosa* bacteremia and sepsis. This animal system recapitulates many of the pathologic processes found in neutropenic patients with gram-negative, bacterial infections.

**Setting:** Academic research laboratory

**Test subjects:** Sprague-Dawley rats

**Methods:** Animals were rendered neutropenic by cyclophosphamide, colonized by oral feeding with *P. aeruginosa*, and then randomized to receive IMT504 over a range of doses and treatment regimens representing early and late therapeutic interventions.

**Results:** IMT504 immunotherapy conferred a significant survival advantage over the 12-day study period compared with placebo-treated animals when administered at the onset of neutropenia and even in the absence of antibiotics and after the onset of fever and systemic infection. Notably, even late salvage IMT504 monotherapy was highly effective (13/14 surviving rats with IMT504 therapy vs. 2/14 controls, p <0.001). Moreover, late salvage IMT504 monotherapy was as effective as antibiotic therapy (13/14 surviving rats vs. 21/21 rats, p=0.88). In addition, no antagonism or loss of therapeutic efficacy was noted with combination therapy of IMT504 plus antibiotics.

**Conclusions:** IMT504 immunotherapy provides a remarkable survival advantage in bacteremia and sepsis in neutropenic animals and deserves further study as a new treatment option in patients with, or at risk for, severe gram-negative bacterial infections and sepsis.

Introduction

Bacteremia and sepsis remain a major cause of morbidity and mortality and a central challenge to the management of critically ill patients worldwide. Despite a considerable...
amount of time and effort, no new therapy for severe sepsis has proven useful other than early resuscitation with intravenous fluids, vasopressors, broad-spectrum antibiotics and expert supportive care (1). Early administration of effective antibiotics has consistently proven to be critical to survival in septic shock, along with attempts to maintain tissue perfusion by intravenous fluid resuscitation (2). However, specific efforts to disrupt the underlying immunopathogenic mechanisms that cause sepsis have not proven to be of significant survival benefit. Most of these interventions have consisted of inhibitors against pro-inflammatory mediators and pro-coagulant strategies, which have failed in clinical testing (3-9).

A looming crisis in the treatment of bacterial infections in general, and gram-negative bacteria in particular, is the progressive spread of antibiotic resistance genes leading to the emergence of multiply-drug resistant (MDR) microbial pathogens (10). While MDR Pseudomonas sp. have always been a significant problem due to intrinsic antimicrobial resistance, other genera of gram-negative bacteria now commonly are highly resistant to antibiotics including Acinetobacter sp. (especially in military hospitals serving returning veterans from Afghanistan and Iraq) (11) and Klebsiella sp., (especially with the development of carbapenem resistance) (12). The MDR pathogens are associated with a very high mortality rate in afflicted patients. Novel non-antibiotic therapies are now in great need to salvage patients with systemic MDR bacterial pathogens. In the experiments described herein a novel immunotherapeutic agent, IMT504, is found to offer highly significant survival advantage on par with antibiotic treatment in an immunocompromised animal model of Pseudomonas aeruginosa sepsis.

IMT504 is a synthetic oligodeoxynucleotide (ODN) that features a specific immunomodulatory sequence consisting of the active motif PyNTTTTGT (Py: C or T, N: A, C, G or T) linked together on a nuclease-resistant phosphorothioate backbone (13). IMT504 has potent effects for the human and primate innate and adaptive immune systems. IMT504 directly activates B cells to produce beneficial cytokines, promotes antibody production and maturation, expands antibody diversity, and potentiates the capacity of B cells to serve as antigen presenting cells (14). IMT504 can also activate plasmacytoid dendritic cells, which are important components of the antigen presenting
cells in the human immune system as well as regulators of the immune response (13,14). Moreover, IMT504 activates CD4 and CD8 T cells, natural killer cells (NK cells and NKT cells) to promote cellular immune functions with activities against specific forms of cancer and virus-infected cells (15). In addition, pre-clinical studies indicate an excellent safety profile (16-18).

IMT504 differs structurally from ODNs containing the unmethylated CpG motifs (cytosine-phosphate-guanosine) but functionally has some similar immunostimulatory activities (13,19). However, IMT504 has a more limited host range than CpGs, being primarily restricted to humans, non-human primates and rats (18). Moreover, CpG ODNs are inducers of type 1 interferons, an activity not seen with IMT504. Additionally, IMT504 are potent inducers of mesenchymal stem cells; this capacity is not known to exist with CpG nucleotides (16,19).

We hypothesized that IMT504 would provide protection in neutropenic animals at risk for systemic infection from the opportunistic bacterial pathogen *Pseudomonas aeruginosa* in our neutropenic rat model. If IMT504 could boost host immune defenses sufficiently to protect against lethality from sepsis in immunocompromised animals, this could be an appealing and novel approach in the care of patients at risk for systemic bacterial infection during cytoablative chemotherapy for neoplastic disease.

**Materials and Methods:**

**Reagents:**

All reagents used in these experiments were purchased from Sigma (St. Louis, MO) unless otherwise stated. The IMT504 was purchased from Trilink (San Diego, CA). 150-200 gram specific pathogen-free, female, Albino, Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in these experiments using our standard neutropenic rat protocol as described previously (20). Isoflurane was purchased from commercial sources (Baxter, Deerfield, IL) as was ceftriaxone (Roche, Nutley, NJ) and cefepime (Sagent, Schaumburg, IL). The rat multiplex cytokine assay was obtained from
The neutropenic rat model:

Animals were housed in environmentally isolated cages and maintained in a constant ambient temperature and humidity in a 12-hour day/night cycling. Animals were provided with an ad-libitum supply of commercial rodent chow and distilled water. The animals were allowed to adjust to the laboratory facilities for at least 7 days before undergoing any experimental procedures. The laboratory complies with NIH guidelines for the use of animals in biomedical research and the protocol was reviewed and approved by the institutional animal care committee before initiating any experimental protocols.

The conditioning phase began with a regimen of 25 mg/kg subcutaneously of ceftriaxone given throughout the experiment starting 4 days before the induction of neutropenia. This broad-spectrum antibiotic assists in disrupting colonization resistance by the endogenous enteric microbiota against the oral challenge strain of *P. aeruginosa*. On day 0, cyclophosphamide was given at a dose of 75 mg/kg IP to induce transient but severe neutropenia (absolute neutrophil count <50/mm³ at day 5 in all five animals tested). Seventy-two hours later, a second dose of cyclophosphamide (25 mg/kg IP) was administered to maintain neutropenia for approximately 4-8 days. At day 0 animals were given an oral challenge of a rodent-virulent strain of *P. aeruginosa* 12.4.4 immunotype 6. The challenge strain was administered by orogastric feeding at a volume of 1 ml at a dose of 10⁷ CFU/ml. The same dose of *P. aeruginosa* 12.4.4 was administered at 48 and 96 hours after the first dose of cyclophosphamide. For the initial dose-finding study (Figure 1), vehicle control (n=26) or IMT504 50 µg (n=24) was given subcutaneously (S.C.) once at day 5 after the initial dose of cyclophosphamide. The mid dose was IMT504 50 µg on day 5, 7 and 9 (150 µg total, n=70) and the high dose regimen was IMT504 100 µg on days 5, 7 and 9 (300 µg total, n=28).
Therapeutic intervention with IMT504 was classified as Early Therapy, Late Therapy and Optimized Late Therapy.

Early Therapy (Figure 4):
Vehicle control administered I.M. daily starting at day 1, 3, 5 (8 rats)
IMT504 150 µg administered I.M. Day 1 (8 rats)
IMT504 50 µg administered I.M. Days 1, 3, 5 (8 rats)
IMT504 50 µg administered I.M. Day 3 (4 rats)
IMT504 50 µg administered I.M. Days 3, 5, 7 (8 rats)

Late Therapy (Figure 5):
Vehicle control administered I.M. daily starting at day 5 to 10 (8 rats)
IMT504 150 µg administered I.M. Day 5 (8 rats)
IMT504 50 µg administered I.M. Day 5, 7, 9 (8 rats)
IMT504 25 µg administered I.M. Days 5 to 10 (16 rats)
IMT504 50 µg administered I.M. Days 5 to 10 (14 rats)

Optimized Late Therapy (Figure 6):
Vehicle control administered I.M. daily starting at day 5 to 10 (14 rats)
IMT504 50 µg administered I.M. daily starting at day 5 to 10 (14 rats)
Cefepime (25 mg/kg S.C.) + vehicle control administered daily starting at day 5 to 10 (21 rats)
IMT504 50 µg administered I.M. daily starting at d 5 to 10 + cefepime (25 mg/kg) (16 rats)

The animals underwent blood sampling at the onset of fever and 24 hours after the onset of fever. These blood samples were obtained from the retro-orbital plexus of the rat under light isoflurane anesthesia. The plasma samples (approximately 250 µL in each sample) were obtained for plasma cytokine determination.

Statistical Analysis
The 14-day survival time between multiple groups were analyzed with a non-parametric
ANOVA (Kruskal-Wallis) test followed by the post-test Dunn’s multiple comparisons
test, to test significance between two groups in each set. The survival function between
groups is presented as a Kaplan-Meier plot and analyzed by the log-rank method. Two
sample tests were analyzed with a Mann-Whitney U test. A two sided p-value <0.05 was
set as the level of statistical significance.

Results:

IMT504 protects against lethality in the neutropenic rat model.

A preliminary dose-finding study with increasing dose levels of IMT504 is summarized
in Figure 1. IMT504 provided highly significant survival benefits ($P<0.001$) at the mid
and high dose regimens, even in the absence of antimicrobial therapy directed at the
infecting microorganism. No significant survival advantage was observed with a single
dose regimen of 50µg IMT504. The optimal dose for survival in the animal model was a
total of $\geq 150$µg IMT504 administered in 3 divided 50µg doses, when given at the onset
of fever (day 5). For this reason, 50µg was chosen as the primary dosage for most of the
remainder of these experiments.

Effects of IMT504 on microbial clearance from tissue sites and interleukin-6 levels.

The quantitative measurement of *P. aeruginosa* concentrations in target tissues in each
dosing group is presented in Figure 2. A strong trend was noted with decreasing colony
counts of almost 2 logs in target tissues with increasing doses of IMT504 ($P=0.062$). We
tested IMT504 for any direct antimicrobial effect against the challenge strain of *P.
aeruginosa* and found no inhibitory effects up to the highest concentration tested
(>500µg/ml IMT504).

In a separate set of animals plasma levels of interleukin-6 were determined at 24 hr after
the onset of fever (day 6) in each animal (Figure 3). The lowest levels of IL-6 levels were
observed in the 150µg IMT504 group (versus the control group, $P<0.0001$) followed by
the 300µg group ($P<0.05$). Other cytokines that were measured at this same time point
were highly variable among the animals and failed to demonstrate any statistically significant differences (data not shown).

Effects of IMT504 on survival with Early Therapy, Late Therapy and Optimized Late Therapy

Early Therapy:
The effects of IMT504 therapy on survival when instituted early in the disease process are presented in Figure 4. Therapeutic intervention with IMT504 began at day 1 or day 3 with varying total amounts of IMT504 administered. All of the rats receiving IMT504 50 µg at Days 1, 3, 5 survived (8/8) vs. 2/8 rats in the placebo-treated group (P < 0.05). Survival rates in the other IMT504-treated groups were not statistically significantly different from the group treated with IMT504 50 µg at Days 1, 3, 5.

Late Therapy:
The effects of IMT504 therapy on survival when instituted late in the disease process are presented in Figure 5. Survival rates between the multiply-dosed IMT504-treated groups at day 5-10 were not statistically significantly different. Thirteen of 14 rats treated with IMT504 50 µg at Days 5-10 survived, 6/8 rats given IMT504 50 µg at days 5,7,9 survived, 13/16 rats treated with IMT504 25 µg at Days 5-10 survived vs. 2/8 placebo-treated controls (all P < 0.05). However, a single dose of IMT504 150 µg given on day 5 alone was not of significant benefit when compared with the placebo control group (4/8 vs. 2/8 survivors).

Optimized Late Therapy:
The effects of optimized late therapy with IMT504 were compared to antibiotic therapy and combination IMT504 plus antibiotics (Figure 6). All three of these optimized late salvage groups had superior survival when compared to placebo-treated controls (all P < 0.001). Comparable high survival rates occurred in all three of the treated groups (13/14,
In addition, no antagonism or loss of therapeutic efficacy was noted with combination therapy of IMT504 plus antibiotics.

**IMT504 does not alter the activity of antimicrobial agents given against the challenge strain of P. aeruginosa**

The results of a combination of IMT504 with the anti-pseudomonal cephalosporin cefepime were compared to IMT504 monotherapy, cefepime alone or the control group (Figure 6). There is no evidence of any loss of therapeutic effect or antagonism when the two agents were combined. Further studies with suboptimal dosing of cefepime in combination with IMT504 will be necessary to determine any potential additive or synergistic effects, and to determine whether combination therapy with IMT504 could salvage cefepime in a drug-resistant setting.

**Discussion:**

Safe immunotherapies could be particularly valuable clinically as multi-antibiotic resistant strains of bacteria are threatening the long-term viability of our current, existing antimicrobial chemotherapy for the treatment of bacterial infections (21). *Pseudomonas aeruginosa* and other gram-negative bacterial pathogens including *Klebsiella* spp. have recently become the focus of much attention as these virulent gram-negative microorganisms are becoming increasingly resistant to standard broad-spectrum beta-lactam agents. Resistance to potent antibiotics such as carbapenems and other extended spectrum beta lactams and beta-lactamase inhibitors has become increasingly commonplace via the acquisition of novel, plasmid-mediated, beta-lactamases such as *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-beta lactamase 1 (NDM1) and other beta-lactamase enzymes (22-24). This expanding population of antibiotic-resistant patients could be an appealing target for therapeutic intervention with IMT504 (25).

The mechanism of protection afforded by IMT504 in this study appears to be a combination of reduction in bacterial load in these immune-compromised animals along
with reduction in systemic inflammatory markers such as interleukin-6 levels. IL-6 has long been known to serve as a predictor of outcome in experimental and human sepsis (26). The capacity to promote GM-CSF expression by IMT504 in the presence of IL-2 might also participate in the protection of animals from neutropenic sepsis (17). GM-CSF likely leads to increased production of granulocytes and macrophages. Additionally, GM-CSF has other salutatory properties that stimulate innate immunity and protect against microbial invasion (27, 28). IMT 504 also activates B and T cells along with mesenchymal stem cells that might provide critical host immune functions during the hypo-inflammatory phase of septic shock (3,15,16,19). A more detailed integration into the mechanism(s) that account for the beneficial effects of IMT504 are necessary to more fully understand the impact of this immunostimulatory oligodeoxynucleotide. IMT504 has no direct antimicrobial activity and therefore could be used with antimicrobial agents as a companion immunotherapy or even as primary therapy when antibiotics have failed.

The administration of IMT504 confers a very substantial survival advantage in neutropenic rats from fatal gram-negative bacteremia from Pseudomonas sepsis, on par with that seen using standard antibiotic treatment. In this report, IMT504 could effectively be combined with cefepime, an anti-pseudomonal antibacterial agent to which the challenge strain of P. aeruginosa is susceptible. Administration of IMT504 could be of considerable therapeutic value in clinical medicine as an immunotherapy for the treatment of neutropenic patients with multi-drug resistant, gram-negative bacillary infections. Pseudomonas aeruginosa was chosen because it is among the worst effectors of gram-negative bacterial infections, in which antibiotic resistance and bad outcomes are commonplace in human infections. Plans are underway to test IMT504 in infections caused by other gram-negative bacteria, including Klebsiella spp., Acinetobacter spp. and other bacteria that contain resistance factors to multiple drugs including carbapenems (e.g., carbapenem-resistant Enterobacteriaceae).

Importantly, recent studies have shown that adjunctive therapy has the potential to salvage antibiotics that would otherwise no longer function in a drug-resistant setting (28-30). IMT504 may offer a novel route to resurrecting the arsenal of antimicrobial agents.
that are rapidly dwindling in utility. Future clinical trials of IMT504 are currently being planned, in particularly vulnerable patient populations like cystic fibrosis and burn victims, where treatment options are limited, and morbidity and mortality due to *Pseudomonas aeruginosa* infection remain quite high (31).
References


Figure 1: Kaplan-Meier survival plots in a dose-finding study in neutropenic rats over the 14-day study period. Legend: CTRL-vehicle control, all other groups are different dosing regimens of IMT504 as listed in the methods section. Both the 150 (50µg given at day 5, day 7 and day 9), and the 300µg (100µg given at day 5, day 7 and day 9) dose groups provided a significant (P<0.001) survival advantage compared to the vehicle control group.

Figure 2: Colony counts in colony forming unit/gram tissue of Pseudomonas aeruginosa 12.4.4. in animals treated with increasing doses of IMT504. Legend: D-dose of IMT504 in µg/ animal; a trend was noted with decreasing colony counts in target tissues with increasing doses of IMT504 but these differences were not significantly different (P=0.062).

Figure 3: Plasma interleukin-6 levels at day 6. The IL-6 levels were significantly lower than the control in each of the IMT504 total dose group of 150µg (given as 3 doses of 50µg IMT504 (P<0.0001) and the 300µg (given as 3 doses 100µg IMT504) dose group (P<0.05) but not the 75µg dose group (given at 75µg IMT504).

Figure 4: Kaplan-Meier survival plot with early intervention with IMT504 at different dosing intervals before onset of fever (day 1 or day 3). Legend: CTRL-vehicle control group, 50 or 150µg dose of IMT504; d-day, *P = 0.032 (Kruskal-Wallis Test); Survival with 50µg on days 1, 3, 5 (d135) than 50µg given once at day 3 (d3) or 50µg given on days 3, 5, 7 (d357) was significantly better (P < 0.05) when compared to control arm (Dunn’s Multiple Comparisons Test).

Figure 5: Kaplan-Meier survival plot with Late intervention with IMT504 at different dosing intervals after onset of fever (day 5). Legend: CTRL-vehicle control group; 25 or 50µg dose of IMT504; d-day, day *P = 0.0097 (Kruskal-Wallis Test); 25µg administered days 5-10, 50µg administered days 5-10 or at day 5,7,9 (d5,7,9) were all protective when
compared to the control group (Dunn’s Multiple Comparisons Test $P<0.05$ for each). In contrast a single 150µg dose of IMT504 administered at day 5 was not protective.

Figure 6: Kaplan-Meier survival plot with IMT504 monotherapy vs. cefepime monotherapy vs. combination therapy with cefepime and IMT504 dosed daily at day 5 after onset of fever. Legend: CTRL-vehicle control group; 50µg dose of IMT504; d-day
Figure 2

Organ Counts (cfu/gm)

Total Dose IMT504

- 300
- 150
- 50
- 0
- 0.1

Spleen
Liver
Lung
Figure 6

- CTRL n=14
- 50ug d5 to10 n=14
- Cefepime treated n=21
- IMT504 plus Cefepime n=16

Survival vs. Hours